$G_{OLF\alpha}$ Mediates Dopamine D_1 Receptor Signaling

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It is generally assumed that the coupling of dopamine D_1 receptors to adenylyl cyclase is mediated by the stimulatory GTP-binding protein G_s. However, the striatum contains little $G_{s\alpha}$ subunit, whereas it expresses high levels of $G_{olf\alpha}$, a close relative of $G_{s\alpha}$ that is also expressed in olfactory receptor neurons. We used $G_{olf\alpha}$ knockout mice to examine the functional coupling of D_1 receptors. We found that these mice showed no hyperlocomotor response to either the D_1 agonist SKF-81297 or the psychostimulant cocaine. Moreover, $G_{olf\alpha}$ knockout mice did not display cocaine-induced c-fos expression in the striatum. Finally, in the absence of $G_{olf\alpha}$, striatal D_1 receptors have a decreased affinity for dopamine. Thus coupling to $G_{olf\alpha}$ appears to mediate D_1 signaling in the striatum.

Key words: dopamine; D_1 receptor; G_{olf}; G_s; striatum; knockout

The dopamine D_1 receptor is the most abundant and widespread of the five known dopamine receptor subtypes. It is highly expressed in the striatum, nucleus accumbens, and olfactory tubercle and is moderately expressed in the cortex, amygdala, hypothalamus, and thalamus (Gingrich and Caron, 1993; Jaber et al., 1996; Missale et al., 1998). The D_1 receptor is also found on the terminals of striatal neurons in the substantia nigra pars reticulata (Altar and Hauser, 1987). D_1 receptors stimulate the formation of cAMP in response to agonists both in intact preparations (Hess et al., 1987; Watts et al., 1993) and in a number of D_1-transfected cell lines (Dearry et al., 1990; Monsma et al., 1990; Zhou et al., 1990). It is thus generally assumed that the coupling of dopamine D_1 receptors to adenylyl cyclase is mediated by G_s. However, several studies have shown that the striatum, despite its high D_1 receptor level, has very little $G_{s\alpha}$ subunit, whereas it does express high levels of another G-protein subunit, $G_{olf\alpha}$ (Drinnan et al., 1991; Herve et al., 1995; Belluscio et al., 1998), which was found originally to mediate olfactory receptor signaling (Jones and Reed, 1989). $G_{olf\alpha}$ and $G_{s\alpha}$ share 88% homology in amino acid composition, both stimulate adenylyl cyclase (Jones and Reed, 1989), and both are substrates for covalent ADP-ribosylation catalyzed by cholera toxin (Jones et al., 1990). In addition, there are no specific $G_{olf\alpha}$ or $G_{s\alpha}$ inhibitors.

Recently, a mouse line deficient in $G_{olf\alpha}$ has been generated ($G_{olf\alpha}$ knockout). These mice are anosmic because of the lack of olfactory receptor signaling (Belluscio et al., 1998). They also provide the means to test the role of $G_{olf\alpha}$ in D_1 signaling. We have found that $G_{olf\alpha}$ knockout mice are deficient in striatal dopamine D_1 receptor-mediated behavioral and biochemical effects, consistent with the hypothesis that $G_{olf\alpha}$ mediates D_1 signaling in the striatum.

MATERALS AND METHODS

Animals. The generation of $G_{olf\alpha}$ knockout mice has been detailed previously (Belluscio et al., 1998). Wild-type and knockout littermates have a mixed (129/Sv X C57BL/6) background and were backcrossed at least three times into the C57BL/6 strain. Only male mice were used in all of the studies. All mice were kept on a 6 A.M.-6 P.M. light cycle. All animal procedures were approved by the Institutional Animal Care and Use Committee.

In situ *hybridization. In situ* hybridization was performed on 20 μm fresh frozen sections with ^{33}P-UTP-labeled riboprobes. cDNA clones encoding $G_{olf\alpha}$ (Jones and Reed, 1989) and $G_{s\alpha}$ (Sullivan et al., 1986) were obtained by RT-PCR. Under the *in situ* hybridization conditions that were used, the $G_{olf\alpha}$ and $G_{s\alpha}$ probes did not cross-hybridize (Belluscio et al., 1998).

Immunohistochemistry. Animals (n = 3 for both genotypes) were deeply anesthetized with ketamine and transcardially perfused with 4% paraformaldehyde. Sections (45 μm) were cut on a freezing-sliding microtome. The primary antibody was directed against the c-fos N-peptide (AB-2; Oncogene Sciences, Mineola, NY) and used at 1:500 dilution. Fos immunoreactivity was visualized with the avidin–biotin–peroxidase method (Vectostatin Elite ABC; Vector, Burlingame, CA). The peroxidase reaction was developed in diaminobenzidine and H_2O_2.

Locomotor activity. All mice (n = 5 for both genotypes) were kept on a 6 A.M.-6 P.M. light cycle. Male mice between 3 and 5 months old were used and tested during the light period. Animals were placed in square open chambers (40 cm long × 40 cm wide × 37 cm high). They were monitored throughout the test session by a video tracking system equipped with infrared beams (PolyTrack, San Diego Instruments) that records the animal's location and path (horizontal activity) as well as the number of rearings (vertical activity). Before each test, the open fields were cleaned to maintain constant olfactory cues.

Drug treatment. Drugs were dissolved in saline and administered intraperitoneally. Animals' locomotor activities were monitored right after the injection (n = 5 for each genotypes). (±)-SKF-81297 hydrobromide was obtained from RBI (Natick, MA), and cocaine was obtained from Sigma (St. Louis, MO).

Autoradiography. Coronal fresh frozen sections were cut at 20 μm and thaw-mounted onto slides. Wild-type and mutant brain (n = 4 for each genotype) sections of comparable brain regions were mounted on the same slides. For dopamine D_1 binding, sections were dried at room temperature, preincubated for 30 min in 50 mM Tris buffer containing (in mM): 120 NaCl, 5 KCl, 2 $CaCl_2$, 1 $MgCl_2$, and 0.001% ascorbic acid, pH 7.4, and then incubated for 90 min in the same buffer supplemented with 2 nM n-methyl-^{3}H-SCH23390 (85.0 Ci/mmol; Amersham, Arlington

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Heights, IL) and 50 nM ketanserin (to block 5-HT2 receptor binding). Nonspecific binding was determined in the presence of 10 μM flupenthixol. For displacement studies, alternate slides were incubated with various concentrations of dopamine (1, 4, 10, 25, 50, 100, 200, 400, 800, and 1600 μM) (Altar and Marien, 1987; Richfield et al., 1989).

Data analysis. Data were analyzed using StatView 4.5 (Abacus Concepts Inc.). Unpaired two-tailed Student's *t* test was used when genotype was the only grouping variable. ANOVA was used when genotype was not the only grouping variable and when data were collected in a single trial of a single session. Repeated measure ANOVA was used when data were collected in multiple trials of a single session. Nested repeated measure ANOVA was used when data were collected in multiple trials in more than one session.

RESULTS

$G_{olf\alpha}$ but not $G_{s\alpha}$ is highly expressed in the striatum

The distribution of $G_{olf\alpha}$ mRNA and $G_{s\alpha}$ mRNA in wild-type mice was studied by *in situ* hybridization with $G_{olf\alpha}$ and $G_{s\alpha}$ RNA probes, respectively. $G_{olf\alpha}$ mRNA is highly expressed in the caudate-putamen, nucleus accumbens, olfactory tubercle, piriform cortex, dentate gyrus, CA3 region of the hippocampus (Fig. 1*a*,*c*), and Purkinje cells of the cerebellum (data not shown). There are low levels of $G_{olf\alpha}$ expression in the thalamus, hypothalamus, lateral septum, bed nucleus of the stria terminalis, preoptic area (Fig. 1*a*,*c*), and substantia nigra (data not shown). In contrast, $G_{s\alpha}$ mRNA is widely expressed except in the caudate-putamen, nucleus accumbens, and olfactory tubercle, where it is barely detectable (Fig. 1*b*,*d*). In $G_{olf\alpha}$ knockout mice, $G_{olf\alpha}$ mRNA is undetectable, whereas the level of $G_{s\alpha}$ is unchanged (Belluscio et al., 1998).

$G_{olf\alpha}$ knockout mice do not display D_1 receptor-dependent locomotor responses

Because striatal D_1 receptor activation leads to behavioral stimulation, we examined the locomotor and rearing activities of $G_{olf\alpha}$ knockout mice both in baseline conditions and in response to direct and indirect D_1 agonists. Both locomotor (Fig. 2*a*,*b*) and rearing activities (data not shown) of knockout mice were significantly higher in three 1 hr open field daily sessions compared with wild-type mice. Nevertheless, knockout mice showed normal within-session (Fig. 2*a*) and between-session habituation (Fig. 2*b*).

As previously reported, the D_1-selective agonist SKF-81297 (8 mg/kg) evoked increases in locomotor activity (Fig. 2*c*,*d*) and rearing (data not shown) in wild-type mice. In contrast, SKF-81297 did not stimulate locomotor activity (Fig. 2*c*,*d*) or rearing in $G_{olf\alpha}$ knockout mice. Similarly, cocaine (20 mg/kg) dramatically increased locomotor activity (Fig. 2*c*,*d*) and rearing (data not shown) in wild-type mice but had no effect in $G_{olf\alpha}$ knockout mice (Fig. 2*c*,*d*). Cocaine also caused mild stereotypy in wild-type mice but not in knockout mice (data not shown).

$G_{olf\alpha}$ knockout mice do not display cocaine-induced c-fos expression in the striatum

The expression of the immediate-early gene c-fos is markedly induced in the striatum in response to psychostimulants such as cocaine (Graybiel et al., 1990; Lucas et al., 1997). Mice were injected with 20 mg/kg body weight cocaine or saline, killed 2 hr later, and studied for c-fos expression by immunohistochemistry. In saline-injected wild-type or $G_{olf\alpha}$ knockout mice, virtually no c-fos expression was detected in the brain (data not shown). In response to cocaine, wild-type but not $G_{olf\alpha}$ knockout mice displayed c-fos expression in the striatum (Fig. 3). In the cingulate cortex, lateral septum (Fig. 3), and piriform cortex (data not shown), c-fos was markedly induced in both genotypes. There were also low levels of cocaine-induced c-fos expression in the thalamus, hypothalamus, parietal cortex, and perirhinal cortex in both genotypes (data not shown).

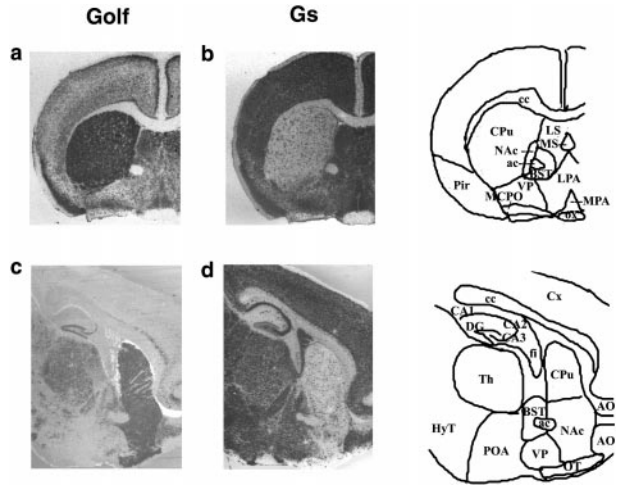


Figure 1. The distribution of $G_{olf\alpha}$ and $G_{s\alpha}$. The distribution of $G_{olf\alpha}$ mRNA (*a*, *c*) and $G_{s\alpha}$ mRNA (*b*, *d*) in wild-type mice was studied by *in situ* hybridization with $G_{olf\alpha}$ and $G_{s\alpha}$ RNA probes, respectively. $G_{olf\alpha}$ mRNA is highly expressed in the *CPu*, *NAc*, *OT*, *Pir*, *DG*, and *CA3* (*a*, *c*). There is a low level of $G_{olf\alpha}$ expression in the *Th*, *HyT*, *LS*, *BST*, and *POA*. $G_{s\alpha}$ mRNA is highly expressed everywhere except in *CPu*, *NAc*, and *OT*, where it is barely detectable. *ac*, Anterior commissure; *AO*, anterior olfactory nuclei; *BST*, bed nucleus stria terminalis; *CA1*, CA1 field of hippocampus; *CA2*, CA2 field of hippocampus; *CA3*, CA3 field of hippocampus; *cc*, corpus callosum; *CPu*, caudate-putamen (striatum); *Cx*, cortex; *DG*, dentate gyrus; *fi*, fimbria hippocampus; *HyT*, hypothalamus; *LPA*, lateral preoptic area; *LS*, lateral septum; *MCPO*, magnocellular preoptic nucleus; *MPA*, medial preoptic area; *MS*, medial septum; *NAc*, nucleus accumbens; *OT*, olfactory tubercle; *ox*, optic chiasm; *Pir*, piriform cortex; *POA*, preoptic area; *Th*, thalamus; *VP*, ventral pallidum.

Decreased affinity of D_1 receptors for dopamine in the striatum of $G_{olf\alpha}$ knockout mice

G-protein coupling is usually necessary for high affinity agonist binding (Adham et al., 1998; Zhao et al., 1998). To compare the state of D_1 receptor coupling in wild-type and $G_{olf\alpha}$ knockout mice, we performed *in vitro* autoradiography on brain sections. We examined the effects of increasing concentrations of the agonist dopamine on binding of the D_1 antagonist ligand ^{3}H-SCH23390. As shown in Figure 4, dopamine is less efficient at displacing the radioligand in the striatum of $G_{olf\alpha}$ knockout mice as compared with wild-type mice. The IC_{50} is significantly higher in $G_{olf\alpha}$ knockout mice than in wild-type mice ($t_{(6)} = 2.6$; $p = 0.04$), consistent with a decreased affinity of striatal D_1 receptors for dopamine. No genotype difference in IC_{50} was found in the nucleus accumbens, olfactory tubercle, or substantia nigra. Specific radioligand binding was the same for both genotypes in all brain regions.

DISCUSSION

Dopamine D_1 receptors are coupled to $G_{olf\alpha}$ in the striatum

The present study provides the first functional evidence that the dopamine D_1 receptor in the striatum is coupled to $G_{olf\alpha}$. Specifically, we show in three ways that striatal D_1 receptors are not functional in mice lacking $G_{olf\alpha}$. First, $G_{olf\alpha}$ knockout mice do not display locomotor responses to the D_1-selective agonist SKF-81297. Second, both the locomotor and the c-fos-inducing effects of cocaine are absent in $G_{olf\alpha}$ knockout mice. Although SKF-81297 directly activates D_1 receptors, cocaine increases extracellular dopamine concentration by blocking dopamine reuptake and acts therefore as an indirect D_1 agonist. It has been shown using D_1 antagonists (Cabib et al., 1991; Young et al., 1991; Ushijima et al., 1995) and D_1 receptor knockout mice (Xu et al., 1994a,b; Drago et al., 1996; Moratalla et al., 1996) that cocaine-

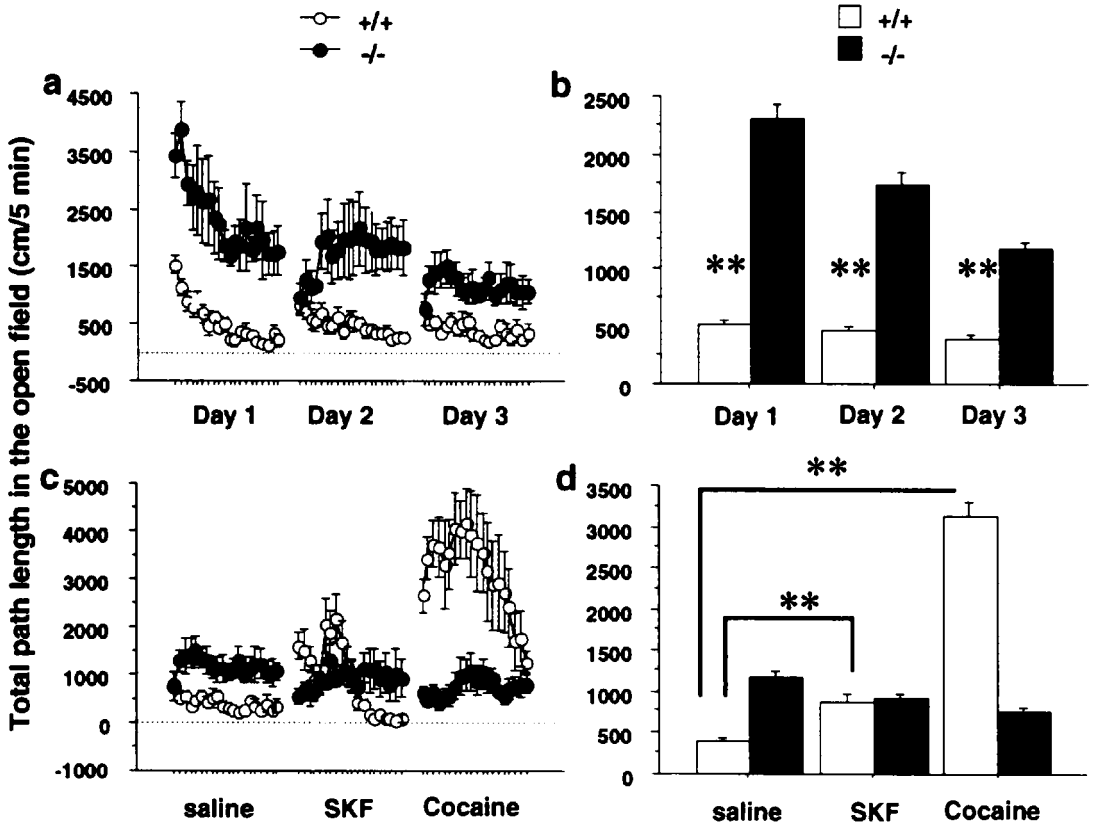


Figure 2. Basal and drug-induced locomotor activity in the open field. Naive animals were exposed to the open field, and their horizontal activity (total path length) was monitored for 1 hr (each *point* represents 5 min in *a* and *c*; the average activity during the hour is shown in *b* and *d*) for 3 consecutive days. Locomotor activity of knockout mice is significantly higher than that of wild-type mice on all 3 d (a, b, $F_{(1,8)} = 26.0$; $p < 0.001$). The D_1-selective agonist SKF-81297 (8 mg/kg) and cocaine (20 mg/kg) elicited a significant increase in locomotor activity in wild-type mice, whereas they had no effect in $G_{olf\alpha}$ knockout mice (c, d). There is a significant genotype × treatment interaction ($F_{(2,16)} = 24.5$; $p < 0.0001$).

induced locomotion and striatal c-fos expression are D_1 dependent. The lack of these responses in the $G_{olf\alpha}$ knockout mice is therefore consistent with an inactivity of striatal D_1 receptors. Cocaine-induced c-fos expression in other brain regions of the $G_{olf\alpha}$ knockout mice such as the cingulate cortex and the lateral septum could be attributable either to intact D_1-signaling in these structures or to D_1-independent mechanisms. The latter alternative is suggested by observations that cocaine induces c-fos expression in the cingulate cortex and lateral septum of D_1 knockout mice (Moratalla et al., 1996).

A third line of evidence suggesting that striatal D_1 receptors are inactive in $G_{olf\alpha}$ knockout mice is the decrease in the affinity of these receptors for dopamine. A decrease in the affinity of a G-protein-coupled receptor for agonists is often associated with G-protein uncoupling (Adham et al., 1998; Zhao et al., 1998). In the absence of $G_{olf\alpha}$, striatal D_1 receptors may not be coupled to a G-protein. The other two regions with high levels of $G_{olf\alpha}$ but not $G_{s\alpha}$, namely the nucleus accumbens and olfactory tubercle, did not show decreased affinity for dopamine, suggesting that D_1 receptors in these regions may be coupled to a different G-protein.

Although Golf knockout and wild-type mice are on a mixed genetic background (C57BL/6 X 129/Sv; see Materials and Methods), it is highly unlikely that the lack of responsiveness to cocaine and dopamine agonist of Golf knockout mice results from differences between two parental strains, for two reasons. First, the experimental animals are littermates and therefore contain similar proportions of both strains. Second, both 129/Sv and C57BL/6 parental strains are similarly responsive to cocaine and dopamine agonists (our unpublished results).

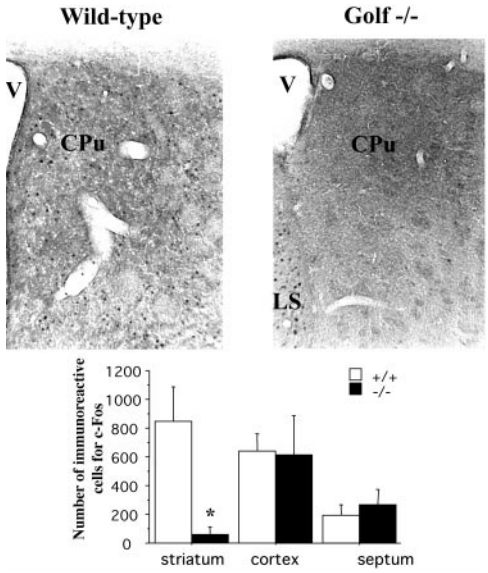
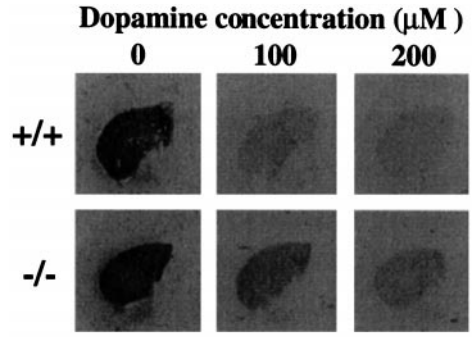


Figure 3. Cocaine-induced c-fos expression. Mice were injected with 20 mg/kg cocaine and killed 2 hr later for immunohistochemical studies. There was a dramatic increase in c-fos immunoreactive nuclear staining in the striatum, cingulate cortex, and lateral septum in the wild-type mice in contrast to saline-injected controls. In $G_{olf\alpha}$ knockout mice, cocaine-induced c-fos expression was seen in the cingulate cortex and lateral septum but not in the striatum (genotype difference in the striatum: $t_{(4)} = 3.1$, $p = 0.035$). *CPu*, Caudate-putamen (striatum); *LS*, lateral septum; *V*, lateral ventricle.



	CPu		NAc		OT		SN	
	+/+	-/-	+/+	-/-	+/+	-/-	+/+	-/-
Specific Binding (fmol/mg)	43.0 ±6.6	41.5 ±4.0	31.8 ±3.1	33.7 ±11	37.9 ±9.2	41.6 ±10	26.9 ±3.9	32.5 ±3.6
IC_{50} (μM)	3.6 ±1.1	9.1 ±1.8*	9.4 ±0.7	7.4 ±2.1	5.8 ±1.3	5.2 ±1.2	7.2 ±1.0	7.0 ±1.4

Figure 4. Displacement of antagonist radioligand binding by agonist. *In vitro* autoradiography was performed on brain sections from both wild-type and $G_{olf\alpha}$ knockout mice. The dopamine D_1 selective antagonist ^{3}H-SCH23390 was used as the radioligand, and different concentrations of dopamine were used to displace ^{3}H-SCH23390 binding. The displacement of the antagonist radioligand by dopamine in the striatum was less efficient in $G_{olf\alpha}$ knockout mice compared with wild-type mice. IC_{50} (concentration of dopamine required to displace one-half of the specific binding sites) is significantly higher in $G_{olf\alpha}$ knockout mice than in wild-type mice in the striatum ($t_{(6)} = 2.6$; $p = 0.04$). There was no genotype difference in specific binding.

Two distinct D_1 signaling pathways

The midbrain dopamine system has three major projections: the nigrostriatal pathway, which is involved in motor function; the mesolimbic pathway, which is involved in reward; and the mesocortical pathway, which is involved in cognitive functions (Gingrich and Caron, 1993; Jaber et al., 1996; Missale et al., 1998). Although D_1 receptors are found in all three projection areas, there appears to be a clear segregation of their downstream pathways. $G_{olf\alpha}$ is highly expressed in the striatum (nigrostriatal pathway), whereas $G_{s\alpha}$ is barely detectable there but highly expressed in the cortex (mesocortical pathway). The mesolimbic pathway, on the other hand, has both kinds of stimulatory G-proteins, with $G_{olf\alpha}$ in the nucleus accumbens and the olfactory tubercle, $G_{s\alpha}$ in the septum, and both $G_{olf\alpha}$ and $G_{s\alpha}$ in the piriform cortex.

Segregation in adenylyl cyclase distribution has also been reported. Specifically, adenylyl cyclase type V (AC5) is found to be restricted to the striatum, nucleus accumbens, and olfactory tubercle, whereas adenylyl cyclase type I (AC1) is barely detectable in these three regions but is widely distributed in other brain regions (Mons et al., 1995; Matsuoka et al., 1997; Shishido et al., 1997). This AC1 versus AC5 segregation matches well with the $G_{s\alpha}$ versus $G_{olf\alpha}$ segregation. It is therefore likely that D_1 signaling in the nigrostriatal pathway is primarily mediated by D_1–$G_{olf\alpha}$–AC5 coupling, whereas D_1 signaling in the mesocortical pathway is mediated by D_1–$G_{s\alpha}$–AC1 coupling.

The distinction between these two D_1 signaling pathways is also seen within the striatum during development. The striatum expresses $G_{s\alpha}$ and AC1 but not $G_{olf\alpha}$ or AC5 before the first postnatal week (Rius et al., 1994; Matsuoka et al., 1997), and there is a progressive switch from D_1–$G_{s\alpha}$–AC1 to D_1–$G_{olf\alpha}$–AC5 during the first 3 postnatal weeks. It will be interesting to investigate whether these two signaling pathways have different functional properties and how such differences may impact on the development and function of the nigrostriatal pathway.

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