D1–D2 dopamine receptor heterooligomers with unique pharmacology are coupled to rapid activation of G_q/11 in the striatum

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We demonstrate a heteromeric D1–D2 dopamine receptor signaling complex in brain that is coupled to G_q/11 and requires agonist binding to both receptors for G protein activation and intracellular calcium release. The D1 agonist SKF83959 was identified as a specific agonist for the heteromer that activated G_q/11 by functioning as a full agonist for the D1 receptor and a high-affinity partial agonist for a pertussis toxin-resistant D2 receptor within the complex. We provide evidence that the D1–D2 signaling complex can be more readily detected in mice that are 8 months in age compared with animals that are 3 months old, suggesting that calcium signaling through the D1–D2 dopamine receptor complex is relevant for function in the postadolescent brain. Activation of G_q/11 through the heteromer increases levels of calcium/calmodulin-dependent protein kinase IIα in the nucleus accumbens, unlike activation of G_s/olf-coupled D1 receptors, indicating a mechanism by which D1–D2 dopamine receptor complexes may contribute to synaptic plasticity.

heterooligomerization | SKF83959 | calcium signaling | calcium/calmodulin-dependent protein kinase IIα

Diverse roles for each of the five dopamine receptors (D1–D5) have been shown to be initiated primarily through stimulation or inhibition of adenylyl cyclase (AC) via G_s/olf or G_i/o signaling proteins, respectively (1). There have been reports, however, of a D1-like receptor in brain that is coupled to G_q/11, stimulating phospholipase C (PLC) and intracellular calcium release (2–5). Activation of this G_q/11-coupled D1-like receptor by specific receptor agonists does not correlate with the ability of these same agonists to activate AC (4), suggesting that the G_q/11-coupled D1-like receptor is a molecular entity distinct from the G_s/olf-coupled D1 receptor.

Molecular identification of the G_q/11-coupled D1-like receptor has proven elusive because D1 receptor coupling to PLC has not been demonstrated in a variety of cell types in which the D1 receptor was expressed. We had postulated that G_q/11 activation by D1 receptor agonists in brain could occur by concurrent activation of the D1 receptor and the D2 receptor (6). We have shown that heterologously coexpressed D1 and D2 dopamine receptors formed heterooligomers (7) and that coactivation of these receptors resulted in a PLC-dependent rise in intracellular calcium (6). We also demonstrated that D1 and D2 receptors could be coimmunoprecipitated from striatal membranes (6). These results suggested the possibility of a unique signaling complex in brain composed of PLC-coupled D1–D2 receptor heterooligomers.

In this work we report the presence of such a D1–D2 dopamine receptor signaling complex in striatum that is coupled to rapid G_q/11 signaling on activation of both receptors and which can be defined by a unique pharmacology. The complex was more readily detected in older mice and could modulate levels of calcium/calmodulin-dependent protein kinase IIα (CaMKIIα) in the nucleus accumbens, indicating a potential role for the D1–D2 heteromer in synaptic plasticity in the postadolescent brain.

Results

We examined calcium signaling through D1 and D2 dopamine receptors that were stably coexpressed in human embryonic kidney cells (D1–$D2_{HEK}$ cells). A robust dose-dependent transient rise in calcium caused by release from intracellular stores was observed after coapplication of the D1 receptor agonist SKF81297 and the D2 receptor agonist quinpirole (Fig. 1 *a* and *b*) or with application of dopamine [supporting information (SI) Fig. 6]. The rise in calcium was abolished by the selective D1 receptor antagonist SCH23390 and by the selective D2 receptor antagonist raclopride (Fig. 1*c*). In D1–$D2_{HEK}$ cells treated with SKF81297, there was a smaller rise in calcium (Fig. 1 *a* and *b*) not seen in cells expressing D1 alone (data not shown), which could also be blocked by SCH23390 or raclopride (Fig. 1*c*). The ability of raclopride to blunt the signal indicated a role for the D2 receptor in the signal generated by SKF81297 and suggested that this agonist could directly activate the D2 receptor. Because treatment of D1–$D2_{HEK}$ cells or $D2_{HEK}$ cells with quinpirole alone did not stimulate calcium release (data not shown), calcium release appeared to depend on coordinated activation of both D1 and D2 receptors.

We demonstrated that the effects of D1–D2 receptor activation occurred by G_q/11 activation of PLC, producing inositol trisphosphate (IP_3), which can act on intracellular IP_3 receptors to release calcium (Fig. 1*d*), and was independent of AC modulation (SI Fig. 7). Treatment of D1–$D2_{HEK}$ cells with the PLC inhibitor U71322 or thapsigargin, a depletor of intracellular calcium stores, eliminated the calcium signal. The signal was also eliminated by 2-aminoethoxydiphenyl borate, an antagonist of intracellular IP_3 receptors. A definitive role for G_q/11 as the initiator of this cascade was established by using the G_q/11 inhibitor YM254890 (8), which abolished rises in calcium in response to SKF81297 and quinpirole (Fig. 1*d*).

D1 receptor agonists have varying abilities to activate AC or phosphoinositide (PI) hydrolysis in brain (2, 4). Although SKF81297 is a potent activator of both AC and PI turnover, SKF83822 has been shown to activate only AC, and SKF83959 selectively triggers PI hydrolysis. To see whether there was a similar

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Abbreviations: AC, adenylyl cyclase; CaMKIIα, calcium/calmodulin-dependent protein kinase IIα; GTPγS, guanosine 5′-γ-thiotriphosphate; IP_3, inositol trisphosphate; PLC, phospholipase C; PTX, pertussis toxin.

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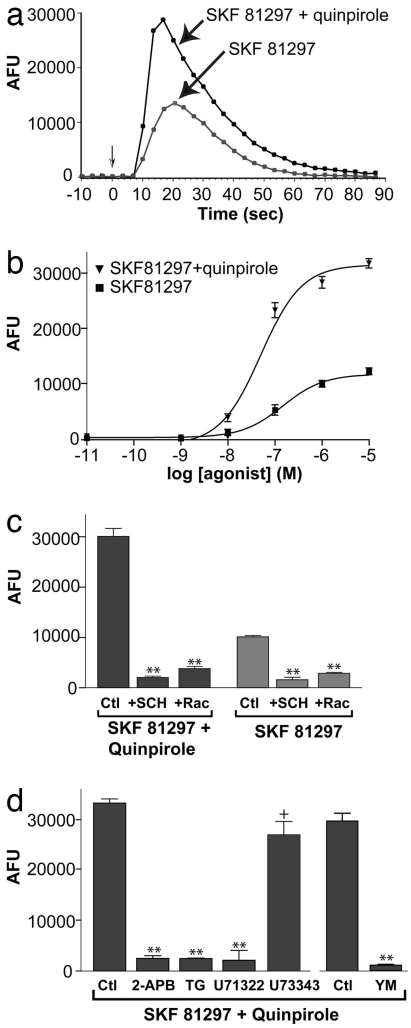

Fig. 1. Calcium signaling through stably expressed D1 and D2 dopamine receptors is caused by G_q/11-mediated activation of PLC. (*a*) Changes in fluorescence corresponding to changes in intracellular calcium levels on treatment of D1–$D2_{HEK}$ cells with SKF81297 (1 μM) or SKF81297 and quinpirole (1 μM each). The time of agonist addition is indicated with open arrow. AFU, arbitrary fluorescence units. (*b*) Dose–response curves of peak calcium levels in response to agonist ($EC_{50(SKF81297+quinpirole)}$, 50.8 ± 8.8 nM, $n = 8$; $EC_{50(SKF81297)}$, 147.6 ± 46.9 nM, $n = 8$). (*c*) Treatment of cells with 10 μM SCH23390 (SCH) or raclopride (Rac) abolished agonist (1 μM)-mediated rises in calcium ($n = 5$). (*d*) Rises in calcium in response to SKF81297 and quinpirole (1 μM each) were eliminated by the IP_3 receptor blocker 2-aminoethoxydiphenyl borate (2-APB; 100 μM) as well as by depletion of intracellular calcium stores with thapsigargin (TG; 1 μM) or inhibition of PLC with U71322 (50 μM) ($n = 6$ for all). The inactive isomer of U71322, U73343, did not abolish the calcium signal, although the effect of SKF81297 and quinpirole was reduced by 18.9 ± 7.9% ($n = 4$). The G_q/11 inhibitor YM-254890 (YM; 100 nM) blocked increases in calcium in D1–$D2_{HEK}$ cells in response to SKF81297 and quinpirole ($n = 5$). Background levels of fluorescence were qualitatively determined from individual fluorescence profiles but were generally considered to be below 3,500 AFU. +, $P < 0.05$; **, $P < 0.0001$; Student's *t* test compared with corresponding control.

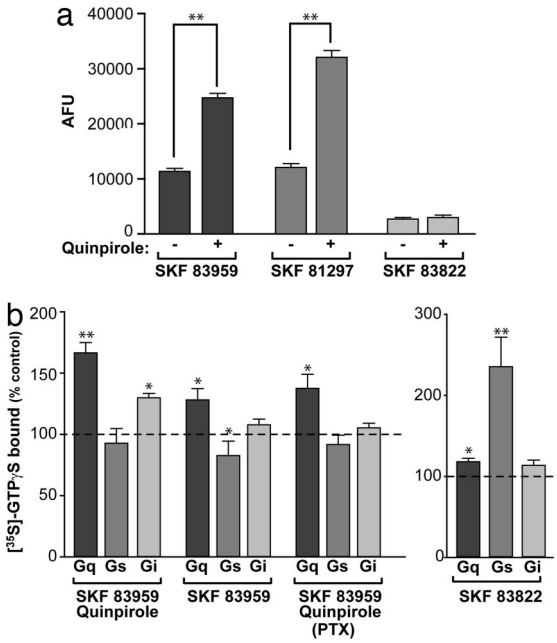

Fig. 2. Specificity of D1 receptor agonists for the D1–D2 calcium signal. (*a*) SKF83959 (1 μM) or SKF81297 (1 μM) stimulated calcium in D1–$D2_{HEK}$ cells ($n = 6$), which was increased by 2.18 ± 0.071-fold and 2.66 ± 0.038-fold, respectively, by the coaddition of 1 μM quinpirole ($n = 8$). SKF83822 (1 μM) application did not stimulate any increases in intracellular calcium ($n = 4$), and coapplication of quinpirole had no effect ($n = 4$). AFU, arbitrary fluorescence units. (*b*) Quantification of ^{35}S-labeled guanosine 5′-γ-thiotriphosphate ([^{35}S]GTPγS) incorporation into immunoprecipitated G proteins demonstrates activation of G_q/11 (67.2 ± 8.4%) and G_{i3} (20.0 ± 3.3%) but not G_s (−6.9 ± 12.1%) in response to SKF83959 and quinpirole (10 μM each) ($n = 4$). SKF83959 alone (10 μM) increased incorporation into G_q/11 (28.6 ± 9.3%), did not significantly affect G_i (8.2 ± 4.6%), and incorporation into G_s was slightly decreased (−17.0 ± 11.0%) ($n = 4$). Treatment of cells with pertussis toxin (PTX) abolished G_{i3} activation by SKF83959 and quinpirole treatment but only slightly affected G_q/11 activation (38.1 ± 11.6%) ($n = 3$). SKF83822 application resulted in robust activation of G_s (140.0 ± 37.0%), modest activation of G_q/11 (15.2 ± 4.3%), and no significant activation of G_{i3} (12.9 ± 6.3%) ($n = 4$). The dashed line represents basal levels of [^{35}S]GTPγS incorporation in the absence of agonist. *, $P < 0.05$; **, $P < 0.005$; Student's *t* test (for *b*, compared with [^{35}S]GTPγS incorporation in the absence of agonist).

agonist selectivity profile for these drugs on the D1–D2 calcium signal, we first confirmed that only SKF81297 and SKF83822 could activate AC through D1 receptors in a dose-dependent manner, whereas SKF83959 could not (SI Fig. 8). The SKF compounds were then compared for their ability to trigger intracellular calcium release in D1–$D2_{HEK}$ cells (Fig. 2*a*). In response to SKF83959 or SKF81297, a rise in calcium was observed that was significantly increased by coapplication of quinpirole. By comparison, SKF83822 did not stimulate any rises in intracellular calcium, with or without coapplication of quinpirole.

The differential ability of SKF83822 and SKF83959 to modulate intracellular levels of cAMP and calcium indicates differences in their ability to activate G_s-coupled D1 receptors and G_q/11-coupled D1–D2 receptor complexes. To test this theory, we treated membranes from D1–$D2_{HEK}$ cells with agonists in the presence of [^{35}S]GTPγS and quantified the incorporation of ^{35}S into immunoprecipitated Gα proteins as a measure of their activation. In response to SKF83959 and quinpirole, [^{35}S]GTPγS incorporation into G_q/11 and G_i was increased over basal levels, whereas there was no change in incorporation into G_s (Fig. 2*b*). Preincubation of membranes with PTX eliminated incorporation into G_i and did not significantly affect incorporation into G_q/11 when compared with that in the absence of PTX, indicating that the effects of quinpirole on calcium release were mediated through potentiation of G_q/11 activation and were independent of G_i/o activation. Treatment with SKF83959 alone increased [^{35}S]GTPγS incorporation into G_q/11 but to a lesser extent than with quinpirole coapplication. Neither G_s nor G_i was activated by SKF83959 alone.

In contrast to SKF83959, SKF83822 minimally activated G_q/11, and it did not affect G_i but robustly activated G_s (Fig. 2*b*).

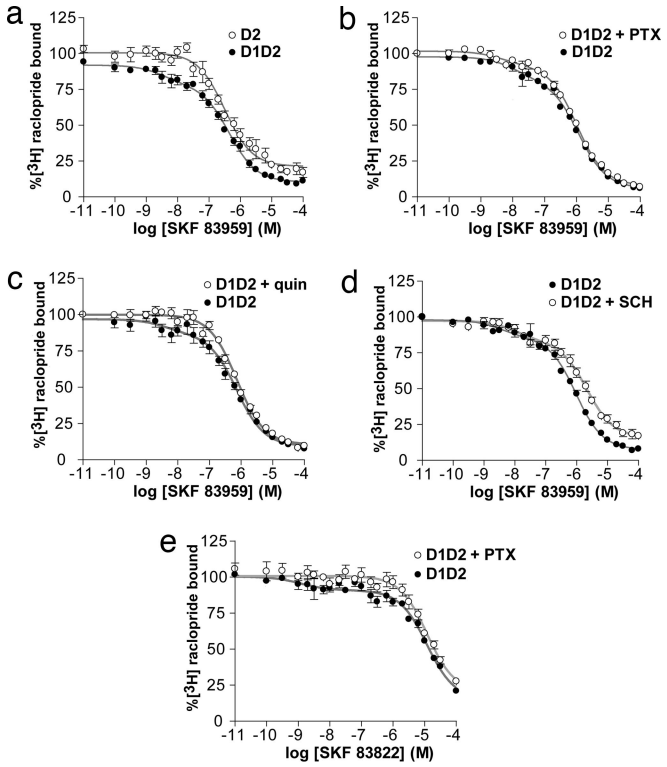

Fig. 3. SKF83959 binds with high affinity to PTX-resistant D2 receptors only in the presence of D1 receptors. Competition of [^{3}H]raclopride binding by SKF83959 or SKF83822 is shown. Data from three to eight independent experiments conducted in duplicate were normalized and fit to one-site or two-site analysis. (*a*) Comparison of binding on membranes from $D2_{HEK}$ cells and D1–$D2_{HEK}$ cells reveals a high-affinity binding site for SKF83959 only in D1–D2 cells (K_H, 2.4 ± 0.8 nM; %K_H, 19 ± 1.5). (*b*) High-affinity binding of SKF83959 to D2 receptors in D1–$D2_{HEK}$ cells was only slightly affected by pretreatment with PTX (K_H, 1.9 ± 1.3 nM; %K_H, 11 ± 3.3). (*c*) Incubation of D1–$D2_{HEK}$ membranes with quinpirole (10 nM) eliminated high-affinity binding of SKF83959. (*d*) Incubation of D1–$D2_{HEK}$ membranes with SCH23390 (10 nM) did not reduce affect high-affinity binding of SKF83959. (*e*) Competition binding of [^{3}H]raclopride by SKF83822 indicated high-affinity binding of the agonist to D2 receptors in D1–$D2_{HEK}$ cells that was eliminated by pretreatment of cells with PTX.

Coapplication of quinpirole with SKF83822 did not affect activation of G_q/11 or G_s (data not shown).

The generation of a raclopride-sensitive calcium signal with SKF81297 or SKF83959 treatment of D1–$D2_{HEK}$ cells suggests that these two drugs can act as agonists for D2 receptors in a manner that depends on the presence and possibly activation of the D1 receptor. To test this hypothesis, the ability of the SKF agonists to displace [^{3}H]raclopride binding to the D2 receptor competitively was examined in D1–$D2_{HEK}$ cells and $D2_{HEK}$ cells (Fig. 3 and Table 1). Competition binding profiles of SKF83959 on [^{3}H]raclopride binding in D1–$D2_{HEK}$ cells revealed a high-affinity binding site for SKF83959 on the D2 receptor that was not observed in $D2_{HEK}$ cells (Fig. 3*a*). Pretreatment of D1–$D2_{HEK}$ cells with PTX modestly reduced but did not eliminate the proportion of high-affinity binding sites for SKF83959 (Fig. 3*b*), demonstrating that the majority of SKF83959 binding was to PTX-resistant and not G_i/o-coupled D2 receptors. This PTX-resistant site overlapped with or was the same as the binding site for quinpirole because incubation of membranes with quinpirole competitively displaced SKF83959 binding from the high-affinity site (Fig. 3*c*) ($n_H \approx 1$) (Table 1). Incubation of membranes with SCH23390 did not affect high-affinity binding of SKF83959 to the D2 receptor (Fig. 3*d*), indicating that the site was distinct from the D1 receptor and that it was present in the D2 receptor basal state in the absence of ligand occupancy or activation of the D1 receptor.

Competition by SKF81297 of [^{3}H]raclopride binding similarly revealed a high-affinity PTX-resistant binding site on the D2 receptor in D1–$D2_{HEK}$ cells (data not shown). However, PTX-sensitive binding of SKF81297 to the D2 receptor in $D2_{HEK}$ cells was also observed. For SKF83822, a proportion of [^{3}H]raclopride binding could be displaced by agonist in both $D2_{HEK}$ cells and D1–$D2_{HEK}$ cells that could be abolished by PTX (Fig. 3*e*), reflecting high-affinity binding to G_i/o-coupled D2 receptors in both cell lines.

The competition binding results reveal a distinct pharmacology of the D2 receptor in D1–$D2_{HEK}$ cells such that the D1 receptor agonists SKF81297 and SKF83959 but not SKF83822 can act as ligands for a PTX-resistant D2 receptor when it is coexpressed with the D1 receptor. Taken together with the calcium-signaling data, binding of the D1 receptor agonists to the D2 receptor site indicates partial agonism of D2 receptors within the G_q/11-coupled D1–D2 receptor complex, therefore allowing a single agonist to activate both members of the heteromer. In accordance with this concept, the addition of quinpirole along with SKF81297 or SKF83959 would result in full agonism at the PTX-resistant high-affinity state of the D2 receptor within the complex and a greater calcium signal.

To determine whether G_q/11-coupled D1–D2 receptor signaling complexes exist in the brain, [^{35}S]GTPγS incorporation into G_q/11 from murine striatal membranes was quantified after membranes had been treated with SKF83959 alone or with equivalent concentrations of quinpirole. Initial experiments used striata from 12-week-old male mice, but G_q/11 activation in response to agonists was not reliably observed. A consistent agonist-dependent increase in [^{35}S]GTPγS incorporation into G_q/11 could be elicited, however, when older animals (≥8 months old) were used (Fig. 4*a*). Treatment with SKF83959 and quinpirole gave significant increases in

Table 1. Competition binding studies with [^{3}H]raclopride

Row	Cell line	Agonist	Treatment	n_H	K_H, nM	K_L, nM	K_i, nM	R_H, %	*n*
a	D1–$D2_{HEK}$	SKF83959	—[†]	−0.68 ± 0.02	2.38 ± 0.80	319 ± 37		19.1 ± 1.5	8
b	$D2_{HEK}$	SKF83959	—	−0.90 ± 0.10*	NA[‡]	NA	346 ± 31	NA	3
c	D1–$D2_{HEK}$	SKF83959	PTX	−0.79 ± 0.06	1.90 ± 1.3*	351 ± 27		11 ± 3.3*	4
d	D1–$D2_{HEK}$	SKF83959	Quinpirole	−0.94 ± 0.08*	NA	NA	246 ± 12	NA	3
e	D1–$D2_{HEK}$	SKF83959	SCH23390	−0.65 ± 0.05	3.49 ± 0.7*	712 ± 38		16.2 ± 1.3*	3
f	D1–$D2_{HEK}$	SKF83822	—	−0.59 ± 0.09	0.39 ± 0.03	3,927 ± 980		11 ± 3.2	3
g	D1–$D2_{HEK}$	SKF83822	PTX	−0.93 ± 0.08**	NA	NA	3,974 ± 609	NA	3

[^{3}H]Raclopride binding to membranes from D2 or D1–D2 cells in the presence of increasing concentrations of SKF83959 or SKF83822 is shown. Data from three to eight independent experiments were analyzed and pooled (n_H, Hill coefficient; K_H, high-affinity dissociation constant; K_L, low-affinity dissociation constant; R_H, percentage of receptors in high-affinity state). In rows b, d, and f, where Hill coefficients were 1.0, binding data were analyzed to fit to a single site, and the K_i was calculated. *, $P < 0.05$ for n_H, K_H, and R_H values compared with those values in row a; **, $P < 0.05$ compared with n_H in row f.

[†]—, not done.

[‡]NA, not applicable.

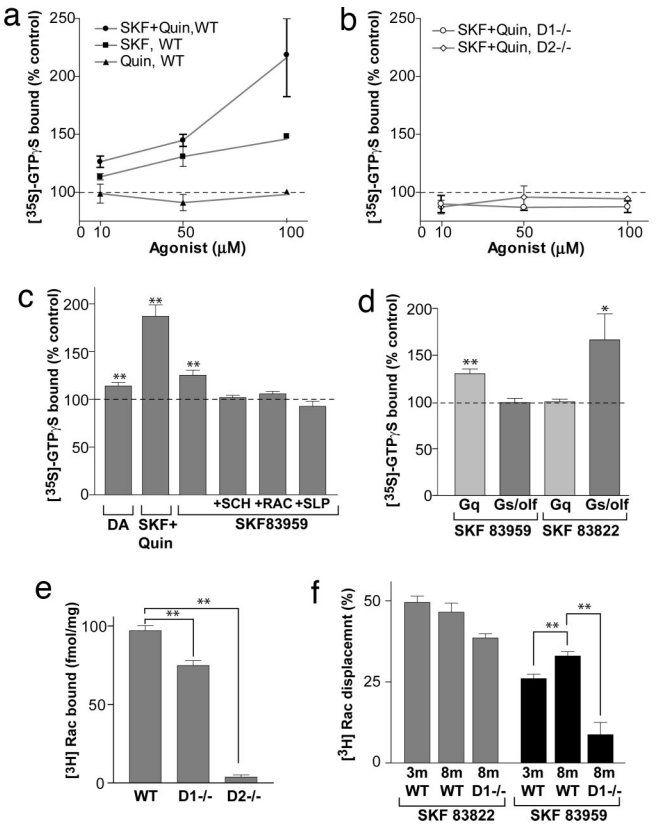

Fig. 4. Coactivation of striatal D1 and D2 dopamine receptors activates G_q/11. Agonist-dependent [^{35}S]GTPγS incorporation into G proteins is shown. (*a*) A dose-dependent increase in activation of G_q/11 was observed after membranes from wild-type (WT) mouse striatum (D1+/+) were treated for 1 min with agonists. SKF83959 stimulated relative increases in [^{35}S]GTPγS incorporation into G_q/11 of 13.3 ± 2.7% for 10 μM, 30.8 ± 8.5% for 50 μM, and 45.9 ± 1.4% for 100 μM agonist (n = 6 for all). Cotreatment with SKF83959 (SKF) and quinpirole [(Quin) 10, 50, and 100 μM each] resulted in increases of 26.3 ± 4.9%, 44.7 ± 5.1%, and 116.2 ± 34.0% over baseline, respectively (n = 6). Quinpirole alone did not stimulate activation of G_q/11 (n = 6). (*b*) No activation of G_q/11 was observed in membranes from D1 or D2 mutant mice (D1−/−; D2−/−) (n = 5, n = 2). Basal level of [^{35}S]GTPγS incorporation is indicated with the dashed line. (*c*) Comparison of G_q/11 activation by dopamine, SKF83959, and quinpirole or SKF83959 alone (100 μM agonist for each). Activation was prevented by pretreatment of membranes with SCH23390 (SCH), raclopride (RAC), or sulpiride (SLP) (n = 6 for each). (*d*) SKF83959 activated G_q/11 but not G_s/olf, in contrast to SKF83822, which activated G_s/olf but not G_q/11 (n = 4 for each). (*e*) Binding of 1 nM [^{3}H]raclopride in striatal membranes from WT mice (97.1 ± 3.3 fmol/mg) was reduced by 23% in D1−/− mice (74.9 ± 3.1 fmol/mg) and was completely absent in D2−/− mice. (*f*) SKF83822 displaced [^{3}H]raclopride (1 nM) binding to similar degrees in 3-month and 8-month-old mice (49.5 ± 1.9% and 46.5 ± 2.8%) (n = 7), and displacement in 8-month-old D1−/− mice (38.5 ± 1.4%) was not statistically different from that in 8-month-old wild type (n = 3). In contrast, there was a 27% increase in displacement of [^{3}H]raclopride binding by SKF83959 in 8-month-old mice (32.9 ± 1.4%) in contrast to 3-month-old mice (26.0 ± 1.3%) (n = 6), and displacement was almost completely eliminated in D1−/− mice (4.87 ± 3.7%) (n = 3). *, $P < 0.05$; **, $P < 0.005$; Student's t test compared with normalized baseline values.

[^{35}S]GTPγS incorporation into G_q/11 over baseline that were greater than with SKF83959 alone. Quinpirole alone did not stimulate activation of G_q/11 at any of the doses tested. Also, it was confirmed that it was the striatal D1 and D2 receptor subtypes that formed this signaling complex because we could not elicit G_q/11 activation with agonist treatments of membranes from mice that lacked functional D1 (D1−/−) or D2 receptors (D2−/−) (Fig. 4*b*). The involvement of the D2 subtype was further confirmed by the absence of specific [^{3}H]raclopride binding in striatal membranes from D2−/− mice (Fig. 4*e*).

For both SKF83959 and SKF83959 plus quinpirole treatments, activation of G_q/11 was prevented by blockade of either D1 or D2 receptors with SCH23390 or raclopride, respectively (Fig. 4*c*). The D2 antagonist sulpiride also blocked activation of G_q/11. These results indicate that in the striatum, activation of both D1 and D2 receptors are necessary for rapid G_q/11 activation and that activation of the D1 receptor alone is not sufficient.

The agonist specificity of the striatal D1–D2 receptor signaling complex was similar to that established in the D1–D2 stable cell line in that SKF83959 could activate G_q/11 signaling through the complex and did not have any effect on G_s/olf activation, whereas SKF83822 robustly activated G_s/olf (Fig. 4*d*). Notably, activation of G_q/11 by SKF83822 in striatum could not be observed, suggesting the small degree of G_q/11 activation by this compound in D1–$D2_{HEK}$ cells is not reflected *in vivo*.

Competition binding experiments of [^{3}H]raclopride and SKF83959 or SKF83822 on murine striatal membranes revealed high-affinity binding of both D1 receptor agonists to D2 receptors, and saturation binding isotherms from striata of 3- and 8-month-old male mice gave similar B_{max} (≈220 fmol/mg) and K_d values (≈1 nM) for [^{3}H]raclopride. The ability of a single dose of SKF83822 or SKF83959 to displace [^{3}H]raclopride (1 nM) was then quantified (Fig. 4*f*), as described in ref. 9. SKF83822 (10 nM) displaced [^{3}H]raclopride to similar degrees in 3- and 8-month-old mice as well as in 8-month-old D1−/− animals. In contrast, there was a 27% increase in displacement of [^{3}H]raclopride binding by 10 nM SKF83959 in 8-month-old mice compared with 3-month-old mice, and displacement was almost completely eliminated in D1−/− mice. Therefore, high-affinity binding of SKF83959 to the D2 receptor in brain depends on the presence of the D1 receptor, unlike binding of SKF83822, and is greater in older animals.

To identify functional consequences of calcium signaling by the D1–D2 receptor complex, the effect of D1–D2 heterooligomer activation on CaMKIIα was examined. CaMKIIα plays a fundamental role in synaptic plasticity, and both its translation and activity can be regulated by increases in intracellular calcium (10, 11), typically subsequent to NMDA receptor activation. Immunohistochemical labeling for CaMKIIα was performed after i.p. dopamine receptor agonist administration to animals (Fig. 5 *a–h*) and quantified (Fig. 5 *i* and *j*) as described. For both total and activated CaMKIIα, a large increase in both the intensity and number of immunolabeled neurons in the nucleus accumbens of adult male rats was observed within 10 min of SKF83959 and quinpirole coadministration (Fig. 5*a*). There was no change in the number of CaMKIIα-positive neurons in response to SKF83959 or quinpirole individually, although there was a moderate increase in the intensity of labeling per cell for either drug (Fig. 5 *b* and *c*). The agonist-mediated increases in CaMKIIα could be blocked by pretreating animals with either SCH23390 or raclopride (Fig. 5 *e* and *f*) indicating the necessity for both D1 and D2 receptors. Furthermore, the agonist-mediated increase was also detected in wild-type mice but was absent in both D1−/− and D2−/− mice (SI Fig. 9). Significantly, there was no increase in CaMKIIα in animals treated with SKF83822 or SKF83822 and quinpirole (Fig. 5*g*), indicating that the effect of dopamine agonist on CaMKIIα was specific to activation of G_q/11-coupled receptor complexes. Pretreatment of animals with the NMDA receptor antagonist MK-801 did not affect the SKF83959- and quinpirole-mediated increase in the number of CaMKIIα-positive neurons, although the increase in the intensity of immunolabel per cell was slightly lower (Fig. 5*h*). Overall, these results point to a role for D1–D2 receptor complexes in direct modulation of CaMKIIα levels through activation of G_q/11 and release of intracellular calcium.

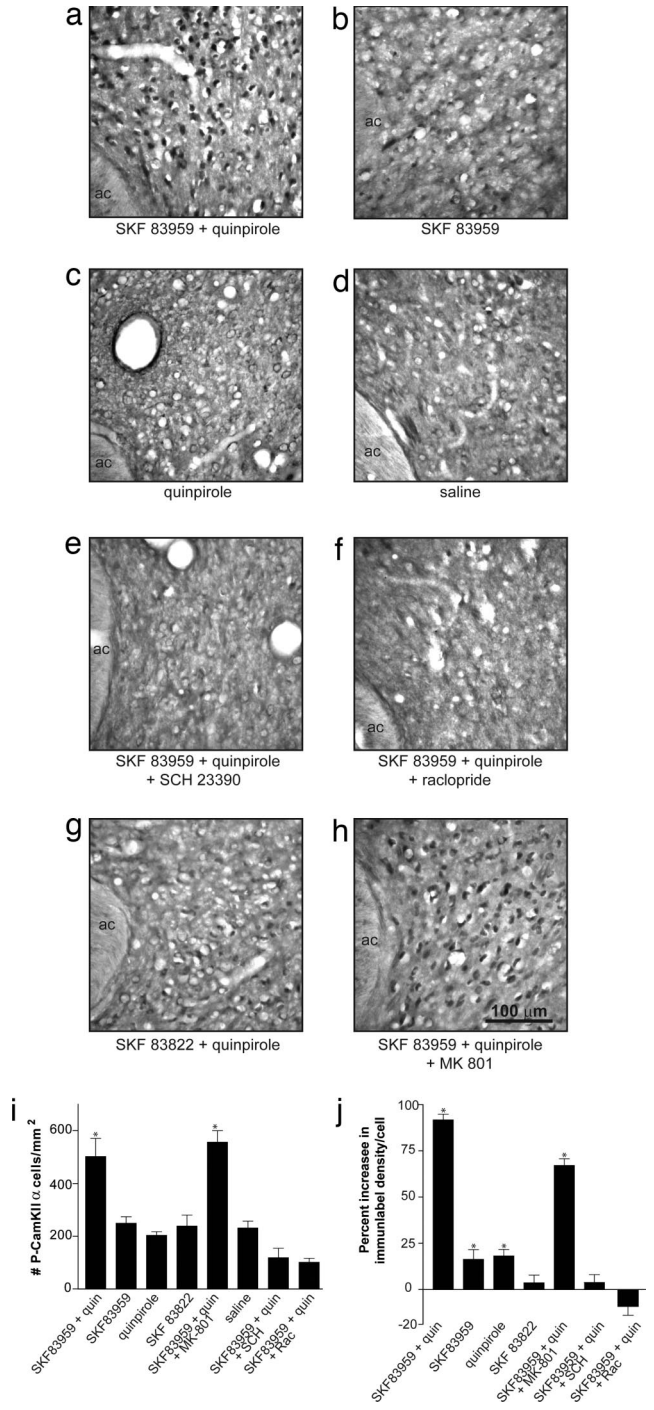

Fig. 5. Activation of G_q/11-coupled D1–D2 dopamine receptor complexes increases CaMKIIα levels in the nucleus accumbens. (*a*) Injection of animals with SKF83959 and quinpirole increased the number of CaMKIIα-immunolabeled cells and the intensity of labeling per cell compared with saline-injected controls (*d*). (*b*) SKF83959 caused a small increase in immunolabel intensity but no change in the number of CaMKIIα-positive neurons. (*c*) Quinpirole gave results similar to those in *b*. (*e* and *f*) Pretreatment with SCH23390 or raclopride prevented the SKF83959- and quinpirole-mediated increase in CaMKIIα. (*g*) Injections of SKF83822 and quinpirole gave no net changes in CaMKIIα. (*h*) The NMDA receptor antagonist MK-801 slightly reduced the immunolabel intensity of the SKF83959/quinpirole-mediated increase in CaMKIIα but did not affect the increase in the number of CaMKIIα-positive neurons. (*i* and *j*) Quantification of data from four independent experiments. Within each experiment, treatments were performed in duplicate, and two or three slices were analyzed from each animal. *, $P < 0.005$; Student's *t* test compared with saline controls. ac, anterior commissure.

Discussion

In this work we have identified a heteromeric signaling complex in brain composed of D1 and D2 dopamine receptor subtypes which rapidly activates G_q/11 on agonist binding to both receptors within the complex. The receptor complex possesses a unique pharmacology such that a specific subset of D1 receptor agonists, SKF81297 and SKF83959, can activate the heteromer by acting concurrently on both the D1 receptor and a distinct conformation of the D2 receptor that depends on the presence of the D1 receptor. Because SKF83959 does not activate AC-coupled D1 or D2 receptors or G_q/11 through D1 receptor homomeric units, we propose that this D1-like receptor agonist is in fact a specific agonist for G_q/11-coupled D1–D2 receptor heterooligomers. We also present evidence indicating that the D1–D2 receptor complex is more prevalent in murine striatum at 8 months of age and that it can increase levels of total and activated CaMKIIα in the nucleus accumbens, providing a distinct mechanism of dopaminergic modulation of neuronal function in later adulthood.

Coimmunoprecipitation of D1 and D2 dopamine receptors from rodent striata had provided direct evidence that these receptors could oligomerize *in vivo* (6). We now show that both the D1 and D2 receptors within the striatal G_q/11-coupled signaling unit possess a distinct pharmacology and rank order of the agonists that can activate the complex, consistent with the creation of unique ligand-binding pockets and G protein coupling resulting from receptor heterooligomerization. Specifically, although each of the D1 agonists tested has equivalent ability to bind with high affinity to the D1 receptor, only SKF81297 or SKF83959, and not SKF83822, could activate G_q/11 through D1 receptors in conjunction with D2 receptor activation by quinpirole. In the absence of quinpirole, SKF81297 or SKF83959 could activate the complex by acting as full agonists for the D1 receptor and partial agonists for the D2 receptor. This unique D2 receptor pharmacology was induced by the presence of D1 receptors and was independent of D1 receptor activation. Strikingly, the ability of the agonists SKF81297 and SKF83959 but not SKF83822 to activate D1–D2 heterooligomers correlates with their specificity in stimulating PI turnover in brain (2, 4).

The apparent increase in G_q/11-coupled D1–D2 receptor complexes in striata from older animals was unexpected because studies have shown that after 60 days of age, the density of binding sites for D1 and D2 receptors in the nucleus accumbens and striatum of male rats either decreases or does not change significantly (12, 13). Because our data indicated no difference in the total density of D2 receptors in the striata of 3-month-old and 8-month-old mice, there appears to be a shift in the proportion of D2 receptors associated with D1 receptors with increasing age. Because most studies of dopamine receptor function in the brain use rodents that are 3–4 months of age, these results may explain the limited reports of putative functions of G_q/11-coupled dopamine receptors in brain. Furthermore, potential changes in the relative proportion of dopamine-activated G_s/olf, G_i/o, and G_q/11 signaling pathways in different brain regions could have important implications for our understanding of the age-related regulation of dopamine function in brain.

Calcium signaling has profound effects on almost all aspects of neuronal function, notably regulation of intercellular communication and neuronal plasticity (14, 15). The possibility that G_q/11-coupled dopamine receptors can modulate synaptic plasticity by activating CaMKIIα has been suggested previously (1, 16). Our results showing that G_q/11-coupled D1–D2 receptor complexes can increase CaMKIIα in the nucleus accumbens provide the molecular basis for a direct link among dopamine action, calcium signaling, and CaMKIIα activation. Furthermore, these data may provide an explanation for reports showing that in the nucleus accumbens both coactivation of D1 and D2 receptors as well as activation of

CaMKIIα are necessary for the induction of behavioral sensitization to psychostimulants such as cocaine (17, 18).

In summary, in brain there is a G_q/11-coupled signaling unit composed of D1 and D2 dopamine receptors that can be identified by its unique pharmacology and that requires concurrent activation of both receptors for signaling. The formation of a distinct dopaminergic signaling unit by two receptors that signal through separate pathways when homooligomeric is significant in that it provides a greater repertoire of signaling pathways by which dopamine can modulate neuronal function than would be possible by each of the five different dopamine receptor subtypes acting solely as separate units. Characterization of changes in this signaling unit with age and the functional consequences of signaling through the complex will increase our understanding of how D1–D2 heteromers contribute to neuronal function as well as the role that this pathway may play in the etiology or pathophysiology of disorders in which altered dopamine signaling is implicated, such as schizophrenia. Notably, a diminished link between D1 and D2 dopamine receptors has been noted in the brains of schizophrenic patients (19), and it has been proposed that disruption in calcium homeostasis is the central factor underlying the molecular pathology of schizophrenia (20). Our data provide a mechanism by which to converge these lines of evidence and significant impetus to determine whether the D1–D2 receptor signaling complex is altered in neuropsychiatric disease.

Materials and Methods

Measurement of Intracellular Calcium Levels. Calcium mobilization assays on stable HEK 293 cell lines expressing human D1 and/or D2 receptors ($\approx$1 pmol/mg protein $\pm$ 0.2 pmol) were performed as described previously (6) with modifications. Cells were seeded at 1.2×10^5 cells per well in 96-well plates and maintained in advanced minimum essential medium (Invitrogen, Carlsbad, CA). All experiments were performed in the presence of EGTA. For responses to SKF83822, a portion of the signal was identified as nondopaminergic because it could not be eliminated by incubation of cells with SCH23390 or raclopride. Dopamine receptor-specific responses were obtained by subtracting the antagonist-resistant signal. For antagonist and inhibition studies, cells were incubated at 37°C in saline plus the appropriate compound for 20 min before the assay. For PTX treatments, cells were incubated before dye loading for 18–24 h in PTX (0.25 μg/ml) diluted in growth medium.

GTPγS Assay. Agonist-mediated [^{35}S]GTPγS incorporation into specific G proteins was assessed as described (21). GDP (final concentration, 1 μM) was added to 100-μg membranes from HEK cells or striata of wild-type, D1 mutant mice (22), or D2 mutant mice (23), and the assay mixture was incubated on ice for 10 min. The mixture was moved to 30°C and then incubated for 5 min before adding [^{35}S]GTPγS (1,250 Ci/mmol) (PerkinElmer, Wellesley, MA) to a final concentration of 2.5 nM. Agonist was added 30 sec after the addition of the radioisotope, and the reaction was allowed to proceed for 1 or 3 min (for G_q/11 and G_s activation). Membranes were collected and solubilized, and 5 μg of anti-Gα antibody was added (G_q/11, G_s, G_s/olf, G_{i3}; Santa Cruz Biotechnology, Santa Cruz, CA). Specificity of the antibodies has been described (21). Immunoprecipitation occurred overnight at 4°C. Protein G–agarose was added, and the reaction was left for an additional 90 min at 4°C. The agarose was washed five times with solubilization buffer, and incorporation of [^{35}S]GTPγS was measured by liquid scintillation spectrometry.

Radioligand-Binding Assays. Binding experiments were performed on 20-μg membrane extracts with 1–2 nM [^{3}H]raclopride or 1 nM [^{3}H]SCH23390 in the presence of agonist as described (8). Data points were analyzed by nonlinear least-squares regression (Prism 3.0 software; GraphPad, San Diego, CA).

Immunohistochemistry. Adult male Sprague–Dawley rats were injected i.p. with saline or agonists SKF83959 (3 mg/kg) or SKF83822 (3 mg/kg), either alone or with quinpirole (2 mg/kg). Stock solutions of drugs were diluted so that the DMSO concentration did not exceed 5%, and the total volume of injection was 500 μl. For antagonist experiments, SCH23390 (1 mg/kg), raclopride (2 mg/kg), MK-801 (1 mg/kg), or saline was injected 10 min before agonist administration. Animals were anesthetized 10 min after injection and perfused intracardially with 4% paraformaldehyde, and 16-μm cryostat sections were prepared as described (7) for immunostaining by using the Elite ABC kit (Vector Laboratories, Burlingame, CA) as indicated by the manufacturer. Primary antibodies for total and activated (i.e., Thr^{286}-phosphorylated) CaMKIIα (rabbit anti-CaMKIIα and rabbit anti-Thr^{286}–CaMKIIα; Santa Cruz Biotechnology) were used at 1:200. Images were obtained by using an Axioplan2 microscope (Carl Zeiss, Thornwood, NY) and quantified by using ImageJ software (National Institutes of Health, Bethesda, MD).

Statistical Analysis. All values are reported as mean $\pm$ SEM. Comparisons of means were performed by using Student's *t* test (two-tailed, unpaired).

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1. Neve KA, Seamans JK, Trantham-Davidson H (2004) *J Rec Sig Trans Res* 24:165–205.
2. Jin LQ, Goswami S, Cai G, Zhen X, Friedman E (2003) *J Neurochem* 85:378–386.
3. Tang TS, Bezprozvanny I (2004) *J Biol Chem* 279:42082–42094.
4. Undie AS, Weinstock J, Sarau HM, Friedman E (1994) *J Neurochem* 62:2045–2048.
5. Wang HY, Undie AS, Friedman E (1995) *Mol Pharmacol* 48:988–994.
6. Lee SP, So CH, Rashid AJ, Varghese G, Cheng R, Lanca AJ, O'Dowd BF, George SR (2004) *J Biol Chem* 279:35671–35678.
7. So CH, Varghese G, Curley KJ, Kong MM, Alijaniaram M, Ji X, Nguyen T, O'Dowd BF, George SR (2005) *Mol Pharmacol* 68:568–578.
8. Takasaki J, Saito T, Taniguchi M, Kawasaki T, Moritani Y, Hayashi K, Kobori M (2004) *J Biol Chem* 279:47438–47445.
9. Torvinen M, Marcellino D, Canals M, Agnati LF, Lluis C, Franco R, Fuxe K (2005) *Mol Pharmacol* 67:400–407.
10. Lisman J, Schulman H, Cline H (2002) *Nat Rev Neurosci* 3:175–190.
11. Scheetz AJ, Nairn AC, Constantine-Paton M (2000) *Nat Neurosci* 3:211–216.
12. Suzuki M, Hatano K, Sakiyama Y, Kawasumi Y, Kato T, Ito K (2001) *Synapse* 41:285–293.
13. Teicher MH, Andersen SL, Hostetter JC, Jr (1995) *Dev Brain Res* 89:167–172.
14. Berridge MJ (1998) *Neuron* 21:13–26.
15. Verkhratsky A (2005) *Physiol Rev* 85:201–279.
16. Zhen X, Goswami S, Abdali SA, Gil M, Bakshi K, Friedman E (2004) *Mol Pharmacol* 66:1500–1507.
17. Capper-Loup C, Canales JJ, Kadaba N, Graybiel AM (2002) *J Neurosci* 22:6218–6227.
18. Pierce RC, Quick EA, Reeder DC, Morgan ZR, Kalivas PW (1998) *J Pharmacol Exp Ther* 286:1171–1176.
19. Seeman P, Niznik HB, Guan HC, Booth G, Ulpian C (1989) *Proc Natl Acad Sci USA* 86:10156–10160.
20. Lidow MS (2003) *Brain Res Rev* 43:70–84.
21. Dowling MR, Nahorski SA, Challis RAJ (2004) in *Receptor Signal Transduction Protocols*, eds Willars GB, Challis RAJ (Humana, Totowa, NJ), pp 197–206.
22. Drago J, Gerfen CR, Lachowicz JE, Steiner H, Hollon TR, Love PE, Ooi GT, Grinberg A, Lee EJ, Huang SP, *et al.* (1994) *Proc Natl Acad Sci USA* 91:2564–2568.
23. Kippin TE, Kapur S, van der Kooy D (2005) *J Neurosci* 25:5815–5823.