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Adenosine A_{2A} Receptor Signaling and G_{olf} Assembly Show a Specific Requirement for the γ_7 Subtype in the Striatum*[S]

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The adenosine A_{2A} receptor ($A_{2A}R$) is increasingly recognized as a novel therapeutic target in Parkinson disease. In striatopallidal neurons, the G-protein α_{olf} subtype is required to couple this receptor to adenylyl cyclase activation. It is now well established that the $\beta\gamma$ dimer also performs an active role in this signal transduction process. In principal, sixty distinct $\beta\gamma$ dimers could arise from combinatorial association of the five known β and 12 γ subunit genes. However, key questions regarding which $\beta\gamma$ subunit combinations exist and whether they perform specific signaling roles in the context of the organism remain to be answered. To explore these questions, we used a gene targeting approach to specifically ablate the G-protein γ_7 subtype. Revealing a potentially new signaling paradigm, we show that the level of the γ_7 protein controls the hierarchial assembly of a specific G-protein $\alpha_{olf}\beta_2\gamma_7$ heterotrimer in the striatum. Providing a probable basis for the selectivity of receptor signaling, we further demonstrate that loss of this specific G-protein heterotrimer leads to reduced $A_{2A}R$ activation of adenylyl cyclase. Finally, substantiating an important role for this signaling pathway in pyschostimulant responsiveness, we show that mice lacking the G-protein γ_7 subtype exhibit an attenuated behavioral response to caffeine. Collectively, these results further support the $A_{2A}R$ G-protein $\alpha_{olf}\beta_2\gamma_7$ interface as a possible therapeutic target for Parkinson disease.

G-protein-coupled receptors represent the single largest family of target proteins for drug development. Their actions require the participation of heterotrimeric guanine nucleotide binding proteins (G-proteins) whose roles in these diverse signaling pathways may be determined by their specific $\alpha\beta\gamma$ subunit combinations. The existence of 16 α, 5 β, and 12 γ subtypes creates the potential to generate a large number of distinct G-protein $\alpha\beta\gamma$ heterotrimers (1, 2). Although their biochemical properties have been well studied (3, 4), key questions regarding which G-protein $\alpha\beta\gamma$ heterotrimers actually exist *in vivo* and determining whether they perform specific signaling roles and biological functions remain to be answered. To address these questions, a gene-targeting approach has been used to delete the various α subunit genes in mice, leading to the identification of physiological functions for most of them (5). By contrast, little attention has focused on the β and γ subunit genes. In particular, several features of the γ subunit genes suggest they may perform heterogeneous functions *in vivo*. Analogous to their α partners, the various γ subtypes show substantial structural diversity and exhibit pleiotropic patterns of expression (2). Accordingly, we have undertaken a gene targeting approach to systematically ablate the individual γ subtypes in mice (6, 7), with the ultimate goal of elucidating their biological functions.

Our recent work has demonstrated that knock-out of *Gng7*, encoding the γ_7 subtype, produces a behavioral phenotype resulting in part from a localized defect in dopamine D_1 receptor (D_1R)[2] signaling in the brain (6). Within the brain, the striatum collects and processes information from the cerebral cortex and thalamus affecting the control of voluntary movements (8). Accounting for >90% of neurons within the striatum, the medium spiny neurons are comprised of two distinct subpopulations that are classified on the basis of their distinct circuitries (9). The striato-nigral (SN) neurons projecting to the substania nigra pars reticulata and entopeduncular nucleus constitute the direct tract, whereas the striato-pallidal (SP) neurons projecting to the lateral part of the globus pallidus comprise the indirect tract (8). Typically, a coordinated balance between these two tracts produces normal movements, whereas a preponderance of one tract over the other is implicated in producing motor abnormalities associated with basal ganglia disorders (10, 11).

In the SN neurons, the D_1R acts through the G-protein α_{olf} subunit to stimulate cAMP production (12). Based on recent analyses of *Gng7*$^{-/-}$ mice, this action is also dependent on the γ_7 subtype (6). Intriguingly, in the SP neurons, the adenosine A_{2A} receptor ($A_{2A}R$) also couples through the G-protein α_{olf} subunit to enhance cAMP production even though this pathway produces the opposite behavioral effect (12). In the present study, we explored whether a specific G-protein $\alpha_{olf}\beta_2\gamma_7$ subunit combination is required for this pathway in SP neurons. Using mice with targeted deletions of *Gng7* or *Gnal*, lacking the

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[2] The abbreviations used are: D_1R, dopamine D1 receptor; $A_{2A}R$, adenosine A_{2A} receptor; SP, striatopallidal; SN, striatonigral; GTPγS, guanosine 5′-3-*O*-(thio)triphosphate.

G-protein γ_7 (6) or α_{olf} (13) subunits, respectively, we showed that levels of the α_{olf} and β_2 proteins were selectively and coordinately reduced in *Gng7*$^{-/-}$ mice, whereas levels of γ_7 were largely unaffected in the *Gnal*$^{-/-}$ mice. Notably, these results indicate that assembly of the $\alpha_{olf}\beta_2\gamma_7$ heterotrimer is an ordered process that is controlled by the amount of the γ_7 subtype. Moreover, loss of the G-protein γ_7 subunit led to defects in both D_1R (6) and A_{2A} receptor activation of adenylyl cyclase without producing any gross alterations in locomotor behavior typical of Parkinson disease. Importantly, these findings contribute to a growing literature that suggests that blockade of $A_{2A}R$ signaling in the striatum may be an effective strategy for treating various neurological and addictive disorders.

EXPERIMENTAL PROCEDURES

Production of Mice—Disruption of *Gng7*, the gene encoding the G-protein γ_7 subunit in mice, was described previously (6). *Gng7*$^{+/-}$ mice were backcrossed to C57BL/6 mice (Jackson Laboratories, Bar Harbor, ME) for 5 generations, or separately to BALB/c mice (Jackson Laboratories) for 5 generations. *Gng7*$^{+/-}$ mice were intercrossed to produce the *Gng7*$^{-/-}$ mice and wild-type littermates used in these experiments. In this work, we will describe these mice as "on a C57BL/6 background," or "on a BALB/c background." However, after only 5 generations of backcrosses, there is still some contribution to their genetic makeup from the original ES cells (129SvEv^{Brd}, Lexicon Genetics, Inc., The Woodlands, TX), from the dam that was bred with the chimera (C57BL/6 albino, Lexicon), and from the Cre recombinase expressing strain that we utilized (BALB/c-TgN(CMV-Cre)#Cgn, Jackson Laboratories). Hence, it was essential to use littermates to control for the possible influence of genetic background to observed responses. On the C57BL/6 background, 16 *Gng7*$^{-/-}$ mice (8 males and 8 females) and 16 wild-type littermates (8 males and 8 females) were studied. On the BALB/c background, 12 *Gng7*$^{-/-}$ mice (6 males and 6 females), and 14 wild-type littermates (4 males and 10 females) were studied. Genotypes were determined by PCR analysis of tail biopsy DNA as described previously (6).

Mice with a disrupted *Gnal* gene, that lack the G-protein α_{olf}, were described previously (13). These were backcrossed for up to 9 generations with C57BL/6 mice to obtain homozygous (*Gnal*$^{-/-}$) and their control littermates (*Gnal*$^{+/+}$). For comparing homozygous mutant and wild-type mice, 6-week-old male and female mice were used for experiments.

Animal Care and Approval—Mice were segregated by sex and group housed in plastic microisolator cages in ventilated racks (Thoren Caging Systems, Inc., Hazelton, PA). Mice were given ad lib access to water and Mouse Diet 9F (Purina Mills, LLC, St. Louis, MO). Environmental factors included temperature and humidity control and a 12-hour light/dark cycle. The animal facility is maintained as virus antibody-free and parasite-free. The Geisinger Clinic Institutional Animal Care and Use Committee approved animal research protocols.

Adenylyl Cyclase Assay—Striatal tissues were homogenized in Buffer A (10 mM Tris, pH 7.4, 1 mM EDTA, 1 mM DTT, 0.3 mM AEBSF, 30 μM leupeptin, 1 μM pepstatin A) with 10% sucrose using a Brinkmann Homogenizer (Brinkmann Instruments Co, Westbury, NY). Membranes were then isolated by centrifugation (65 min at 100,000 $\times$ *g*) onto a cushion of Buffer A with 44.5% (w/v) sucrose. The membranes at the interface were transferred to a new tube and twice washed with Buffer A and collected by centrifugation (30 min at 100,000 $\times$ *g*). Protein concentrations were determined with Coomassie Plus (Thermo Fisher Scientific, Inc., Rockford, IL). Adenylyl cyclase activity was determined by incubating membrane protein (20 μg) at 30 °C for 15 min in 0.1 ml of buffer containing 50 mM HEPES (pH 7.4), 1 mM EGTA, 5 mM $MgCl_2$, 0.1 mM ATP, 1×10^6 cpm of [α-^{32}P]ATP, 10 μM rolipram, 1 unit/ml adenosine deaminase, 5 mM creatine phosphate, 50 units/ml creatine phosphokinase, and various agonists as indicated in the text. Reactions were terminated by addition of 0.1 ml of 2% SDS, 40 mM ATP, 1.4 mM cAMP, 10,000 cpm of [^{3}H]cAMP, and heating to 100 °C for 3 min. [^{32}P]cAMP was isolated by chromatography on Dowex and Alumina columns, using [^{3}H]cAMP as a recovery marker, and quantified by liquid scintillation counting.

Radioligand Binding Assays—Radioligand binding to $A_{2A}R$ in striatal membranes prepared from *Gng7*$^{-/-}$ mice and wild-type littermates was performed using either the radiolabeled agonist [^{125}I]2-[2-(4-amino-3-iodo-phenyl)ethylamino]adenosine (^{125}I-APE) or the radiolabeled antagonist ^{125}I-ZM241385 as described previously (14, 15). Binding was performed using striatal membranes prepared from wild type or *Gng7*$^{-/-}$ mice in buffer containing 10 mM HEPES pH 7.4, 1 mM EDTA, 5 mM $MgCl_2$, and 1 unit/ml adenosine deaminase. The agonist, ^{125}I-APE binds to two affinity states of the $A_{2A}R$, a high affinity state corresponding to receptor-G-protein complexes, and a low affinity state corresponding to receptors uncoupled from G-proteins (15). Because GTPγS added to membranes uncouples receptors from G-proteins, 50 μM GPTγS was added to some membranes to measure agonist binding to largely uncoupled receptors. In the absence of added GTPγS, ^{125}I-APE binds preferentially to G-protein coupled receptors. ^{125}I-APE also binds to uncoupled receptors, but may under measure total receptor number because a fraction of the radioligand may dissociate for the low affinity site during washing of filters. Hence, the total number of receptors (B_{max}) was more accurately detected as the number of specific binding sites for the high affinity antagonist, ^{125}I-ZM241385. Nonspecific radioligand binding was measured in the presence of 50 μM *N*-ethylcarboxamidoadenosine (NECA).

Immunoblot Analysis—To examine the expression of G-protein subunits in mouse striatum, Western blot analysis was performed on cholate-solublized membranes that were prepared as described previously (6). Antisera for $G\alpha_s$ was used at a 1:500 dilution, for $G\alpha_{olf}$ (16) at 1:2000, and for Ras (BD Biosciences, Palo Alto, CA) at a 1:2000 dilution. Antisera for β_1 (1:500), β_2 (1:500), γ_2, γ_3, γ_5 (1:100), and γ_7 were described previously (17, 18, 19) and were used at a 1:200 dilution, except as indicated. His-tagged G-protein β and γ subunits (CytoSignal Research Products, Irvine, CA) and His-tagged α_{olf} (12) were used as standards for quantitative immunoblotting.

Real-time RT-PCR—To examine levels of mRNA for α_{olf}, β_2, γ_7, and DARPP-32, RNA was prepared from striatum of six *Gng7*$^{-/-}$ mice and six wild-type littermates at the N24 back-

cross to C57BL/6 and real-time RT-PCR was conducted as described previously (20). Primers were as follows: *Gnal* (CCT TCC TAC TTG CCT GAC CGC; TGA CGA TAG TGC TTT TCC CGG), *Gng7* (GCT GGG ATC GAA CGC ATC AAG; CAG GAA GAT CCC GGC ATT CAC), *Gnb2* (TCA TAG GTC ACG AGT CGG ACA TCA; ATG GCA TCC CAG ATG TTG CAG TTG), *Ppp1r1b* (CAC CAC CCA AAG TCG AAG AGA; CGA AGC TCC CCT AAC TCA TCCT), and to correct for variation in cDNA yield *Eef1a1* (GGA ATG GTG ACA ACA TGC TG; CGT TGA AGC CTA CAT TGT CC).

Locomotor Activity—Locomotor activity was quantified in CLAMS cages (Columbus Instruments, Columbus, OH). The cages consist of clear plastic boxes (20 cm × 10 cm × 12.5 cm) fitted with three rows of 8 photoelectric sensors (x, y, and z directions). The mice were placed in the CLAMS cages at 11 am and remained in the cages for 4 h. During this time the mice had *ad lib* access to water. Every minute the numbers of individual (total) and consecutive (ambulatory) photobeam breaks for each of 3 sensor arrays, and the number of contacts with the sipper tube were recorded. For caffeine trials, mice were removed from the cages after 1 h and given an intraperitoneal injection of saline or caffeine (5 ml/kg). Mice that had been habituated to the CLAMS cages and to the injection procedure with saline were used in drug trials. The locomotor response to drug is expressed as an increment over the response to saline. Locomotor activities were studied at age 10.0 ± 0.8 weeks for C57BL/6 background mice and age 8.5 ± 0.3 weeks for BALB/c background mice. The response to caffeine was studied at age 23.6 ± 3.0 weeks for C57BL/6 background mice and age 14.6 ± 0.3 weeks for BALB/c background mice.

Statistical Analysis—Sample statistics and Student's *t*-tests were computed using Excel (Microsoft). Data are presented as means ± S.E. of the mean. Locomotor activity was compared by repeated measures multivariate analysis of variance (MANOVA), using JMP (SAS Institute Inc., Carey, NC).

RESULTS

The $A_{2A}R$ is primarily responsible for the psychostimulant action of caffeine (21, 22). Because blockade of this receptor has been shown to reverse the hypolocomotor phenotype resulting from dopamine deficiency (23) and dysfunctional dopamine signaling (24), the $A_{2A}R$ signaling components are increasingly recognized as valid therapeutic targets for treating Parkinson disease and for reducing the side effects of levodopa therapy (25, 26). In SP neurons, the G-protein α_{olf} subunit has been shown to positively couple this receptor to stimulation of cAMP production (27, 12). However, little information is available on the obligatory β and γ components involved in this context. By applying biochemical and behavioral approaches to a novel mouse model, we found that the G-protein γ_7 subunit is specifically required for both $A_{2A}R$ signaling and psychostimulant response to caffeine. Identifying a mechanistic basis for this requirement, we show that the γ_7 protein drives the preferential assembly of a G-protein $\alpha_{olf}\beta_2\gamma_7$ heterotrimer in the striatum that is involved in a key signaling pathway controlling locomotion and reward.

Defective $A_{2A}R$ Signaling in Mice Lacking the γ_7 Protein—Regulation of cAMP production in medium spiny neurons represents a primary target of many neurotransmitters and psychoactive drugs that affect short- and long-term locomotor responses (28). Virtually all medium spiny neurons, including SN and SP neurons, express substantial levels of γ_7 mRNA (29, 30, 31). Because adenylyl cyclase signaling by the D_1R was shown to be dependent on γ_7 expression in SN neurons (6), we explored whether adenylyl cyclase signaling by the $A_{2A}R$ is similarly dependent on expression of γ_7 in SP neurons. As a biochemical assay, we used the selective $A_{2A}R$ agonist, CGS-21680, to stimulate adenylyl cyclase activity in striatal membranes prepared from *Gng7*$^{-/-}$ mice. By comparison to their wild type littermates, adenylyl cyclase activity in response to 10 μM or 25 μM CGS-21680 was reduced by 30–40% in striatal membranes from *Gng7*$^{-/-}$ mice (Fig. 1*A*). Furthermore, cAMP production in response to 100 μM forskolin was also reduced by ~35% in striatal membranes from *Gng7*$^{-/-}$ mice (Fig. 1*B*).

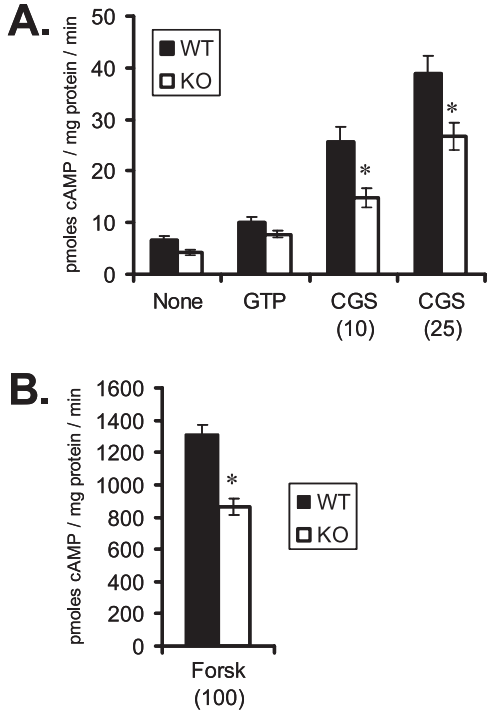

FIGURE 1. **Adenylyl cyclase activity of membranes prepared from striata of *Gng7*$^{-/-}$ mice (*KO*) and wild-type littermates (*WT*), expressed as pmol of cAMP per mg of membrane protein per min.** *A*, adenylyl cyclase activity in response to no added agonist (*None*), 1 μM GTP alone (*GTP*), or with 10 μM CGS-21680 (*CGS*) or 25 μM CGS-21680. *B*, in response to 100 μM forskolin (*Forsk*), adenylyl cyclase activity is significantly reduced in *Gng7*$^{-/-}$ striatal membranes ($n = 9$ mice in each group, *, $p < 0.01$ by Student's *t*-test).

Because forskolin-stimulated adenylyl cyclase activity was reduced, we could not assess whether the impaired response to the $A_{2A}R$ agonist was due to a defect in G-protein coupling and/or adenylyl cyclase activation. Therefore, as a second biochemical assay, we used high affinity agonist binding to directly measure the actual interaction between the $A_{2A}R$ and the G-protein. Initial saturation binding experiments were performed with the $A_{2A}R$ agonist, ^{125}I-APE, on pooled samples of striatal membranes from either *Gng7*$^{-/-}$ mice or their wild-type littermates (12 mice in each group). In the wild-type sample, the addition of GTPγS dramatically reduced agonist binding by greater than 75%, indicating a significant portion of the $A_{2A}R$ was associated with G-protein (Fig. 2*A*). In contrast, in

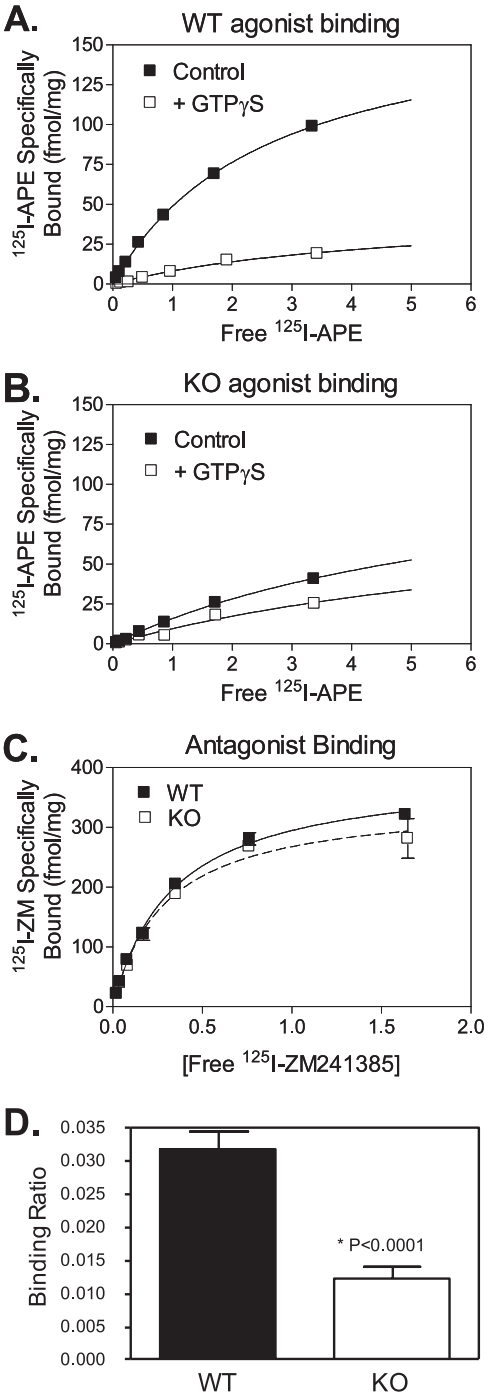

FIGURE 2. **Radioligand binding to striatal membranes.** Pooled membranes from striata of 12 wild-type littermates (*A*) or 12 *Gng7*$^{-/-}$ mice (*B*), showing ^{125}I-APE specifically bound at various concentrations in the absence (*filled boxes*) or presence (*open boxes*) of GTPγS. The difference ± GTPγS is defined as *GTPγ-S sensitive binding*. Note reduced GTPγS-sensitive binding to *Gng7*$^{-/-}$ membranes compared with controls. The total number of receptors was determined from binding of the antagonist, ^{125}I-ZM241385 (*C*). The ratio of specific binding of ^{125}I-APE ± GTPγS/^{125}I-ZM241385 was measured in 12 individual membrane preparations from wild type or *Gng7*$^{-/-}$ mice, as an index of receptor coupling to G proteins (*D*).

the knock-out sample, addition of GTPγS produced little reduction in A_{2A}R agonist binding (Fig. 2*B*), suggesting most A_{2A}R was no longer coupled to G-protein. Finally, there was no significant difference in the binding of the A_{2A}R antagonist, ^{125}I-ZM241385, between the samples (Fig. 2*C*), indicating the total number of A_{2A}R was comparable between the two genotypes. Taken together, these results indicate a striking reduction in the fraction of the A_{2A}R that was coupled to G-protein in striatal membranes from *Gng7*$^{-/-}$ mice. Subsequent binding studies were performed on striatal membranes from individual mice representing each genotype. To calculate the fraction of the A_{2A}R pool that was coupled to G-protein, we determined the ratio of specific GTPγS-sensitive ^{125}I-APE agonist binding sites relative to ^{125}I-ZM421385 antagonist binding sites in striatal membranes from both genotypes. By comparison to their wild-type littermates, the fraction of the A_{2A}R pool that was coupled to G-protein was markedly reduced in striatal membranes from *Gng7*$^{-/-}$ mice ($p < 0.001$) (Fig. 2*D*). Collectively, these results confirm that A_{2A}R signaling shows a specific requirement for G-protein γ_7 expression and that its loss is associated with an impaired ability of this receptor to couple to G-protein.

Impaired Assembly of G_{olf} Heterotrimer in Mice Lacking the γ_7 Protein—One mechanism that could account for the observed defects in both G-protein coupling and adenylyl cyclase activation is a coordinate reduction in the cellular amount of the G-protein α_s or α_{olf} subunit. These two structurally related isoforms are both able to stimulate cAMP production (12) and are both expressed in the striatum (32). Previously, we showed that loss of the γ_7 subunit coordinately reduced levels of the α_{olf} protein in the striatum (6). To confirm and extend this finding, we performed immunoblot analysis on micropunch samples from dorsal striatum (caudate) and ventral striatum (nucleus accumbens) of *Gng7*$^{-/-}$ mice on two different genetic backgrounds (*i.e.* C57BL/6 and BALB/c). By comparison to their wild-type littermates, α_{olf} protein levels were strikingly reduced by >85% in both dorsal and ventral striatal membranes from knock-out animals on a C57BL/6 genetic background (Fig. 3*A*). The effect was remarkably specific in that α_s protein levels (*i.e.* 45- and 52-kDA forms) were not affected in the dorsal striatum and were reduced by only 20% in the ventral striatum of knock-out mice (Fig. 3*B*). Attesting to the generality of this finding, immunoblot analysis of the corresponding regions of knock-out mice on a BALB/c genetic background yielded similar results (supplemental Fig. S1). Taken together, these results demonstrate that the cellular level of the α_{olf} but not the α_s subunit is dependent on expression of the γ_7 subunit.

Next, we investigated how loss of the γ_7 protein impacts the levels of particular β protein(s) in the striatum. Because targeting of the $\beta\gamma$ dimer to the plasma membrane is dependent upon post-translational lipid modifications of the γ subunit (33, 34, 35), we reasoned that loss of the γ_7 protein could affect the level of a specific β subtype in striatal membranes from *Gng7*$^{-/-}$ mice. By comparison to their wild-type littermates, β_2 protein levels were selectively reduced by 31% with no significant changes in the amounts of β_1 and β_4 proteins (Fig. 3*C*). Taken together, these findings show both coordinate and selective suppression of the α_{olf}, β_2, and γ_7 subunits at the protein level.

To assess whether the α_{olf} subunit plays a reciprocal role in this process, we used the *Gnal*$^{-/-}$ mouse model (13) to determine whether loss of the α_{olf} protein causes a corresponding

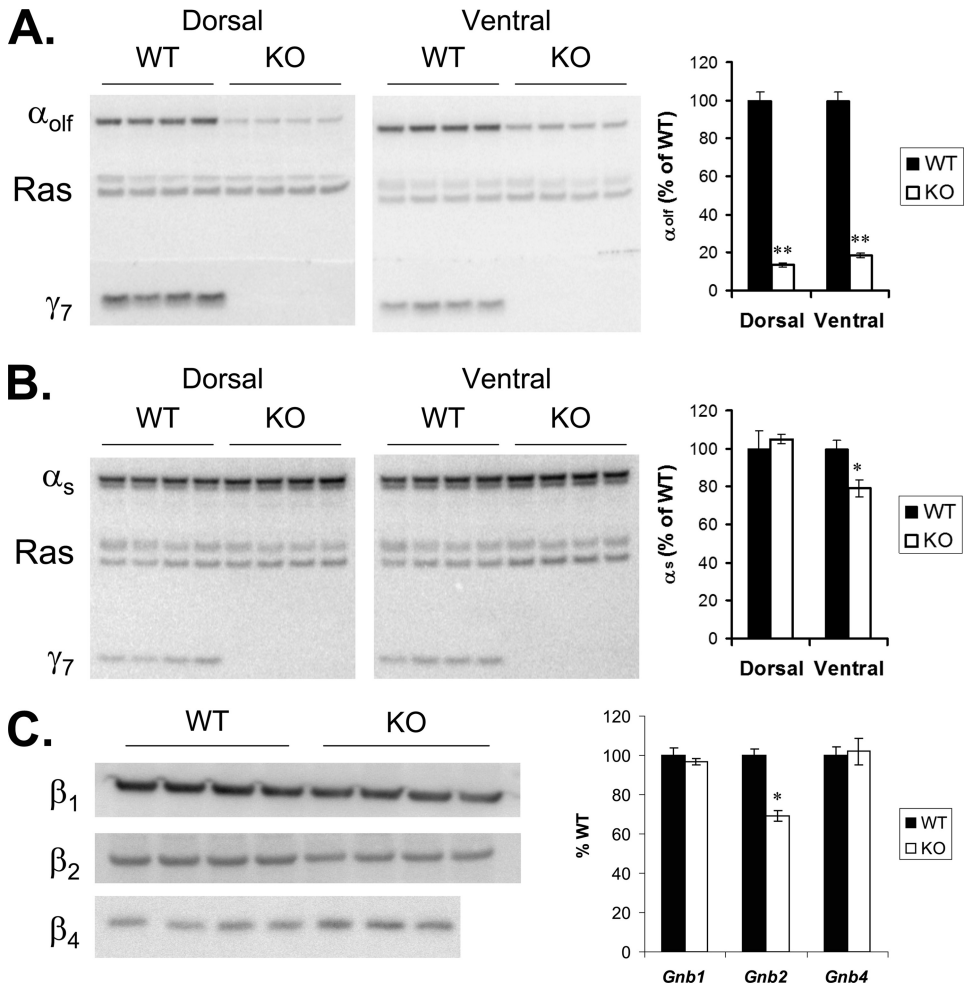

FIGURE 3. *A*, immunoblot of α_{olf}, γ_7, and Ras on membranes prepared from micropunch samples of dorsal striatum (15 μg/lane) and ventral striatum (10 μg/lane) of four *Gng7*$^{-/-}$ mice (*KO*) and four wild-type littermates (*WT*) on a C57BL/6 genetic background. *B*, immunoblot of α_s, γ_7, and Ras in membranes described above. *C*, immunoblot of β_1, β_2, and β_4 on membranes prepared from whole striatum of *Gng7*$^{-/-}$ mice and wild-type littermates (20 μg/lane) on a C57BL/6 genetic background. Graphs depict quantitation of 1 or 2 immunoblots for each subunit, values are normalized to Ras for each lane, then expressed as percent of wild-type (% WT) (n = 4 to 8 mice in each group; *, $p < 0.01$; **, $p < 1 \times 10^{-6}$).

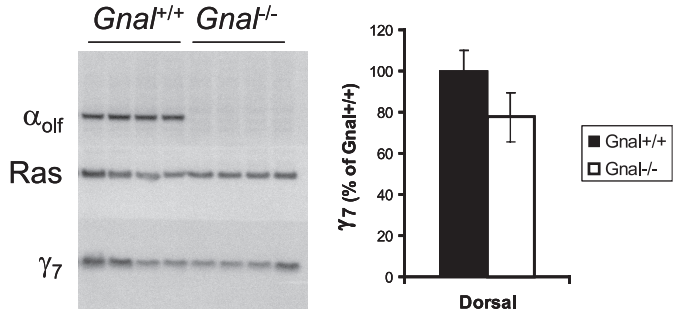

FIGURE 4. **Immunoblot of membranes (15 μg/lane) prepared from micropunch samples of dorsal striatum of four *Gnal*$^{+/+}$ mice, and four *Gnal*$^{-/-}$ mice, blotted with antisera for α_{olf} (*top*), Ras (*middle*), or γ_7 (*bottom*).** *Right panel* shows quantitation of γ_7 normalized to Ras for each lane, then expressed as percent of *Gnal*$^{+/+}$.

suppression of the γ_7 protein. By comparison to their wild type littermates, γ_7 protein levels were not significantly different in striatal membranes from *Gnal*$^{-/-}$ mice (Fig. 4), indicating that the expression of the γ_7 but not the α_{olf} protein drives the assembly of a specific G-protein heterotrimer in the striatum.

Mechanism for Coordinate Suppression of the G-protein α_{olf} Subunit in Mice Lacking the γ_7 Subunit—One mechanism that could account for suppression of the α_{olf} protein is a reduced level of the corresponding mRNA transcript. To test this possibility, we performed real time RT-PCR analysis on striatal tissue from *Gng7*$^{-/-}$ mice and their wild-type littermates on the C57BL/6 background. Despite the loss of α_{olf} protein (Fig. 4), the level of α_{olf} mRNA was not significantly reduced in the striatum of *Gng7*$^{-/-}$ mice (Fig. 5*A*). Moreover, the level of β_2 mRNA was not significantly different in wild type and *Gng7*$^{-/-}$ striatum. Finally, using a well validated marker of medium spiny neurons, the level of DARPP-32 (*Ppp1r1b*) mRNA was not significantly reduced in the striatum of *Gng7*$^{-/-}$ mice (Fig. 5*A*), indicating that there was no overt loss of medium spiny neurons from the brains of knock-out mice. These findings point to a post-transcriptional mechanism responsible for the coordinate suppression of the α_{olf} and β_2 proteins in mice lacking the γ_7 protein. Because plasma membrane binding of the α subunit is facilitated by $\beta\gamma$ association (36, 37), we examined whether the decreased amount of α_{olf} protein in the striatal membrane fractions was associated with increased accumulation of this protein in the corresponding cytosolic fractions. Despite loss from the membrane fraction (Fig. 5*B*), no α_{olf} protein was detectable in the cytosolic fraction of *Gng7*$^{-/-}$ mice (Fig. 5*C*).

Basis for γ_7 Selectivity—To determine why γ_7 has a special place in the assembly of the G_{olf} heterotrimer in the native context, we performed immunoblot analysis to provide a quantitative accounting of the G-protein subunits that are expressed in the striatum of wild-type and *Gng7*$^{-/-}$ mice (Table 1). The wild-type striatum contains roughly equimolar levels of α_{olf} and γ_7 proteins (supplemental Fig. S2*A* and Table 1). Therefore, in the *Gng7*$^{-/-}$ striatum, the 85% reduction in α_{olf} could be almost completely accounted for by loss of γ_7. Likewise, the wild-type striatum contains a >2-fold molar excess of β_2 relative to γ_7 (supplemental Fig. S2*A* and Table 1). Therefore, in the knock-out striatum, the 30% reduction in β_2 could also be attributed to loss of γ_7. While our accounting is necessarily limited by the availability of G-protein subunit antibodies and standards, these results strongly suggest the existence of a specific G-protein $\alpha_{olf}\beta_2\gamma_7$ complex that normally functions downstream of the $A_{2A}R$ in SP neurons. To explore the mech-

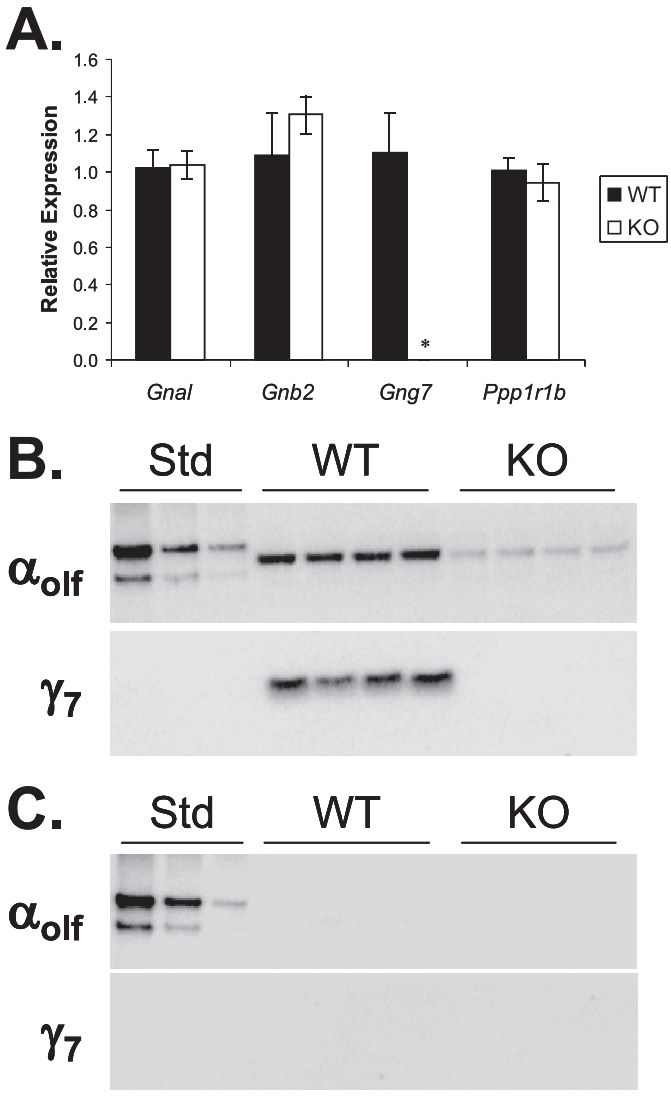

FIGURE 5. **Real time RT-PCR amplification of α_{olf} (*Gnal*), β_2 (*Gnb2*), γ_7 (*Gng7*), and DARPP-32 (*Ppp1r1b*) from RNA prepared from dorsal striatum of *Gng7*$^{-/-}$ mice (*KO*) and wild-type littermates (*WT*), on the C57BL/6 genetic background.** Relative expression was calculated as $2^{-(\Delta Ct-\Delta Ctavg)}$, where ΔCt is the threshold cycle for the gene of interest minus the threshold for *Eef1a1*, and ΔCtavg is the average ΔCt of the wild-type samples ($n = 6$ in each group, *, $p < 0.001$ by Student's *t*-test). *B*, immunoblot of membrane fraction (15 μg/lane) prepared from micropunch samples of dorsal striatum of *Gng7*$^{-/-}$ mice (*KO*) and wild-type littermates (*WT*), blotted with antisera for α_{olf} (*top*) or γ_7 (*bottom*). The first three lanes contain 100 ng, 50 ng, or 25 ng of recombinant α_{olf} protein (*Std*). *C*, immunoblot of cytosol fraction (15 μg/lane) from samples in *B*.

TABLE 1

G-protein subunit expression in wild-type and *Gng7*$^{-/-}$ dorsal striatum as determined by quantitative immunoblotting

	Measured wild type concentration	Formula weight	Calculated wild type concentration	Measured change in *Gng7*$^{-/-}$	Calculated change in *Gng7*$^{-/-}$
	ng/μg membrane protein		*fmol/μg membrane protein*	*%*	*fmol/μg membrane protein*
γ_2	2.84 ± 0.09	7850	362 ± 11	−18 ± 4	−65 ± 16
γ_3	0.71 ± 0.10	8305	85 ± 7	+39 ± 11	+33 ± 10
γ_7	0.65 ± 0.10	7611	86 ± 8	−100	−86 ± 8
β_2	8.19 ± 0.40	37331	219 ± 11	−31 ± 4	−68 ± 9
α_{olf}	3.18 ± 0.12	44308	72 ± 2	−85 ± 5	−61 ± 4

anistic role of the γ_7 protein in this process, we examined the relative levels of the various γ subtypes in the striatum. Compared with the γ_7 protein, there is a 4-fold molar excess of the γ_2 protein and an equimolar amount of the γ_3 protein in the striatum (7). Moreover, in the knock-out striatum, there is a further increase in the level of the γ_3 protein (supplemental Fig. S2*B*). Hence, the combined levels of the γ_2 and γ_3 proteins appear to be sufficient to compensate for loss of the γ_7 protein, suggesting other γ subtypes are not functionally interchangeable.

Alterations in Locomotor Activity in Mice Lacking γ_7—To assess the functional consequences of impaired $A_{2A}R$ signaling and G_{olf} assembly, we examined the behavioral responses of *Gng7*$^{-/-}$ mice. Because the $A_{2A}R$ is primarily responsible for the psychostimulant actions of caffeine (21, 22), we reasoned that *Gng7*$^{-/-}$ mice on the C57BL/6 background might exhibit an attenuated response to the locomotor enhancing effects of this drug. As a quantitative measure of locomotor activity, *Gng7*$^{-/-}$ mice and their wild-type littermates were placed in CLAMS cages equipped with photobeams to record their movements. After allowing sufficient time for acclimation to their surroundings, the locomotor responses for both groups of mice were calculated as the difference between the response to caffeine *versus* saline injection. Although showing similar responses to low dose caffeine (Fig. 6*A*), *Gng7*$^{-/-}$ mice showed a significantly attenuated response to high dose caffeine that was particularly apparent at later time points (Fig. 6*B*; MANOVA analysis: $F_{1,30} = 7.4$, $p = 0.01$). Because locomotor behavior is influenced by genetic background (38), these studies were repeated on *Gng7*$^{-/-}$ mice on the BALB/c background to assess the generality of this finding. Even more strikingly, *Gng7*$^{-/-}$ mice on the BALB/c background exhibited significantly reduced responses to both low and high dose caffeine (Fig. 6, *C* and *D*; MANOVA analyses: for the low dose, $F_{1,24} = 16.4$, $p = 0.0005$ and for the high dose, $F_{1,24} = 8.6$, $p = 0.007$). Taken together, these results show a clear association between defective $A_{2A}R$ signaling and impaired locomotor response to caffeine in *Gng7*$^{-/-}$ mice.

In addition to psychostimulant response, we also measured the basal locomotor activity that reflects the coordinated output from the SN and SP tracts (10, 11) in these animals. Previously, we showed that the basal locomotor activity of *Gng7*$^{-/-}$ mice was not impaired despite reduced striatal D_1R signaling (6). Because increased D_1R signaling is generally associated with a higher level of locomotor activity (39), this finding was somewhat surprising. To exclude the possibility that the background strain was obscuring any defect due to loss of γ_7, we compared the basal locomotor activities of *Gng7*$^{-/-}$ mice and their wild-type littermates on two different genetic backgrounds. On the C57BL/6 background, both wild-type and knock-out mice displayed comparably high levels of locomotor activity when introduced into CLAMS cages (Fig. 7*A*) and similar abilities to acclimate to the novel environment (Fig. 7*B*). That is to say, the initially high levels of locomotor activity observed in both groups decreased similarly over the 3 h following initial exposure to the CLAMS cages (Fig. 7*A*) and over the course of 4 days upon repeated exposure to the CLAMS cages (Fig. 7*B*). On the BALB/c background, *Gng7*$^{-/-}$ mice exhibited

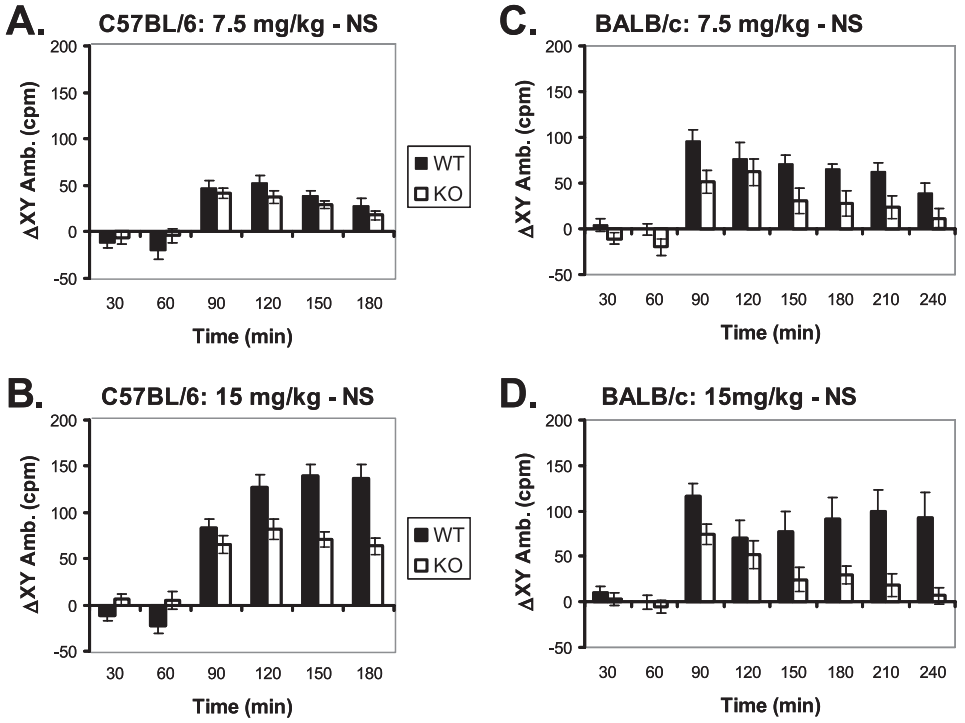

FIGURE 6. **Increase in locomotor activity in CLAMS cages in response to an intraperitoneal injection, at 60 min (*arrow*), of caffeine 7.5 mg/kg (*A* and *C*) or 15 mg/kg (*B* and *D*) for *Gng7*$^{-/-}$ mice (*KO*) and wild-type littermates (*WT*) on either the C57BL/6 background (*A* and *B*) or the BALB/c background (*C* and *D*).** Data are expressed as the average ambulatory activity in both the x- and y-dimensions, consecutive photobeam breaks per minute, over each 30-min interval for a trial with drug injection minus the average over the same interval for a trial with saline injection (Δ*XY Amb.*). On the C57BL/6 background, genotype was a significant factor in a repeated measures MANOVA only for the high dose ($F_{1,30} = 7.4$, $p = 0.01$), on the BALB/c background genotype was significant for both the low dose ($F_{1,24} = 16.4$, $p = 0.0005$) and the high dose ($F_{1,24} = 8.6$, $p = 0.007$).

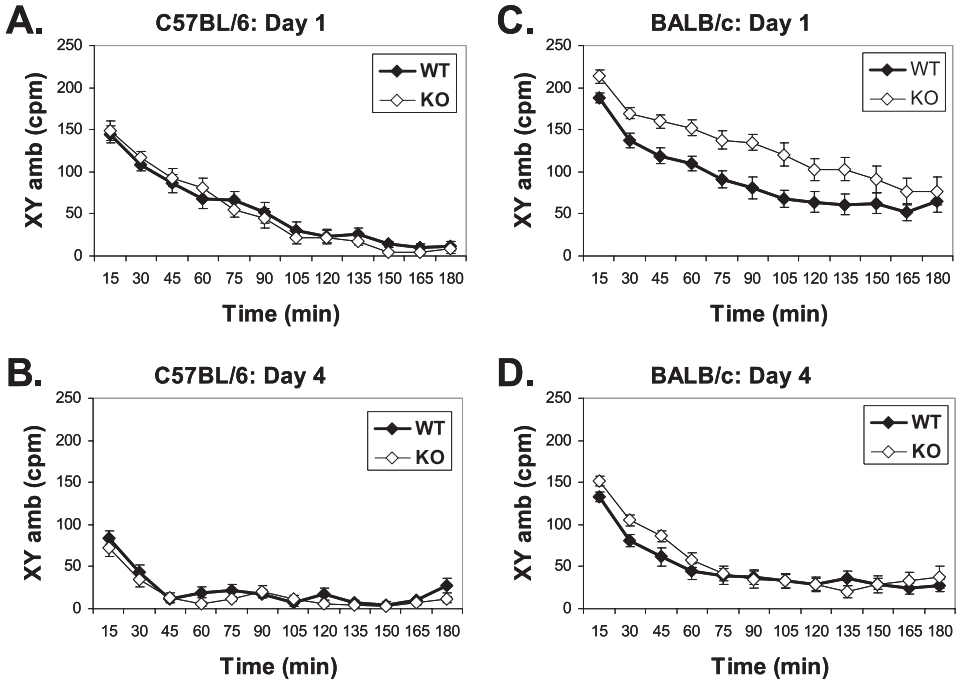

FIGURE 7. **Locomotor activity in CLAMS cages during 3 h trials, expressed as the ambulatory activity per minute in both the x- and y-dimensions (*XY amb*) averaged over 15 min intervals.** Results are for trials on the first (*A* and *C*) and the fourth (*B* and *D*) consecutive day, for *Gng7*$^{-/-}$ mice (*KO*) and wild-type littermates (*WT*) on either the C57BL/6 background (*A* and *B*) or the BALB/c background (*C* and *D*). In a repeated measures MANOVA, day ($F_{1,136} = 108$, $p < 0.0001$), genotype ($F_{1,136} = 7.9$, $p = 0.006$) and background ($F_{1,136} = 85.3$, $p < 0.0001$) were all significant factors, and there was a significant genotype × background interaction ($F_{1,136} = 9.7$, $p = 0.002$). On the BALB/c background, *Gng7*$^{-/-}$ mice were more active than wild-type littermates on Day 1 ($F_{1,38} = 11.3$, $p = 0.002$).

a significantly higher locomotor activity than their wild-type littermates when introduced into CLAMS cages (Fig. 7*C*; MANOVA analysis: $F_{1,38} = 11.3$, $p = 0.002$) but showed no differences in their abilities to acclimate to the novel environment (Fig. 7, *C* and *D*). Collectively, these findings support the possibility that combinatorial disruption of both D_1R and $A_{2A}R$ signaling pathways secondary to loss of the γ_7 protein produces overtly normal motor activity.

DISCUSSION

Despite their molecular cloning more than a decade ago (2), the functional significance for the large diversity of G-protein γ subunits is still not known. From analysis of *Gng7*$^{-/-}$ mice, we now show that loss of the G-protein γ_7 subtype produces both biochemical and behavioral consequences. In the process of studying these effects, we also identify a fundamental role for the γ_7 subtype in driving the preferential assembly of a G-protein $\alpha_{olf}\beta_2\gamma_7$ heterotrimer that is required for $A_{2A}R$ signaling and its locomotor inhibitory effect in the striatum. These results support a growing body of data pointing to the effectiveness of $A_{2A}R$ blockade to better normalize motor activity in Parkinson patients (40, 41).

G_{olf} Assembly Is Specifically Regulated by the γ_7 Subtype—The functions of G-protein $\alpha\beta\gamma$ heterotrimers are dependent on their proper assembly and trafficking to the plasma membrane (37, 42). However, very little is known regarding which $\alpha\beta\gamma$ heterotrimers exist in the intact cell setting and how they are actually assembled. Previously, our analysis of *Gng7*$^{-/-}$ mice provided the first *in vivo* demonstration that loss of the γ_7 protein disrupts the assembly of the G_{olf} but not the G_s heterotrimer in the striatum (6). Now, we extend this finding by showing through reciprocal analysis of *Gnal*$^{-/-}$ mice that loss of the α_{olf} protein does not substantially impact the level of the γ_7 protein. Taken together, these results point

to a hierarchical order of G_{olf} formation that begins with the γ_7 subunit. In hindsight, the γ_7 subtype has several characteristics consistent with a primary role in this process. Spanning >66 kb in size, the mouse *Gng7* gene produces multiple mRNA transcripts that encode the same protein, suggesting complex regulation of its expression. Moreover, the mouse γ_7 protein encompasses only 69 amino acids that assume an α-helical structure in solution (43) that approximates that seen in the crystal structure (44). This suggests that unlike its α (45) and β (46, 47) partners, chaperone-type proteins may not be required for proper folding of the γ_7 protein. Finally, indicating that a "γ subunit first" hierarchy may be generally applicable to other G-protein $\alpha\beta\gamma$ heterotrimers, loss of the γ_{t1} subtype has recently been shown to disrupt assembly of a specific G-protein $\alpha_{t1}\beta_1\gamma_{t1}$ heterotrimer in the retina (48).

At this time, we can only hypothesize as to the mechanism by which the γ_7 protein sets the level of the G_{olf} heterotrimer. Because the corresponding mRNAs were not altered, the simplest explanation for coordinately reduced α_{olf} and β_2 protein levels is a post-transcriptional requirement for the γ_7 in the stabilization and/or trafficking of G_{olf} to the plasma membrane. Because loss of γ_7 protein was not associated with accumulation of unassembled α_{olf} protein in the cytosol (Fig. 5), we speculate that formation of this G_{olf} results from stabilization of the α_{olf} and β_2 subunits by the γ_7 subunit, and that in the absence of this component, the unassembled α_{olf} and β_2 subunits undergo active degradation.

Likewise, at this stage, we can only speculate as to the basis for the unique requirement for γ_7 subtype in this process. Refuting the long-standing dogma that most γ subunits are functionally interchangeable (reviewed in Ref. 2), the γ_7 subtype must possess unique features that cannot be replaced by the other γ subtypes. To begin to identify such features, we examined the possibility that the reportedly high abundance of the γ_7 subtype in the striatum (49, 50) could provide an explanation. In fact, the γ_2 and γ_3 proteins are present together in 4-fold molar excess to the γ_7 subtype (supplemental Fig. S2 and Table 1). Therefore, abundance does not seem to be the answer. Next, we considered the possibility that the γ subtypes might be sequestered between different neuronal types making up the striatum. Two recent studies (30, 31) comparing the translational mRNA profiles of SN and SP neurons reveal that both cell types express multiple γ forms (supplemental Fig. S3). Therefore, cell type specific expression does not explain the failure of other γ subtypes to substitute for the role of γ_7 in assembly process. Finally, we are contemplating the possibility that the γ_7 subtype could be localized within a particular subcellular compartment in neurons, similar to that shown for the γ_5 subtype in focal adhesions (51). Such compartmentation could result from a structural feature that is unique to either the γ_7 mRNA or protein. In this regard, it is notable that the γ_7 mRNA contains a very long 3′-UTR, a region that has been implicated in translational regulation and subcellular targeting of dendritically targeted proteins (52).

A_{2A}R Signaling Is Dependent on a Specific G-protein $\alpha_{olf}\beta_2\gamma_7$ Heterotrimer—The A_{2A}R activates adenylyl cyclase activity in SP neurons (27, 12, 53, 54). Consistent with a requirement for the $\alpha_{olf}\beta_2\gamma_7$ heterotrimer in this pathway, both A_{2A}R-G-protein coupling and adenylyl cyclase activation are markedly reduced in knock-out membranes (Figs. 2 and 1). In looking for a functional connection, it is tempting to compare the >75% reduction in A_{2A}R-G-protein coupling with the 85% reduction in α_{olf} protein (Fig. 3 and supplemental Fig. S1). Because the A_{2A}R number (Fig. 2, as determined by antagonist binding) is several orders of magnitude lower than the total G_{olf} content (Table 1, as determined by quantitative immunoblotting), the random collision coupling model (55) would predict that the receptor represents the rate-limiting step for adenylyl cyclase activation. However, such a model is incompatible with several published reports. Notably, only about 20% of the A_{2A}R is actually coupled to G-protein in platelets (56) and striatum (14, 57). Furthermore, in these tissues, the G-protein appears to control the rate of adenylyl cyclase activation (58). Thus, despite the apparent excess of G_{olf}, it appears that the A_{2A}R interacts with only a limited pool of G_{olf} and that the latter controls the cellular response. Although not yet identified, the cellular mechanisms responsible for limiting their interaction could include post-translational modification and/or subcellular localization. In HEK293 cells, A_{2A}R coupling to G_s and activation of adenylyl cyclase has been shown to occur within cholesterol-rich microdomains (59). In future studies, it will be interesting to investigate the functional significance for the poor coupling between the A_{2A}R and G_{olf} in the striatum and to elucidate the underlying mechanism. Finally, in an analogous fashion, the D_1R activates adenylyl cyclase activity in SN neurons (12). Because this receptor utilizes the same G-protein $\alpha_{olf}\beta_2\gamma_7$ heterotrimer (6), it will be interesting to explore whether poor coupling of the D_1R is also observed in the striatum and whether the G_{olf} represents the rate-limiting step in adenylyl cyclase activation. Supporting the latter possibility, our previous analyses of mutant *Drda1a* and *Gnal* mice have revealed that the G-protein rather than the receptor controls psychostimulant responses (60).

Taken in conjunction with our earlier studies, the G-protein γ_7 subtype seems to play a special role in adenylyl cyclase stimulation in various cellular contexts. In HEK293 cells, the γ_7 subtype is responsible for driving the assembly of a particular G_s heterotrimer required for β adrenergic and D_1R signaling but not for PGE_2 and D_5R signaling (61, 62); and in the physiological relevant context of the brain, it is responsible for driving the assembly of a specific G_{olf} heterotrimer required for D_1R signaling in SN neurons (6) and for A_{2A}R signaling in SP neurons (Figs. 1 and 2). In addition to its role in assembly, it remains to be determined whether the γ_7 subunit performs additional roles in the signal transduction process. In this regard, the $\beta\gamma$ dimer has been suggested to contribute to recognition of the upstream receptor (63, 15), as well as downstream regulation of effectors such as the striatal-enriched adenylyl cyclase type 5 isoform (64, 65). Addressing whether the γ_7 subunit affects these processes will require the use of complementary approaches that bypass the requirement for the γ_7 subtype in the assembly of G_{olf}.

Altered Caffeine Responsiveness Is Associated with Impaired A_{2A}R Signaling—Previous studies of the locomotor stimulating effect of caffeine have demonstrated the primary involvement of the A_{2A}R (66, 21). Supporting its function acting down-

stream of this receptor, *Gng7*$^{-/-}$ mice lacking the γ_7 subtype showed an attenuated response to caffeine that was particularly apparent on the BALB/c background (Fig. 7). Moreover, consistent with their functioning as components of the same heterotrimeric G-protein, *Gnal*$^{-/-}$ mice lacking the α_{olf} subtype also exhibited a reduced response to caffeine (16). Finally, in agreement with a role for G_{olf} in adenylyl cyclase stimulation and protein kinase activation, *Ppp1r1b*$^{-/-}$ mice lacking DARPP-32, which is phosphorylated by protein kinase A (67), also showed an impaired response to caffeine (68). Taken together, these results establish the importance of the A_{2A}R-$G\alpha_{olf}\beta_2\gamma_7$-AC5-PKA-DARPP-32 pathway in mediating the psychostimulant properties of caffeine.

Clinical Significance—Despite defects in both D_1R and A_{2A}R signaling, deficiency of the G-protein γ_7 subtype has little impact on basal locomotor behavior (Fig. 7). This finding is similar to reported findings for *Drd1a*$^{-/-}$ and *Ador2a*$^{-/-}$ mice, *i.e.* slightly increased or decreased locomotor activity (69, 21). In contrast, *Drd2*$^{-/-}$ mice display a markedly reduced locomotor activity (70–72). In this regard, the ability of A_{2A}R signaling to modulate D_2R signaling could prove beneficial for the treatment of Parkinson disease. For instance, several groups have observed attenuated stimulation of locomotor activity in response to caffeine in *Drd2*$^{-/-}$ mice (73), and a partial reversal of acute D_2R antagonist-induced catalepsy in *Ador2a*$^{-/-}$ mice (66). Such results form the basis for clinical trials of adenosine antagonists in the treatment of Parkinson disease (26). In these trials, Istradefylline has been shown to reduce symptoms in patients with Parkinson disease on levodopa therapy (40, 41). Our studies further support targeting of the A_{2A}R signaling pathway as an augmentative strategy for treating Parkinson disease and provide a better mechanistic understanding of the apparent effectiveness of this treatment.

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