The D3 dopamine receptor is endocytosed through a heterologous mechanism mediated by phorbol esters. Here, we show that following this endocytosis the D3 dopamine receptors fail to recycle and are instead targeted for degradation through an interaction with the G protein-coupled receptor (GPCR)-associated sorting protein-1 (GASP-1). Furthermore, we identified a specific binding motif in the C terminus common to the D2 and D3 that confers GASP-1 binding. shRNA knockdown of GASP-1 delayed post-endocytic degradation of both the D2 and D3 dopamine receptors. In addition, mutation of the D2 and D3 receptor C termini to resemble the D4, which does not interact with GASP-1, not only inhibited GASP-1 binding but slowed degradation after endocytosis. Conversely, mutation of the C terminus of the D2 receptor D3 to resemble that of the D4, which does not interact with GASP-1, did not inhibit GASP-1 binding but slowed degradation after endocytosis. Members of these families that do not bind GASP (10), are recycled rather than degraded after endocytosis. Members of these families that do not bind GASP (10), are recycled rather than degraded after endocytosis. Post-endocytic degradation of GPCRs by GASP-1 has been shown to have behavioral relevance in vivo. For example, GASP-1 knock-out mice show reduced tolerance to the cannabinoid WIN55,212-2 as a consequence of reduced CB1 down-regulation (11). In addition, GASP-1 knock-out mice also show reduced sensitization to the locomotor activating effects of repeated cocaine as a consequence of reduced D2 receptor degradation (12).

Unlike the D1 and D2 receptors, D3 receptors do not endocytose in response to activation by dopamine (13, 14). Instead, endocytosis of the D3 receptor appears to occur through a heterologous mechanism mediated by PKC (15), requiring both the β and δ PKC isoforms, actin-binding motif 280 and flamin A (16). This endocytosis is dependent on dynamin, indicating a clathrin component, but it appears to be GPCR kinase- and arrestin-independent (16).

Importantly, no prior study has examined the post-endocytic fate of the D3 receptor. Here, we report that the D3 receptor is targeted for degradation following endocytosis in response to phorbol ester activation of PKC and that it does...
so through a specific interaction with GASPT.1. Furthermore, we report, for the first time, a specific binding domain in the C terminus of the D2 and D3 that facilitates GASPT binding and confers the degradative fate. In addition, in contrast to some previous studies, we find that D4s can undergo significant endocytosis in response to dopamine. Furthermore, we show that, unlike the other two members of the G, class of dopamine receptor, the D4 receptor does not bind GASPT-1 and remains stable following endocytosis and prolonged dopamine exposure. Importantly, mutation of the D4 receptor to resemble the D2 and D3 receptors in the domain we show here binds GASPT-1, confers GASPT-1 binding, and promotes post-endocytic degradation of the D4 receptor.

**MATERIALS AND METHODS**

**Drugs and Reagents**

The horseradish peroxidase (HRP)-conjugated anti-rabbit and anti-mouse antibodies were purchased from New England Biolabs. The anti-GASPT antibodies were as described previously (6). FLAG antibodies, dopamine, quinpirole, raclopride, spiperone, bisindolylmaleimide I (BIM), and phorbol 12-myristate 13-acetate (PMA) were purchased from Sigma.

**Cell Culture Constructs and Transfections**

cDNAs of D2, D3, and D4 (Missouri S&T cDNA Resource Center) were amplified and ligated into N-terminal signal sequence FLAG-tagged vectors. D2NR, D3NR, and D4KL mutant constructs were introduced by site-directed mutagenesis (Stratagene).

**Culture and Immunocytochemistry**

Human embryonic kidney (HEK) 293 cells (American Type Culture Collection) were grown in DMEM (Invitrogen) supplemented with 10% fetal bovine serum (HyClone). N-terminal FLAG-tagged constructs were stably expressed in HEK293 cells. For generation of clonal stable cell lines, single colonies were chosen and propagated in the presence of selection-containing media. The antibody-feeding immunocytochemistry experiments were carried out as described previously (17). Briefly, cells stably expressing FLAG-tagged D2, D3, and D4 receptors or their mutant counterparts were grown on coverslips to 50% confluency. Live cells were incubated with M1 antibody (Sigma) directed against the FLAG tag (1:1,000 for 15 min. Cells were quenched with Tris-HCl buffer (pH 7.4) for 1 h at room temperature and transferred to a purification column where conjugated antibody (lower band) was eluted (PBS containing 10 mM KPO4, 150 mM NaCl (pH 7.2) with 2 mM sodium azide) and collected.

**Flow Cytometry**

**Antibody Conjugation—Anti-FLAG M1 antibody was conjugated to Alexa Fluor® 647 dye according to the manufacturer’s instructions (Invitrogen). Briefly, 0.5 ml of M1 antibody (2 mg/ml) was mixed with 50 μM of 1 M sodium bicarbonate and added to a vial of Alexa Fluor® 647 reactive dye. The reaction was mixed for 1 h at room temperature and transferred to a

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**Biotin Protection Degradation Assay**

HEK293 cells stably expressing N-terminal FLAG-tagged D2, D3, D4, D2NR, D3NR, or D4KL were grown to 100% confluence in 10-cm poly-D-lysine-coated plates and subjected to the biotin protection degradation assay protocol as described previously (20). Briefly, cells were treated with 3 μg/ml disulfide-cleavable biotin (Pierce) for 30 min at 4°C. Cells were then washed in PBS and placed in pre-warmed media for 15 min before treatment with ligand (or no treatment) for 30, 90, or 180 min with either 10 μM dopamine (D2, D2NR, D3, and D4KL) or 100 μM PMA (D3 and D3NR). Concurrent with ligand treatment, 100% and strip plates remained at 4°C. After ligand treatment, plates were washed in PBS, and the remaining cell surface-biotinylated receptors were stripped in 50 mM glutathione, 0.3 mM NaCl, 75 mM NaOH, 1% FBS at 4°C for 30 min. Cells were quenched with Tris-HCl buffer (pH 7.4) and then lysed in immunoprecipitation buffer (IPB), 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris-HCl (pH 7.4), with protease inhibitors (Roche Applied Science). Cleared lysates were immunoprecipitated with anti-FLAG M2 anti-
bodies overnight at 4 °C and incubated for 2 h the following
day with protein G-Sepharose (Invitrogen). Samples were
washed four times in 1 ml of IPB and twice in 1 ml of 10 mM
Tris-HCl (pH 7.4) and deglycosylated by incubation with pep-
tide:N-glycosidase F (New England Biolabs) for 1 h at 37 °C,
resolved by SDS-PAGE, and visualized with streptavidin over-
lay (Vectastain ABC immunoperoxidase reagent, Vector
Laboratories).

**In Vitro Transcription/Translation**

The full-length coding sequence of GASP-1 or the C-termi-
nal region, cGASP-1, was subcloned into the mammalian ex-
pression vector pcDNA3.1 (Invitrogen) as described previ-
ously (6). In vitro translation of these constructs was
performed in the presence of 35S-labeled methionine (Amer-
sham Biosciences) using the T7 RNA polymerase promoter
and a coupled in vitro transcription/translation system (Pro-
mega, Madison, WI).

**GST Fusion Protein Affinity Chromatography**

Oligonucleotides were designed and annealed to generate
GST fusion proteins containing the cytoplasmic receptor tails
for D2 (GST, FNIEFRKAFKLHC), D3 (GST, FNIEFRKAF-
LLSC), and D4 (GST, FRNVFRKALRACC), cloned into
pGEX-4T1 vector (GE Healthcare). The mutations for D2NR,
D3NR, and D4KL were introduced using oligonucleotide-
mediated site-directed mutagenesis. GST fusion proteins
were prepared as described previously (6). The GST fusion
protein loads of individual constructs were determined before
the GST pulldown experiment and confirmed by Coomassie
stain of the gel. For affinity determination of in vitro trans-
lated GASP-1 on the different receptor tails, 30 μl of the GST

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**FIGURE 1. Endocytosis of the D2, D3, and D4 receptors.** A, HEK293 cells stably expressing N-terminally FLAG-tagged D2, D3, or D4 were incubated with anti-
FLAG antibody and then were either left untreated or treated with 10 μM dopamine, 10 μM quinpirole, or 100 nM PMA for 45 min. Cells were then fixed, per-
meabilized, and incubated with Alexa Fluor® 488-conjugated anti-mouse antibody. Shown are representative confocal images. B–D, endocytosis of the D2-
like receptors at 10, 20, 45, and 90 min was quantified using flow cytometry (see “Materials and Methods”). D2 and D4 receptors endocytosed in response to
dopamine (B) and quinpirole (C), whereas D3 receptors endocytosed only in response to PMA (D). Data are represented as the mean of at least four inde-
pendent experiments performed in duplicate analyzed using one-way ANOVA with Bonferroni t test. ***, p ≤ 0.001, D2, D3, and D4 compared with un-
treated controls.
fusion protein-loaded resins (50% (v/v) suspensions) were pre-blocked in binding buffer (20 mM Hepes (pH 7.4), 100 mM KCl, 5 mM MgCl₂, 0.1% Triton X-100) with 10 mg/ml bovine serum albumin (BSA) for 30 min at room temperature. In vitro translated, [35S]methionine-labeled GASP proteins were incubated with the GST fusion protein resins in binding buffer for 1 h at room temperature. Resins were washed four times with binding buffer and eluted with SDS-PAGE sample buffer for analysis by SDS-PAGE. GASP-1 binding was analyzed by autoradiography.

Co-immunoprecipitation from HEK293 Cells

HEK293 cells stably expressing D₂, D₃, or D₄, or no heterologous receptor were grown to confluency and washed twice with PBS, and lysates were prepared as described previously (6) in 0.1% Triton X-100, 150 mM NaCl, 25 mM KCl, 10 mM Tris-HCl (pH 7.4), with protease inhibitors. Cleared lysate was incubated with M2 anti-FLAG antibody (Sigma) overnight at 4 °C. Samples were then incubated with protein G-Sepharose (Invitrogen) for 2 h at 4 °C, washed extensively, and deglycosylated with peptide-N-glycosidase F for 1 h. Precipitates were resolved on a 4–20% gradient Tris-HCl precast gel (Bio-Rad) and transferred to nitrocellulose, and the blots were cut below the 75-kDa marker band to separately immunoblot for either receptor (membrane below 75 kDa) or GASP (membrane 75 kDa and above). GASP blots were incubated for 1 h with rabbit anti-GASP antibody (1:1,000) and then visualized with streptavidin overlay (Vector ABC reagents, Vector Laboratories) and ECL plus. Lysate samples were probed for GASP and equal protein loading was determined by incubation with antibody against β-actin (mouse anti-β-actin, 1:4,000 for 1 h at room temperature followed by 1 h with HRP-conjugated anti-mouse antibody (New England Biolabs), 1:4,000 for 1 h at room temperature).

Knockdown of GASP-1

HEK293 cells stably expressing N-terminal FLAG-tagged D₂ or D₃ receptors were transfected with either scrambled shRNA (shScr) or one of two GASP-1 shRNA plasmids (shGASP-1a or shGASP-1b). Both scrambled and shGASP-1a constructs were generated by Santa Cruz Biotechnology. For shGASP-1b constructs, GASP-1-specific oligonucleotides designed according to the Addgene protocol (forward primer 5'-CCGGGAGTACCTTGACTGACGATCAGCTATCCTCAGAGCGGATCTTTTTTTG-3' and reverse primer 5'-AAGTACCTGTGAGAGACTCAGAGCTTATCCTCAGAGCGGATCTTTTTTGAATT-3') were annealed and ligated into the vector pLKO.1 (Addgene). For generation of clonal stable cell lines, single colonies were chosen and propagated in the presence of Zeocin- (receptor) and puromycin (shRNA)-containing media, and knockdown of GASP-1 expression was analyzed by immunoblot.

RESULTS

Endocytosis of D₂, D₃, and D₄ Dopamine Receptors—To investigate the endocytic properties of the D₂-like family of dopamine receptors, live cells were incubated with M1 antibody directed against the extracellular FLAG-tagged N termi-
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D4 receptor has been reported to show little endocytosis and has been protected previously (Fig. 1A) from dopamine (DA) or quinpirole (Fig. 1C) (as indicated by increased fluorescence) as early as 20 min but not to PMA even after extended time points (Fig. 1D). Conversely, the $D_3$ receptor showed no significant endocytosis in response to treatment with dopamine or quinpirole even after 90 min but showed robust endocytosis when treated with PMA for 45 min (Fig. 1D).

Pretreatment of $D_2$- or $D_4$-expressing cells with dopamine receptor antagonist (ant) (10 μM raclopride or spiperone, respectively), prevented dopamine (DA)-induced endocytosis (Fig. 2, A, and C, compare DA with ant-DA), although the PKC inhibitor BIM (1 μM) had no effect (Fig. 2, A and C, compare DA with BIM-DA). In contrast, pretreatment of $D_3$-expressing cells with dopamine receptor antagonist (10 μM spiperone) failed to prevent PMA-induced endocytosis (Fig. 2B, compare PMA with ant-PA). Incubation of the $D_2$-expressing cells with the PKC inhibitor BIM, on the other hand, abolished receptor endocytosis induced by PMA (Fig. 2B, compare PMA with BIM-PA).

It has been shown previously that $D_2$ receptors, once internalized, are unable to recycle back to the plasma membrane even in the presence of antagonist (5). However, the post-endocytic fate of the $D_3$ and $D_4$ receptors is unknown. To monitor post-endocytic recycling, we again used a modified version of the flow cytometry assay (see "Materials and Methods"). In this case, loss of fluorescence is indicative of recycling because receptors returning to the surface are stripped of antibody. In agreement with our previous observations, $D_2$ receptors that were endocytosed in response to dopamine failed to recycle to the plasma membrane (Fig. 2D), even in...
the presence of dopamine antagonist (Fig. 2D, compare DA with DA-ant), although D₁ receptors were efficiently recycled (supplemental Fig. 1A). Furthermore, PKC inhibition did not alter D₂ receptor recycling (Fig. 2D, compare DA with DA-BIM). Like the D₂ receptors, D₃ receptors failed to recycle back to the plasma membrane after PMA-mediated endocytosis even when either dopamine receptor antagonist or PKC inhibitor was present (Fig. 2E, compare PMA with PMA-ant and PMA-BIM). The D₃ receptor also failed to recycle after dopamine-mediated endocytosis (Fig. 2F, compare DA with DA-ant). Thus, all three G₁-coupled dopamine receptors fail to recycle after endocytosis, regardless of whether endocytosis is promoted by agonist ligand or PMA.

**D₂ and D₃ Receptors Degrade following Prolonged Agonist Treatment**—We next examined whether the D₂-like receptors that failed to recycle were targeted for degradation. To investigate the stability of the D₂-like receptors following endocytosis, we used the biotin protection/degradation assay that is specifically designed to follow the stability of a pool of receptors endocytosed from the plasma membrane (see “Materials and Methods”). Cells expressing D₂, D₃, or D₄ receptor were biotinylated with thio-cleavable biotin and incubated for 30, 90, or 180 min with either dopamine (DA, 10 μM, D₂, D₃) or PMA (100 nM, D₃), and residual surface biotin was stripped leaving only the internalized protected receptors labeled with biotin. Using this assay, receptors that are degraded show a reduction in the protected pool over time. In agreement with previous reports (5), D₂ receptors were extensively degraded within 90 min of agonist treatment, and after 180 min the signal was no different from no treatment (Fig. 3, A and D). The recycling D₁ receptor remained stable for the entire 180 min in this assay (supplemental Fig. 1B). Like the D₂ receptor, the D₃ receptor was also extensively degraded within 90 min and undetectable by 180 min in the presence of the PKC activator PMA (Fig. 3, B and E). In contrast, even though they are not recycled (Fig. 2F), the pool of D₄ receptors that were endocytosed in response to dopamine remained stable even following prolonged dopamine treatment (Fig. 3, C and F).

Previous work has identified a GPCR-associated sorting protein, GASP-1, that is responsible for targeting some GPCRs to the lysosomal pathway for degradation. Indeed, it has already been shown that D₂ receptors, but not D₁ receptors, bind GASP in vitro and are targeted for degradation both in vitro (5) and in vivo (12). We thus investigated whether GASP-1 played a role in the post-endocytic degradation of the D₃ receptor. We first determined which of the D₂-like receptors bound to GASP-1 in vitro. In agreement with previous reports, the cytoplasmic tail of the D₂ receptor, but not the D₁ receptor bound to the receptor binding domain of GASP-1, cGASP-1, with high affinity (Fig. 4A). In addition, the cytoplasmic tail of the D₃ receptor also bound cGASP with high affinity, consistent with the ability of this receptor to degrade after endocytosis (Fig. 4A). However, the cytoplasmic tail of the D₄ receptor did not bind cGASP (Fig. 4A), consistent with the failure of this receptor to be degraded after endocytosis (Fig. 3, C and F) even though it is not efficiently recycled (Fig. 2F). Thus, the D₄ receptor has a trafficking profile similar to that described for the truncated form of the D₁ receptor, which is neither recycled nor degraded after endocytosis and does not bind GASP (see Fig. 4A, D₁-Tr) (17, 21). We next investigated whether GASP-1 was interacted with full-length D₂, D₃, and D₄ receptors in living cells. GASP-1 (and also GASP-2) co-immunoprecipitated with D₂ and D₃ receptors but not to either D₄-expressing cells or empty human embryonic kidney cells (Fig. 4B, upper panel).

To further assess the involvement of GASP-1 in the down-regulation of the D₃ receptor, cell lines were generated that stably express the D₁ receptor (or the D₃ receptor) and co-express either one of two GASP-1 target-specific shRNAs (shGASP-1a or shGASP1b) or a scrambled (shScr) shRNA. In these cell lines, the shGASP-1a and shGASP-1b efficiently knocked down the expression of GASP-1 leaving GASP-2 unaffected, although the scrambled shRNA had no influence on GASP expression and served as the control (see Fig. 5, A for D₂ and B for D₃). Using these stable cell lines and the biotin protection degradation assay, we assessed the post-endocytic stability of the D₂ and D₃ receptors when GASP-1 expression was knocked down. Overexpression of shGASP-1a and shGASP-1b (Fig. 5C, middle and lower panels), but not the shScr (Fig. 5C, upper panels), prevented post-endocytic degradation of the D₂ receptor. In addition, although GASP-1 knockdown did not completely inhibit D₃ receptor degradation, it signifi-
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FIGURE 5. GASP-1 knockdown reduces D₂ and D₃ receptor degradation. Knockdown of GASP-1 by shRNA directed against GASP-1 (shGASP-1a and shGASP-1b) or a scrambled shRNA (shScr) in D₂ (A) and D₃ (B)-expressing cells. HEK293 cells stably expressing either D₂ (C) or D₃ (D) plus shScr (upper panel), shGASP-1a (middle panel), or shGASP-1b (lower panel) were surface-biotinylated and incubated in the absence or presence of either DA (10 μM) or PMA (100 nM) for 30, 90, or 180 min. The fate of the surface-labeled endocytosed receptors was assessed after immunoprecipitation of receptor using anti-FLAG (D₂) or anti-HA (D₃) antibodies followed by SDS-PAGE and streptavidin overlay. 100% lane shows total surface receptor labeled. Strip lane shows efficiency of thio cleavage. A representative immunoblot is shown. NT, no treatment.

FIGURE 6. Mapping of the GASP-1 binding domain in D₂ and D₃ receptor cytoplasmic tails. A, alignments of the cytoplasmic tails of the D₂, D₃, and D₄ receptors are highly homologous (Fig. 6A). Nevertheless, because the D₃ receptor did not bind GASP and was not degraded after endocytosis, we hypothesized that either the D₂ and D₃ receptors contain a GASP-1 binding domain absent in the D₄ receptor or that the D₄ receptor contains domains that inhibit GASP-1 binding. To examine these possibilities, we generated GST fusion proteins of several mutant receptor cytoplasmic tails and assessed GASP-1 binding in vitro. Mutation of Lys435 and Leu438 in the D₂ receptor tail to asparagine and arginine, respectively, to resemble the D₄ receptor (Fig. 6A) generated a mutant GST-D₂NR that no longer bound GASP-1 (Fig. 6B). Conversely, substitution of Asn409 and Arg412 in the D₄ receptor tail with lysine and leucine to resemble the D₂ and D₃ receptors generated a mutant GST-D₄KL that now bound to GASP-1 (Fig. 6B).
Endocytosis and Post-endocytic Stability of GASP-1 Binding Mutants—We generated full-length FLAG-tagged cDNAs of the D2NR, D3NR, and D4KL mutant receptors and generated cells lines stably expressing these receptors to assess whether the mutations affected endocytosis or degradation. There were no differences in the endocytic properties of the D2NR, D3NR, and D4KL mutant receptors and generated cells lines stably expressing these receptors to assess whether the mutations affected endocytosis or degradation. There were no differences in the endocytic properties of the D2NR, D3NR, and D4KL receptor mutant as assessed by immunofluorescence assay or quantified by flow cytometry (Fig. 7, A–D). However, consistent with the importance of GASP-1 binding for degradation of the D2 and D3 receptor, both the D2NR (Fig. 8, A and D) and D3NR (Fig. 8, B and E) receptor were substantially more stable after endocytosis as assessed by biotin protection/degradation assay (compared with Fig. 3). Disrupting GASP-1 binding almost completely stabilized the D2 receptor (Fig. 8, A and D) and delayed degradation of the D3 receptor (Fig. 8, B and E), a result consistent with the effects of GASP-1 knockdown on the degradation of wild type D2 and D3 receptors (Fig. 5, C and D). Conversely, the D4KL receptor was now degraded after endocytosis (Fig. 8, C and E), suggesting facilitating GASP-1 binding to the D4 is sufficient to promote post-endocytic degradation of the receptor.

We next examined whether the D2NR and D3NR that were not degraded were able to recycle. Somewhat surprisingly, disruption of GASP-1 binding was not sufficient to promote recycling of the receptors (Fig. 9, A–C). These results suggest that the D2 and D3 receptors do not recycle by default even when they are not targeted for degradation. These results are consistent with the trafficking profile of the D3 receptor, which, although it does not bind GASP and degrade, is not recycled after endocytosis (Figs. 2F and 3, C and F).

DISCUSSION

Here, we elucidate the endocytic and post-endocytic trafficking properties of the members of the D2-like class of dopa-
mine receptors. We identify a specific GASP-1 interaction motif present in the D2 and D3 receptor C-tails, but not found in D4, rendering its subsequent resistance to dopamine-mediated down-regulation. Although GASP-1 can interact with a number of GPCRs, the amino acids identified in this study are not conserved among these other receptors. This suggests the interaction motif is likely to be more complicated than a simple primary amino acid sequence possibly involving tertiary structure. Surprisingly, despite the ability of the D3 receptor to interact with GASP-1, this receptor was also resistant to dopamine-mediated down-regulation. This can be attributed to a failure of D3 to endocytose in response to dopamine. D3 down-regulation mediated by PKC activation, however, was sensitive to GASP-1 expression.

The regulation of post-endocytic sorting of the dopamine receptors, and differential post-endocytic sorting in particular, could have profound implications in neuropsychiatric disease. For example, drugs of abuse cause a reduction in the availability of D2-like receptors (22–26), and D2-like receptors also appear to be down-regulated relative to D3-like receptors in other neuropsychiatric disorders, including schizophrenia (27, 28). Previous work in our laboratory has shown that D2 down-regulation in response to drugs of abuse is mediated by interaction with GASP-1 (5). Here, we show that, like the D2 receptor, the D3 receptor also binds GASP-1 and is targeted for degradation after endocytosis, although the D4 is not targeted for degradation. By extension, in the presence of only dopamine (and not heterologous activation of PKC), one would expect selective degradation of D2 but not D3 or D4.

Conversely, activation of PKC would produce selective down-regulation of D3 but not D2 or D4 receptors.

Many Gq-coupled GPCRs and tyrosine kinase receptors influence PKC activity through diacylglycerol accumulation. Such activation could indirectly lead to the selective removal of D3 receptors from the plasma membrane of neurons and thereby decrease signal transduction from this receptor. Specifically, if PKC activation leads to endocytosis and subsequent degradation of the D3 receptor, then there will be a concomitant reduction in all the signaling associated with D3 function. Importantly, dopamine receptor antagonist failed to prevent PKC-mediated D3 endocytosis. In effect, this provides a means whereby D3 receptors can be removed from the plasma membrane without prior activation by dopamine (or exogenous ligand). Thus, one could expect completely different patterns of receptor expression/stability dependent on dopamine tone, PKC activity, and the presence of exogenous and endogenous dopaminergic drugs, both agonists and antagonists.

Heterologous endocytosis and post-endocytic degradation of D3 receptors by GASP-1 could also have profound implications for the treatment of neuropsychiatric disorders because alterations in the levels of the D3 receptor have been implicated in several different pathological states. For example, a reduction in D3 receptor-binding sites has been reported in the striatum and the substantia nigra of monkeys following 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine treatment (29), a model of Parkinson disease. Also, positron emission tomography studies in Parkinson disease patients have revealed a...
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reduction in (+)-[^14]C]propyl-hexahydronaphtho-oxacin binding in the brain regions associated with high D₃ receptor expression (30).

The loss of dopaminergic neurons in the substantia nigra, which contributes to the onset of Parkinson disease, is mediated by the persistent activation of PKCδ (31). Administration of the PKCδ inhibitor, rottlerin, prevents neuronal cell loss in primary cultures of 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-treated mice and provides neuroprotection from 1-methyl-4-phenyl-1,2,3,6-tetrahydropyridine-induced locomotion (31). Intriguingly, PKCδ is the isofrom of PKC reported to be responsible for heterologous D₃ receptor endo-

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