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<td>Complete List of Authors</td>
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Selectivity and Anti-Parkinson’s Potential of Thiadiazolidinone RGS4 inhibitors

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Keywords: Regulator of G-protein signaling, Parkinson’s Disease, PPI, RGS, Protein-Protein interaction, thiadiazolidinone
Abstract: Many current therapies target G protein coupled receptors (GPCR), transporters, or ion channels. In addition to directly targeting these proteins, disrupting the protein-protein interactions that localize or regulate their function could enhance selectivity and provide unique pharmacologic actions. Regulators of G protein Signaling (RGS) proteins, especially RGS4, play significant roles in epilepsy and Parkinson’s disease. Thiadiazolidinone (TDZD) inhibitors of RGS4 are nanomolar potency blockers of the biochemical actions of RGS4 in vitro. Here we demonstrate substantial selectivity (8- to >5000-fold) of CCG-203769 for RGS4 over other RGS proteins. It is also 300-fold selective for RGS4 over GSK-3β, another target of this class of chemical scaffolds. It does not inhibit the cysteine protease papain at 100 µM. CCG-203769 enhances Gαq-dependent cellular Ca++ signaling in an RGS4-dependent manner. TDZD inhibitors also enhance Gαi-dependent δ-OR inhibition of cAMP production in SH-SY-5Y cells which express endogenous receptors and RGS4. Importantly, CCG-203769 potentiates the known RGS4 mechanism of Gαi-dependent muscarinic bradycardia in vivo. Furthermore, it reverses raclopride-induced akinesia and bradykinesia in mice, a model of some aspects of the movement disorder in Parkinson’s disease. A broad assessment of compound effects revealed minimal off-target effects at concentrations necessary for cellular RGS4 inhibition. These results expand our understanding of the mechanism and specificity of TDZD RGS inhibitors and support the potential for therapeutic targeting of RGS proteins in Parkinson’s disease and other neural disorders.
**Introduction:** G protein-coupled receptors (GPCRs) remain key drug targets for therapeutic use\(^{(1-3)}\). The recent crystal structures of numerous GPCRs have improved the ability to develop subtype-selective ligands\(^{(4, 5)}\). Also, allosteric modulators of GPCRs have provided new degrees of control of signaling with the potential for more refined therapeutic agents\(^{(2, 6)}\). In some cases, however, a single GPCR may mediate both desired and unwanted actions. This is especially true for agonists (e.g. clonidine and adenosine) active in the central nervous system (CNS) where side effects are major limitations to use. It would be highly advantageous to have a mechanism to improve the selectivity of existing GPCR ligands. The downstream actions of GPCRs are modulated by the family of Regulator of G protein Signaling proteins (RGS proteins). Many of the 20 members of this family are abundantly expressed in the brain\(^{(7-13)}\). Consequently, they have been proposed as intriguing CNS drug targets\(^{(7-13)}\). RGS proteins act intracellularly by speeding the deactivation of G\(\alpha_i\) and G\(\alpha_q\) family G proteins. Inhibition of RGS proteins would thus be expected to potentiate the actions of GPCR agonists. Furthermore, the differential tissue distribution of RGS proteins could provide a novel way to selectively enhance GPCR agonist action in a tissue-specific or neuron-subtype specific manner.

RGS4, in particular, assembles in a complex with A1 adenosine receptors and has been proposed to suppress the anticonvulsant action of adenosine\(^{(14)}\). Suppression of RGS4 also mediates long-term depression by dopamine through D2 receptors in medium spiny neurons\(^{(15)}\) and RGS4 knockout mice have reduced impairment in 6-OHDA mouse models\(^{(15)}\). Furthermore, loss of RGS4 appears to suppress abnormal involuntary movements in mice which represents a model for DOPA-induced dyskinesias\(^{(16)}\). According to the recent model\(^{(15)}\),
a key action of dopamine is to suppress RGS4 function. Thus, direct, chemical inhibition of the hyperactive RGS4 would eliminate the need for dopamine and could provide a novel dopamine-independent approach to Parkinson’s disease. Consequently, therapeutic targeting of RGS4 could be of great interest.

It has been challenging to effectively disrupt RGS4/Gα and other protein-protein interactions, especially in the CNS, but progress is being made\(^{(17-19)}\). Indeed, the emerging consensus in the field is that effective inhibitors of protein-protein interactions (iPPIs) may not meet the “standard” pharmaceutical criteria for drug-like molecules\(^{(20, 21)}\). In spite of this, numerous compounds with MW > 500\(^{(20-22)}\) and several covalent protein modifiers\(^{(23, 24)}\) are now in clinical trials. We recently described a series of nanomolar-potency TDZD inhibitors of RGS proteins\(^{(25, 26)}\) that can disrupt RGS binding to Gα subunits in HEK-293 cells. As is seen for some other potent iPPIs, they covalently modify cysteine residues in RGS proteins\(^{(25)}\) but, surprisingly, they have high specificity for RGS4 vs. other cysteine-dependent proteins such as some kinases and cysteine proteases.

In this study, we assess the specificity of TDZD RGS4 inhibitors and demonstrate cellular activity on RGS4 actions on Gαq-mediated cellular Ca\(^{++}\) signaling and delta-opioid regulation of adenylyl cyclase. Furthermore, CCG-203769 shows in vivo activity on muscarinic control of heart rate as well as in reversing raclopride-induced Parkinson’s-like effects. These results represent the first demonstration of in vivo effects of TDZD RGS4 inhibitors and implicate a potential role in Parkinson’s disease and other neuropsychiatric disorders.
Results and Discussion:

Identifying selective small molecule inhibitors of protein-protein interactions with activity in the central nervous system remains a key challenge to the expansion of the current therapeutic repertoire beyond receptors, transporters, and kinases. Our previously described RGS4 inhibitor CCG-203769 blocks the RGS4-Gαo protein-protein interaction in vitro with an IC$_{50}$ value of 17 nM in the Flow Cytometry Protein Interaction Assay (FCPIA, Figure 1A). More importantly, it also displays dramatic selectivity for RGS4 over other RGS proteins (Table 1). The closely related RGS8 is very weakly inhibited (IC$_{50}$ >60 µM) providing >4500-fold selectivity for RGS4 (Figure 1A, Table 1). This difference is greater than that seen for our earlier TDZD inhibitor, CCG-50014, which was only ~350-fold selective for RGS4 over RGS8$^{(25, 26)}$. CCG-203769 inhibits RGS19 with an IC$_{50}$ of 140 nM (8-fold selective for RGS4) and 6 µM for RGS16 (350-fold). As with previously reported RGS4 inhibitors, CCG-203769 does not inhibit RGS7, which lacks cysteines in the RGS domain (Table 1).

In addition to inhibiting RGS4/Gα binding, CCG-203769 also blocks the GTPase accelerating protein (GAP) activity of RGS4. In single-turnover and steady-state GTPase experiments with Gαo and Gα1i, the rate of GTP hydrolysis is strongly stimulated by RGS4 and this effect is inhibited by CCG-203769 with an IC$_{50}$ < 1 µM (Figure. 1B, C). As previously shown for the related TDZD compound CCG-50014$^{(25)}$, our new compound is irreversible in non-reducing buffers (Figure 1D). This, as well as the complete lack of effect on RGS7, is consistent with CCG-203769 having the same thiol-modification mechanism as CCG-50014$^{(25)}$.

Beyond RGS protein specificity, CCG-203769 is highly selective for RGS4 vs other thiol-dependent proteins. To assess effects on a protein with a catalytic cysteine residue, we
tested CCG-203769 for inhibition of the cysteine protease papain. The general thiol-reactive reagent iodoacetamide strongly inhibited papain-mediated hydrolysis of the fluorescent casein substrate at 30 µM. CCG-203769 at the same concentration had no effect on the protease activity of papain (Figure 1E). Related TDZD compounds are inhibitors of glycogen synthase kinase 3β\(^{(27-29)}\). Indeed, they are currently being evaluated in clinical trials for depression and Alzheimer’s disease based on this proposed mechanism. Using a radiometric assay, we show that CCG-203769 inhibits GSK-3β with an IC\(_{50}\) value of 5 µM (Figure 1F). This represents 300-fold selectivity of our compound for RGS4 vs. GSK-3β.

The nanomolar potency on RGS4 in vitro translates to respectable cellular activity. We first examined the RGS4/G\(_{α_o}\) interaction in HEK293 cells. RGS4 is typically present in the cytoplasm but translocates to the membrane when co-expressed with a G\(_α\) subunit as demonstrated previously\(^{(25)}\). Similar to prior results with CCG-50014\(^{(25)}\), CCG-203769 also reverses the G\(_{α_o}\)-induced membrane translocation of GFP-tagged RGS4 (Figure 2). This demonstrates inhibition of the RGS4-G\(_{α_o}\) interaction in cells. The functional consequences of CCG-203769 were further investigated using a controlled system where induced expression of RGS4 suppresses G\(_{α_q}\)-mediated Ca\(^{++}\) signaling activated by the M3 muscarinic receptor. Doxycycline treatment induces RGS4 expression (Figure S1A) reducing the Ca\(^{++}\) transient induced by 1 nM carbachol by 63% (Figures S1B&C & 3A). At concentrations of 1 and 3 µM, CCG-203769 has no effect on intracellular Ca\(^{++}\) responses to carbachol stimulation of the M3 muscarinic receptor in the absence of RGS4. However, at the same concentrations, it partially reverses the RGS4-mediated muscarinic suppression. At higher concentrations, there may be an off-target effect as the compound appears to inhibit the Ca\(^{++}\) transient induced by
carbachol. This is similar to previously observed, though more dramatic, effects of CCG-50014 to disrupt Ca\(^{++}\) handling in HEK cells\(^{(26)}\).

We used SH-SY-5Y neuroblastoma cells to study endogenously expressed RGS and opioid receptors. Wang et al\(^{(30)}\) had previously shown that RGS4 specifically regulates delta-opioid receptor (DOP) signaling while having little to no effect on mu-opioid receptor (MOP) signaling. CCG-50014 significantly potentiates SNC-80 effects on cAMP accumulation through DOP (Figure 3C). Consistent with the previously determined specificity of RGS4, there was no significant effect of the compound on MOP-regulated cAMP levels though a trend toward potentiation of morphine activity was observed. It is not clear if this is due to effects on RGS4 or on other RGS proteins in the SH-SY5Y cells to regulate the MOP signal transduction cascade. These cellular studies demonstrate that CCG-203769 can potentiate RGS4-regulated signaling pathways, regardless of whether they are G\(_{\alpha_o}\) or G\(_{\alpha_q}\)-mediated processes.

Although the TDZD inhibitors have cellular activity, specificity is always a key question regarding compounds with a covalent mechanism of action. As noted above, CCG-203769 has low potency against GSK-3\(\beta\) as well as producing no inhibition of the activity of the thiol-protease papain at 30 \(\mu\)M. We also assessed off-target effects through broad activity profiling at the NIMH PDSP program (University of North Carolina, Chapel Hill)\(^{(31)}\). The compound showed no activity at 10 \(\mu\)M against a series of receptors, transporters, etc (Table 2 and S1). CCG-203769 had modest activity to inhibit ligand binding in membrane preparations for a small subset of the tested systems (\(\alpha2\) adrenergic, D3 dopamine, and opioid receptors). Using the Glowsensor assay\(^{(32, 33)}\) for inhibition of cAMP in cells by these G\(_{\alpha_o}/\alpha_o\)-coupled receptors, the TDZD compounds showed no agonist or antagonist activity at concentrations up to 10 \(\mu\)M in cells (Table 2).
A critical step toward translation of new therapeutics is the demonstration of *in vivo* activity as well as avoidance of off-target effects. RGS4 is expressed in the sino-atrial node where it functions to regulate heart rate. Accordingly, RGS4 knockout mice show enhanced carbachol-induced bradycardia\(^{34}\). To determine whether this genetic disruption of RGS4 function could be replicated pharmacologically, we tested CCG-203769 for effects on carbachol-mediated bradycardia in conscious, unrestrained rats. Carbachol (0.1 mg/kg, IP) produces a modest decrease in heart rate (Figure 4) compared to a saline vehicle control. CCG-203769 (10 mg/kg, IV) had no significant effect upon heart rate when given alone (Figure 4). However, CCG-203769, administered immediately prior to carbachol, significantly potentiated the bradycardic effect (Figure 4, \(p<0.05\), 2-way ANOVA).

Given the functional role of RGS4 in Parkinson’s disease models\(^{15}\), we tested CCG-203769 in a pharmacologic model of D2 antagonist-induced bradykinesia. Raclopride administration in rats causes increased hang time in the bar test (Figure 5A) which was rapidly reversed by doses of CCG-203769 ranging from 0.1-10 mg/kg. The lowest dose, 0.01 mg/kg had no effect while 0.1 mg/kg produced a sub-maximal effect. The higher doses, 1 and 10 mg/kg produced equivalent effects. Similarly, the raclopride-induced paw drag in mice (as indicated by reduced numbers of steps), was reversed by 0.1-10 mg/kg CCG-203769 (Figure 5B).

In this report, we have characterized the first TDZD RGS inhibitor with physiological activity. CCG-203769, has nanomolar potency against RGS4 and RGS19 *in vitro* and is almost 5000-fold selective for RGS4 over the closely related RGS8, making CCG-203769 the most selective RGS4 inhibitor identified to date. As expected, cellular activity is less potent with half-maximal effects occurring in the 1-3 \(\mu\)M range in cells. The inducible RGS4 system provided
us with strong evidence of actions on RGS4 rather than other mechanisms that could also result in potentiation of the M3 muscarinic signaling response (e.g. M3 muscarinic allosteric modulation or effects on cellular Ca\(^{++}\) handling). There are, however, additional actions of CCG-203769 at higher concentrations that are not fully understood.

Despite its cysteine-reactive mechanism of action, CCG-203769 selectively targets RGS4 over the known TDZD target GSK3β, the cysteine protease papain, and a large number of receptors and ion channels. The mechanism underlying the RGS selectivity of this compound over other cysteine-dependent processes has yet to be fully elucidated, however the available data allow for a potential explanation. We previously showed that the covalent modification of RGS4 was through opening of the thiadiazolidinone ring to form a disulfide bond with cysteine residues on the protein\(^{(25, 26)}\). This reactivity would presumably provide a non-specific mechanism of action. However, dynamic modeling studies \(^{(35)}\) indicate that the target cysteines on RGS4 are buried in a hydrophobic environment that is only transiently accessible to solvent. This suggests that the cysteines in RGS4 may be in a unique environment that facilities the high potency of CCG-203769. Also, in the reducing environment of the cell, the disulfide-bonded compound interaction is likely reversible, as shown \textit{in vitro} with addition of reducing agents\(^{(25)}\). Further studies are required to confirm these hypotheses.

In this report, we show that a TDZD RGS4 inhibitor, despite a covalent mechanism of action, is very selective for RGS4 over other RGS proteins as well over other sulfhydryl-dependent enzymes and a wide range of CNS receptors. Furthermore, it has \textit{in vivo} activity on RGS4-dependent control of heart rate and produces beneficial effects in a D2 antagonist-mediated akinesia and bradykinesia. In conjunction with the genetic evidence that RGS4 knockout mice have reduced defects after 6-hydroxy dopamine injury\(^{(15)}\), these results suggest
that CCG-203769 and other related RGS4 inhibitors may have potential as novel antiparkinsonian therapies.
Materials and Methods:
Sources: Compounds were obtained from sources previously reported for carbachol\(^{(36)}\), doxycycline, \([\gamma^{32}P]\) GTP\(^{(25)}\), forskolin, morphine, and SNC80\(^{(30)}\). Raclopride was purchased from Tocris Bioscience (Bristol, UK). CCG-203769 and CCG-50014 were synthesized as previously described\(^{(26)}\). Fluo4 NW kits were obtained from (InVitrogen, Carlsbad, CA). RGS proteins and G\(\alpha\) subunits were expressed, purified, and labelled as previously described\(^{(37)}\). GSK-3\(\beta\) was obtained from Sigma (catalog #G1663). HEK-293T cells expressing the M3 muscarinic receptor and inducible RGS4 (M3-R4 cells) were described previously\(^{(38)}\).

RGS/G\(\alpha\) binding studies: The binding of biotinylated RGS proteins to fluorescently labeled G\(\alpha_o\) and the reversibility of RGS4 inhibitor compound actions were measured by Flow Cytometry Protein Interaction Assay (FCPIA) as previously described\(^{(37, 39, 40)}\).

Single-turnover GAP assay: Single turnover GTPase acceleration experiments were performed as previously described using purified his\(_6\)-tagged G\(\alpha_o\)\(^{(25)}\).

Steady-state GAP assay: Steady-state hydrolysis of unlabeled GTP was measured using malachite green in a receptor-independent assay utilizing a mutant G\(\alpha_i\) (R178M, A326S)\(^{(38, 41)}\). These mutations facilitate the release of GDP from the enzyme making the GTP hydrolysis step rate-limiting\(^{(41)}\). GTP hydrolysis was measured by mixing 6 \(\mu\)M mutant G\(\alpha_i\) with 300 \(\mu\)M GTP in 100 \(\mu\)L in 96-well plates in the presence or absence of 200 nM RGS4 and CCG-203769 or DMSO (vehicle control). All assay components were diluted in a buffer comprising 50 mM HEPES at pH 7.4, 100 mM NaCl, 0.01% Lubrol, 5 mM MgCl\(_2\), and 10 \(\mu\)g/mL BSA. The reaction was allowed to proceed for 2 hours at room temperature and then was quenched with 60 \(\mu\)L of an HCl/malachite green dye solution. Immediately after addition of malachite green, 10 \(\mu\)L of 32% w/v sodium citrate was added as a colorimetric stabilizer, followed by incubation at room temperature for 20 minutes. Released inorganic phosphate was measured as an
increase in absorbance ($A_{630}$) from the complex of phosphate with malachite green$^{(42)}$. Background control samples lacking Gα were used to determine the rate of non-enzymatic GTP hydrolysis which was subtracted.

Papain inhibition: Experiments were performed using fluorescein-isothiocyanate-labeled casein as the fluorescent substrate as previously described$^{(25)}$.

GSK-3β inhibition: Purified GSK-3β (0.5 U, Sigma G1663) was incubated with the indicated concentration of compound for 15 minutes at room temperature. Substrate peptide (300 nM, Enzo #BML-P151) was added along with 1 mM [$\gamma^{32}$P] ATP. After a 15 minute incubation at 30°C, the reaction was quenched by addition of 4 ml of 1% phosphoric acid. The amount of phosphorylated peptide was determined by filtration on P81 phosphocellulose filters which were washed three times with 4 ml of 1% phosphoric acid to remove unincorporated radioactivity. Incorporated radionuclide was quantified by liquid scintillation counting.

Opioid inhibition of cellular cAMP: SH-SY5Y cells were grown in DMEM containing 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 µg/ml) under 5% CO$_2$ at 37°C. Cells were plated into 24-well plates to reach ~ 90% confluency on the day of assay and washed once with fresh serum-free medium. Medium was replaced with 1 mM IBMX (3-isobutyl-1-methylxanthine) in serum-free medium for 15 minutes at 37°C, and then changed to medium containing 1 mM IBMX, 30 µM forskolin, and 100 nM of either morphine or SNC80 with or without test compound for 5 min at 37 °C. Reactions were stopped by replacing the medium with ice-cold 3% perchloric acid and samples were kept at 4 °C for at least 30 minutes. An aliquot (0.4 ml) from each sample was removed, neutralized with 0.08 ml of 2.5 M KHCO$_3$, vortexed, and centrifuged at 15,000 x g for 1 minute to pellet the precipitates. Accumulated cAMP in a 10-15 µl aliquot of the supernatant from each sample was measured
by radioimmunoassay following the manufacturer’s instructions (cAMP radioimmunoassay kit, GE Healthcare, Piscataway, NJ). Data are from four separate experiments, each carried out in duplicate and calculated as percent inhibition. The basal cAMP accumulation with forskolin alone with or without CCG-50014 did not differ.

Calcium signaling transients: The M3-R4 cell line with regulated expression of RGS4\(^{(38)}\) was based upon the HEK-293 Flp-In T-Rex cell line (Invitrogen, Carlsbad, CA). It stably expresses the muscarinic M3 receptor and has human RGS4 (stabilized C2S mutant, C-terminal HA tagged) expression under doxycycline control. Cells were maintained in DMEM supplemented with 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 µg/ml) under 5% CO\(_2\) at 37°C. For experiments, cells were split into 96-well black, clear bottom, poly-D-lysine coated microtiter plates (Nunc, Cat. # 152037) at a density of 20,000 cells/well in DMEM containing 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 µg/ml). RGS4 expression was induced by supplementing the medium with 1 µg/mL doxycycline for 24-48 hours before experimentation. Cells were loaded with Fluo-4 No-Wash dye (Invitrogen, Carlsbad, CA) in loading buffer for 30 minutes at 37°C. Compounds were then added and incubated for 30 minutes at room temperature prior to carbachol stimulation. Plates were transferred to a FlexStation 3 plate reader (Molecular Devices, Sunnyvale, CA) and carbachol (1 nM final) was injected into the wells and the fluorescence intensity was measured as a function of time. Peak fluorescence intensity was calculated from a 120 second kinetic measurement as a percent increase above the initial fluorescence during the pre-injection period.

RGS4 membrane localization: Assays were performed as previously described\(^{(25)}\). Briefly, HEK-293T were cells grown to 80-90% confluence in 6-well dishes in DMEM supplemented
with 10% fetal bovine serum and Penicillin (100 units/ml)-Streptomycin (100 µg/ml) under 5% CO₂ at 37°C. RGS and Gα_0 were transiently co-transfected (250 ng of a plasmid encoding full-length human RGS4 with a C-terminal GFP fusion RGS4pDEST47 and 250 ng of pcDNA3.1 or pcDNA3.1 encoding wild-type human Gα_0). Transfected cells were split onto poly-D-lysine coated glass coverslips and cultured for 24-48 hours before live cell imaging. Images were acquired on an Olympus Fluoview 500 confocal microscope with a 60 x 1.40 numerical aperture oil objective. Images were obtained by taking a series of stacks every 0.5 µm through the cell and combined into a composite image. The light source for the fluorescence studies was a 488 nm laser with a 505-525 nm bandpass filter. Images were quantified using NIH ImageJ software version 1.43r.

**Activity Profiling of CCG-203769:** Detailed assay protocols for primary and secondary radioligand binding studies as well as functional cell-based assays can be found on the PDSP website: http://pdsp.med.unc.edu/.

**Carbachol-induced bradycardia:** These studies were reviewed and approved by the University Committee on Use and Care of Animals at the University of Michigan. Under ketamine (90 mg/kg, i.p.) and xylazine (10 mg/kg, i.p.) anesthesia, rats were implanted with indwelling venous catheters (Micro-Renathane tubing, Braintree Scientific Inc., Braintree, MA, USA) and telemetric BP and ECG transmitters (Model C50-PXT, Data Sciences, Transoma Medical, Inc., St. Paul, MN, USA) at the same time under aseptic conditions. Venous catheters were inserted 3 cm into the right or left jugular vein and sutured to the vein and to the surrounding tissue at 3-4 points to secure catheter placement. The remaining tubing (approximately 9-12 cm) was threaded subcutaneously to a dorsal incision and held in place by suture to musculature directly below the incision. Telemeters were implanted subcutaneously in the rat and secured
to the abdominal wall. The catheter extending from the base of the transmitter was placed 3 cm into the left femoral artery. Electrodes from the bottom of the transmitter were threaded subcutaneously and one was sutured to the muscle above the xiphoid process and the other was sutured to the right of the clavicle. All rats were singly housed and allowed at least 7 days to recover before testing.

The telemetry system consisted of battery-operated transmitters and receivers (Data Sciences, TransomaMedical, St. Paul, MN, USA). Mean arterial pressure (MAP) and heart rate (beats per min, bpm) were acquired using Dataquest A.R.T. 3.01, collected every 10 sec and then averaged over 1 minute periods. A rat’s home cage was placed in the receiver at least one hour prior to testing to allow for habituation. CCG-203769 is an oil and was solublized in sterile saline by vigorous vortexing. All compounds were administered in vivo in a volume of 1 ml/kg by routes of administration indicated above. After habituation, rats received CCG-203769 or saline (by i.v. infusion through the indwelling venous catheter over 30 sec) while freely moving in their homecage. One minute later, saline or 0.1 mg/kg carbachol (i.p.) was administered. Before and after i.v. infusions, catheters were flushed with approximately 0.5 ml of heparinized saline (50 U/ml) to check catheter patency and flush treatments from dead space in catheter. Following all experiments, rats were euthanized by i.v. pentobarbital (150 mg/kg) to ensure catheter patency. Statistical significance was evaluated by 2-way ANOVA with a significance cut-off of 0.05.

Raclopride-induced movement suppression: These experimental protocols were approved by the Italian Ministry of Health (license n. 171/2010-B) and Ethical Committee of the University of Ferrara Young male (20-25 g; 8-9 weeks) C57BL/6J mice, were purchased from Harlan Italy (S. Pietro al Natisone, Italy) and were housed with free access to food and water with a 12-h
light/dark cycle with lights on between 07:00 and 19:00. Prior to pharmacological testing, mice were handled for 1 week by the same operator to reduce stress, and trained daily for a week on the behavioral tests until their motor performance became reproducible. On the day of experiment drugs were administered systemically (i.p.); CCG-203769 was administered 30 min after raclopride.

Motor activity was evaluated by means of different behavioral tests (bar and drag) specific for different motor abilities, as previously described\(^{43, 44}\). The different tests are useful to evaluate motor functions under static or dynamic conditions. Akinesia appears as an abnormal absence or poverty of movements, that is associated with loss of the ability to move the forepaw when placed on blocks (bar test). Bradykinesia is slowness of movement with difficulties of adjusting in response to backwards dragging (drag test). The tests were repeated in a fixed sequence (bar and drag test) before (control session) and after (30 minutes) raclopride injection, then 20 and 90 minutes after CCG-203769 injection.

The bar test or catalepsy test\(^{45}\), measures the ability of the animal to respond to an externally imposed static posture. Each mouse was placed gently on a table and the right and left forepaws were placed alternately on blocks of increasing heights (1.5, 3 and 6 cm). The immobility time (in seconds) on the blocks was recorded (cut-off time 20 seconds per step, 60 seconds maximum). Time was recorded as total time spent on the blocks. The drag test is a modification of the “wheelbarrow” test\(^{46}\). Each mouse was gently lifted by the tail (allowing the forepaws on the table) and dragged backwards at a constant speed (about 20 cm/sec) for a fixed distance (100 cm). The number of touches made by each forepaw was counted by two separate observers (mean between the two forepaws).
Data are expressed as means ± SEM of n determinations per group. Statistical analysis was performed using one-way repeated measures (RM) ANOVA followed by the Newman-Keuls test. P values <0.05 were considered to be statistically significant. Both raclopride and CCG-203769 were freshly dissolved in the vehicle just prior to use.
Author Information
Levi Blazer, Andrew Storaska, and Sue Wade designed and performed the biochemical and cell-based assays. Qin Wang and John Traynor designed the opioid functional studies which were performed by Qin Wang. Emily Jutkiewicz and Levi Blazer designed and performed the studies on compound effects on muscarinic bradycardia. Emma Turner and Stephen Husbands designed and synthesized CCG-203769. Mariangela Calcagno and Michele Morari designed and Mariangela Calcagno performed the Parkinson’s model studies. Xi-Ping Huang designed and performed the target screening studies at the PDSP. Richard Neubig contributed to the design of the overall project and all individual experiments. He and Levi Blazer wrote the manuscript with text and suggestions contributed by all authors.

Conflicts of Interest
Richard Neubig is founder and owner of Argessin LLC which has licensed rights to TDZD RGS4 inhibitors from the University of Michigan.
Acknowledgements

The authors would like to thank Jian Mei for assistance with the FCPIA studies and Roger Sunahara and John Tesmer for valuable discussions. Funding was provided by NIH RO1 DA023252 (RRN), the Chemical Biology Interface training program NIH T32GM008597 (LLB) and core facilities supported by the Michigan Diabetes Research and Training Center NIH P60 DK020572 and University of Michigan Comprehensive Cancer Center Support Grant NIH P30 CA046592. Receptor binding profiles, and agonist and antagonist functional data was generously provided by the National Institute of Mental Health's Psychoactive Drug Screening Program, Contract # HHSN-271-2013-00017-C (NIMH PDSP). The NIMH PDSP is directed by Bryan L. Roth MD, PhD at the University of North Carolina at Chapel Hill and Project Officer Jamie Driscoll at NIMH, Bethesda MD, USA. For experimental details please refer to the PDSP web site http://pdsp.med.unc.edu/.

Supporting Information Available

A brief characterization of the M3-R4 Flp-in cell line is presented as supporting information for the manuscript. This information is available free of charge via the internet at http://pubs.acs.org/.
Figure Legends

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assessed 20 or 90 minutes after DMSO or CCG-203769. Values are mean +/- SEM with differences from baseline indicated by ** (p < 0.01) and differences from Raclopride + DMSO indicated by ## (p < 0.01).
Table 1. Selectivity of CCG-203769. The binding of RGS proteins to $\alpha_o$ was measured using FCPIA. CCG-203769 inhibited RGS/$\alpha_o$ binding in an RGS-selective manner. Functional data for non-RGS activities are described in the text. Data are presented as mean from three independent experiments performed in duplicate. Fold-selectivity is presented as the ratio of the IC$_{50}$ of CCG-203769 towards a given target versus its IC$_{50}$ against RGS4.

<table>
<thead>
<tr>
<th>RGS Protein</th>
<th>IC$_{50}$ (µM)</th>
<th>Fold Selectivity (RGS4)</th>
</tr>
</thead>
<tbody>
<tr>
<td>RGS4</td>
<td>0.017</td>
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<tr>
<td>RGS19</td>
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<tr>
<td>RGS16</td>
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<tr>
<td>RGS8</td>
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<td>4650</td>
</tr>
<tr>
<td>RGS7</td>
<td>&gt;100</td>
<td>&gt;6000</td>
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<tr>
<td>GSK3β</td>
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</tr>
<tr>
<td>Papain</td>
<td>&gt;100</td>
<td>&gt;6000</td>
</tr>
</tbody>
</table>
Table 2 Specificity analysis of CCG-203769.

The compound was tested for activity in a wide variety of ligand binding or functional assays through the NIH PDSP laboratory. Targets for which activity was found in primary or secondary binding assays were then examined in cell-based functional studies. In the latter, assessment of both agonist and antagonist activity was done. Assay protocols are available on the PDSP web site (https://pdspdb.unc.edu).

<table>
<thead>
<tr>
<th>Target</th>
<th>Biochemical assays</th>
<th>Cellular assays</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Primary Binding (% Inhibition @ 10 µM)</td>
<td>Secondary Binding (IC$_{50}$ nM)</td>
</tr>
<tr>
<td>5-HT1A,B,D,E; 5-HT2A,B,C, 3, 5A, 7 β-AR1,2,3; α1-AR A, B, D D2, D4, H1-4; M1-5; Benzodiazepine; GABA-A; DAT, NET, SERT Sigma1,2</td>
<td>&lt;50%</td>
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<td>5-HT6</td>
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<td>α2A adrenergic</td>
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<tr>
<td>α2B adrenergic</td>
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<tr>
<td>α2C adrenergic</td>
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<tr>
<td>MOR</td>
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<td>2,680</td>
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ND = Not determined.
Literature references


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