Modulation of μ -Opioid Receptor Signaling by RGS19 in SH-SY5Y Cells

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ABSTRACT

Regulator of G-protein signaling protein 19 (RGS19), also known as $G\alpha$ -interacting protein (GAIP), acts as a GTPase accelerating protein for $G\alpha_z$ as well as $G\alpha_i/o$ subunits. Interactions with GAIP-interacting protein N-terminus and GAIP-interacting protein C-terminus (GIPC) link RGS19 to a variety of intracellular proteins. Here we show that RGS19 is abundantly expressed in human neuroblastoma SH-SY5Y cells that also express μ - and δ -opioid receptors (MORs and DORs, respectively) and nociceptin receptors (NOPRs). Lentiviral delivery of short hairpin RNA specifically targeted to RGS19 reduced RGS19 protein levels by 69%, with a similar reduction in GIPC. In RGS19-depleted cells, there was an increase in the ability of MOR (morphine) but not of DOR [(4-[(R)-[(2S,5R)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-N,N-diethylbenzamide (SNC80)] or NOPR (nociceptin) agonists to inhibit forskolin-stimulated adenylyl cyclase and increase mitogen-activated protein kinase (MAPK) activity. Overnight treatment with either

MOR [D-Ala, N-Me-Phe, Gly-ol⁵-enkephalin (DAMGO) or morphine] or DOR (D-Pen⁵-enkephalin or SNC80) agonists increased RGS19 and GIPC protein levels in a time- and concentration-dependent manner. The MOR-induced increase in RGS19 protein was prevented by pretreatment with pertussis toxin or the opioid antagonist naloxone. Protein kinase C (PKC) activation alone increased the level of RGS19 and inhibitors of PKC 5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile and mitogen-activated protein kinase kinase 1 2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one, but not protein kinase A (H89), completely blocked DAMGO-induced RGS19 protein accumulation. The findings show that RGS19 and GIPC are jointly regulated, that RGS19 is a GTPase accelerating protein for MOR with selectivity over DOR and NOPR, and that chronic MOR or DOR agonist treatment increases RGS19 levels by a PKC and the MAPK pathway-dependent mechanism.

Introduction

Regulator of G-protein signaling (RGS) proteins comprise a family of more than 20 molecules that act as GTPase accelerating proteins, or GAPs, to control the duration of G protein-coupled receptor (GPCR)-mediated signaling. They do this by virtue of a conserved RGS homology domain (RH) that binds the GTP-bound form of $G\alpha$ proteins (Hepler et al., 1997; Hollinger and Hepler, 2002; Neubig and Siderovski, 2002; Traynor and Neubig, 2005). There is considerable evidence that RGS proteins are effective modulators of opioid signaling, and this results in altered behavioral responses to morphine (reviewed in Traynor, 2012).

RGS19, or $G\alpha$ -interacting protein (GAIP) (De Vries et al., 1995), is a small RGS protein (~24 kDa) and a member of the A/Rz subfamily of RGS proteins (Ross and Wilkie, 2000).

RGS19 transcripts are expressed ubiquitously throughout the brain and are found in structures that also express μ -opioid receptors (MOR), δ -opioid receptors (DOR), or κ -opioid receptors as well as nociceptin receptors (NOPR), a member of the opioid receptor family that binds the endogenous neuropeptide nociception/orphanin FQ (Grafstein-Dunn et al., 2001; Garzon et al., 2004; Xie et al., 2005). RGS19 consists of the RH domain and a cysteine string motif that binds the N-terminal leucine-rich region of GAIP-interacting protein N-terminus (Fischer et al., 2003). Its C-terminus possesses a PDZ-binding motif (SEA) that interacts with GAIP-interacting protein C-terminus (GIPC) to link a variety of signaling molecules (De Vries et al., 1998; Lou et al., 2002).

RGS19 has been postulated to act as a GAP for $G\alpha$ proteins coupled to NOPR because the RGS19 gene is found lying head to head with the NOPR gene and the two genes share a bidirectional transcriptional promoter (Ito et al., 2000; Xie et al., 2003). Consistent with this, RGS19 has been shown to act as a GAP for NOPR signaling in COS-7 cells (Xie et al.,

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ABBREVIATIONS: AC, adenylyl cyclase; DAMGO, D-Ala², N-Me-Phe⁴, Gly-ol⁵-enkephalin; DOR, δ -opioid receptor; DPDPE, D-Pen², D-Pen⁵-enkephalin; GAP, GTPase accelerating protein; GAIP, G-alpha interacting protein; GFP, green fluorescent protein; GIPC, GAIP-interacting protein C-terminus; Go6976, 5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrile; GPCRs, G protein-coupled receptors; HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; MAPK, mitogen-activated protein kinase; MEK1, mitogen-activated protein kinase kinase 1; MOR, μ -opioid receptor; NLX, naloxone; NOPR, nociceptin receptor; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one; PKC, protein kinase C; PTX, pertussis toxin; RGS, regulator of G-protein signaling; RH, RGS homology domain; RT-PCR, reverse-transcription polymerase chain reaction; shRNA, short hairpin RNA; SNC80, 4-[(R)-[(2S,5R)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-N,N-diethylbenzamide.

2003, 2005), although as far as we are aware, there are no reports of such action in other systems. On the other hand, evidence for an action of RGS19 on MOR and DOR signaling is mixed. RGS19 does not show significant GAP activity toward *Gai/o* proteins involved in MOR signaling in COS-7 cells (Xie et al., 2005), yet it increases the ability of the MOR agonist D-Ala, N-Me-Phe, Gly-ol⁵-enkephalin (DAMGO) to inhibit adenylyl cyclase (AC) in human embryonic kidney (HEK) 293 cells (Xie et al., 2007) and modulates the antinociceptive actions of morphine and DAMGO in mice (Garzon et al., 2004). RGS19 does not act as a GAP for DOR-coupled *Gai/o* proteins in HEK293 (Xie et al., 2007) or in COS-7 cells (Xie et al., 2005). In addition, antisense knockdown of RGS19 or its binding partner GIPC does not alter DOR agonist-mediated antinociceptive behavior in mice (Garzon et al., 2004). In contrast, RGS19 has been reported to colocalize with DOR and *Gai3* in clathrin-coated pits (Elenko et al., 2003).

The use of different heterologous expression systems and comparison with endogenous systems may account for the inconsistent findings with RGS19 in relation to MOR and DOR signaling. In addition, there is a paucity of studies on the role of RGS19 as a GAP for NOPR signaling. Therefore, we re-examined the GAP activity of RGS19 on opioid receptor-G protein-mediated signaling. Human neuroblastoma SH-SY5Y cells endogenously express MOR, DOR, and NOPR (Wang et al., 2009; Levitt et al., 2011), which couple to *Gai/o* proteins. Here we show that these cells also abundantly express RGS19. Consequently, SH-SY5Y cells represent an ideal model system to answer the question of whether RGS19 acts as a GAP for signaling initiated by MOR, DOR, or NOPR agonists and whether there is receptor specificity. Moreover, agonists at MOR, DOR, or NOPR signal via *G β γ* subunits to protein kinase C (PKC) and the mitogen-activated protein kinase (MAPK) pathway, which have been implicated in posttranslational phosphorylation of RGS19 (De Vries et al., 1995; Ogier-Denis et al., 2000). Since phosphorylation by various mechanisms has been reported to increase stability, membrane association and GAP activity of RGS19 (De Vries et al., 1995; Fischer et al., 2000; Ogier-Denis et al., 2000), we also asked whether agonist action at these receptors leads to altered abundance and/or activity of RGS19.

We show that specific knockdown of RGS19 using short hairpin RNA (shRNA) increases MOR, but not DOR or NOPR signaling, suggesting a selective GAP action of RGS19 at MOR. Furthermore, chronic treatment of SH-SY5Y cells with a MOR or a DOR agonist significantly increased the levels and activity of RGS19. Effects of shRNA and opioid agonists on RGS19 levels were accompanied by parallel changes in its binding partner, GIPC.

Materials and Methods

Materials and Drugs. Morphine, SNC80 (4-[(*R*)-[(2*S*,5*R*)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-*N,N*-diethylbenzamide), and naloxone (NLX) were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI). DAMGO, nociceptin, D-Pen², D-Pen⁵-enkephalin (DPDPE), leupeptin, retinoic acid, IBMX (3-isobutyl-1-methylxanthine), forskolin, phorbol 12-myristate 13-acetate, dimethyl sulfoxide (DMSO), actinomycin D, and all other chemicals, unless stated, were from Sigma-Aldrich (St. Louis, MO). Protease inhibitor cocktail tablets (Complete Mini, EDTA-free) were from Roche Diagnostics (Indianapolis, IN) and Go6976 (5,6,7,13-tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-*a*]pyrrolo[3,4-*c*]

carbazole-12-propanenitrile), PD98059 [2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one], and H89 were from Calbiochem (La Jolla, CA). Cyclic AMP radioimmunoassay kits were from GE Healthcare (Piscataway, NJ). Tissue culture medium, LipofectAMINE 2000 reagent, OPTI-MEM medium, fetal bovine serum, penicillin-streptomycin, and trypsin were from Invitrogen (Carlsbad, CA). Antibodies were from the indicated sources: anti-phospho-p44/42 MAPK (ERK1/2) and anti-p44/42 MAPK (ERK1/2) (Cell Signaling Technology, Beverly, MA), anti- β -actin and anti- α -tubulin (Sigma-Aldrich), and anti-mouse and anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-RGS19 N-terminus antiserum was a gift from Dr. Marilyn Gist Farquhar (University of California San Diego, La Jolla, CA). Monoclonal anti-human GIPC antibody (clone AT1G10) was purchased from ATGen Co., Ltd. (Gyeonggi-do, South Korea). SuperSignal West Pico chemiluminescent substrate was from Pierce (Rockford, IL). Immobilon-P transfer membranes (0.45 μ m pore size) were from Millipore Corporation (Bedford, MA).

Cell Culture. Human SH-SY5Y cells, C6 rat glioma cells, HEK293 cells, and PC12 cells were purchased from ATCC (Manassas, VA). Cells were grown in Dulbecco's modified Eagle's medium containing 10% fetal bovine serum and penicillin (100 units/ml)-streptomycin (100 μ g/ml) under 5% CO₂ at 37°C.

Reverse-Transcription Polymerase Chain Reaction (RT-PCR). Total RNA was prepared from SH-SY5Y cells, rat brain, HEK293 cells, or C6 cells using the VersaGENE RNA purification system (Gentra Systems, Minneapolis, MN) and then subjected to RT-PCR with SuperScript One-Step RT-PCR System according to the supplier's manual (Invitrogen). Primers for detection of RGS19 were designed from the RGS19 coding region as follows: sense primer, 5'-GGATCCCCACCCCGCATGAGGCTGA-3'; antisense primer, 5'-GTCGACCTAGGCCTCGGAGGAGGACTGTG-3'. The primers were first checked by amplifying human RGS19 plasmid DNA to ensure that the correct size of the PCR product (663 base pairs) was detected. Total RNA (200 ng) was used with primers (0.3 μ M each) and MgSO₄ (1.2 mM) in a 25- μ l volume. The reverse transcription was performed by incubating RNA at 45°C for 30 minutes, followed by PCR with 30 cycles at 95°C for 30 seconds, 55°C for 45 seconds, and 72°C for 1 minute. The RT-PCR products were separated by electrophoresis on a 1.8% agarose gel, stained with ethidium bromide, and photographed using a Kodak Image Station 440, Eastman Kodak, Rochester, NY).

Design and Construction of Lentivirus Encoding shRNA Against RGS19. The shRNA lentiviral delivery system developed by Dr. Didier Trono (Wiznerowicz and Trono, 2003) was used. In brief, four targeting sites were designed based on the human RGS19 gene (MN-005873) as follows: site 1-5' TGTCCAGTCATGATACAGC 3', site 2-5' CAGCGAGGAGAACATGCTC 3', site 3-5' TCCTGTCCCCAAGGAGGT 3', and site 4-5' GCTGCAGATCTACACGCTC 3'. The four shRNA oligos against RGS19 were constructed into the pLVTHM lentivector by direct cloning of annealed shRNA at MluI-ClaI sites. The gene for green fluorescent protein (GFP) is encoded by the vector pLVTHM. Lentiviruses were produced, concentrated, and titrated as previously described (Wang et al., 2009). There were approximately 3×10^7 – 10^8 transducing units per milliliter for each lentivirus.

Generation of SH-SY5Y Cell Lines Stably Expressing RGS19 shRNA. Cells plated (at ~80% confluency) in 35 mm dishes were infected with a mixture of the four lentiviral stocks encoding shRNA against RGS19 with 6 μ g/ml of polybrene in Dulbecco's modified Eagle's medium. A control cell line was obtained using lentivirus stock encoding shRNA against GFP. The stable cell lines were generated by passaging cells into larger dishes, and GFP expression was used as an indicator for the presence of shRNA throughout cell culture maintenance, using GFP fused in frame with RGS19 shRNA in the lentivector pLVTHM.

Western Blot Analysis. Whole-cell lysates were prepared from SH-SY5Y cells, C6 cells, and PC12 cells as previously described (Wang et al., 2009). Briefly, cells were suspended in ice-cold radio-immunoprecipitation assay lysis buffer containing protease inhibitor cocktail (Wang et al., 2009), then homogenized, and centrifuged at 20,000 $\times$ g

for 10 minutes. The supernatant (~20 μ g) was subjected to SDS-PAGE on a 12% mini-gel and transferred to an Immobilon-P transfer membrane. The membrane was blocked with 1% bovin serum albumin in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 hour and incubated with RGS19-specific antiserum at a 1:8,000 dilution or GIPC-specific antibody at a 1:1,000 dilution overnight in the cold room. After three consecutive washes, the membrane was incubated with 1:20,000 dilution of secondary antibody (goat anti-rabbit IgG-horseradish peroxidase for RGS19) or (goat anti-mouse IgG-HRP for GIPC) for 1 hour at room temperature. Prestained SDS-PAGE protein standards (Bio-Rad, Precision Plus Protein Standards, Kaleidoscope) were used to determine the size of detected proteins. The membranes were stripped and reblotted with anti- β -actin antibody at 1:2000 dilution or anti- α -tubulin antibody at 1:5,000 dilution as an internal control for protein loading. In some experiments, the membranes were cut at the 37-kDa marker, and the upper membrane was blotted with anti- α -tubulin antibody at 1:5000 dilution as an internal control for protein loading. Proteins were visualized by chemiluminescence with SuperSignal West Pico (Pierce) and exposed to X-ray film or using the ODYSSEY FC imaging system (LI-COR, Inc., Lincoln, NE).

cAMP Accumulation Assay. Retinoic acid treated (10 μ M for 6–7 days) SH-SY5Y cells at 80–90% confluency were washed once with fresh serum-free medium, and the medium was replaced with 1 mM IBMX in serum-free medium for 15 minutes at 37°C and then replaced with medium containing 1 mM IBMX, 30 μ M forskolin with submaximum doses of agonists (100 nM morphine, 100 nM SNC80 or 10 nM nociceptin) for 5 minutes 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 $\times g$ for 1 minute to pellet the precipitates. Accumulated cAMP was measured by radioimmunoassay in a 15 μ l aliquot of the supernatant from each sample following the manufacturer's instructions. Percent of forskolin-stimulated cAMP for each agonist was calculated.

MAPK Assay. Cells were plated in 24-well plates for 6–7 days without retinoic acid treatment, serum-starved for 30–48 hours, and then washed once with fresh serum-free medium and stimulated with submaximum doses of agonists (100 nM morphine, 100 nM SNC80, or 10 nM nociceptin) for 5 minutes at 37°C. The reaction was stopped by adding 0.1 ml of ice-cold SDS sample buffer (62.5 mM Tris-HCl, pH 6.8, 2% SDS, 10% glycerol, 50 mM DTT, and 0.01% bromophenol blue). Samples were removed from the wells, boiled for 5 minutes, and then subjected (15 μ l each) to electrophoresis using a 12% SDS-PAGE mini-gel, followed by transfer to an Immobilon-P membrane for Western blotting. The blot was probed with 1:6000 dilution of antiphospho-p44/42 MAPK (Thr202/Tyr204) antibody and visualized using horseradish peroxidase-conjugated anti-rabbit IgG, followed by enhanced chemiluminescence detection (Kodak X-ray film, Carestream Health, Rochester, NY) to measure the activated phospho-ERK. To ensure equal loading, the same membranes were stripped and reblotted with a 1:4000 dilution of anti-p44/42 MAPK antibody to measure total ERK levels. MAPK activity was calculated as normalized arbitrary units of phosphorylated MAPK (ERK1/2) over total ERK 1/2 by densitometry analysis of films in the linear range of exposure using a Kodak Image Station 440. Percent of agonist-stimulated phospho-ERK over basal was calculated.

Data and Statistical Analysis. Data from at least three separate experiments are presented as means $\pm$ S.E. Data were compared by two-way analysis of variance with Bonferroni posttest analysis of variance, and differences considered significant if $P < 0.05$.

Results

RGS19 Expression in Human SH-SY5Y Cells. RGS19 expression in SH-SY5Y cells was confirmed by both RT-PCR and Western blot analysis (Fig. 1). A main band at 663 bp was

Fig. 1. RGS19 expression in SH-SY5Y cells. (A) Expression of RGS19 mRNA by RT-PCR. Total RNAs prepared from rat brain (rB), human HEK293 (HEK), human SH-SY5Y (SY5Y), rat PC12, and rat C6 glioma cells (C6) were subjected to RT-PCR using RGS19-specific primers as described under Materials and Methods. The PCR product was separated on a 1.8% agarose gel, stained, and photographed. Human RGS19 cDNA (cDNA) was used as positive control for the primers. The expected 663-bp PCR product was detected in all cells tested. The human HEK and SH-SY5Y cells showed two bands. (B) Expression of RGS19 protein by Western blot analysis. Whole-cell lysates were prepared from SH-SY5Y (SY5Y), PC12, and C6 glioma cells (C6) and resolved on a 12% SDS-PAGE mini-gel for Western blot analysis using a specific anti-RGS19 antibody, as described under Materials and Methods.

detected in rat brain, rat PC12, rat C6 glioma, and human HEK293 and SH-SY5Y cells. Human RGS19 cDNA was included as positive control. In both human HEK293 and SH-SY5Y cell lines, a lower-molecular-weight band was detected, likely as a result of a human splice variant, as previously reported (Xie et al., 2003, 2005). RGS19 protein was validated by SDS-PAGE followed by Western blot analysis using a previously characterized anti-RGS19-specific antibody [anti-GAIP (N)] (Elenko et al., 2003). A strong band was detected at ~25 kDa (estimated full-length RGS19 protein, MW 24,600) (De Vries et al., 1995).

Effect of Knockdown of Endogenous RGS19 on MOR, DOR, and NOPR Signaling. Reliable measures of opioid signaling are the inhibition of AC and stimulation of the MAPK pathway. To study the functional role of RGS19, we developed a SH-SY5Y cell line stably expressing shRNA against RGS19 to block endogenous RGS19 protein expression continuously. Four lentiviral stocks encoding shRNA targeted to four different sites on the RGS19 gene with a GFP marker were used to infect SH-SY5Y cells. More than 90% of the SH-SY5Y cells were infected with lentivirus, as indicated by visualization of the GFP marker (Wang et al., 2009). The stable SH-SY5Y cell line expressing shRNA against RGS19 showed much reduced RGS19 protein expression representing approximately $69 \pm 4\%$ knockdown, providing a RGS19-deficient cell line (Fig. 2A). In contrast, RGS19 protein was easily detectable in SH-SY5Y control cells stably expressing shRNA against GFP and in C6 glioma cells (Fig. 2A).

SH-SY5Y cells express AC types 1 and 8 that are inhibited by GTP-bound $\text{G}\alpha_i/o$ proteins (Hirst and Lambert, 1995; Sunahara and Taussig, 2002). To measure AC activity, RGS19-deficient and control SH-SY5Y cells were first differentiated with 10 μ M retinoic acid for 6–7 days, and then AC activity was measured as the accumulation of forskolin (30 μ M)-stimulated cAMP in the presence of the phosphodiesterase inhibitor IBMX (1 mM). The levels of forskolin-stimulated cAMP accumulation were similar in control and RGS19-deficient cells. The degree of

Fig. 2. Effect of RGS19 knockdown on cAMP and MAPK signaling. (A) Development of a SH-SY5Y cell line stably expressing shRNA against RGS19. A mixture of four lentiviruses encoding four shRNA targeting four different sites on the RGS19 gene was used to infect SH-SY5Y cells as described under Materials and Methods. The control cell line was generated by infecting lentiviral shRNA against the gene for GFP. Western blot analysis (as in Fig. 1) showed a 69% decrease in the RGS19-deficient cells (RGS19) compared with the control cells expressing GFP shRNA (GFP) determined from three individual experiments. RGS19 in C6 cells is a control (C6). (B) Inhibition of cAMP accumulation. Using the established stable cell lines following 6–7 days of treatment with 10 μ M retinoic acid, the percent of forskolin-stimulated cAMP accumulation by morphine (Mor, 100 nM), SNC80 (100 nM), and nociceptin (NOC, 10 nM) was measured as described under Materials and Methods. *** $P < 0.001$ ($n = 5-7$), for the RGS19-deficient RGS19 shRNA cell line compared with the control cells expressing shRNA against GFP. (C) MAPK activation. Serum-starved (48 hours) cells were treated with vehicle (Ctr), morphine (Mor, 100 nM), SNC80 (SNC, 100 nM), or nociceptin (NOC, 10 nM) for 5 minutes. MAPK activation was measured as described under Materials and Methods. *** $P < 0.001$ ($n = 4$) for the RGS19-deficient RGS19 shRNA cell line, compared with control cells expressing shRNA against GFP. Data are presented as percent of vehicle-treated control.

inhibition of forskolin-stimulated cAMP accumulation by the MOR agonist morphine at 100 nM, a submaximal concentration, was $16 \pm 4\%$ in control cells expressing shRNA against GFP, but this was significantly increased to $40 \pm 5\%$ in the RGS19-deficient cell line ($P < 0.001$; Fig. 2B). In

contrast, the degree of inhibition of forskolin-stimulated cAMP accumulation by the DOR opioid agonist SNC80 at 100 nM, a submaximal concentration, was similar in the RGS19-deficient cell line ($43 \pm 5\%$) compared with control cells ($35 \pm 2\%$) expressing shRNA against GFP. Inhibition of cAMP accumulation by the NOPR agonist nociceptin at a submaximal concentration of 10 nM did not significantly differ between the RGS19-deficient cell line ($57 \pm 2\%$) and control cells ($48 \pm 4\%$).

To determine whether the differential effect of RGS19 on MOR agonist signaling was specific to the $G_{\alpha i/o}$ -mediated inhibition of AC, we examined whether MAPK regulation, a $G\beta\gamma$ -mediated effect, was also modulated by RGS19. Stimulation of ERK phosphorylation by submaximal concentrations of morphine (100 nM), SNC80 (100 nM), and nociceptin (10 nM) was measured in RGS19-deficient SH-SY5Y cells compared with control SH-SY5Y cells expressing shRNA against GFP (Fig. 2C). The basal level of ERK phosphorylation, measured as normalized arbitrary units of phosphorylated ERK over total ERK, was similar in both cell lines. Morphine did not stimulate ERK phosphorylation in control cells expressing shRNA against GFP. However, the level of ERK phosphorylation by morphine in the RGS19-deficient cells was markedly increased to $191 \pm 14\%$ ($P < 0.001$). On the other hand, the level of ERK phosphorylation by SNC80 and nociceptin was similar in the RGS19-deficient cells ($113 \pm 10\%$ and $113 \pm 11\%$, respectively) and control cells ($116 \pm 10\%$ and $117 \pm 13\%$, respectively).

We have previously shown that DOR-mediated signaling, but not MOR signaling, is modulated by RGS4 in SH-SY5Y cells (Wang et al., 2009). We therefore examined whether RGS4 was also able to modulate NOPR signaling. However, no change was seen in the ability of nociceptin (10 nM) to inhibit AC signaling in SH-SY5Y cells expressing shRNA against RGS4, which results in a $\sim 90\%$ knockdown of RGS4 protein ($35 \pm 6\%$ inhibition) compared with control SH-SY5Y cells expressing shRNA against GFP ($38 \pm 7\%$ inhibition; data not shown). Together, these data indicate a specific role for RGS19 in modulating MOR signaling, but not DOR and NOPR signaling, in SH-SY5Y cells.

Regulation of RGS19 Protein by MOR or DOR Agonists. Here we test the hypothesis that exposure to MOR agonist regulates the level of RGS19 protein via activation of the MAPK pathway. Treatment of SH-SY5Y cells overnight with 10 μ M of DAMGO or 10 μ M of morphine increased the intensity of the RGS19 protein band on Western blot by $324 \pm 90\%$ and $269 \pm 13\%$, respectively (Fig. 3A). The DAMGO-induced increase in RGS19 protein was concentration- (Fig. 3B) and time-dependent (Fig. 3C), although the response was slow and no difference was observed until 6 h.

As a control receptor that is not regulated by RGS19 in these cells, we also investigated the effect of the DOR agonists DPDPE and SNC80. Surprisingly, both DPDPE and SNC80 increased RGS19 protein levels over untreated cells by $346 \pm 63\%$ and $229 \pm 5\%$, respectively. In contrast to the increase in protein, overnight treatment with DAMGO or DPDPE did not change the level of RGS19 mRNA (Fig. 3D).

To evaluate the role of functional $G_{\alpha i/o}$ heterotrimeric GTP-binding proteins and opioid receptors in the agonist-mediated RGS19 up-regulation, SH-SY5Y cells were pretreated with or without 100 ng/ml pertussis toxin (PTX) for 6 h before and during overnight incubation in the absence or presence of

Fig. 3. Increased RGS19 protein expression, but not mRNA, after overnight treatment with MOR or DOR agonists. (A) RGS19 protein was increased by overnight treatment with DAMGO (DA), DPDPE (DP), morphine (Mor), or SNC80 (SNC). Whole-cell lysates were prepared from SH-SY5Y cells treated with opioid agonist (10 μ M, overnight), and RGS19 protein was identified by Western blot as described in Fig. 1. RGS19 protein expression was increased 2- to 3-fold in opioid agonist-treated cells compared with vehicle-treated cells (Ctr, 100%). (B,C) Time- and concentration-dependent increases in RGS19 protein after DAMGO treatment. SH-SY5Y cells were treated with 10 μ M DAMGO for 0 to 24 h or with varying concentrations of DAMGO overnight and then lysed for Western blot analysis as in Fig. 1. The Western blots are representative of three experiments. β -actin used as a loading control was not changed. (D) RGS19 mRNA levels detected by RT-PCR as described under Materials and Methods were not changed after overnight DAMGO or DPDPE treatment. EF-1 α was the loading control.

10 μ M DAMGO or DPDPE. Treatment with PTX alone did not change the level of RGS19 protein. However, PTX completely blocked DAMGO- or DPDPE-induced upregulation of RGS19 (Fig. 4). When the cells were treated overnight with DAMGO or DPDPE in the presence of the opioid antagonist NLX (10 μ M), the opioid agonist-induced increase in RGS19 protein levels was completely prevented (Fig. 4).

ERK1/2-dependent and PKC phosphorylation of RGS19 is known (De Vries et al., 1995; Ogier-Denis et al., 2000). Moreover, MOR agonists activate the MAPK pathway and the PLC pathway (Gutstein et al., 1997; Clark et al., 2003; Mathews

Fig. 4. (A) Blockade of DAMGO- and DPDPE-induced RGS19 protein upregulation by PTX and NLX. SH-SY5Y cells were preincubated for 6 hours with PTX (100 ng/ml) or 1 hour with NLX (10 μ M), followed by overnight incubation in the absence or presence of DAMGO or DPDPE (10 μ M each). RGS19 protein was identified by Western blot as described in Fig. 1. (B) Quantified data from three independent experiments, ***, $P < 0.001$. β -actin loading controls were not changed.

et al., 2008). Here we examined the role of these two kinases in the opioid-mediated increase in RGS19 protein in SH-SY5Y cells. Treatment with the MEK1 pathway inhibitor PD98059 (50 μ M) completely blocked DAMGO-induced upregulation of RGS19 protein, reducing the increase from $151 \pm 8\%$ to $96 \pm 2\%$ (Fig. 5A). Similarly, the PKC inhibitor Go6976 (0.1 μ M) completely blocked DAMGO-induced upregulation of RGS19 protein compared with cells treated with DAMGO alone (from $144 \pm 9\%$ to $80 \pm 33\%$) (Fig. 5B). In addition, treatment of SH-SY5Y cells with the PKC activator phorbol 12-myristate 13-acetate time and concentration dependently increased RGS19 protein levels (Fig. 5, C and D). The presence of either PD98059 or Go6976 reduced the morphine-induced activation of MAPK activity ($180 \pm 24\%$) back to control values (Fig. 6), suggesting that MOR agonist activation of the MAPK pathway is via PKC in these cells. The PKA inhibitor H89 (1.0 μ M) did not affect the ability of DAMGO to upregulate RGS19 protein ($149 \pm 18\%$) compared with DAMGO alone ($144 \pm 9\%$) (Fig. 5B).

The slow time course of opioid-mediated RGS19 upregulation suggests that either the effect of MOR and DOR agonists is not direct phosphorylation of RGS19 or that new synthesis of RGS19 is required. To test this hypothesis, SH-SY5Y cells were treated with the transcription inhibitor actinomycin D (5 μ g/ml, overnight), with or without DAMGO (10 μ M). Both Western blot (Fig. 7A) and RT-PCR (Fig. 7B) analyses showed that levels of RGS19 protein and mRNA were significantly reduced by actinomycin D treatment. Under these conditions RGS19 protein levels were not increased by DAMGO.

GIPC is an Obligate Partner of RGS19. GIPC interacts specifically with the C terminus of RGS19 (DeVries, et al., 1998), and the two proteins are coexpressed in rat brain regions and colocalized at the plasma membrane (Jeanneteau, et al., 2004). Furthermore, it has been demonstrated that knockdown of GIPC enhances morphine antinociception in the mouse (Garzon et al., 2004). As expected, therefore, GIPC

Fig. 5. Involvement of ERK1/2 and PKC, but not PKA, in DAMGO-induced RGS19 protein upregulation. SH-SY5Y cells were treated with 10 μ M DAMGO for 6 hours in the absence or presence of (A) the MEK1 inhibitor PD98059 (50 μ M) or (B) the PKC inhibitor Go6976 (0.1 μ M) or the PKA inhibitor H89 (1.0 μ M). Data are presented as percent of vehicle-treated controls. ***, $P < 0.001$, $n = 3$; **, $P < 0.01$, $n = 5$. (C, D) Effect of the PKC activator [phorbol 12-myristate 13-acetate (PMA)]. SH-SY5Y cells were treated with PMA (100 nM) for 0–4 hours (C) or over the concentration range 10^{-8} to 10^{-6} M (D) for 1 hour. RGS19 protein was measured by Western blot as described in Fig. 1. Quantified data are presented on the right side of the figure. α -Tubulin as loading control was not changed.

was expressed in SH-SY5Y cells as a single strong band at ~ 37 KDa. Cells expressing shRNA against RGS19 showed a $50 \pm 2\%$ reduced level of GIPC expression compared with cells expressing shRNA against GFP (Fig. 8A), similar to the level of knockdown of RGS19 (Fig. 2A). When SH-SY5Y cells were treated with or without DAMGO overnight, GIPC was increased by $47 \pm 9\%$ in cells treated with DAMGO compared

with control without DAMGO treatment (Fig. 8B). These data indicate a tight regulation between RGS19 with its partner GIPC.

Discussion

In this study, we showed that SH-SY5Y cells express RGS19 and that reduction in the level of RGS19 protein leads to an increase in MOR, but not DOR or NOPR, signaling to AC and the MAPK pathway, thus affecting both $G\alpha$ and $G\beta\gamma$ -mediated pathways. This finding complements our earlier work showing specificity of RGS4 for DOR in these same cells and also highlights the advantages of using a system in which all components are endogenously expressed. However, in contrast to the selective GAP activity of RGS19 for MOR, we demonstrate that the levels of RGS19 protein, but not RGS19 mRNA, are increased in SH-SY5Y cells by either chronic MOR or DOR agonist treatment in a PTX- and NLX-sensitive manner involving PKC and MAPK pathways. Changes in RGS19 expression by shRNA targeted to RGS19 or by treatment with opioid agonists were accompanied by similar alterations in GIPC expression, indicating a close relationship between these two proteins.

The finding that RGS19 acts as a GAP at MOR, but not DOR or NOPR, in SH-SY5Y cells and so negatively modulates MOR agonist-induced activation of ERK1/2 phosphorylation and inhibition of AC adds to our previous data showing that RGS4 acts as GAP for DOR, but not MOR (Wang et al., 2009)

Fig. 6. Role of PKC in morphine-mediated MAPK activation. SH-SY5Y cells stably expressing RGS19 shRNA were serum starved for 30 hours and then pretreated with the PKC inhibitor (Go6976, 0.1 μ M) or the MEK1 inhibitor (PD98059, 50 μ M) for 6 hours. MAPK activation was measured in the presence of 100 nM morphine for 5 minutes as described under Materials and Methods. ***, $P < 0.001$, $n = 4$. Total ERK was not changed.

Fig. 7. Effect of actinomycin D treatment on RGS19 protein and mRNA. SH-SY5Y cells were treated with actinomycin D (AM, 5 μ g/ml, overnight) or vehicle together with (+) or without (-) DAMGO (10 μ M). Then RGS19 protein (A) and mRNA (B) were identified by Western blot and RT-PCR as described in Fig. 1. Data are presented as percent of vehicle-treated controls. ***, $P < 0.001$, $n = 3$. Loading controls, α -tubulin and EF-1 α , were not changed.

or NOPR (Xie et al., 2005) signaling. Moreover, our findings support in vivo work that RGS19 acts as a negative modulator of MOR, but not DOR, agonists in controlling supraspinal antinociception in the mouse as measured by the tail-flick test (Garzon et al., 2004). These findings must, however, be considered in the light of reports of other RGS proteins modulating MOR signaling and behavior (Traynor, 2010), especially the important role for RGS9-2. Thus, RGS9-2 negatively regulates MOR signaling (Psifogeorgou et al., 2007) and knockout or knockdown of RGS9-2 enhances responses to morphine in a variety of behaviors in mice (Garzon et al., 2003; Zachariou et al., 2003), although RGS9-2 positively modulates fentanyl and methadone antinociception (Psifogeorgou et al., 2011). RGS9-2 has preferential GAP activity toward $G_{\alpha o}$ and is highly, though not exclusively, expressed in the striatum (Gold et al., 1997). In contrast, RGS19 is a nonselective GAP for G_{α} subunits and is highly expressed in the hippocampus, with expression also in the

Fig. 8. GIPC protein changes with RGS19. Whole-cell lysates were prepared from SH-SY5Y cells stably expressing either GFP shRNA or RGS19 shRNA as described under Materials and Methods. GIPC was detected as a single band at ~37 KDa. This was decreased to ~50% of control level in cells expressing shRNA against RGS19 compared with cells expressing shRNA against GFP (A). When SH-SY5Y cells were treated with (+) DAMGO (10 μ M) overnight, GIPC was increased ~50% compared with cells without DAMGO treatment (B). Quantified data from three sets of individual experiment are presented on the right side of the figure. Loading controls (α -tubulin) were not changed.

ventral tegmental area and pontine nucleus (Grafstein-Dunn et al., 2001), regions that do not express RGS9-2 (Gold et al., 1997). Therefore, RGS19 will be likely to act at MOR coupled G_{α} proteins other than $G_{\alpha o}$ and in regions where it predominates over RGS9-2. Thus, the findings reinforce the importance of G_{α} specificity and expression patterns in the characterization of RGS protein activity.

However, the GPCR itself must also play an important role in RGS selectivity since RGS19 does not act as a GAP for DOR or NOPR signaling in SH-SY5Y cells, yet RGS4 in the same cell is an effective GAP for DOR. This cannot be ascribed to GPCR specificity for different G_{α} subunits since both MOR and DOR activate the same $G_{\alpha i/o}$ protein subtypes (Prather et al., 1994a,b; Chakrabarti et al., 1995) and in SH-SY5Y cells, MOR, DOR, and NOPR share the same pool of heterotrimeric G proteins (Alt et al., 2002; Levitt et al., 2011). RGS19 and RGS4 are both small RGS proteins that lack large protein-protein binding domains and have approximately 60% homology in the RH domains (Hollinger and Hepler, 2002). Furthermore, comparison of the RH regions suggests that the principal structural characteristics are conserved (de Alba et al., 1999). Indeed, both RGS19 and RGS4 efficiently act as GAPs toward members of the $G_{\alpha i/o}$ and $G_{\alpha z}$ families. Thus, the apparent selectivity of RGS19 as a GAP for MOR signaling and RGS4 as a GAP for DOR signaling is unexpected. However, there is accumulating evidence for GPCRs contributing to the specificity of RGS protein action (Xu et al., 1999; Saitoh et al., 2002; Wang et al., 2002, 2009; Bernstein et al., 2004), and we (Wang et al., 2009) and others (Georgoussi et al., 2006; Xie et al., 2007) have suggested a role for the C-terminus of opioid receptors in providing specificity of RGS action at DOR versus MOR. Moreover, outside the RH domain, RGS19 has a more complex structure than RGS4 with an N-terminal cysteine string that binds to the N-terminal leucine-rich region of GAIP-interacting protein N-terminus (Fischer et al.,

2003) and a PDZ-binding motif that interacts with GIPC at its C-terminus (De Vries et al., 1998; Lou et al., 2002). These more complex regions may contribute to the observed selectivity of RGS19 for MOR.

It was somewhat surprising to find that RGS19 did not modulate nociceptin-mediated inhibition of AC or stimulation of the MAPK pathway given that the RGS19 and NOPR genes are physically linked and share a bidirectional transcriptional promoter (Ito et al., 2000; Xie et al., 2003). Indeed, RGS19 has been reported to regulate NOPR signaling as measured by its effectiveness to increase nociceptin-stimulated GTPase activity and to antagonize nociceptin-induced inhibition of cAMP accumulation when both RGS19 and NOPR are coexpressed in COS-7 cells (Xie et al., 2005). However, we have previously shown with RGS4 that overexpression of RGS proteins can lead to nonphysiologic findings. For example, as noted already, endogenous RGS4 does not act as a GAP for endogenous MOR signaling in SH-SY5Y cells (Wang et al., 2009) and the RGS4 knockout mouse shows only very minor phenotypic responses to morphine (Grillet et al., 2005; Han et al., 2010). On the other hand, RGS4 has been reported to act negatively to modulate MOR signaling in various heterologous systems (Ippolito et al., 2002; Garnier et al., 2003; Xie et al., 2005; Georgoussi et al., 2006; Xie et al., 2007; Talbot et al., 2010). NOPR couples to *Gai/o* proteins and so is expected to be sensitive to the GAP activity of RGS proteins (Xie et al., 2005). Since neither endogenous RGS4 nor RGS19 modulates NOPR signaling in SH-SY5Y cells, it remains to be determined whether there is an RGS protein in these cells that controls NOPR signaling. Certainly, SH-SY5Y cells possess many alternative candidate RGS proteins, including RGS 2, 3, 5, 6, 7 and 8, as determined by RT-PCR (Wang and Traynor, unpublished data).

In contrast to RGS4, which is downregulated after overnight treatment with opioid agonists (Wang and Traynor, 2011), we observed that RGS19 levels were elevated following overnight treatment with either a MOR or a DOR agonist. This increase was prevented by the opioid antagonist NLX and by pretreatment with PTX, indicating an action via *Gai/o*-coupled opioid receptors. There was no accompanying change in RGS19 mRNA. It was surprising that DOR agonists also increased RGS19 level when we were unable to show activity of these RGS proteins toward DOR signaling. However, we have previously reported downregulation of RGS4 by MOR agonists in these cells, even though MOR agonist action is not regulated by RGS4 (Wang and Traynor, 2011).

The opioid agonist-mediated increase in RGS19 protein was completely blocked by both the PKC inhibitor Go6967 or the ERK1/2 inhibitor PD98059. Moreover, Go6967 also prevented the phosphorylation of ERK1/2, suggesting that the MOR agonist-mediated stabilization of RGS19 protein in SH-SY5Y cells is via PKC activation of ERK1/2; activation of the MAPK pathway via PKC is known in several systems (Booth and Stockand, 2003; Cote-Velez et al., 2008). RGS19 contains two potential PKC phosphorylation sites and several S/TP motifs as possible sites for MAPK-mediated phosphorylation. Moreover, phosphorylation is known to stabilize RGS19 protein (De Vries et al., 1995; Fischer et al., 2000; Ogier-Denis et al., 2000). However, the extended time course needed to observe the DAMGO-mediated increase in RGS19 protein is slow and inconsistent with a simple phosphorylation of RGS19. On the other hand, treatment of cells with actinomycin D for 18 h

reduced the levels of RGS19 mRNA and protein and prevented the DAMGO-induced upregulation, which suggests that the catabolism of RGS19 occurs within this time frame, leading to a reduction in RGS19 protein, and indicates that DAMGO does not stabilize RGS19 protein but rather drives new synthesis by a PKC/MAPK mechanism.

Overall, the current findings demonstrate that endogenous RGS19 is regulated together with its binding partner, GIPC, and modulates the ability of MOR agonists to inhibit AC and stimulate the MAPK pathway in SH-SY5Y cells without altering the actions of DOR or NOPR agonists on these pathways. Chronic treatment of SH-SY5Y cells with either a MOR or a DOR agonist increases RGS19 and GIPC protein levels via a PKC/MAPK pathway. It can be hypothesized that by acting as a negative regulator, RGS19 will decrease MOR signaling. This increased RGS19 may contribute to the development of MOR tolerance while sparing other *Gai/o*-coupled systems in the cell. Finally, the results clarify previous findings and demonstrate the advantages of using a system that endogenously expresses relevant signaling components when studying RGS function.

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Authorship Contributions

Participated in research design: Wang, Traynor.

Conducted experiments and data analysis: Wang.

Wrote or contributed to the writing of the manuscript: Wang, Traynor.

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