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

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Running title: RGS19 Modulates Mu-Opioid Receptor Signaling

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ABBREVIATIONS: GPCRs, G protein coupled receptors; RGS, regulator of G protein signaling; shRNA, short hairpin RNA; GFP, green fluorescent protein; HEK, human embryonic kidney; IBMX, 3-isobutyl-1-methylxanthine; MAPK, mitogen-activated protein kinase; AC, adenylyl cyclase; PMA, phorbol 12-myristate 13-acetate; MOR, μ opioid receptor; DOR, δ opioid receptor; NOPR, nociceptin receptor; GAP, GTPase accelerating protein; RH, RGS homology domain; DAMGO, D-Ala, N-Me-Phe, Gly-ol^5-Enkephalin; DPDPE, D-Pen^2, D-Pen^5-enkephalin; GIPN, GAIP-interacting protein N-terminus; GIPC, GAIP-interacting protein C-terminus; SNC80, 4-[(*R*)-[(2*S*,5*R*)-4-allyl-2,5-dimethylpiperazin-1-yl](3-methoxyphenyl)methyl]-*N*,*N*-

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diethylbenzamide; Go6976, 5,6,7,13-Tetrahydro-13-methyl-5-oxo-12H-indolo[2,3-a]pyrrolo[3,4-c]carbazole-12-propanenitrileand; PD98059, 2-(2-amino-3-methoxyphenyl)-4H-chromen-4-one; ANOVA, analysis of variance; PTX, pertussis toxin; NLX, naloxone.

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ABSTRACT

Regulator of G protein signaling protein 19 (RGS19), also known as Gα interacting protein (GAIP) acts as a GTPase accelerating protein (GAP) for Gαz as well as Gαi/o subunits. Interactions with GIPN (GAIP-interacting protein N-terminus) and GIPC (GAIP-interacting protein C-terminus), 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 (MOR and DOR, respectively) and nociceptin receptors (NOPR). Lentiviral delivery of short hairpin RNA (shRNA) 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 DOR (SNC80) or NOPR (nociceptin) agonists to inhibit forskolin-stimulated adenylyl cyclase and increase MAPK activity. Overnight treatment with either MOR (DAMGO or morphine) or DOR (DPDPE 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. PKC activation alone increased the level of RGS19 and inhibitors of PKC (Go6976) and MEK1 (PD98059), but not PKA (H89), completely blocked DAMGO-induced RGS19 protein accumulation. The findings show that RGS19 and GIPC are jointly regulated, that RGS19 is a GAP 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.

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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 Gα proteins (Helper et al., 1997; Hollinger and Helper, 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 Gα-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 found in structures that also express mu- (MOR), delta- (DOR), or kappa- (KOR) opioid receptors as well as nociceptin receptors (NOPR), a member of the opioid receptor family that binds the endogenous neuropeptide nociception/orphanin FQ (Xie et al., 2005; Grafstein-Dunn et al., 2001; Garzon et al., 2004). RGS19 consists of the RGS homology domain (RH domain) and a cysteine string motif which binds the N-terminal leucine-rich region of GIPN (GAIP-interacting protein N-terminus (Fisher et al., 2003). Its C-terminus possesses a PDZ-binding motif (SEA) that interacts with GIPC (GAIP-interacting protein C-terminus) 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 Gα proteins coupled to NOPR because the RGS19 gene is found lying head to head with the NOPR gene and the two genes share a bi-

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directional 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., 2003, 2005), although as far as we are aware there are no reports 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 towards Gαi/o proteins involved in MOR signaling in COS-7 cells (Xie et al., 2005), yet increases the ability of the MOR agonist DAMGO to inhibit adenylyl cyclase (AC) in HEK293 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 Gαi/o proteins in HEK293 (Xie at 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 co-localize with DOR and Gαi3 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) that couple to Gαi/o proteins. Here we show 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/or if there is receptor specificity. Moreover, agonists at MOR, DOR or NOPR signal via Gβγ subunits to PKC and the MAPK pathway, which have been

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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; Fisher et al., 2000; Ogier-Denis et al., 2000), we also asked if 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, and naloxone were obtained through the Opioid Basic Research Center at the University of Michigan (Ann Arbor, MI). DAMGO, nociceptin, DPDPE, leupeptin, retinoic acid, IBMX (3-isobutyl-1-methylxanthine), forskolin, phorbol 12-myristate 13-acetate (PMA), 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, PD98059, 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,

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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, St Louis, MO); anti-mouse and anti-rabbit (Santa Cruz Biotechnology, Santa Cruz, CA). Anti-RGS19 N-terminus antiserum was a kind gift from Dr. Marilyn Gist Farquhar (University of California San Diego, La Jolla). 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^{TM}-P transfer membranes (0.45 μm 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 DMEM medium containing 10% fetal bovine serum and penicillin (100 units/ml)-streptomycin (100 μg/ml) under 5% CO_2 at 37 ^{o}C.

RT-PCR

Total RNA was prepared from SH-SY5Y cells, rat brain, HEK293 cells, or C6 cells using the VersaGENE^{TM} RNA purification system (Gentra Systems, Minneapolis, MN) and then subjected to RT-PCR with SuperScript^{TM} One-Step^{TM} RT-PCR System according to the supplier's manual (Invitrogen, Carlsbad, CA). Primers for detection of RGS19 were designed from the RGS19 coding region as follows: sense primer, 5' -GGATCCCCCACCCCGCATGAGGCTGA- 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

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PCR product (663 bp) was detected. Total RNA (200 ng) was used with primers (0.3 μM each) and $MgSO_4$ (1.2 mM) in a 25 μl volume. The reverse transcription was performed by incubating RNA at 45 °C for 30 min followed by PCR with 30 cycles at 95 °C for 30 s, 55 °C for 45 s, and 72 °C for 1 min. 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.

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' TCCTGTCCCCCAAGGAGGT 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 Mlu1-Cla1 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 x 10^7-10^8 transducing units (TU/ml) 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 μg/ml of polybrene in DMEM 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.

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Western Blots 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 RIPA lysis buffer containing protease inhibitor cocktail, then homogenized and centrifuged at 20,000 x g for 10 min. The supernatant (~20 μg) was subjected to SDS-polyacrylamide (SDS-PAGE) on a 12% mini-gel and transferred to an Immobilon™-P transfer membrane. The membrane was blocked with 1% BSA in TBST (10 mM Tris-HCl, pH 8.0, 150 mM NaCl, 0.05% Tween 20) for 1 h 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-HRP for RGS19) or (goat anti-mouse IgG-HRP for GIPC) for 1h 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 1:2,000 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 1:5,000 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 μM 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 (3-isobutyl-

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1-methylxanthine) in serum-free medium for 15 min at 37 °C, and then replaced with medium containing 1 mM IBMX, 30 μM forskolin with submaximum doses of agonists (100 nM morphine, 100 nM SNC80 or 10 nM nociceptin) 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 min. 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 min to pellet the precipitates. Accumulated cAMP was measured by radioimmunoassay in a 15 μl 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 h 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 min 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% bromphenol blue). Samples were removed from the wells, boiled for 5 min, and then subjected (15 μl each) to electrophoresis using a 12% SDS-PAGE mini gel, followed by transfer to an Immobilon^{TM}-P membrane for Western blotting. The blot was probed with 1:6,000 dilution of anti-phospho-p44/42 MAPK (Thr202/Tyr204) antibody and visualized using horseradish peroxidase-conjugated anti-rabbit IgG, followed by enhanced chemiluminescence detection (Kodak X-ray film) to measure the activated phospho-ERK. To assure equal loading, the same membranes were stripped and re-blotted with a 1:4,000 dilution of anti-p44/42 MAPK antibody to measure

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total ERK levels. MAPK activity was calculated as normalized arbitrary units (a.u.) 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 were presented as means ± S.E. Data were compared by two-way ANOVA 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 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 due to 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 lengh 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

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pathway. To study the functional role of RGS19, we developed a SH-SY5Y cell line stably expressing shRNA against RGS19 to continuously block endogenous RGS19 protein expression. 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. Over 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 ± 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 cells glioma cells (Fig. 2A).

SH-SY5Y cells express adenylyl cyclase (AC) types 1 and 8 that are inhibited by GTP-bound Gαi/o proteins (Sunahara et al., 2002; Hirst et al., 1995). To measure AC activity, RGS19-deficient and control SH-SY5Y cells were first differentiated with 10 μM retinoic acid for 6-7 days, then AC activity was measured as the accumulation of forskolin (30 μM)-stimulated cAMP in the presence of the phosphodiesterase inhibitor IBMX (3-isobutyl-1-methylxanthine, 1 mM). The levels of forskolin-stimulated cAMP accumulation were similar in control and RGS19-deficient cells. The degree of inhibition of forskolin-stimulated cAMP accumulation by the MOR agonist morphine at 100 nM, a submaxium concentration, was 16 ± 4% in control cells expressing shRNA against GFP, but this was significantly increased to 40 ± 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 submaxium concentration, was similar in the RGS19-deficient cell line (43 ± 5%) compared to control cells (35 ± 2%) expressing shRNA against GFP. Inhibition of cAMP accumulation by the NOPR

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agonist nociceptin at a submaximal concentration of 10 nM was not significantly different between the RGS19-deficient cell line (57 ± 2%) and control cells (48 ± 4%).

To determine if the differential effect of RGS19 on MOR agonist signaling was specific to the Gαi/o-mediated inhibition of AC, we examined whether MAPK regulation, a Gβγ-mediated effect, was also modulated by RGS19. Stimulation of ERK phosphorylation by submaximum concentrations of morphine (100 nM), SNC80 (100 nM) and nociceptin (10 nM) was measured in both RGS19-deficient SH-SY5Y cells in comparison to control SH-SY5Y cells expressing shRNA against GFP (Fig. 2C). The basal level of ERK phosphorylation, measured as normalized arbitrary units (a.u.) 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 ± 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 ± 10% and 113 ± 11%, respectively) and control cells (116 ± 10% and 117 ± 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 ~90% knockdown of RGS4 protein (35 ± 6% inhibition) compared to control SH-SY5Y cells expressing shRNA against GFP (38 ± 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.

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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 μM DAMGO or 10 μM morphine increased the intensity of the RGS19 protein band on Western blot by 324 ± 90% and 269 ± 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 proteins levels over untreated cells by 346 ± 63% and 229 ± 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 Gαi/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 pertusis toxin (PTX) for 6 h prior to and during overnight incubation in the absence or presence of 10 μM DAMGO or DPDPE. Treatment with PTX alone did not change the level of RGS19 protein. However, PTX completely blocked DAMGO- or DPDPE-induced up-regulation of RGS19 (Fig. 4). When the cells were treated overnight with DAMGO or DPDPE in the presence of the opioid antagonist naloxone (NLX, 10 μM), the opioid agonist-induced increase in RGS19 protein levels was completely prevented (Fig. 4).

Erk1/2-dependent and PKC phosphorylation of RGS19 is known (Ogier-Denis et al., 2000; De Vries et al., 1995). Moreover, MOR agonists activate the MAPK pathway and the PLC

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pathway (Gutstein et al., 1997; Clark et al., 2003; Mathews 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 μM) completely blocked DAMGO-induced up-regulation of RGS19 protein reducing the increase from 151 ± 8% to 96 ± 2% (Fig. 5A). Similarly, the PKC inhibitor Go6976 (0.1 μM) completely blocked DAMGO-induced up-regulation of RGS19 protein compared to cells treated with DAMGO alone (from 144 ± 9% to 80 ± 33%) (Fig. 5B). In addition, treatment of SH-SY5Y cells with the PKC activator PMA time- and concentration-dependently increased RGS19 protein levels (Figs. 5C and 5D). The presence of either PD98059 or Go6976 reduced the morphine-induced activation of MAPK activity (180 ± 24%) back to control values (Fig. 6), suggesting MOR agonist activation of the MAPK pathway is *via* PKC in these cells. The PKA inhibitor H89 (1.0 μM) did not affect the ability of DAMGO to upregulate RGS19 protein (149 ± 18%) compared to DAMGO alone (144 ± 9%) (Fig. 5B).

The slow time course of opioid-mediated RGS19 up-regulation suggests either that the effect of MOR and DOR agonists is not direct phosphorylation of RGS19, or the new synthesis of RGS19 is required. In order to test this hypothesis, SH-SY5Y cells were treated with the transcription inhibitor actinomycin D (5 μg/ml, overnight), with or without DAMGO (10 μM). 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 co-expressed in rat brain regions and

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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 was expressed in SH-SY5Y cells as a single strong band at ~37 KDa. Cells expressing shRNA against RGS19 showed a 50 ± 2% reduced level of GIPC expression compared to 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 ± 9% in cells treated with DAMGO compared to control without DAMGO treatment (Fig. 8B). These data indicate a tight regulation between RGS19 with its partner GIPC.

Discussion

In this study, we show 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 Gα and Gβγ-mediated pathways. This 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 naloxone-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.

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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) or NOPR (Xie et al., 2005; this manuscript) 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 importnat 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 towards Gαo and is highly, though not exclusively, expressed in the striatum (Gold et al., 1997). In contrast, RGS19 is a non-selective GAP for Gα subunits and is highly expressed in the hippocampus, with expression also in the 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 Gα proteins other than Gαo and in regions where it predominates over RGS9-2. Thus, the findings reinforce the importance of Gα 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 Gα

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subunits since both MOR and DOR activate the same Gαi/o protein subtypes (Chakrabarti et al., 1995; Prather et al., 1994a,b) and in SH-SY5Y cells, MOR, DOR and NOPR share the same pool of heterotrimeric G proteins (Alt et al., 2000; 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 the principle structural characteristics are conserved (de Alba et al., 1999). Indeed, both RGS19 and RGS4 efficiently act as GAPs towards members of the Gαi/o and Gαz 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; Wang et al., 2002; Bernstein et al., 2004; Saitoh et al., 2002; Wang et al., 2009) and we (Wang et al., 2009) and others (Xie et al., 2007; Georgoussi et al., 2006) have suggested a role for the C-terminus of opioid receptors in providing specificity of RGS action at DOR versus MOR. Moroever, outside of 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 GIPN (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 bi-directional 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-

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induced inhibition of cAMP accumulation, when both RGS19 and NOPR are co-expressed in COS-7 cells (Xie et al., 2005). However, we have previously shown with RGS4 that overexpression of RGS proteins can lead to non-physiological findings. For example, as noted above, 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 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 Gαi/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 if there is an RGS protein in these cells that controls NOPR signaling. Certainly, SH-SY5Y cells posses 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 down-regulated following overnight treatment with opioid agonists (Wang et al., 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 naloxone and by pretreatment with PTX, indicating an action *via* Gαi/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 this RGS proteins towards DOR signaling. However, we have previously reported downregulation of

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RGS4 by MOR agonists in these cells, even though MOR agonist action is not regulated by RGS4 (Wang et al., 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; Fisher 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 18h reduced the levels of RGS19 mRNA and protein and prevented the DAMGO-induced up-regulation. This suggests that the catabolism of RGS19 occurs within this time frame leading to 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

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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 Gαi/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 and Traynor.

Conducted experiments and data analysis: Wang.

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

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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.*

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 above established stable cell lines following 6-7 days treatment with 10 μM retinoic acid, percent of forskolin-stimulated cAMP

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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 to the control cells expressing shRNA against GFP. C. MAPK activation. Serum-starved (48 h) cells were treated with vehicle (Ctr), morphine (Mor, 100 nM), SNC80 (SNC, 100 nM), or nociceptin (NOC, 10 nM) for 5 min. MAPK activation was measured as described under *Materials and Methods*. ***, $P < 0.001$ (n = 4), for the RGS19-deficient RGS19 shRNA cell line, compared to the control cells expressing shRNA against GFP. Data are presented as percent of vehicle-treated control.

Fig. 3. Increased RGS19 protein expression but not mRNA following 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 μM, overnight) and RGS19 protein was identified by Western blot as decsribed in Fig. 1. RGS19 protein expression was increased 2-3 fold in opioid agonist-treated cells, compared to vehicle-treated cells (Ctr, 100 %). B and C, Time- and concentration-dependent increases in RGS19 protein following DAMGO treatment. SH-SY5Y cells were treated with 10 μM 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 following overnight DAMGO or DPDPE treatment. EF-1α was the loading control.

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Fig. 4. Blockade of DAMGO- and DPDPE-induced RGS19 protein upregulation by PTX and NLX. SH-SY5Y cells were preincubated for 6 h with PTX (100 ng/ml) or 1 h with naloxone (NLX, 10 μM), followed by overnight incubation in the absence or presence of DAMGO or DPDPE (10 μM each). RGS19 protein was identified by Western blot as described in Fig. 1. ***, $P < 0.001$, n = 3. β-actin loading controls were not changed.

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

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

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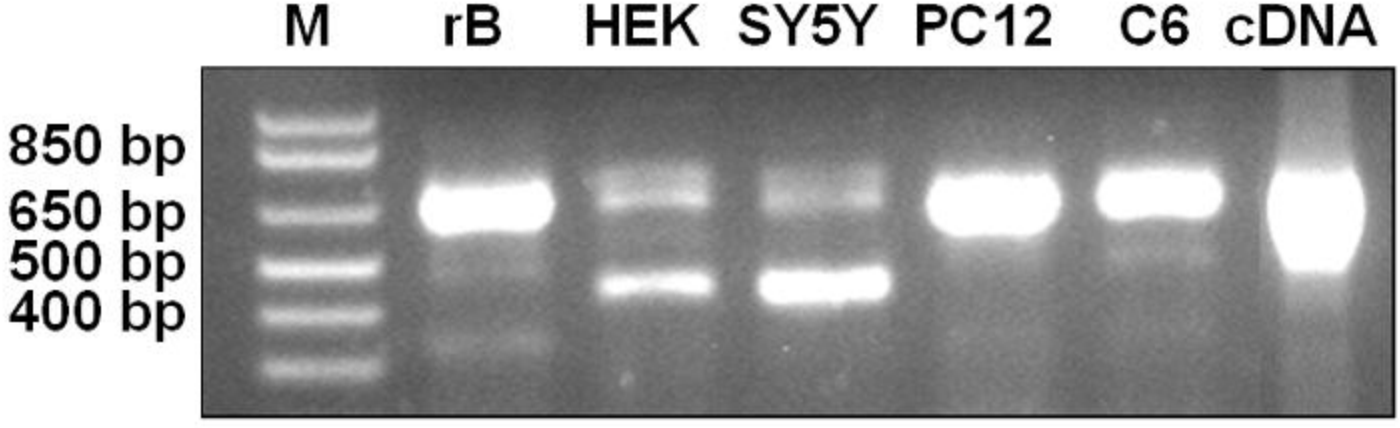
Fig. 7. Effect of actinomycin D treatment on RGS19 protein and mRNA. SH-SY5Y cells were treated with actinomycin D (AM, 5 μg/ml, overnight) or vehicle together with (+) or without (-) DAMGO (10 μM), then RGS19 protein (A) and mRNA (B) were identified by Western blot and RT-PCR as decsribed in Fig. 1.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

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 to cells expressing shRNA against GFP (A). When SH-SY5Y cells were treated with (+) DAMGO (10 μM) overnight, GIPC was increased ~50% compared to cells without DAMGO treatment (B). Quantified data from three sets of individual experiment are presented on the right. Loading controls (α-tubulin) were not changed.

A.


M
rB
HEK
SY5Y
PC12
C6
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850 bp
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500 bp
400 bp

B.

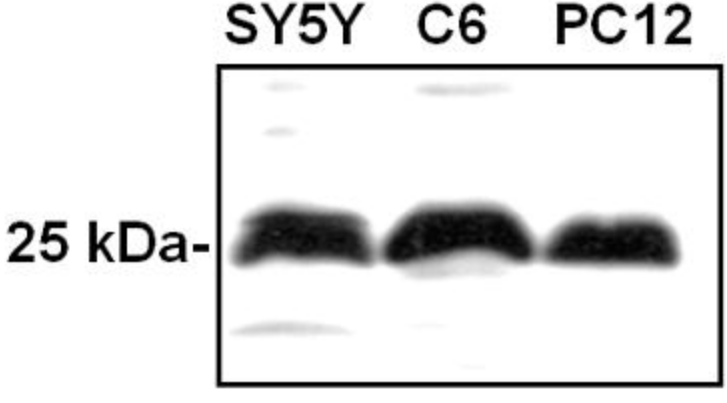
SY5Y
C6
PC12
25 kDa-

Figure1

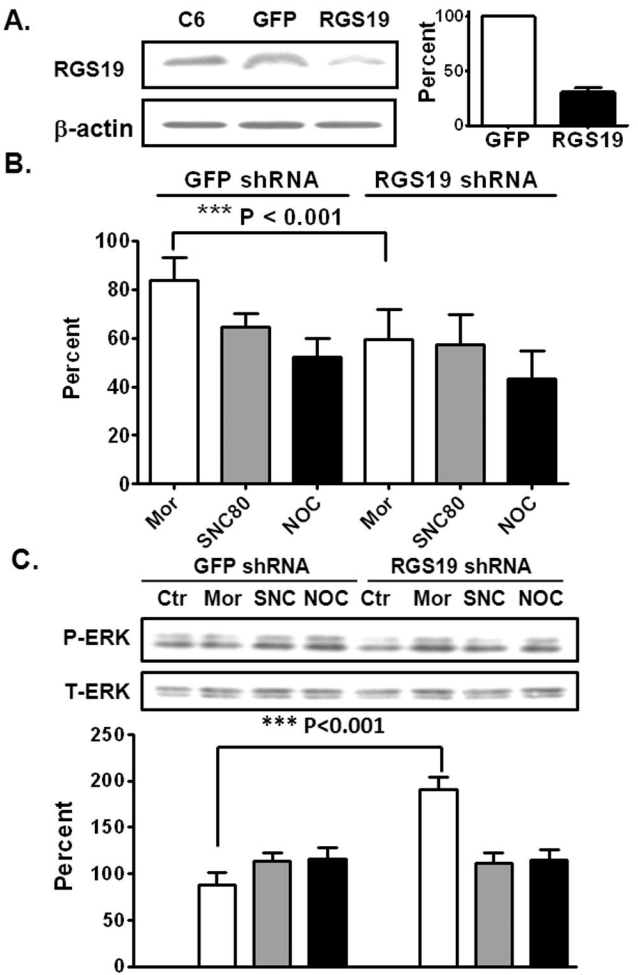
A.
C6
GFP
RGS19
RGS19
β-actin
Percent
100
50
0
GFP
RGS19
B.
GFP shRNA
RGS19 shRNA
*** P < 0.001
Percent
100
80
60
40
20
0
Mor
SNC80
NOC
Mor
SNC80
NOC
C.
GFP shRNA
RGS19 shRNA
Ctr
Mor
SNC
NOC
Ctr
Mor
SNC
NOC
P-ERK
T-ERK
*** P<0.001
Percent
250
200
150
100
50
0

Figure 2

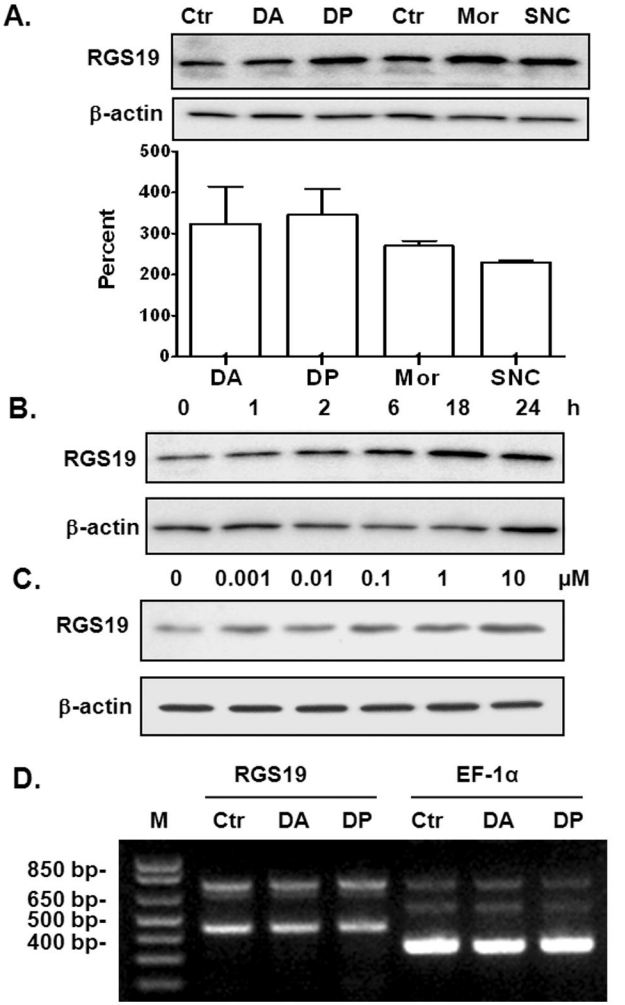
A.
Ctr
DA
DP
Ctr
Mor
SNC
RGS19
β-actin
Percent
500
400
300
200
100
0
DA
DP
Mor
SNC
B.
0
1
2
6
18
24
h
RGS19
β-actin
C.
0
0.001
0.01
0.1
1
10
μM
RGS19
β-actin
D.
RGS19
EF-1α
M
Ctr
DA
DP
Ctr
DA
DP
850 bp-
650 bp-
500 bp-
400 bp-

Figure 3

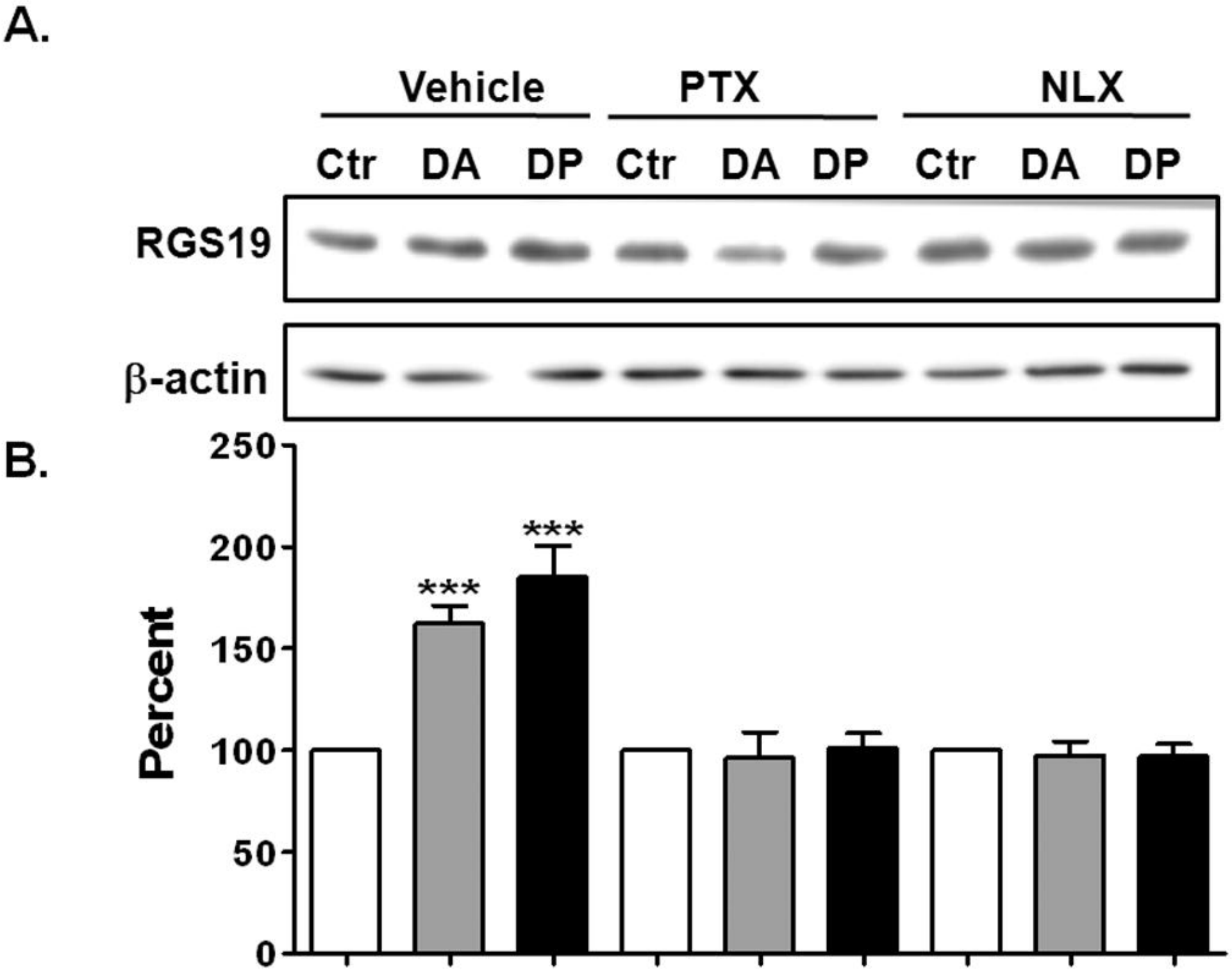
A.
Vehicle
PTX
NLX
Ctr
DA
DP
Ctr
DA
DP
Ctr
DA
DP
RGS19
β-actin
B.
250
200
150
100
50
0
Percent
***
***

Figure 4

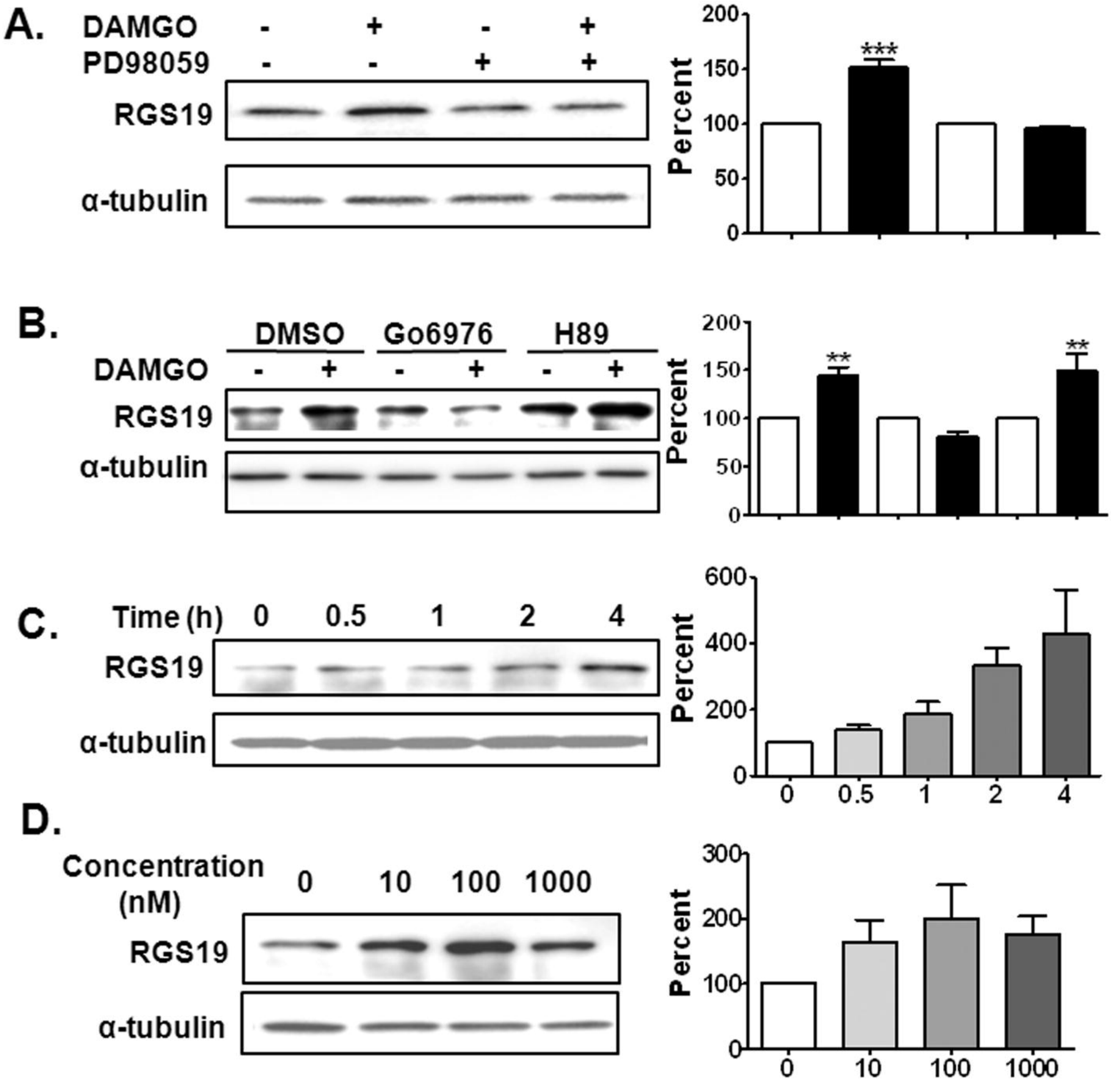
A.
DAMGO - + - +
PD98059 - - + +
RGS19
α-tubulin
Percent
200
150
100
50
0
***
B.
DMSO Go6976 H89
DAMGO - + - + - +
RGS19
α-tubulin
Percent
200
150
100
50
0
**
**
C.
Time (h) 0 0.5 1 2 4
RGS19
α-tubulin
Percent
600
400
200
0
0 0.5 1 2 4
D.
Concentration (nM) 0 10 100 1000
RGS19
α-tubulin
Percent
300
200
100
0
0 10 100 1000

Figure 5

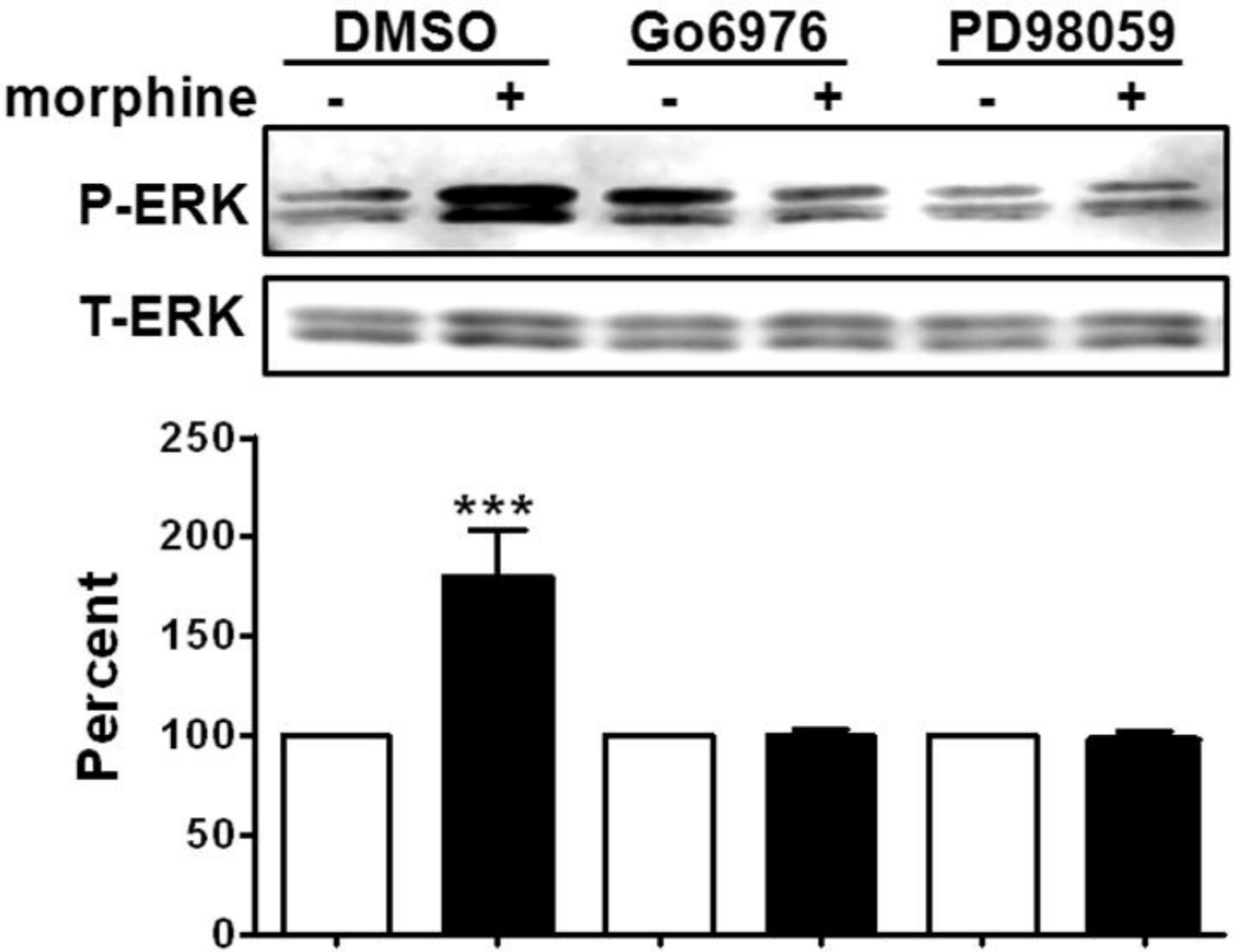
DMSO
Go6976
PD98059
morphine
-
+
-
+
-
+
P-ERK
T-ERK
250
200
150
100
50
0
Percent
***

Figure 6

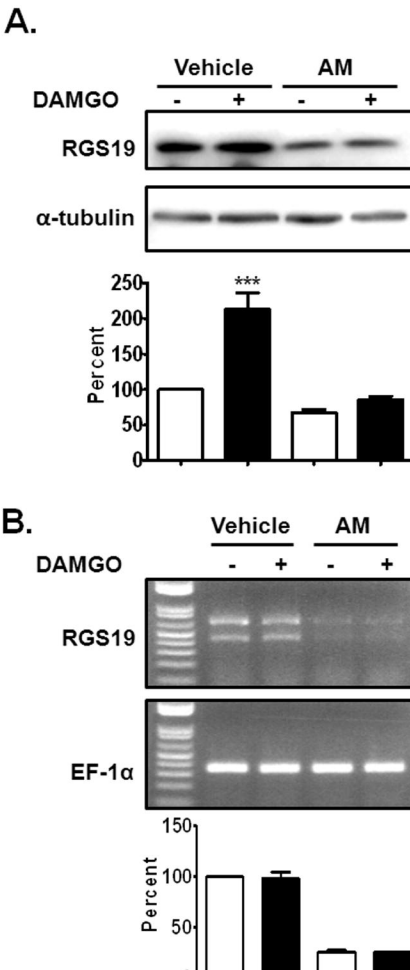
A.
Vehicle
AM
DAMGO
-
+
-
+
RGS19
α-tubulin
250
200
150
100
50
0
Percent
***
B.
Vehicle
AM
DAMGO
-
+
-
+
RGS19
EF-1α
150
100
50
0
Percent

Figure 7

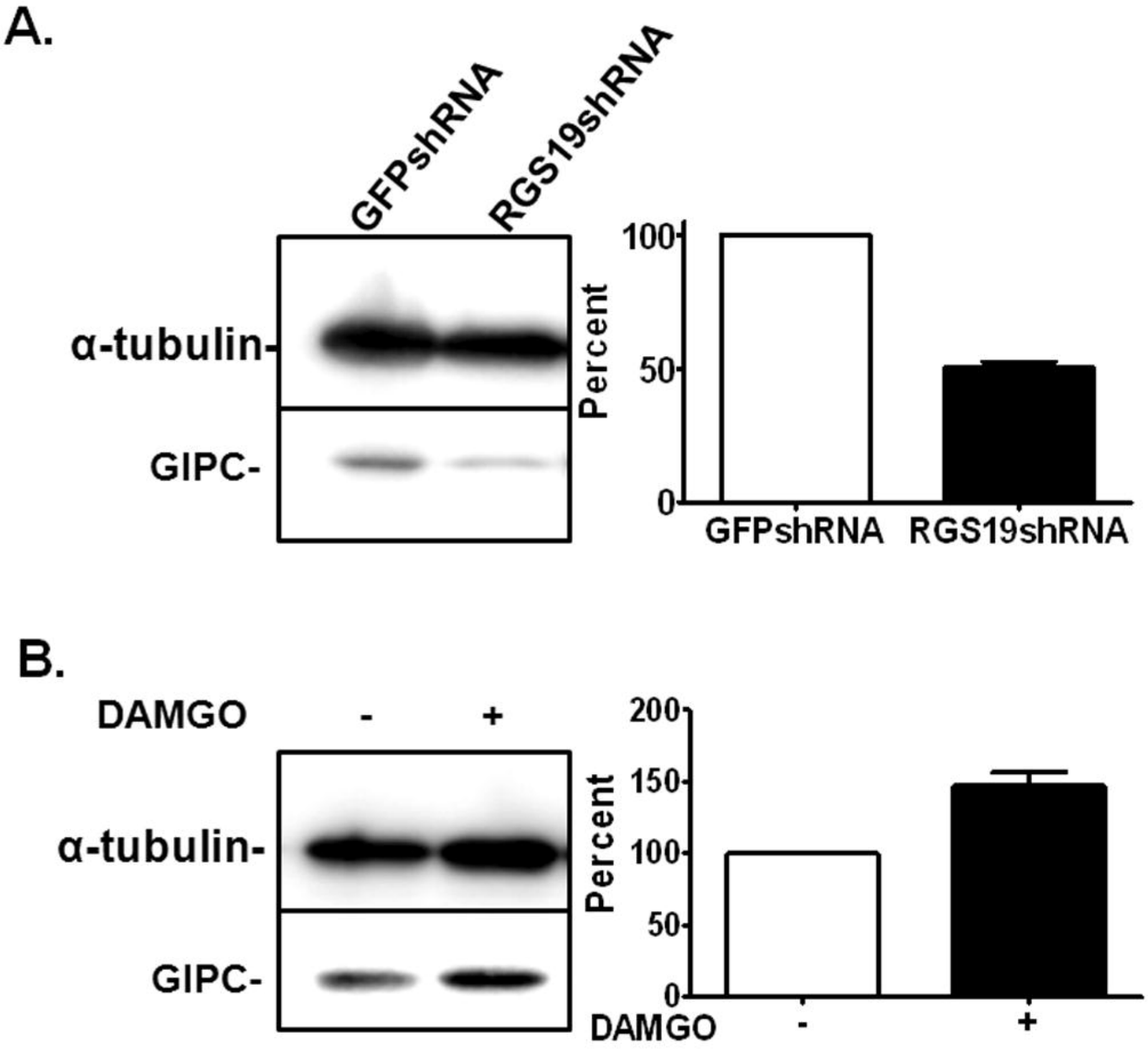
A.
GFPshRNA
RGS19shRNA
α-tubulin-
GIPC-
Percent
100
50
0
GFPshRNA
RGS19shRNA
B.
DAMGO
-
+
α-tubulin-
GIPC-
Percent
200
150
100
50
0
DAMGO
-
+

Figure 8