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A regional and projection-specific role of RGSz1 in the ventrolateral periaqueductal grey in the modulation of morphine reward.

Running title: RGSz1 in the periaqueductal gray regulates morphine actions

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Abstract

Opioid analgesics exert their therapeutic and adverse effects by activating mu opioid receptors (MOPR), however functional responses to MOPR activation are modulated by distinct signal transduction complexes within the brain. The ventrolateral periaqueductal gray (vlPAG) plays a critical role in modulation of nociception and analgesia, but the exact intracellular pathways associated with opioid responses in this region are not fully understood. We previously showed that knockout of the signal transduction modulator RGSz1 (RGSZ1KO) enhanced analgesic responses to opioids, while it decreased the rewarding efficacy of morphine. Here, we applied viral mediated gene transfer methodology and delivered AAV-Cre viruses to the vlPAG of RGSz1$^{fl\fl}$ mice to demonstrate that downregulation of RGSz1 in this region decreases sensitivity to morphine in the place preference paradigm, under pain-free as well as neuropathic pain states. We also utilized retrograde viral vectors along with flipase-dependent Cre vectors to conditionally downregulate RGSz1 in vlPAG projections to the ventral tegmental area (VTA) and show that downregulation of RGSz1 prevents the development of place conditioning to low morphine doses. Consistent with the role for RGSz1 as a negative modulator of MOPR activity, RGSz1KO enhances opioid-induced cAMP inhibition in PAG membranes. Furthermore, using a new generation of BRET sensors, we demonstrate that RGSz1 modulates Gαz but not other Gαi family subunits, and selectively impedes MOPR-mediated Gαz signaling events invoked by morphine and other opioids. Our work highlights a regional and circuit-specific role of the G protein signaling modulator RGSz1 in morphine reward, providing insights on midbrain intracellular pathways that control addiction-related behaviors.

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Significance Statement

In this study we used advanced genetic mouse models to highlight the role of the signal transduction modulator named RGSz1 in responses to clinically used opioid analgesics. We show that RGSz1 controls the rewarding efficacy of opioids by actions in vlPAG projections to the ventral tegmental area, a key component of the midbrain dopamine pathway. These studies highlight novel mechanisms by which pain-modulating structures control the rewarding efficacy of opioids.

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Non-standard abbreviation list

AAV= Adeno Associated Virus

cAMP= Cyclic Adenosine Monophosphate

BRET= Bioluminescence Resonance Energy Transfer

BSA= Bovine Serum Albumin

CMV= cytomegalovirus

GPCR= G Protein Coupled Receptor

GTP= guanosine triphosphate

HEPES= (4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid

EGFP=Enhanced Green fluorescent protein

MOPR = Mu Opioid Receptor

NAc= Nucleus Accumbens

vlPAG = Ventrolateral Periaqueductal Gray

RGS = Regulator of G protein signaling

Syn= Synapsin

VTA= Ventral Tegmental Area

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Introduction

Regulators of G-protein signaling (RGS) are multifunctional signal transduction modulators with critical roles in physiological processes and disease pathophysiology. RGS proteins may modulate the activity of G-protein coupled receptors (GPCRs) by acting as GTPase accelerators for activated Gα subunits (Sjogren et al., 2010; Terzi et al., 2009) or by various other mechanisms. For example, RGS proteins act as effector antagonists for Gα subunits or they may modulate transcriptional or translational activity (Hepler, 1999; Sakloth et al., 2020b; Terzi et al., 2009). RGSz1 (also known as *RGS20) (Barker et al., 2001)* shows a wide expression in the brain (Wang et al., 1998) and produces several splice variants. RGSz1 has been shown to accelerate the GTPase activity of the Gαz subunit by 600-fold, and to also compete with other members of the RGS family (such as Axin2) for Gα association (Gaspari et al., 2018; Glick et al., 1998; Wang et al., 1998).

In vitro evidence suggests that RGSz1 negatively modulates mu-opioid receptor (MOPR) functional responses (Ajit et al., 2007). More recent data from our lab suggests that RGSz1 regulates the rewarding and analgesic efficacy of opioids in a bi-directional way. Specifically, RGSz1KO enhances the analgesic efficacy of morphine, methadone, and fentanyl (Gaspari et al., 2018) under pain-free and chronic pain states while it decreases sensitivity to the rewarding and locomotor activating effects of the drug. Our earlier work also demonstrated that morphine treatment affects the expression of RGSz1 selectively in the vlPAG, and identified a critical role for this molecule in the function of the beta-catenin pathway, which involved competition with Axin2 for binding to Gαz (Gaspari et al., 2018). Our behavioral experiments showed that downregulation of RGSz1 in the vlPAG delays the development of tolerance in both male and female mice.

In this study, we hypothesized that RGSz1 affects the rewarding effects of opioids by modulating the activity of vlPAG projections to brain reward circuitry components, such as the

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VTA. We applied conditional knockout models for brain region and circuit-specific downregulation of RGSz1 in order to understand the functional role of RGSz1 in the vlPAG and in vlPAG projections to the VTA in the rewarding efficacy of morphine. We show that downregulation of RGSz1 in the vlPAG decreases sensitivity to the rewarding actions of morphine in pain-free as well as in chronic pain states. Furthermore, we demonstrate that downregulation of RGSz1 in the vlPAG/VTA circuit decreased the rewarding efficacy of morphine. Using cAMP inhibition assays, we show that RGSz1 in the PAG acts as a negative modulator of cAMP mediated responses, in accord with its role as a Gαz negative modulator. We further show that RGSz1 selectively modulates MOPR-stimulated Gαz signaling but not that of other Gαi/o proteins in an in vitro assay using bioluminescence resonance energy transfer (BRET) Gαβγ biosensors. Moreover, RGSz1 negatively modulates Gαz signaling by clinically prescribed opioids targeting MOPR.

In summary, our studies highlight novel intracellular pathways in the vlPAG that control the rewarding efficacy of morphine and demonstrate that RGSz1/Gαz interactions potently modulate functional responses to clinically prescribed opioids. While several RGS proteins modulate the actions of opioids throughout the brain and peripheral sites, RGSz1 negatively modulates analgesia while promoting morphine reward. This unique property of RGSz1 may provide novel avenues for therapeutic interventions that optimize the actions of opioid analgesics.

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Materials and methods

Animals

We used two to three-month old male RGSz1$^{fl/fl}$ mice (Gaspari et al., 2018) derived from homozygote breeding. The RGSz1 locus targeting strategy and the primers used for genotyping have been previously described (Gaspari et al., 2018). Mice were housed with a 12-h dark/light cycle and they were provided with food and water ad libitum. Housing, husbandry, experiments and procedures, were performed according to the Animal Care and Use Committee of the Icahn School of Medicine at Mount Sinai. For all behavioral assays, experimenters are blinded to the genotype.

Membrane preparation

Membranes were prepared from the ventral PAG and striatum of male RGSz1WT and RGSz1KO mice as described previously (Gomes et al., 2016). Briefly, tissues were homogenized in 25 volumes (1 g wet weight/25 ml) of ice-cold 20 mM Tris-Cl buffer containing 250 mM sucrose, 2 mM EGTA, and 1 mM $MgCl_2$ (pH 7.4) followed by centrifugation at 27,000g for 15 min at 4°C. The pellet was resuspended in 25 ml of the same buffer, and the centrifugation step was repeated. The resulting membrane pellet was resuspended in 40 volumes (of original wet weight) of 2 mM Tris-Cl buffer containing 2 mM EGTA and 10% glycerol (pH 7.4). The protein content of the homogenates was determined using the Pierce BCA Protein Assay Reagent, after which homogenates were stored in aliquots at −80°C until use.

cAMP assay

Membranes (2 µg/10 µl per well of a 96 well plate) from the PAG and striatum of male RGSz1WT and from RGSz1KO mice were incubated for 30 min at 37°C with morphine (0 to 10 µM final concentration) in assay buffer (50 mM HEPES, 10 mM MgCl2, 100 mM NaCl, 200 µM ATP, 10 µM GTP, pH 7.4) containing 40 µM forskolin, 1X protease inhibitor cocktail and 100 µM

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IBMX (3-isobutyl-1-methylxanthine); final assay volume was 50 µl. cAMP levels were measured using the HitHunter cAMP detection kit for biologics from DiscoveRx as described in the manufacturer's protocol. In a separate set of experiments, membranes from PAG or NAc were pre-treated with 100 ng/ml activated pertussis toxin (PTX) for 30 min at RT before incubation with 10 µM (final concentration) of morphine.

RT-qPCR validation

Total ventral PAG RNA was isolated separately for each animal using Trizol reagent (Gaspari et al., 2018). Reverse transcription was performed using qScript®reverse transcriptase (Quantabio) according to the manufacturer's instructions. cDNAs were then diluted to 10 ng/µL and amplified using PerfeCTa SYBR Green SuperMix (Quantabio) on a QuantStudioTM 7 Flex Real Time PCR system. Primers used for RGSz1: ACTCCAGCCGGAAGAAATGC (forward) and GTTCTTCACAGGCCATCCAGA (reverse). GAPDH was used as a reference gene (TGTAGACCATGTAGTTGAGGTCA (forward), AGGTCGGTGTGAACGGATTTG (reverse)).

Conditioned Place Preference Test

An automated unbiased place-conditioning procedure was performed using a two-chamber place-conditioning system (Med Associates, Inc.) as previously described (Gaspari et al., 2017; Gaspari et al., 2018). Mice were habituated in the room for at least 30 min before administration of either morphine or saline and placing them in the conditioning chambers. On day 1, baseline preference was assessed for 20 min. On days 2-7, animals were conditioned to the drug-paired or saline-paired side for 45 min on alternate days. On day 8, animals were tested for 20 min. The results are presented as the time spent in the drug-paired compartment at baseline compared with that on test day. For SNI groups of mice, sensory hypersensitivity was assessed a week after the end of the CPP assay. Data are presented as preference for drug-paired side at baseline and after conditioning.

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Stereotaxic Viral Injection Surgery

Conditional deletion of RGSz1 was achieved by bilateral stereotaxic injections of AAV2-CMV-CRE-EGFP (University of North Carolina Vector Core Facility, titer $4.4x10^{12}$ virus molecules/ml) into the vlPAG of $RGSz1^{fl/fl}$ mice, as described (Gaspari et al., 2018). Control animals received injections of AAV2-CMV-EGFP viruses (University of North Carolina Vector Core Facility, $4.4\text{-}5x10^{12}$ virus molecules/ml). For circuit specific RGSz1 downregulation, the VTA of $RGSz1^{fl/fl}$ mice was bilaterally injected with pAAV-EF1a-fDIO-Cre (Addgene $9.2.x10^{12}$ virus molecules/ml) and the vlPAG with pAAV-EF1a-mCherry-IRES-Flpo (AAV1) viruses (Addgene, 7.8 $x10^{12}$ virus molecules/ml). Control mice were injected with the same virus in the VTA but with pAAV-hSyn-EGFP (AAV1) (Addgene) in the vlPAG. Stereotaxic coordinates for viral vector injections were as follows: vlPAG (with respect to lambda): AP, +0.6 mm; mediolateral (ML), +0.8 mm; and DV, −2.8 mm at 22° from the midline; VTA (with respect to bregma): AP, -3.2 mm; mediolateral (ML), +0.9 mm; and DV, −4.7 mm at 7° from the midline.

Spared nerve injury surgery

Spared nerve injury (SNI) was performed in the left sciatic nerve, as previously described (Mitsi et al., 2015; Sakloth et al., 2020a). Surgery was performed using a stereomicroscope. A skin and muscle incision of the left hind limb at mid-thigh level was performed to reach the sciatic nerve. The common peroneal and the sural nerves were carefully ligated with 6.0 silk suture (Patterson Veterinary), transected and 1–2 mm sections of these nerves were removed, while the tibial nerve was left intact. Skin and muscle were then closed with 4.0 silk suture (Patterson Veterinary).

TRUPATH BRET Gαz signaling assay

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Assays were performed in HEK293T cells (ATCC). Cells were maintained and passaged in Dulbecco's Modified Eagle Medium (DMEM Corning) containing 10% FBS (BioTC), 100 U/ml penicillin (Invitrogen), and 100 µg/ml streptomycin (Invitrogen) in a 37 °C incubator with 5% CO_2.

TRUPATH sensors based on human Gαβγ proteins were obtained from Addgene, and both human MOPR and human RGSz1 (isoform 5; UniProtKB O76081-6) were cloned into pcDNA3.1 vectors. Assays were essentially performed as described (Olsen et al., 2020). Briefly, cells were plated in 10 cm dishes in DMEM containing 10% FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. 24 hrs later media was exchanged for DMEM containing 1% dialyzed FBS, 100 U/ml penicillin, and 100 µg/ml streptomycin. Cells were then transfected with DNA (600 ng of human MOPR, 4 µg of Gz or Gi/o TRUPATH sensors, and 4 ug of hRGSz1 where indicated) using polyethyleneimine (Alfa Aesar). The next day, cells were trypsinized and plated in poly-D-lysine-coated 96-well assay plates (Corning) at a density of 30,000–50,000 cells per well. One day after plating, the medium was carefully aspirated and replaced immediately with 60 µl of assay buffer (1× Hank's balanced salt solution (HBSS), 20 mM HEPES, pH 7.4, 0.1% w/v BSA, 0.01% w/v ascorbic acid). The cells were then treated with 30 µl of opioid drugs at different dilutions in assay buffer. 30 min after drug treatment, 10 µl of freshly prepared 25 µM coelenterazine 400a (Goldbio) in assay buffer was added. Data was then immediately recorded in an LB940 Mithras plate reader (Berthold Technologies) with 410 nm (RLuc8) and 515 nm (GFP2) emission filters, at integration times of 1 s per well. BRET2 ratios were computed as the emission ratio of GFP2/RLuc8. All experiments were performed in duplicates and triplicates, and data was normalized and averaged from four to eight independent experiments in GraphPad prism.

Statistical Analyses

Statistical Analyses

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Experiments reported here were not designed to test a statistical null hypothesis and data analysis is thus considered exploratory. Data were analyzed using Graph Pad Prism 9 software. TRUPATH concentration response data and scatterplots are shown with 95% confidence intervals, and EC50s and pEC50s are reported as mean±SD. Statistical differences between conditions in RGSz1 WT and RGSz1KO was done either via two-way ANOVA using Sidak's multiple comparisons test (Fig. 3A, B), or by one-way ANOVA with Tukey's multiple comparison test.(Fig. 3C, D). For the experiments monitoring behavioral responses over time, we used two-way repeated-measures ANOVAs followed by Sidak's post hoc test. For data containing a single independent variable, we used unpaired two-tailed t tests. For the ANOVA post-tests, compared groups were selected before data collection.

Results

RGSz1 in the vlPAG regulates morphine's rewarding effects.

To gain insight into the brain region-specific actions of RGSz1 we first tested the hypothesis that RGSz1 in the vlPAG positively modulates the rewarding actions of morphine. The experimental outline is shown in Fig. 1A. To downregulate RGSz1 in the vlPAG of adult mice, we infected the vlPAG of male RGSz1$^{fl/fl}$ mice with AAV2-CMV-CRE-EGFP. Fig 1B shows the qPCR validation of RGSz1 downregulation in PAG punches at ten days after viral infection. Control mice were infected with AAV2-CMV-EGFP. Two weeks post-surgery, when maximum viral expression was achieved, mice were tested in the CPP assay. Downregulation of RGSz1 in the vlPAG prevented the rewarding actions of morphine (1mg/kg s.c.) suggesting a positive modulatory role of this protein in the rewarding actions of opioids by actions outside the midbrain dopamine system (Fig. 1C).

RGSz1 in the vlPAG regulates morphine reward under neuropathic pain conditions

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Since the vlPAG plays a critical role in the processing of nociception and analgesia (Ossipov et al., 2014), and chronic pain states may affect responsiveness to opioid analgesics, we next tested if RGSz1 modulates morphine reward under chronic pain states. We utilized the spared nerve injury (SNI) model to confirm that similar to our observations with pain-free states, RGSz1 acts as a positive modulator of morphine reward under neuropathic pain states. For this set of studies we bilaterally infected the vlPAG of RGSz1$^{fl/fl}$ mice with AAV2-CMV-CRE-EGFP or with AAV2-CMV-EGFP vectors and two weeks after viral infusions we performed the SNI operation. Four weeks after the SNI surgery, when mice had transitioned to chronic neuropathic states, we tested them in the CPP paradigm using low doses of morphine (0.5 mg/kg s.c.). Consistent with our hypothesis, downregulation of RGSz1 in the vlPAG prevents place preference to a low morphine dose (0.5 mg/kg, Fig. 1D). The neuropathic state was confirmed by use of the von Frey assay a week after the completion of the CPP experiment.

RGSz1 in the vlPAG-VTA circuit positively modulates morphine reward.

The vlPAG is connected to several brain regions with modulatory roles in analgesia and nociception. We hypothesized that RGSz1 modulates morphine reward by actions in neuronal projections to the midbrain dopamine pathway. Recent studies in rodents have documented a role for vlPAG-VTA projections in responses to drugs of abuse (Ntamati et al., 2018; St Laurent et al., 2020). In order to determine if downregulation of RGSz1 in vlPAG-VTA projections impacts morphine reward, we used RGSz1$^{fl/fl}$ mice and infected their VTA with pAAV-EF1a-fDIO-Cre and their vlPAG with pAAV-EF1a-mCherry-IRES-Flpo (AAV1). Control mice were injected with the same virus in the VTA but they received pAAV-hSyn-EGP (AAV1) in the vlPAG. qPCR analysis demonstrates that this intervention results in a significant reduction of RGSz1 mRNA expression in PAG punches (Fig. 2B). As shown in Fig. 2C, downregulation of RGSz1 in this circuit prevents the rewarding effects of morphine at lower doses (0.3 mg/kg, s.c.). At higher doses, both genotypes show similar place conditioning behaviors (Fig. 2D).

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Knockout of RGSz1 promotes opioid-induced cAMP inhibition in PAG membranes.

We next used striatal and ventral PAG membranes to define the impact of RGSz1KO on morphine-induced cAMP inhibition. We find that in striatal membranes, morphine causes a dose dependent inhibition of cAMP levels in RGSz1WT mice (EC_{50} 1.1±1.4 nM, pEC_{50} 9.0±0.08 and E_{max} of 72±2 % of basal). RGSz1KO mice exhibit a weak but significant change in the potency (EC_{50} 0.3±1.4 nM, pEC_{50} 9.5±0.08; $p<0.0001$, unpaired t-test) but not efficacy (E_{max} of 71±2 % of basal) compared to RGSz1WT mice (Fig. 3A). We detect changes in the potency and efficacy ($p<0.0001$, unpaired t-test) of morphine in PAG membranes from RGSz1KO mice (EC_{50} 1.3±1.4 nM, pEC_{50} 8.9±0.1 and E_{max} of 53±2 % of basal) compared to membranes from RGSz1WT mice (EC_{50} 12±2 nM; pEC_{50} 7.9±0.09 and E_{max} of 71±2 % of basal) (Fig. 3B); this indicates that lack of RGSz1 enhances opioid-induced cAMP inhibition in PAG membranes. Next, we used pertussis toxin treatment to identify the contribution of Gαi/o (pertussis toxin sensitive) and Gαz (pertussis toxin insensitive) to morphine-mediated decreases in cAMP levels. We find that in nucleus accumbens (NAc) membranes from RGSz1WT mice, pertussis toxin treatment completely blocks signaling, while ~5% signaling is pertussis toxin-insensitive in RGSz1KO mice (Fig. 3C). In PAG membranes from RGSz1WT mice, we find that ~10% signaling is pertussis toxin-insensitive and this increases to ~ 25% in RGSz1KO membranes (Fig. 3D). Together, these results indicate that while morphine-mediated decreases in cAMP levels in the NAc of RGSz1WT and RGSz1KO occur primarily via Gαi/o, in the PAG there is an involvement of Gαz in addition to Gαi/o.

Opioid analgesics-induced Gαz activation is impaired by RGSz1 association

To gain more insight on the effects of RGSz1 at the molecular level, we used a new generation of bioluminescence resonance energy transfer (BRET) sensors to monitor MOPR-mediated activation of different inhibitory G proteins by clinical opioid drugs in HEK293T cells

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(Olsen et al., 2020) (Fig. 4A). Since we and others have identified Gαz as a key mediator of opioid-related analgesia (Gaspari et al., 2018; Yang et al., 2000), we first investigated whether RGSz1 selectively affects Gαz-mediated signaling. We thus monitored MOPR-mediated activation of the different inhibitory G proteins in the presence and absence of RGSz1 in our BRET assay. Using DAMGO as the canonical MOPR ligand, RGSz1 appears to selectively reduce potency in activation of the Gαz sensor, but none of the other inhibitory G proteins (Fig. 4B-D).

We next wanted to investigate, whether RGSz1 negatively modulates Gαz signaling stimulated by morphine and other clinically relevant MOPR opioids including Fentanyl and Methadone. The different opioids displayed distinct potencies in MOPR-mediated Gαz activation, with DAMGO (EC_{50} 2.81±1.00 nM, pEC_{50} 8.72±0.19) and Fentanyl (EC_{50} 1.17±0.52 nM, pEC_{50} 9.10±0.24) exhibiting higher potencies compared to Morphine (EC_{50} 26.11±8.36 nM, pEC_{50} 7.68±0.13) and Methadone (EC_{50} 19.86±5.24 nM, pEC_{50} 7.80±0.14) (Fig. 5A, B). Overall, co-transfection of RGSz1 decreased the potency of all opioids tested in our assay by approximately 10-fold. This is in agreement with previous evidence of the RGSz1 modulation of Gαz activity (Gaspari et al., 2018; Wang et al., 1998). In our assay, RGSz1 behaves as a negative modulator likely due to its ability to restore the Gαz subunit to the GDP-bound inactive state. Our in vitro data thus adds further evidence that RGSz1 is a negative modulator of opioid stimulated Gαz signaling via MOPR, a pathway that likely plays a key role in both the analgesic and rewarding properties of clinically used opioid drugs.

Discussion

Our study demonstrates a dynamic role of RGSz1 in the vlPAG in behavioral and biochemical responses to clinically used opioid analgesics. Our in vitro BRET assays show that RGSz1 associates with Gαz but not with other inhibitory Gα subunits in response to opioid treatment. Furthermore, cAMP inhibition assays in PAG membranes further support a role of

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RGSz1 in modulation of cAMP formation upon MOPR activation. Importantly, our study identified the regional and circuit-specific role of RGSz1 in morphine reward sensitivity. We show that downregulation of RGSz1 in the vlPAG prevents morphine place preference, and that this phenotype is recapitulated by conditional downregulation of RGSz1 in vlPAG-VTA projections. Thus, our work reveals a role of RGSz1 within vlPAG-VTA circuitry in reward sensitivity, providing new insight on mechanisms by which nociception/analgesia circuitry controls the rewarding effects of opioids.

The VTA is implicated in the processing of both rewarding and aversive behaviors and plays a pivotal role in responses to drugs of abuse, including opioid analgesics (Doyle and Mazei-Robison, 2021). Importantly, the VTA has also been shown to modulate nociceptive responses (Mitsi and Zachariou, 2016; Watanabe et al., 2018; Zhang et al., 2017). Here, we show that projections from the vlPAG to the VTA affect sensitivity to morphine reward, highlighting a role of midbrain nociceptive circuitry in the modulation of the mesolimbic dopamine system. Understanding the function and regulation of this circuitry by chronic pain conditions, may provide important insight on mechanisms affecting vulnerability to opioids. Similarly, it will be important to assess the function of vlPAG-VTA projections in subjects that have developed physical dependence to opioids.

The PAG is organized into different sub-regions (Bandler and Shipley, 1994), each of which connected by distinct neuronal projections to different parts of the brain, thereby modulating a number of sensory and affective behaviors (Bandler and Keay, 1996; Bandler and Shipley, 1994; Lefler et al., 2020; Vander Weele et al., 2018; Yaksh et al., 1976). In regards to opioid actions, the PAG has been shown to be involved in the modulation of analgesia (Jacquet and Lajtha, 1974; Morgan et al., 2014; Smith et al., 1992), analgesic tolerance (Bagley et al., 2005; Gaspari et al., 2018; Morgan et al., 2006; Tortorici et al., 1999), physical dependence (Laschka and Herz, 1977), and reward (Olmstead and Franklin, 1997). Recent studies have demonstrated that the vlPAG to VTA circuit plays a role in morphine's locomotor effects (St

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Laurent et al., 2020). Consistent with these recent studies that used electrophysiology, optical and chemogenetic approaches to demonstrate the functional role of vlPAG-VTA connections, we show that G protein modulation in vlPAG-VTA projections affects the rewarding efficacy of morphine in the CPP assay, providing potential new avenues towards the optimization of opioid actions and safer use in chronic pain populations.

Several previous studies characterized mouse lines lacking RGS protein members globally or in specific circuitry/brain regions. RGS9, is enriched in the striatum, and plays a dynamic role in the modulation of opioid and psychostimulant reward. We have shown that prevention of RGS9 action in the NAc increased the sensitivity to morphine in the CPP paradigm by tenfold (Zachariou et al., 2003), and also affected the locomotor sensitizing effects of opioids. Furthermore, prevention of RGS9 action enhances analgesic responses to morphine, and delays tolerance, while it decreases response to fentanyl, oxycodone and methadone (Gaspari et al., 2017; Psifogeorgou et al., 2011). RGS7 is also present abundantly in the striatum, but shows a wider range of expression compared to RGS9, and it is also present in noradrenergic brainstem nuclei. RGS7 knockout mice show increased sensitivity to the rewarding and analgesic actions of morphine, and at least part of this phenotype is related to RGS7 actions in the striatum (Sutton et al., 2016). RGS4 is present in a number of brain regions modulating opioid reward and analgesia, including the striatum, the locus coeruleus and the prefrontal cortex. Knockout of RGS4 in the NAc triggers a small but significant increase in sensitivity to the rewarding effects of morphine (Han et al., 2010), but does not influence the analgesic responses to the drug. Notably RGS4 shows agonist-specific function as it acts as a positive modulator of fentanyl and methadone analgesia. RGSz1KO mice do not show the agonist biased modulatory role that we observed with RGS9KO and RGS4KO mice in opioid analgesia assays. As shown in previous studies, RGSz1KO mice respond to much lower doses of morphine, fentanyl and methadone, compared to their wildtype counterparts (Gaspari et al., 2018). Future work will define the circuitry mediating analgesic responses and the cellular

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pathways downstream of RGSz1. We expect that RGSz1 actions in descending vlPAG projections to the rostroventral medial medulla modulate analgesic responses to opioids, but additional circuitry may also contribute to this action. Consistent with earlier findings from constitutive RGSz1KO mice we demonstrate that downregulation of RGSz1 in the vlPAG attenuates the rewarding actions of morphine in the CPP paradigm. Importantly, this phenotype is maintained under chronic neuropathic pain states. These actions are mediated by distinct neuronal populations than those involved in descending inhibitor control, namely, neuronal projections from vlPAG to the VTA. Circuit-specific interventions in the RGSz1$^{fl\fl}$ line also required lower morphine doses compared to regional RGSz1 vlPAG knockdown as this intervention targets only a subset of RGSz1 cells and a reward-sensitivity phenotype can be revealed with very low drug doses. Future work will also determine the impact of vlPAG RGSz1 in the reinstatement of morphine CPP and in the reinforcing actions or morphine and other opioid analgesics in male and in female mice.

Our in vitro assays in PAG membranes and transfected cells provided insights into the mechanism of RGSz1 action. Consistent with our hypothesis and earlier findings, RGSz1 in the PAG acts as a canonical RGS protein and negatively modulates opioid-induced inhibition of cAMP accumulation. Several studies in the past have demonstrated the ability of RGS proteins to modulate inhibition of the cAMP pathway in response to opioids (Clark et al., 2003; Senese et al., 2020). Using human RGSz1 clones we show that RGSz1 preferentially modulates Gαz compared to other Gα subunits. Moreover, we show that this modulation has direct consequences on the signaling of all tested opioid drugs in Gαz associated pathways. In fact, RGSz1 reduces morphine-mediated Gαz activation potency by about 10-fold, which directly correlates with a ~10-fold increase in morphine-mediated cAMP reduction potency in PAG membranes isolated from RGSz1KO animals. At the cellular level, both reward and analgesia mechanisms involve inhibition of cAMP formation. In the vlPAG, MORs are expressed in various

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cell populations which project to distinct brain regions. RGSz1 expressing neurons that are components of the descending inhibitory pathway (Gogas et al., 1991), and project to the rostroventromedial medulla and the spinal cord, play a prominent role in analgesic responses. A different subpopulation of vlPAG neurons that express RGSz1, respond to morphine, and project to the VTA, modulate the rewarding and possibly locomotor actions of opioids.

Overall, this work provides novel information on the circuit-specific function of RGSz1 in the modulation of opioid reward and reveals that the mechanism of RGSz1 actions involves association with Gαz and negative modulation of opioid-induced inhibition of cAMP formation. Thus, inhibition of RGSz1 or downstream pathways may provide a way to administer low doses of opioids for the management of pain, without the risk of physical dependence or addiction-like effects.

Authorship contributions:

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Wrote or contributed to writing the manuscript: F. Sakloth, A. Ruiz, O. Sanchez Reyes, R.A, Serafini, D. Wacker, I. Gomes, L. A. Devi and V. Zachariou.

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Footnotes

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Conflict of interest statement- The authors declare no conflicts of interest

Figure Legends

Figure 1. RGSz1 actions in the vlPAG modulate morphine reward. **A**. Cartoon summarizing viral treatment and experimental timeline. **B.** qPCR analysis of PAG punches reveals a significant downregulation of RGSz1 transcript in AAV-Cre treated groups (n=6 for AAV-GFP and-9 for AAV-CRE, *p=0.01, unpaired t-test). **C.** RGSz1 knockdown in vlPAG neurons by stereotaxic infection with AAV-Cre virus, decreases the rewarding effects of morphine (1mg/kg s.c.) in pain-free groups of mice.. **D.** RGSz1 knockdown in the vlPAG decreases the rewarding effects of morphine (0.5 mg/kg s.c.) in mice suffering from peripheral nerve injury. SNI surgery was performed 30 days before CPP. For the 1 mg/kg dose: *n*=5 for AAV-GFP and 8 for AAV-Cre per group, r.m. two-way ANOVA Test Day Factor F(1,11)=13.39, p=0.0038; Sidak's m.c. AAV-GFP Baseline v Test t=3.356, df=11, p=0.0128. For the 0.5 mg/kg dose, n=8 for the AAV-GFP group and n= 5 for the AAV-Cre group, r.m. two-way ANOVA Test Day Factor F(1,11)=11.06, p=0.0068; Sidak's m.c. AAV-GFP Baseline v Test t=3.156, df=11, p=0.0182. Results are shown as mean±SEM.

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Figure 2. RGSz1 in the vlPAG-VTA circuit regulates morphine's rewarding actions in the conditioned place preference assay. **A.** Cartoon depicting the experimental design and timeline**. B.** qPCR analysis demonstrates a significant downregulation of RGSz1 in the vlPAG at 4 weeks after viral infection (n=8 for AAV-EGFP and 7 for AAV-Ef1a-FlpO, *p=0.005, unpaired t-test). **C**. RGSz1$^{fl/fl}$ mice with downregulated *RGSz1* in vlPAG-VTA circuitry show decreased place preference compared to their controls in response to 0.3 mg/kg morphine (s.c). **D**. At higher doses, both genotypes show similar CPP responses.). For the 0.3 mg/kg dose: *n*=12 for AAV-EGFP and 11 for AAV-Cre per group, r.m. two-way ANOVA Test Day Factor F(1,21)=5.913, p=0.0241; Sidak's m.c. AAV-EGFP Baseline v Test t=2.683, df =21, p=0.0277. For the 1mg/kg dose, n=8 for the AAV-EGFP group and n= 6 for the AAV-Ef1a-Flpo group, r.m. two-way ANOVA Test Day Factor F(1,12)=115.8, p<0.0001; Sidak's m.c. AAV-EGFP Baseline v Test t=9.342, df=12, pp<0.0001; AAV-Ef1a-Flpo Baseline v Test t=6.145, df=12, p<0.0001. *p<0.05, ***p<0.0001 Results are shown as mean±SEM.

Figure 3. Knockout of the RGSz1 gene in the PAG enhances the efficacy of morphine in a cAMP inhibition assay. (**A-B**) Membranes (2μg) from the striatum (**A**) or PAG (**B**) from RGSz1WT and form RGSz1KO mice were incubated for 30 min at 37°C with morphine (0 to 10 μM final concentration) and cAMP levels were measured as described in Methods. Values in the absence of drugs was taken as 100%. Results are shown as mean±SD (n=8 animals/group). Two-Way ANOVA comparing RGSz1WT v/s RGSz1KO at all doses; *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001. (**C-D**) Membranes (2 μg) from the striatum (**C**) or PAG (**D**) from RGSz1WT and form RGSz1KO mice were pre-treated without or with 100 ng/ml activated pertússis toxin (PTX) for 30 min at RT followed by treatment with 10 μM (final concentration) of morphine for 30 min at 37°C and cAMP levels were measured as described in Methods. Values

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in the absence of drugs was taken as 100%. Results are shown as mean±SD (n=6 animals/group). One-Way ANOVA comparing all groups; $p<0.05$; ****$p<0.0001$.

Figure 4. Selective inhibition of Gαz signaling by RGSz1. A, Schematic of TRUPATH BRET assay to test MOPR-mediated activation of different G proteins in HEK293T cells. MOPR (blue) activates G protein heterotrimer comprised of RLuc8-tagged Gα proteins (purple, salmon), Gβ proteins (light green), and eGFP-tagged Gγ proteins (green, grey). G protein activation/dissociation can be assayed as a function of RLuc8-eGFP proximity eGFP fluorescence/Rluc8 luminescence). **B**, pEC50 analysis of concentration response data indicates selective weakening of Gz signaling by RGSz1. Scatterplot data are displayed as geometric mean with 95% confidence intervals calculated from four to eight independent experiments performed in duplicates. One-way ANOVA using Sidak's multiple comparisons test was used to determine significance of differences between compound potencies in the presence and absence of RGSz1 with only the Gz sensor showing statistically significant effects (p=0.0007). **C, D,** Full DAMGO concentration response curves of MOR-mediated activation of different G proteins in the presence and absence of RGSz1. Data is shown as normalized Net BRET of eGFP fluorescence/Rluc8 luminescence and displayed with 95% confidence intervals. Gi1 (EC50=38.35±19.75 nM, pEC50=7.69±0.31, n=4); Gi1+RGSz1 (EC50=51.71±25.54 nM, pEC50=7.60±0.35, n=4); Gi2 (EC50=41.37±22.14 nM, pEC50=7.69±0.28, n=5); Gi2+RGSz1 (EC50=58.11±28.97 nM, pEC50=7.72±0.41, n=5); Gi3 (EC50=23.16±11.90 nM, pEC50=8.05±0.26, n=7); Gi3+RGSz1 (EC50=19.64±8.38 nM, pEC50=7.97±0.21, n=7); GoA (EC50=11.83±4.32 nM, pEC50=8.11±0.17, n=7); GoA+RGSz1 (EC50=10.63±2.27 nM, pEC50=8.05±0.12, n=7); GoB (EC50=6.12±1.66 nM, pEC50=8.06±0.24, n=7); GoB+RGSz1 (EC50=14.25±3.23 nM, pEC50=7.86±0.07, n=7); Gz (EC50=1.40±0.31 nM, pEC50=8.92±0.09, n=8); Gz+RGSz1 (EC50=16.02±3.84 nM, pEC50=7.89±0.011, n=8). EC50 values are calculated as mean±SEM, pEC50 values are calculated as mean±SEM.

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Figure 5. RGSz1 negatively modulates Gαz signaling by opioid drugs. A, BRET assay showing opioid concentration response of MOR-mediated activation of Gαz in the presence and absence of RGSz1. Data is shown as normalized Net BRET of eGFP fluorescence/Rluc8 luminescence. **B**, pEC50 analysis indicating that RGSz1 modulates MOR-mediated Gαz signaling by DAMGO ($p<0.0001$), Fentanyl ($p=0.0003$), Morphine ($p<0.0001$), and Methadone ($p<0.0001$). DAMGO (EC50=2.81±1.00 nM, pEC50=8.72±0.19, n=6); DAMGO+RGSz1 (EC50=26.36±9.14 nM, pEC50=7.70±0.15, n=6); Fentanyl (EC50=1.17±0.52 nM, pEC50=9.10±0.24, n=4); Fentanyl+RGSz1 (EC50=10.90±2.10 nM, pEC50=7.99±0.10, n=4); Morphine (EC50=26.11±8.36 nM, pEC50=7.68±0.13, n=6); Morphine+RGSz1 (EC50=310.68±85.25 nM, pEC50=6.59±0.12, n=6); Methadone (EC50=19.86±5.24 nM, pEC50=7.80±0.14, n=6); Methadone+RGSz1 (EC50=185.72±37.42 nM, pEC50=6.78±0.09, n=6). EC50 values are calculated as mean±SEM, pEC50 values are calculated as mean±SEM. Concentration response data and scatterplot data are displayed as geometric mean with 95% confidence intervals calculated from four to six independent experiments performed in duplicates. One-way ANOVA using Sidak's multiple comparisons test was used to determine significance of differences between compound potencies in the presence and absence of RGSz1.

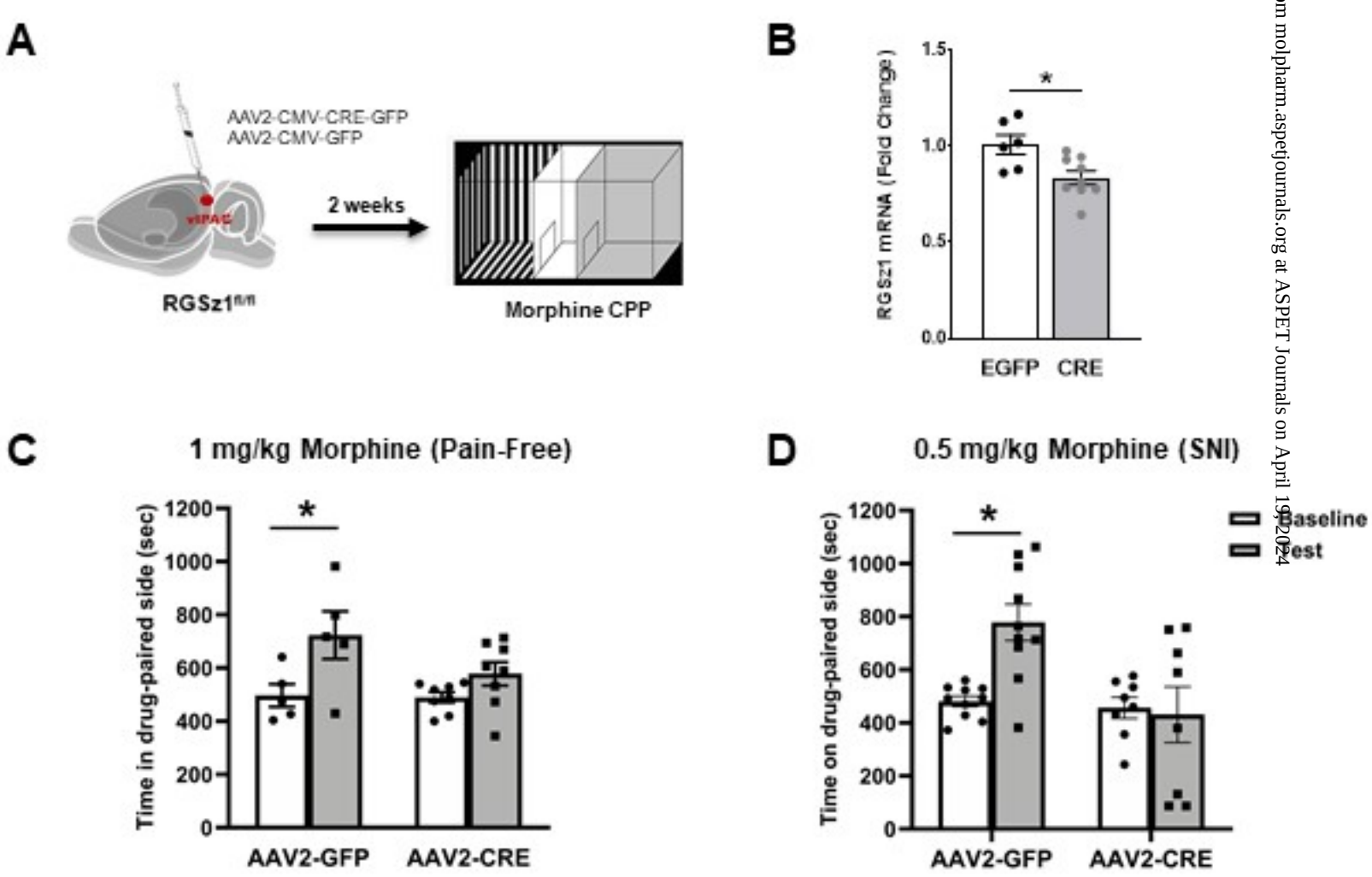


Figure 1

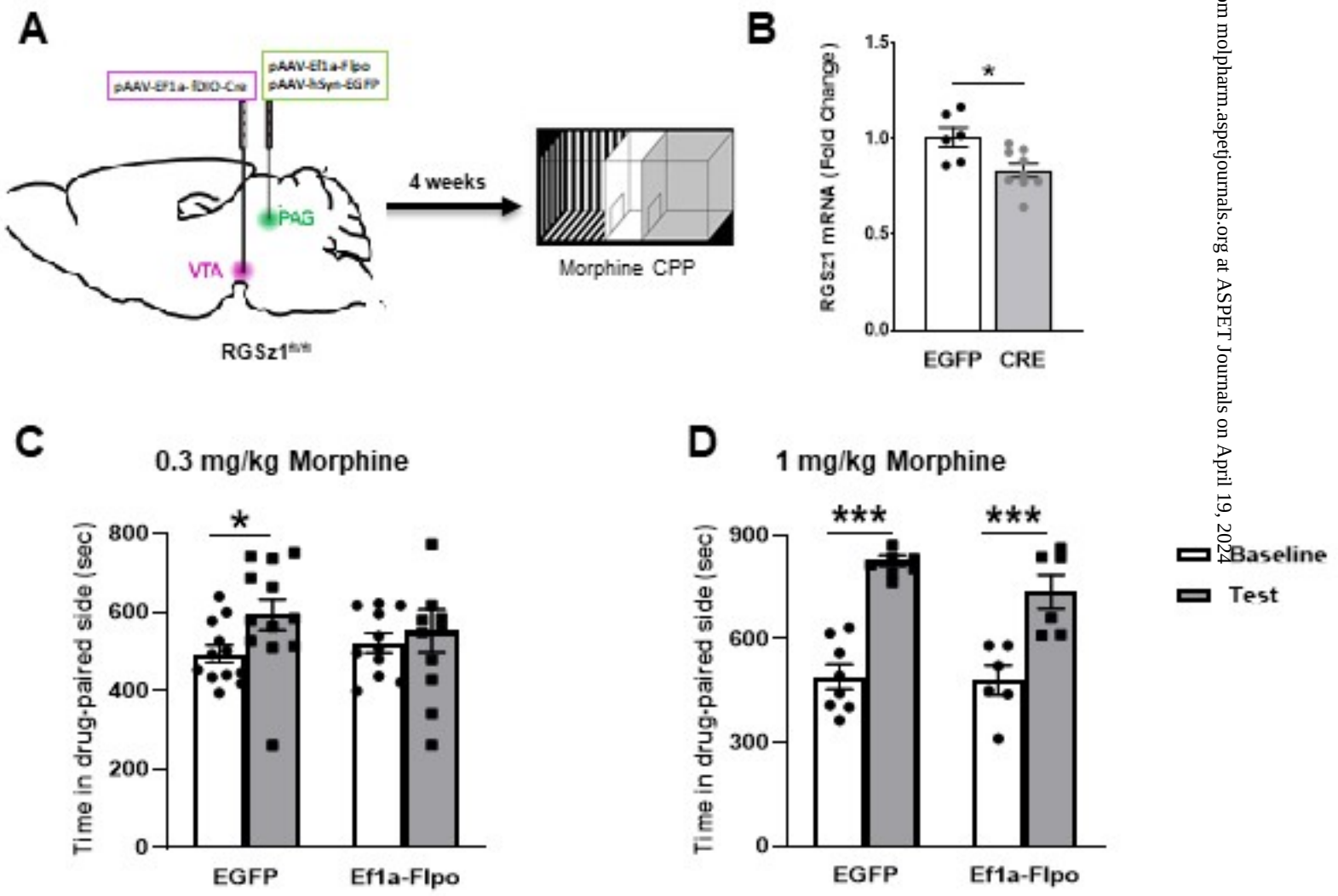


Figure 2

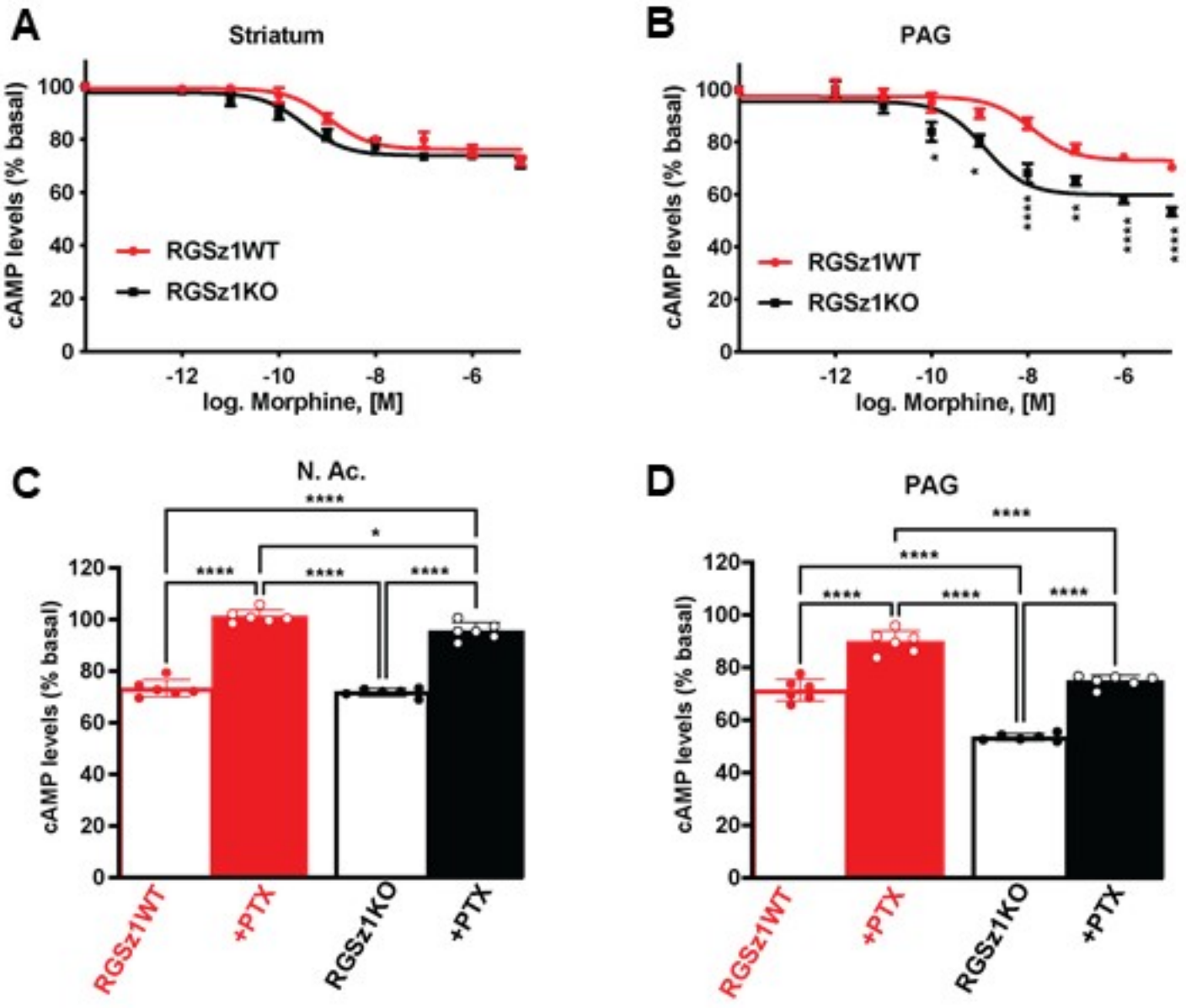


Figure 3

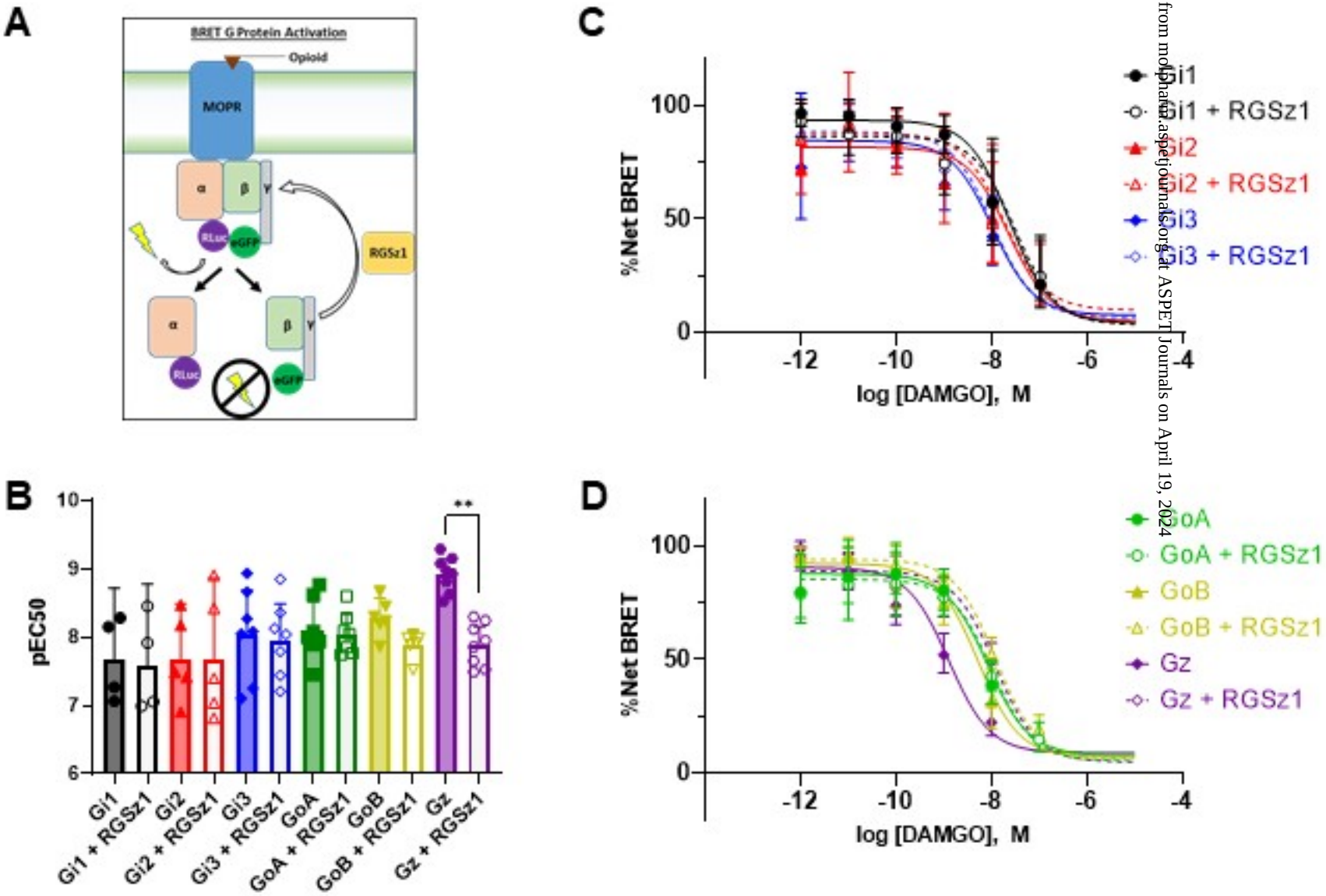
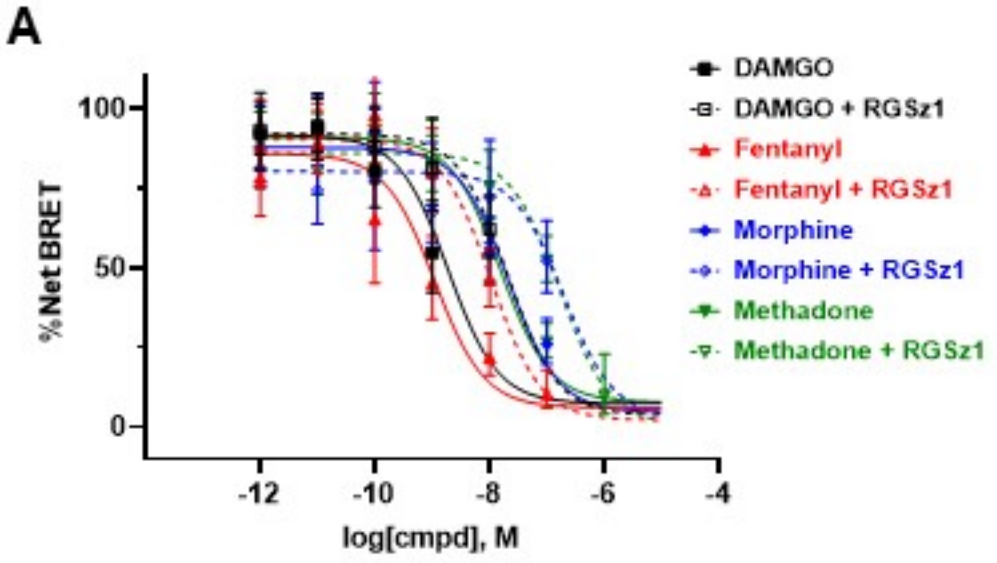


Figure 4



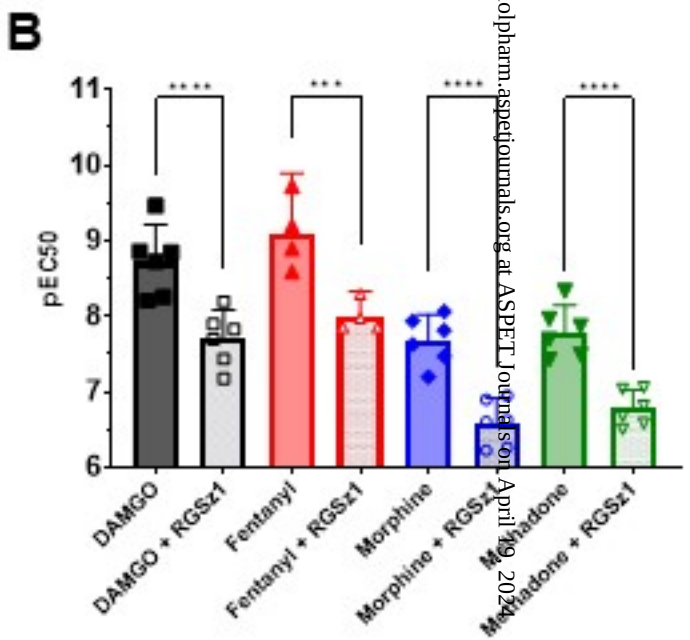


Figure 5