

## Title Page

# **RGS-insensitive mice define roles of mu opioid receptor (MOR)-G $\alpha$ o and G $\alpha$ i subunit coupling in inhibition of presynaptic GABA release**

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Running title page

## Presynaptic MOR-RGS regulation of GABA release

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Nonstandard abbreviations: [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin (DAMGO); G protein-coupled inwardly rectifying potassium channel (GIRK); G protein-coupled receptor (GPCR); met-enkephalin (ME); mu opioid receptor (MOR); heterozygous (het); Regulators of G protein signaling (RGS); ventrolateral periaqueductal gray (VIPAG); wildtype (WT)

## Abstract

Regulators of G protein signaling (RGS) proteins modulate signaling by G protein-coupled receptors (GPCRs). Using a knock-in transgenic mouse model with a mutation in  $G\alpha_o$  that does not bind RGS proteins (RGS-insensitive), we determined the effect of RGS proteins on presynaptic mu opioid receptor (MOR)-mediated inhibition of GABA release in the ventrolateral periaqueductal gray (vlPAG). The MOR agonists [D-Ala<sup>2</sup>, N-MePhe<sup>4</sup>, Gly-ol]-enkephalin (DAMGO) and met-enkephalin (ME) inhibited evoked inhibitory postsynaptic currents (eIPSCs) in the RGS-insensitive mice compared to wildtype (WT) littermates, respectively. Fentanyl inhibited eIPSCs similarly in both WT and RGS-insensitive mice. There were no differences in opioid agonist inhibition of spontaneous GABA release between the genotypes. To further probe the mechanism underlying these differences between opioid inhibition of evoked and spontaneous GABA release, specific myristoylated  $G\alpha$  peptide inhibitors for  $G\alpha_{o1}$  and  $G\alpha_{i1-3}$  that block receptor-G protein interactions were used to test the preference of agonists for MOR- $G\alpha$  complexes. The  $G\alpha_{o1}$  inhibitor reduced DAMGO inhibition of eIPSCs but  $G\alpha_{i1-3}$  inhibitors had no effect. Both  $G\alpha_{o1}$  and  $G\alpha_{i1-3}$  inhibitors separately reduced fentanyl inhibition of eIPSCs, but had no effects on ME inhibition.  $G\alpha_{i1-3}$  inhibitors blocked the inhibitory effects of ME and fentanyl on mIPSC frequency, but both  $G\alpha_{o1}$  and  $G\alpha_{i1-3}$  inhibitors were needed to block the effects of DAMGO. Finally, baclofen-mediated inhibition of GABA release is unaffected in the RGS-insensitive mice and in the presence of  $G\alpha_{o1}$  and  $G\alpha_{i1-3}$  inhibitor peptides suggesting that GABA<sub>B</sub> receptor coupling to G proteins in vlPAG presynaptic terminals is different than MOR coupling.

### **Significance statement**

Presynaptic mu opioid receptors (MORs) in the ventrolateral periaqueductal gray (vlPAG) are critical for opioid analgesia and are negatively regulated by RGS proteins. These data in RGS-insensitive mice provide evidence that MOR agonists differ in preference for  $G_{\alpha o}$  versus  $G_{\alpha i}$  and regulation by RGS proteins in presynaptic terminals, providing a mechanism for functional selectivity between agonists. The results further define important differences in MOR and GABA<sub>B</sub> receptor coupling to G proteins that could be exploited for new pain therapies.

## Introduction

Regulators of G protein signaling (RGS) proteins accelerate the hydrolysis of GTP to GDP, terminating G protein signaling. These proteins comprise a large family of proteins that differ in structure and function and are expressed in various tissues with overlapping distributions (Traynor & Neubig, 2005). RGS proteins bind to active G proteins to regulate both temporal and spatial signaling to downstream effectors (Hollinger & Hepler, 2002; Neubig, 2015). In addition, RGS proteins recognize specific  $G\alpha$  proteins (Masuho, et al., 2020) highlighting the importance of understanding RGS- $G\alpha$  interactions within discrete neural circuits. Specific RGS protein knockout mouse models have been generated to probe regulation of GPCR signaling by RGS proteins but there is evidence of strong compensation by redundant RGS proteins in various knockout lines (Grillet, et al., 2005). To circumvent this issue, we use a mutant mouse line that has a knock-in mutation in the  $G\alpha_o$  subunit (G184S) that does not bind to any RGS proteins (RGS-insensitive) (Goldenstein, et al., 2009).

Mu opioid receptors (MORs) are GPCRs that typically couple to inhibitory G proteins, including  $G\alpha_o$  and  $G\alpha_i$  subunits (Gaibelet, et al., 1999). However, opioid analgesia is dependent on MOR coupling to  $G\alpha_o$ , but not  $G\alpha_i$  (Lamberts, Jutkiewicz, Mortensen, & Traynor, 2011; Lamberts, et al., 2013). In addition, different MOR agonists preferentially bind MORs coupled to specific G protein subunits (Clark, Furman, Gilson, & Traynor, 2006; Massotte, Brillet, Kieffer, & Milligan, 2002). This differential coupling constitutes one determinant of functional selectivity of opioid agonists and differential activation of G proteins by MOR agonists could have important impacts in understanding opioid-

mediated behaviors. The ventrolateral periaqueductal gray (vlPAG) is a supraspinal site important for opioid-induced analgesia. MORs expressed postsynaptically on a subpopulation of vlPAG neurons are coupled to GIRK channels that hyperpolarize the cells (Chieng & Christie, 1994; Ingram, Fossum, & Morgan, 2007; Ingram, Macey, Fossum, & Morgan, 2008). We observed in our prior studies that MOR coupling to GIRK channels was reduced in the RGS-insensitive mice indicating that RGS proteins support signaling to some effectors (McPherson, et al., 2018), in addition to their well-known negative regulation via GTPase accelerating activity (Clark, Harrison, Zhong, Neubig, & Traynor, 2003; Clark, Linderman, & Traynor, 2008; Lamberts, et al., 2013). High efficacy synthetic agonists DAMGO and fentanyl were less effective in the RGS-insensitive mice but the GIRK currents induced by the peptide agonist met-enkephalin (ME) were unaffected (McPherson, et al., 2018). These effects were further confirmed using selective peptide inhibitors of  $G\alpha_o$  and  $G\alpha_i$  subunits showing that ME-induced GIRK currents could be inhibited only with the  $G\alpha_i$  peptide inhibitor. Taken together, these results support the idea that different opioid agonists recruit or prefer receptors bound to specific G proteins, similar to observations in cell lines (Clark & Traynor, 2006; Milligan, McKenzie, McClue, Mitchell, & Mullaney, 1990a; Moon, et al., 2001). However, the loss in MOR coupling to GIRK channels in the RGS-insensitive mice does not explain the enhanced analgesia observed in these mice (Lamberts, et al., 2013) so we have continued to examine presynaptic MOR signaling in the vlPAG.

MORs expressed on presynaptic terminals are coupled to phospholipase A2 resulting in inhibition of neurotransmitter release (Ingram, Vaughan, Bagley, Connor, & Christie, 1998; Vaughan, Ingram, Connor, & Christie, 1997). RGS proteins negatively regulate

presynaptic MORs that inhibit GABA release (Lamberts, et al., 2013) but the G proteins that are involved in presynaptic MOR signaling have not been identified previously. In these studies, we have examined several MOR agonists for their ability to activate MOR signaling via  $G\alpha_o$  or  $G\alpha_i$ , using the RGS-insensitive mice to further define MOR signaling in GABAergic terminals within the vIPAG. Based on observations that RGS-insensitive mice display enhanced antinociception, we hypothesized that MOR inhibition of presynaptic GABA release is enhanced in these mice. Further, we expected to find differences between agonists in the presence of the selective  $G\alpha_o$  and  $G\alpha_i$  peptide inhibitors.

## Materials and Methods

These studies used male and female heterozygous (RGS-insensitive Het) mice for a mutation in the  $G\alpha_o$  protein (G184S) that is insensitive to RGS protein binding (Goldenstein, et al., 2009) and wildtype (WT) 129S1/SvImJ littermates. Homozygous knock-in mice die *in utero*, so WT mice were compared with Het mice. WT mice were used in the studies assessing the effect of G protein peptide inhibitors. Mice were group housed with unlimited access to food and water. Lights were maintained on a 12 h light/dark cycle (lights on at 7:00 A.M.). Mice were sacrificed and cellular recordings were conducted during the light phase of this cycle. The Institutional Animal Care and Use Committee at Oregon Health & Science University approved all experimental procedures. Experiments were conducted in accordance with the United States National Research Council *Guide for the Care and Use of Laboratory Animals* (National Research Council, 2011).

*Electrophysiological recordings.* Mice (postnatal day >25) were anesthetized with isoflurane, brains were removed, and brain slices containing the vIPAG were cut with a vibratome (180–220  $\mu\text{m}$  thick) in sucrose cutting buffer containing the following (in mM): 75 NaCl, 2.5 KCl, 0.1  $\text{CaCl}_2$ , 6  $\text{MgSO}_4$ , 1.2  $\text{NaH}_2\text{PO}_4$ , 25  $\text{NaHCO}_3$ , 2.5 dextrose, 50 sucrose and placed in a holding chamber with artificial cerebral spinal fluid (ACSF) containing the following (in mM): 126 NaCl, 21.4  $\text{NaHCO}_3$ , 11.1 dextrose, 2.5 KCl, 2.4  $\text{CaCl}_2$ , 1.2  $\text{MgCl}_2$ , and 1.2  $\text{NaH}_2\text{PO}_4$ , pH 7.35, equilibrated with 95%  $\text{O}_2$ /5%  $\text{CO}_2$  until moved into a recording chamber. In experiments using myristoylated  $\text{G}\alpha\text{o}$  and  $\text{G}\alpha\text{i}$  peptide inhibitors, slices were incubated for at least 30 min in ACSF plus inhibitors (1–10  $\mu\text{M}$ ) before recording. Recordings were made with electrodes pulled to 2–4 M $\Omega$  resistance with an internal solution consisting of the following (in mM): 140 CsCl, 10 HEPES, 10 KCl, 1  $\text{MgCl}_2$ , 1 EGTA, 0.3  $\text{CaCl}_2$ , 4 MgATP, and 3 NaGTP, pH 7.4. Neurons were voltage-clamped at -70 mV. Junction potentials of 5 mV were corrected at the beginning of the experiments. Access resistance was monitored throughout the experiments. Data were collected with Axopatch 200B microelectrode amplifier (Molecular Devices) at 5 kHz and low-pass filtered at 2 kHz. Currents were digitized with InstruTECH ITC-18 (HEKA), collected via AxoGraph data acquisition software and analyzed using AxoGraph (Axograph Scientific). The Het mice tend to be smaller, so experimenters were not blind to genotype; however, data analysis was done blind to genotype. In experiments using  $\text{G}\alpha\text{o}$  and  $\text{G}\alpha\text{i}$  inhibitor peptides, all mice were WT, but the analyses of peak drug effects were measured blind to slice treatment.



*Reagents.* [D-Ala(2),N-Me-Phe(4),Gly(5)-ol]-enkephalin (DAMGO), met-enkephalin acetate salt hydrate (ME) and fentanyl citrate salt (fentanyl) were obtained from Sigma-Aldrich, and (*R,S*)-baclofen and CGP 55845 hydrochloride were purchased from Abcam. Myristoylated G $\alpha$  peptide inhibitors were synthesized by GenScript (Piscataway, NJ), as follows: G $\alpha$ o1 (MGIANNLRGCGLY), G $\alpha$ i1/2 (MGIKNNLKDCGLP), and G $\alpha$ i3 (MGIKNNLKECGLT) according to sequences for mini-gene vectors designed by the Hamm laboratory (Vanderbilt University Medical Center, Nashville TN; (Gilchrist, Li, & Hamm, 2002). We were unable to obtain the peptide for G $\alpha$ o<sub>2</sub> at sufficient purity (<60%) to use in slice experiments. The G $\alpha$ i inhibitors were combined as a cocktail.

*Statistical analyses.* All data are expressed as mean & standard deviation (SD). Data were analyzed with Prism 9 (GraphPad Software). Each electrophysiological recording from a single neuron is treated as an individual observation because the vIPAG contains heterogenous cell populations; however, all datasets contain recordings from at least three separate animals. Drug effects were reversed by specific antagonists, and peak drug effects were measured as an increase in current from the average of baseline and washout or the presence of antagonists. Differences between groups were assessed using Student's t-test or ANOVA when appropriate (significance is denoted as \* $p < 0.05$ , \*\* $p < 0.01$ , \*\*\*\* $p < 0.0001$ ).

## Results

### *Opioid inhibition of evoked GABA release*

In order to test the hypothesis that RGS proteins affect opioid signaling in presynaptic terminals, we compared the ability of several opioid agonists to inhibit evoked

GABAergic inhibitory postsynaptic currents (eIPSCs) in vIPAG neurons of wildtype (WT) and RGS-insensitive Het mice. The studies used concentrations of opioid agonists that were consistent with our previous study examining opioid activation of GIRK channels in the vIPAG (McPherson, et al., 2018). For comparison, we also tested maximal concentrations of each agonist so we could assess the efficacy of each agonist at inhibiting presynaptic GABA release (Fig. 1). The maximal % inhibition was the same for all three opioid drugs. Het mice had similar effects to WT mice at the maximal concentration (mean  $\pm$  SD; DAMGO:  $54 \pm 5\%$ ,  $n = 6$ ; ME:  $67 \pm 28\%$ ,  $n = 5$ ; fentanyl:  $55 \pm 10\%$ ,  $n = 4$ ). Interestingly, differences between WT and RGS-insensitive Het mice appeared at sub-maximal concentrations of these MOR agonists. The selective MOR agonist DAMGO inhibited eIPSCs 37% more in neurons from the RGS-insensitive Het mice (95% CI = 54 to 73; Fig 1A,B). The nonselective agonist ME inhibited the eIPSCs 88% more in cells from the RGS-insensitive Het mice (95% CI = 38 to 52; Fig. 1C). These results are consistent with our prior report showing an increase in morphine and ME inhibition of eIPSCs in RGS-insensitive Het mice (Lamberts, et al., 2013). In contrast, fentanyl inhibited GABAergic eIPSCs similarly in neurons from both WT and RGS-insensitive Het mice (Fig. 1D).

In a subset of experiments, a paired pulse protocol (2 stimuli, 50-100 ms apart) was used to examine the probability of GABA release from presynaptic terminals in WT and RGS-insensitive Het mice. Paired-pulse ratios (PP ratio =  $P2/P1$ ) for eIPSCs in slices from Het mice ( $0.6 \pm 0.2$ ;  $n = 8$ ) were lower than WT mice ( $1.1 \pm 0.1$ ;  $n = 12$ ;  $t_{(18)} = 2.2$ ,  $*p = 0.04$ ). A lower PP ratio indicates a higher release probability in the RGS-insensitive

Het mice. This change in release probability could be due to changes in endogenous opioid tone, so we tested whether an endogenous opioid tone could be measured in slices from either the WT or RGS-insensitive Het mice using naloxone (5 – 10  $\mu$ M). Spontaneous IPSCs (in the absence of tetrodotoxin) were similar in the absence and presence of naloxone for both genotypes (% change  $\pm$  SD in naloxone for WT: 103  $\pm$  20%; One sample t-test,  $t_5 = 0.4$ ,  $p = 0.7$  and Het: 95  $\pm$  19%; One-sample t-test,  $t_6 = 0.8$ ,  $p = 0.5$ ) indicating a lack of endogenous opioid tone in either genotype.

In order to determine whether the difference between the agonists in the RGS-insensitive mice was due to a preference for  $G\alpha_o$  versus  $G\alpha_i$  subunits coupling to MORs in presynaptic terminals, selective peptide inhibitors of each subunit binding sites were tested. Inhibitor peptides corresponding to the carboxy terminal amino-acids of the  $G\alpha$  subunit compete for binding to the receptor, inhibiting activation of the G proteins (Gilchrist, et al., 2002). The effect of DAMGO was reduced in the presence of the  $G\alpha_o$  inhibitor but the  $G\alpha_i$  inhibitor had no effect (Fig. 2A). Adding all of the peptide inhibitors together essentially abolished DAMGO-mediated inhibition of eIPSCs. Neither the  $G\alpha_o$  or  $G\alpha_i$  peptide inhibitors significantly reduced ME inhibition (Fig. 2B); however the combined inhibitors also significantly reduced ME inhibition of eIPSCs. Finally, both  $G\alpha_o$  and  $G\alpha_i$  inhibitor peptides superfused alone reduced fentanyl inhibition (Fig. 2C). These data suggest that DAMGO preferentially activates MOR- $G\alpha_o$  in presynaptic terminals in the vPAG, but ME and fentanyl are less selective.

Male and female mice were used throughout the studies. DAMGO recordings from WT mice had approximately equal numbers of recordings from male and female mice with similar inhibition in both sexes (males:  $49 \pm 10\%$  (SD);  $n = 8$  versus females:  $41 \pm 10\%$  (SD),  $n = 7$ ;  $t_{(13)} = 1.4$ ,  $p = 0.19$ ). In addition, no noted differences were observed with the other agonists. The lack of sex differences is consistent with our prior study (McPherson, et al., 2018).

#### *Opioid inhibition of spontaneous GABA release*

The change in PP ratio indicated that the RGS-insensitive Het mice have altered evoked GABA release so we were interested in whether spontaneous release (in the presence of TTX) was altered in the knock-in mouse line. Inter-event intervals of mIPSCs measured in the presence of NBQX ( $5 \mu\text{M}$ ) and TTX ( $500 \text{ nM}$ ) were similar in WT ( $0.33 \pm 0.22 \text{ s}$  (SD),  $n = 27$ ) and Het mice ( $0.24 \pm 0.15 \text{ s}$  (SD),  $n = 27$ ;  $t_{(52)} = 1.6$ ,  $p = 0.1$ ).

All three opioid agonists inhibited spontaneous mIPSC frequency to similar degrees in both the WT and Het mice (Fig. 3). The mIPSC amplitude, as well as rise and decay kinetics (data not shown) were also not different between agonists indicating that the opioid modulation of mIPSC frequency was due to presynaptic modulation of release. There were no sex differences in the amount of inhibition induced by any of the 3 opioid agonists (data not shown). These results suggest that either RGS proteins have little impact on opioid modulation of spontaneous release in presynaptic terminals or that inhibition of spontaneous release is not dependent on  $G\alpha_o$  since the RGS-insensitive knock-in mutation is specific for  $G\alpha_o$ . In order to test whether MOR- $G\alpha_i$  coupling is

involved in opioid inhibition of spontaneous GABA release in WT mice, we examined the effects of the specific  $G\alpha_o$  and  $G\alpha_i$  peptide inhibitors. Neither of the inhibitors superfused alone reduced inhibition of mIPSC frequency by DAMGO (Fig. 4A), but the inhibitors applied to slices together reduced DAMGO-mediated inhibition by 83% (95% CI = -5 to 27) compared to control. In contrast, inhibition by ME and fentanyl was reduced in the presence of the  $G\alpha_i$  inhibitors but unaffected in the presence of the  $G\alpha_o$  inhibitor peptide (Fig. 4B,C). These results are consistent with results in the RGS-insensitive Het mice which have a mutation specifically in  $G\alpha_o$  that perturbs RGS protein binding and subsequent GTP-hydrolysis and indicate that MOR- $G\alpha_i$  coupling is important for the inhibition of spontaneous GABA release.

*GABA<sub>B</sub>-mediated inhibition of GABA release is unaffected by  $G\alpha_o$  or  $G\alpha_i$  peptide inhibitors*

Our previous study found no difference in the amount of inhibition of evoked GABA release induced by a maximal concentration of the GABA<sub>B</sub> agonist baclofen (20  $\mu$ M) between slices from WT and RGS-insensitive Het mice (McPherson, et al., 2018). Because RGS proteins have less influence on high efficacy agonists, especially at maximal concentrations (Clark, et al., 2008), we repeated the studies using a lower concentration of baclofen (5  $\mu$ M). This concentration of baclofen also inhibited evoked GABA release to a similar extent in slices from the two genotypes (Fig. 5A). Consistent with these results, incubation of slices in the  $G\alpha_o$  and  $G\alpha_i$  inhibitors did not alter the responses to either concentration of baclofen (5  $\mu$ M or 20  $\mu$ M; Fig. 5B and 5C, respectively). Baclofen was typically tested on the same cells before or after an opioid

response that was affected by either the  $G\alpha_o$  or  $G\alpha_i$  inhibitor indicating that these peptide inhibitors were effective in blocking binding of the  $G\alpha$  subunits in a given experiment and providing positive controls.

Both concentrations of baclofen were also tested for inhibition of spontaneous release of GABA (Fig. 6). The data show that baclofen inhibition of mIPSC frequency is similar in both the WT and RGS-insensitive Het mice and the inhibition is unaffected by the  $G\alpha_o$  and  $G\alpha_i$  peptide inhibitors. Similar results were obtained at both 5 and 20  $\mu$ M concentrations of baclofen.

## Discussion

These studies used a transgenic knock-in mutant mouse model with a mutation in  $G\alpha_o$  (G148S) that blocks RGS protein binding (Goldenstein, et al., 2009). The advantage of this model is that it is unbiased with regard to RGS protein subtypes because compensatory expression of RGS proteins can obscure RGS regulation in knockout mice (Grillet, et al., 2005). Opioid analgesia is reduced in  $G\alpha_o$  knockout mice (Lamberts, et al., 2011) providing evidence that MOR couples to  $G\alpha_o$  in analgesia pathways. Consistent with the knockout data, RGS-insensitive Het mice display enhanced supraspinal morphine analgesia (Lamberts, et al., 2013). Since MOR inhibition of GABA release in the vIPAG is important for opioid analgesia (Bobeck, Chen, Morgan, & Ingram, 2014; Budai & Fields, 1998; Moreau & Fields, 1986; Vaughan & Christie, 1997), we expected that inhibition of GABA release by opioid agonists would be increased in the RGS-insensitive Het mice. Importantly, we observed differences

between MOR agonists in the RGS-insensitive Het mice suggesting that RGS regulation plays a role in functional selectivity of MOR agonists. In addition, MORs activate different  $G\alpha$  subunits to inhibit evoked and spontaneous GABA release. Finally,  $GABA_B$ -mediated inhibition of GABA release is not altered in the Het mice and is unaffected by peptide inhibitors of either  $G\alpha_o$  or  $G\alpha_i$  subunits.

We first examined opioid inhibition of evoked GABA release in the vIPAG. DAMGO and ME, but not fentanyl, inhibited GABA release more in the RGS-insensitive Het mice. Since sub-maximal concentrations were used for each of the agonists, the lack of increase with fentanyl in the recordings from RGS-insensitive Het mice was not attributed to a ceiling effect. These data are consistent with recent data showing that inhibition of RGS4 in the PAG enhanced morphine, but not fentanyl, antinociception (Morgan, Tran, Wescom, & Bobeck, 2020). There is evidence that RGS protein GTPase accelerating activity is more evident with low compared to high efficacy MOR agonists (Clark, et al., 2008); however, the maximal inhibition by all agonists was comparable. Thus, the differences between agonists in inhibiting GABA release in the two genotypes are likely due to a different mechanism, such as the ability of fentanyl-bound MORs to couple to  $G\alpha_i$ .

MORs activate pertussis-toxin (PTX)-sensitive  $G\alpha_o$  and  $G\alpha_i$  subunits (Williams, et al., 2013). Analgesia induced by morphine (Lutfy, et al., 1991; Parenti, Tirone, Giagnoni, Pecora, & Parolaro, 1986; Shah, Duttaroy, Davis, & Yoburn, 1994) and DAMGO (Sanchez-Blazquez & Garzon, 1988) is reduced in the presence of PTX. In order to

probe the signaling of specific G proteins in vIPAG presynaptic terminals further, we used myristoylated peptide inhibitors of  $G_{\alpha o}$  and  $G_{\alpha i}$  subunits. Incubation of slices with the  $G_{\alpha o}$  peptide inhibitor reduced the inhibition by DAMGO and fentanyl, but not ME.  $G_{\alpha o}$  inhibition of  $Ca^{2+}$  channels is more potent than  $G_{\alpha i}$  (Hescheler, Rosenthal, Trautwein, & Schultz, 1987), and there are differences in coupling between  $G_{\alpha}$  subunits and effectors (McKenzie & Milligan, 1990; Milligan, et al., 1990a; Milligan, Mitchell, Mullaney, McClue, & McKenzie, 1990b; Moon, et al., 2001). Thus, it is reasonable that inhibition by DAMGO and fentanyl was reduced by the  $G_{\alpha o}$  peptide inhibitor since evoked release is dependent on voltage-gated  $Ca^{2+}$  channels (Hubbard, Jones, & Landau, 1968). Incubation of slices with  $G_{\alpha i}$  peptide inhibitors reduced fentanyl, but not DAMGO or ME, inhibition of eIPSCs. These results indicate that coupling to  $G_{\alpha i}$  subunits is equally effective at inhibiting eIPSCs in the vIPAG, and that DAMGO and fentanyl form different MOR complexes in presynaptic terminals. Combining the peptide inhibitors reduced the effects of both DAMGO and ME compared to incubating slices in either inhibitor alone. Together with the knowledge that only small differences exist in the potency of DAMGO to stimulate different  $G_{\alpha i}$  versus  $G_{\alpha o}$  subtypes (Clark, et al., 2006), these results indicate there is redundancy of  $G_i/o$  proteins for activation by MOR. It is interesting to note that the endogenous peptide ME is less sensitive to both peptide inhibitors given alone compared to DAMGO and fentanyl suggesting that ME-bound MORs couple equally well to  $G_{\alpha o_1}$  and  $G_{\alpha i_{1-3}}$  subunits. The data highlight the importance of G protein subunit expression and levels as a factor in MOR coupling to effectors (Connor & Christie, 1999).



A surprising finding in these studies was the difference in G protein subunits involved in MOR inhibition of spontaneous GABA release. Inhibition by all three opioid agonists was similar in both the WT and RGS-insensitive Het mice, and the  $G\alpha_o$  peptide inhibitor did not affect opioid inhibition of mIPSC frequency. Instead, the  $G\alpha_{i1-3}$  peptide inhibitors applied alone decreased inhibition by ME and fentanyl without affecting DAMGO-mediated inhibition. However, DAMGO inhibition was reduced in the presence of all inhibitors. This pattern supports the results with DAMGO on evoked release and further suggests that DAMGO preferentially couples to MOR- $G\alpha_o$  subunits (Chakrabarti, Prather, Yu, Law, & Loh, 1995; Clark, et al., 2006; Laugwitz, Offermanns, Spicher, & Schultz, 1993). The data presented here indicate that opioid inhibition of spontaneous release is mediated by  $G\alpha_i$  subunits, explaining why opioid inhibition of spontaneous GABA release was unaffected in the RGS-insensitive Het mice. Thus, these studies are not able to determine if RGS proteins regulate MOR inhibition of spontaneous release. The molecular mechanisms involved in MOR regulation of spontaneous release are not completely understood but there is data to support direct G protein  $\beta\gamma$  subunit regulation of release machinery (Zurawski, Rodriguez, Hyde, Alford, & Hamm, 2016; Zurawski, et al., 2019).

$GABA_B$  receptors also readily inhibit evoked and spontaneous GABA release in the vIPAG (Vaughan, et al., 1997). In the RGS-insensitive Het mice, baclofen inhibited both evoked and spontaneous GABA release similarly to WT mice. Since the  $G\alpha_o$  and  $G\alpha_i$  peptide inhibitors were ineffective at blocking baclofen inhibition, even when applied together, we are not able to make a statement regarding the ability of RGS proteins to

modulate GABA<sub>B</sub> signaling. The results are interesting considering data that GABA<sub>B</sub> coupling to voltage-gated Ca<sup>2+</sup> channels is abolished by PTX (Connor & Christie, 1998). However, GABA<sub>B</sub>-Gi protein coupling has different structural features compared to other GPCR classes. Agonists at this receptor do not induce outward movement of transmembrane domain 6 to provide a cavity for the binding of the C-terminus of the G proteins (Shen, et al., 2021). Consequently, the peptide inhibitors used in this study, designed to mimic the G $\alpha$ -C-terminal interaction with the receptor core, may not bind to the GABA<sub>B</sub> receptor to block G protein binding. Alternatively, G $\alpha$ z is a G protein with 60% sequence homology to the Gi family (Tsu, Ho, Yung, Joshi, & Wong, 1997), is densely expressed in the vIPAG and couples to MOR (Garzon, Castro, & Sanchez-Blazquez, 1998; Garzon, Rodriguez-Munoz, Lopez-Fando, & Sanchez-Blazquez, 2005; Gaspari, et al., 2018). The G $\alpha$ z residues that bind to MOR have not been identified so it is possible the peptide inhibitors would not block G $\alpha$ z coupling to MOR or GABA<sub>B</sub> receptors, especially given substitution of tyrosine in the place of the PTX-sensitive cysteine in the G $\alpha$ z C-terminus.

The descending pain modulatory pathway is sexually dimorphic (Loyd, Morgan, & Murphy, 2008; Loyd & Murphy, 2006, 2014) and MOR agonists are more efficacious in males than females (Fullerton, Doyle, & Murphy, 2018). There were no sex differences in either genotype in opioid agonist inhibition of evoked and spontaneous GABA release. Thus, sex differences in opioid signaling are not explained by RGS-mediated regulation of signaling, at least via G $\alpha$ o subunits. This is consistent with the lack of sex

differences in MOR coupling to GIRK channels in the WT and RGS-insensitive Het mice (McPherson, et al., 2018).

Our results showing enhanced MOR inhibition of presynaptic GABA release by several opioid agonists in the RGS-insensitive mice, in addition to morphine which we examined in our previous paper (Lamberts, et al., 2013), provide a mechanism for the increase in opioid antinociception on the supraspinal hot plate test observed in RGS-insensitive mice (Lamberts, et al., 2013). There is substantial evidence that opioid inhibition of GABA release in the vIPAG activates descending pain modulatory circuits that produce analgesia (Cheng, Fields, & Heinricher, 1986; Moreau & Fields, 1986). We previously reported that postsynaptic MOR coupling to GIRK channels is reduced in RGS-insensitive mice (McPherson, et al., 2018), possibly through loss of a scaffolding function of RGS proteins (Zhong, et al., 2003). Although it is tempting to argue that postsynaptic MORs in the vIPAG do not play a role in opioid-induced antinociception, an equally valid interpretation is that MOR coupling to GIRK channels opposes supraspinal antinociceptive circuits and removal of this MOR signaling supports opioid analgesia in the RGS-insensitive Het mice (Lamberts, et al., 2013). Indeed, blocking both GIRK channels and presynaptic MOR signaling decreases morphine antinociception (Morgan, et al., 2020). Inhibition of RGS4 in the vIPAG enhances opioid-induced antinociception suggesting that RGS4 may play an important role in regulating presynaptic MOR signaling through  $G\alpha_o$ . However, RGS gene expression in the PAG includes RGS4, RGS7, RGS8, RGS10, RGS17 and RGS20 (<https://alleninstitute.org/legal/citation-policy/> Allen Brain Atlas), and these RGS proteins bind preferentially to different G

proteins (Masuho, et al., 2020). Thus, additional RGS proteins may also regulate opioid analgesia through MOR coupling in the PAG.

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### **Authorship Contributions**

Participated in research design: Traynor, Ingram

Conducted experiments: Bouchet, McPherson, Li, Ingram

Contributed new reagents or analytic tools: Traynor

Performed data analysis: Bouchet, McPherson, Ingram

Wrote or contributed to the writing of the manuscript: Bouchet, McPherson, Traynor, Ingram

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## Footnotes

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The authors have no conflicts of interest.

## Legends

**Figure 1.** Opioid agonist inhibition of evoked IPSCs are differentially affected in RGS-insensitive mice. A. Representative traces depicting inhibition of eIPSCs by DAMGO (5  $\mu$ M) in wildtype (WT) and RGS-insensitive (Het) mice. The inhibition is reversed by naloxone. B. Combined experiments of % inhibition ( $\pm$  SD) by a maximal DAMGO concentration (20  $\mu$ M; gray bar) and a sub-maximal concentration (5  $\mu$ M) in WT compared to Het mice (One way ANOVA;  $F_{(2, 25)} = 4.9$ ,  $p = 0.02$ ; Dunnett's, \* $p < 0.05$ ). C. Combined experiments of % inhibition ( $\pm$  SD) by a maximal met-enkephalin concentration (30  $\mu$ M; gray bar) and a sub-maximal concentration (10  $\mu$ M) in WT compared to Het mice (One way ANOVA;  $F_{(2, 15)} = 17.7$ ,  $p = 0.0001$ ; Dunnett's, \* $p < 0.05$ ; \*\*\* $p < 0.0001$ ). D. Combined experiments of % inhibition ( $\pm$  SD) by a maximal fentanyl concentration (10  $\mu$ M; gray bar) and a sub-maximal concentration (1  $\mu$ M) in WT compared to Het mice (One way ANOVA;  $F_{(2, 18)} = 4.1$ ,  $p = 0.03$ ; Dunnett's, \* $p < 0.05$ ). Symbols in bars denote recordings and numbers denote number of animals used in each group.

**Figure 2.** MOR agonists differentially activate  $G\alpha$  subunits to inhibit evoked GABA release. A. DAMGO (5  $\mu$ M)-mediated inhibition of eIPSCs in the absence (control) and presence of  $G\alpha_o$  peptide inhibitor and  $G\alpha_i$  peptide inhibitors (One way ANOVA,  $F_{(3, 22)} = 19.1$ ,  $p = 0.0001$ ; Dunnett's, \*\* $p < 0.001$ , \*\*\*\* $p < 0.0001$ ). B. ME (10  $\mu$ M)-mediated inhibition of eIPSCs in absence and presence of inhibitors (One way ANOVA,  $F_{(3, 19)} = 4.2$ ;  $p = 0.02$ ; Dunnett's, \*\* $p < 0.001$ ). C. Fentanyl (1  $\mu$ M)-mediated inhibition of eIPSCs in absence and presence of inhibitors (One way ANOVA,  $F_{(2, 14)} = 7.3$ ,  $p = 0.007$ ,

Dunnett's, \* $p < 0.05$ , \*\*  $p < 0.001$ ). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

**Figure 3.** Opioid inhibition of GABAergic mIPSCs is not altered in RGS-insensitive mice. A. Combined experiments of % inhibition ( $\pm$  SD) by a maximal DAMGO concentration (20  $\mu$ M; gray bar) and a sub-maximal concentration (5  $\mu$ M) in WT compared to Het mice (One way ANOVA;  $F_{(2, 21)} = 0.3$ ,  $p = 0.8$ ). B. Combined experiments of % inhibition ( $\pm$  SD) by a maximal met-enkephalin concentration (ME 30  $\mu$ M; gray bar) and a sub-maximal concentration (10  $\mu$ M) in WT compared to Het mice (One way ANOVA,  $F_{(2, 24)} = 0.5$ ,  $p = 0.6$ ). C. Combined experiments of % inhibition ( $\pm$  SD) by by a maximal fentanyl concentration (10  $\mu$ M; gray bar) and a sub-maximal concentration (1  $\mu$ M) in WT compared to Het mice (One way ANOVA;  $F_{(2, 25)} = 1.2$ ,  $p = 0.3$ ). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

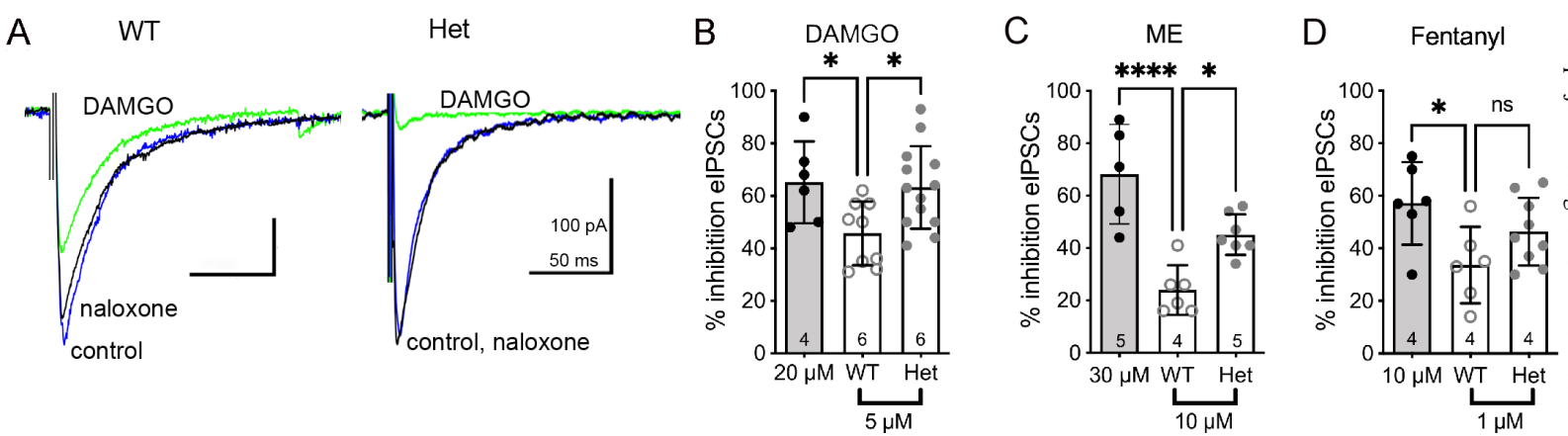
**Figure 4.** MOR- $G\alpha_i$  coupling is more important for inhibition of spontaneous GABA release. A. Inhibition of mIPSCs by DAMGO (5  $\mu$ M) is unaffected by  $G\alpha_o$  and  $G\alpha_i$  inhibitors (One way ANOVA;  $F_{(3, 32)} = 12.6$ ,  $p = 0.0001$ ; Dunnett's, \*\*\*\* $p = 0.0001$ ). B. Inhibition by ME is reduced in the presence of  $G\alpha_i$  inhibitors, but not by the  $G\alpha_o$  inhibitor ( $F_{(2, 19)} = 11.8$ ,  $p = 0.001$ , Dunnett's, \*\* $p < 0.01$ ). C. Inhibition by fentanyl is reduced in the presence of  $G\alpha_i$  inhibitors, but not by the  $G\alpha_o$  inhibitor ( $F_{(2, 19)} = 6.2$ ,  $p = 0.01$ , Dunnett's, \*\* $p < 0.01$ ). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

**Figure 5.** Baclofen-mediated inhibition of evoked GABA release is not affected in slices from RGS-insensitive mice or by  $G\alpha o/i$  peptide inhibitors. A. Baclofen (5  $\mu\text{M}$ ) inhibition is similar in wildtype (WT) and RGS-insensitive (Het) mice ( $t_{(12)} = 1.3$ ,  $p = 0.2$ ). B. Baclofen (5  $\mu\text{M}$ )-mediated inhibition is not altered in the presence of peptide inhibitors ( $F_{(3, 20)} = 0.2$ ,  $p = 0.9$ ). C. Baclofen (20  $\mu\text{M}$ )-mediated inhibition is not altered by the peptide inhibitors ( $F_{(2, 24)} = 0.3$ ,  $p = 0.7$ ). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

**Figure 6.** Baclofen-mediated inhibition of spontaneous GABA release in slices is not affected in RGS-insensitive mice or in the presence of  $G\alpha o/i$  peptide inhibitors. A. Baclofen (5  $\mu\text{M}$ ) inhibition is similar in wildtype (WT) and RGS-insensitive (Het) mice ( $t_{(11)} = 1.8$ ,  $p = 0.1$ ). B. Baclofen (5  $\mu\text{M}$ )-mediated inhibition is not altered in the peptide inhibitors ( $F_{(2, 14)} = 0.4$ ,  $p = 0.7$ ). C. Baclofen (20  $\mu\text{M}$ ) inhibition is similar in wildtype (WT) and RGS-insensitive (Het) mice ( $t_{(17)} = 0.005$ ,  $p = 1.0$ ). B. Baclofen (20  $\mu\text{M}$ )-mediated inhibition is not altered in the peptide inhibitors ( $F_{(2, 46)} = 0.03$ ,  $p = 1.0$ ). Symbols in bars denote recordings and numbers denote number of animals used in each bar.

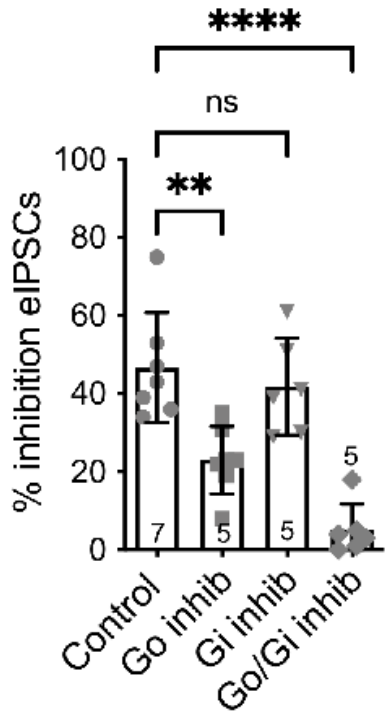


Figure 1

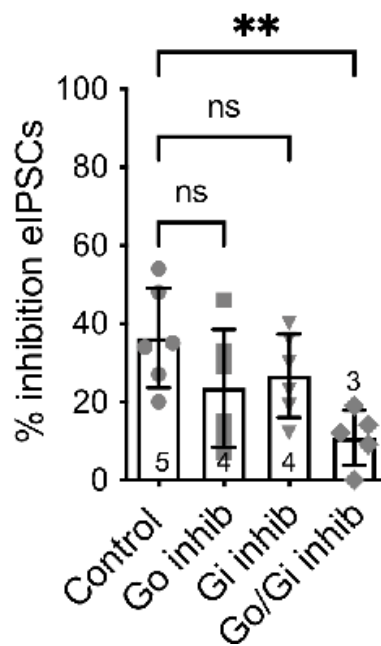


## Figure 2

**A** DAMGO (5  $\mu$ M)



**B** ME (10  $\mu$ M)



**C** Fentanyl (1  $\mu$ M)

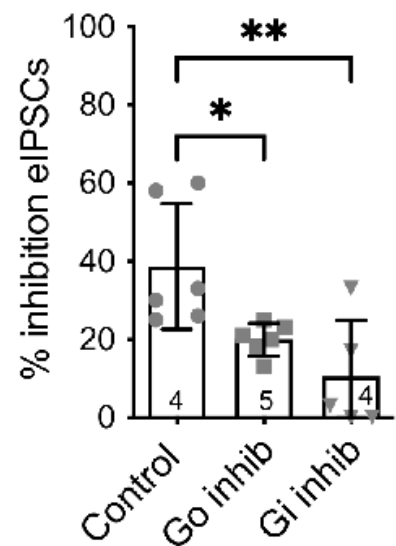


Figure 3

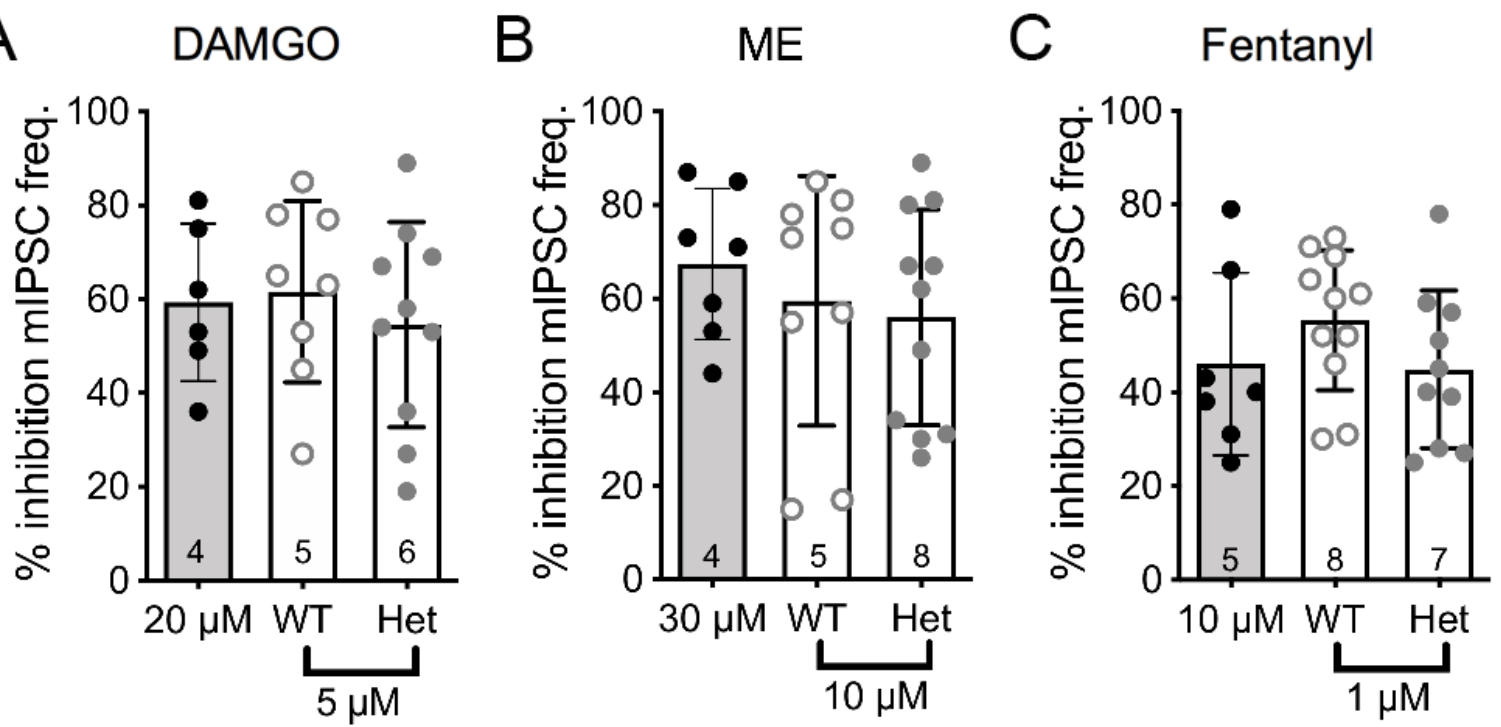


Figure 4

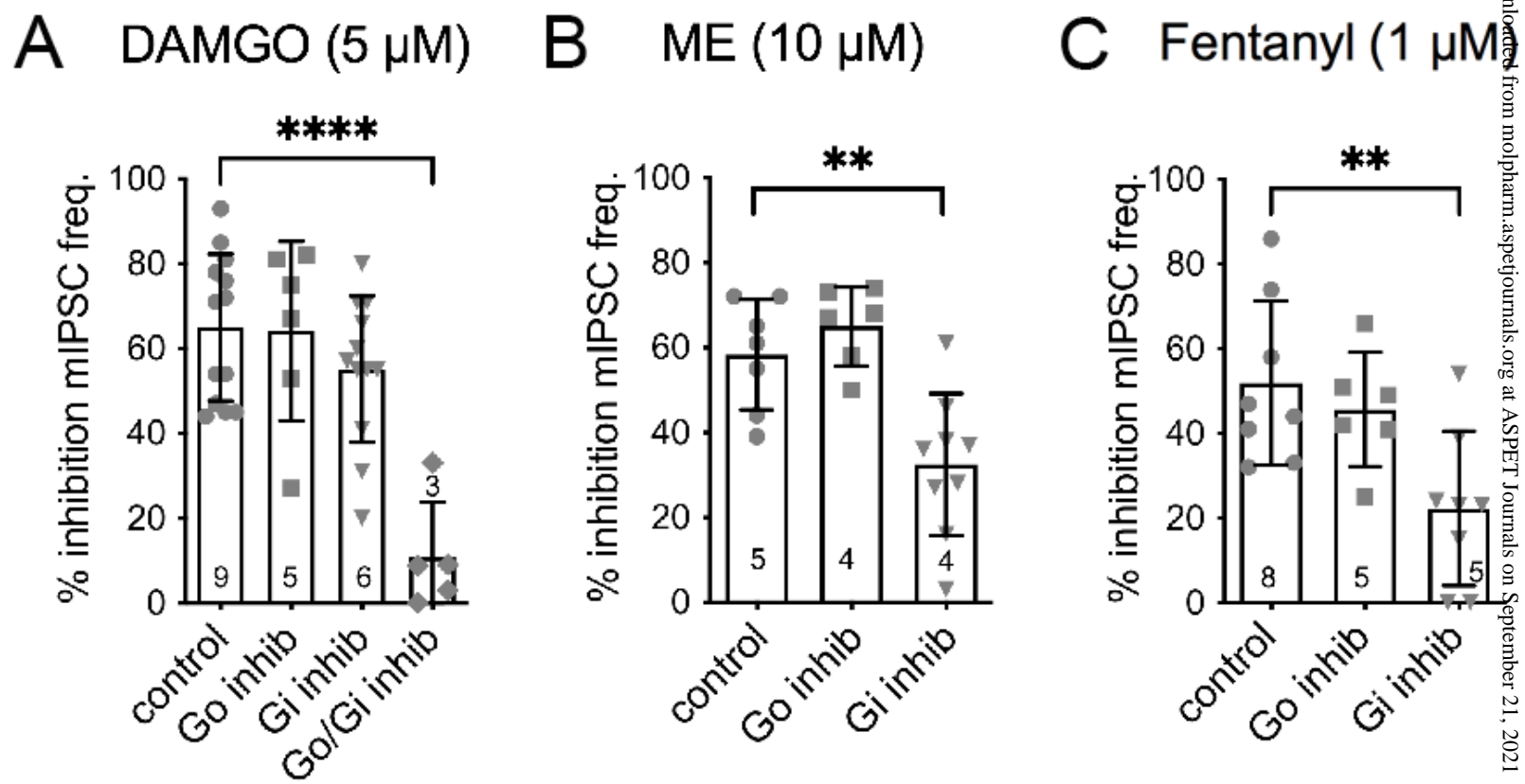
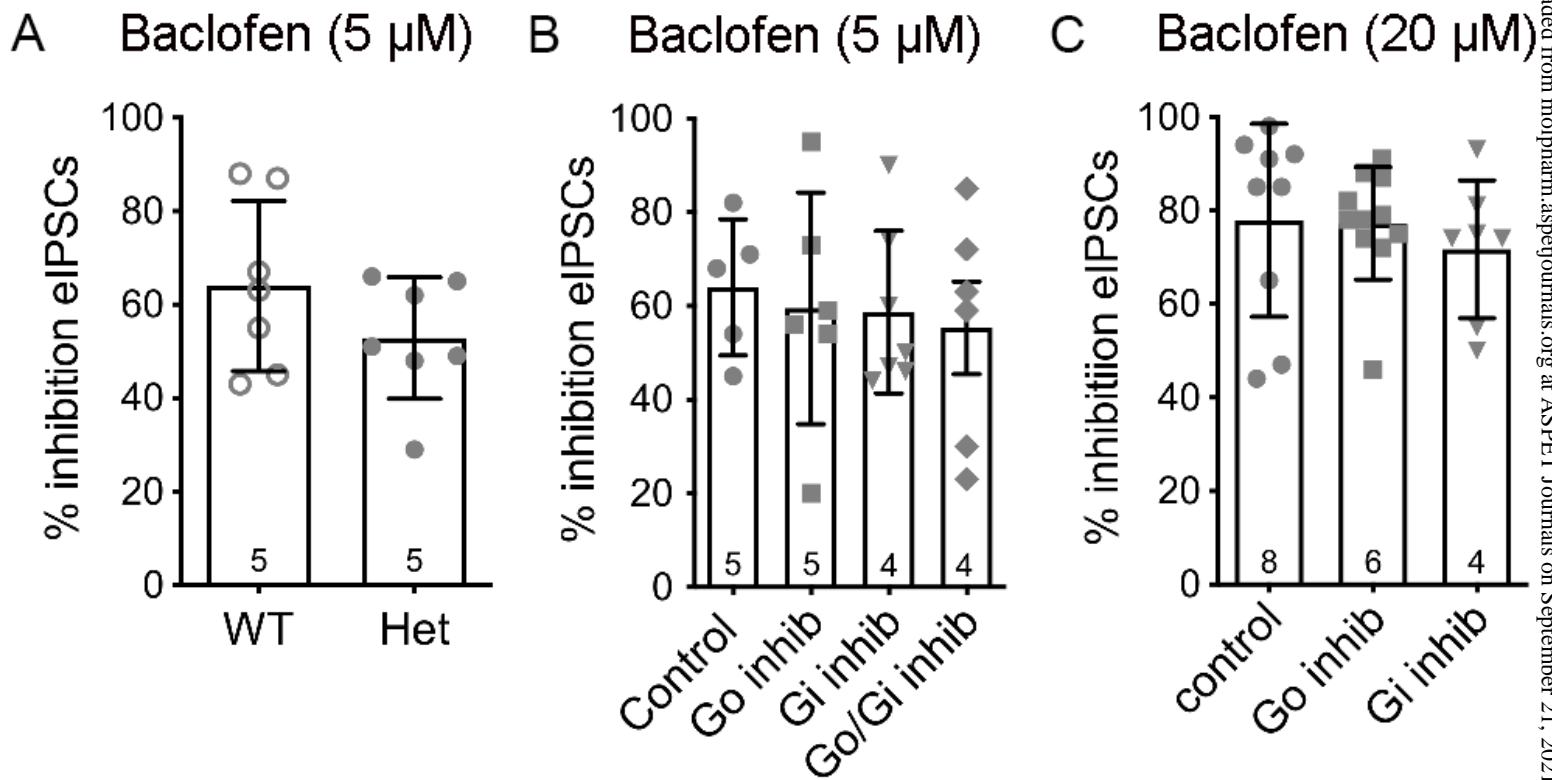


Figure 5



# Figure 6

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