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Contribution of G-Protein α-Subunits to Analgesia, Hyperalgesia, and Hyperalgesic Priming Induced by Subanalgesic and Analgesic Doses of Fentanyl and Morphine

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While opioids produce both analgesia and side effects by action at μ-opioid receptors (MORs), at spinal and supraspinal sites, the potency of different opioids to produce these effects varies. While it has been suggested that these differences might be because of bias for signaling via β-arrestin versus G-protein α-subunits (Gα), recent studies suggest that G-protein-biased MOR agonists still produce clinically important side effects. Since bias also exists in the role of Gα subunits, we evaluated the role of $G\alpha_{i/o}$ subunits in analgesia, hyperalgesia, and hyperalgesic priming produced by fentanyl and morphine, in male rats. We found that intrathecal treatment with oligodeoxynucleotides antisense (AS-ODN) for $G\alpha_i2$, $G\alpha_i3$, and $G\alpha_o$ markedly attenuated hyperalgesia induced by subanalgesic dose (sub-AD) fentanyl, while AS-ODN for $G\alpha_i1$, as well as $G\alpha_i2$ and $G\alpha_i3$, but not $G\alpha_o$, prevented hyperalgesia induced by sub-AD morphine. AS-ODN for $G\alpha_i1$ and $G\alpha_i2$ unexpectedly enhanced analgesia induced by analgesic dose (AD) fentanyl, while $G\alpha_i1$ AS-ODN markedly reduced AD morphine analgesia. Hyperalgesic priming, assessed by prolongation of prostaglandin E_2-induced hyperalgesia, was not produced by systemic sub-AD and AD fentanyl in $G\alpha_i3$ and $G\alpha_o$ AS-ODN-treated rats, respectively. In contrast, none of the $G\alpha_{i/o}$ AS-ODNs tested affected priming induced by systemic sub-AD and AD morphine. We conclude that signaling by different $G\alpha_{i/o}$ subunits is necessary for the analgesia and side effects of two of the most clinically used opioid analgesics. The design of opioid analgesics that demonstrate selectivity for individual $G\alpha_{i/o}$ may produce a more limited range of side effects and enhanced analgesia.

Key words: **analgesia; fentanyl; G-protein; hyperalgesic priming; morphine; opioid-induced hyperalgesia**

Significance Statement

Biased μ-opioid receptor (MOR) agonists that preferentially signal through G-protein α-subunits over β-arrestins have been developed as an approach to mitigate opioid side effects. However, we recently demonstrated that biased MOR agonists also produce hyperalgesia and priming. We show that oligodeoxynucleotide antisense to different $G\alpha_{i/o}$ subunits play a role in hyperalgesia and analgesia induced by subanalgesic and analgesic dose (respectively), of fentanyl and morphine, as well as in priming. Our findings have the potential to advance our understanding of the mechanisms involved in adverse effects of opioid analgesics that could assist in the development of novel analgesics, preferentially targeting specific G-protein α-subunits.

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The authors declare no competing financial interests.

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Introduction

Approximately 30% of medicines in use today are G-protein-coupled receptor (GPCR) ligands, as are 20% of drugs in clinical trials (Hauser et al., 2017). GPCRs mediate their effects through the activation of guanine nucleotide binding proteins (G-proteins; Childers, 1988; Reisine and Bell, 1993; Uhl et al., 1994; Pasternak and Standifer, 1995; Standifer et al., 1996) and β-arrestins (Gainetdinov et al., 2004; Williams et al., 2013). G-proteins are composed of three distinct subunit families (α, β, and γ), which couple GPCRs to downstream second messengers and effectors. α-Subunits are pharmacologically relevant because of their

intrinsic GTPase activity (Standifer and Pasternak, 1997). Different α-subunits (e.g., $G\alpha_i$, $G\alpha_o$, $G\alpha_s$, $G\alpha_q$, Gx/α_z) establish the differential modulation of cAMP by GPCRs (Standifer and Pasternak, 1997). Activated $G\alpha$ proteins have a variety of effects, including activation of mitogen-activated protein kinase (MAPK) and phosphatidylinositol 3-kinase (Leurs et al., 2005). Activation of the enzyme phospholipase (PL) A2 may also occur, which induces the release of arachidonic acid, as well as the inhibition of the Na^+/H^+ exchanger in the plasma membrane, and the lowering of intracellular Ca^{2+} (Leurs et al., 2005). How these various downstream signaling pathways are differentially activated by GPCRs remains to be elucidated.

Oligodeoxynucleotides (ODNs) antisense (AS) for α-subunit mRNA have been used to implicate different α-subunits in various opioid receptor-mediated functions (Pasternak and Standifer, 1995; Rossi et al., 1995; Standifer et al., 1996; Hadjimarkou et al., 2002; Silva et al., 2002; Wainford and Kapusta, 2012). For example, while intracerebroventricular administration of AS-ODN directed against either $G\alpha_i2$ or $G\alpha_o$ reduces morphine, but not morphine-6β-glucuronide (M6G) analgesia, AS-ODN directed against $G\alpha_i1$ or Gx/α_z reduces M6G, but not morphine analgesia (Rossi et al., 1995; Standifer et al., 1996). In contrast, fentanyl displays high potency for activation of $G\alpha_i1$ and $G\alpha_o$ (Saidak et al., 2006).

While opioids produce both analgesia and side effects by action at the μ-opioid receptor (MOR), their relative potencies that produce these diverse effects vary between opioids (Araldi et al., 2018a, c, 2019; Ferrari et al., 2019; Khomula et al., 2021). It has been suggested that this difference between opioid analgesics might be because of biased signaling by GPCR via β-arrestin versus $G\alpha_i$ (Raehal and Bohn, 2014). However, several recent studies of MOR agonists biased toward G-proteins indicate that they still produce substantial side effects, such as constipation, tolerance, and respiratory depression (Viscusi et al., 2016; Olson et al., 2017; Hill et al., 2018; Gillis et al., 2020). We recently found that two such biased MOR agonists (i.e., TRV130 and PZM21) still produce hyperalgesia [opioid-induced hyperalgesia (OIH)] and hyperalgesic priming (opioid-induced hyperalgesic priming), as well as analgesia (Araldi et al., 2018c). In this study, we tested the hypothesis that spinal opioid-induced analgesia, OIH, and hyperalgesic priming produced by two clinically important opioid analgesics, fentanyl and morphine, are mediated by different $G\alpha_i/\alpha_o$ proteins.

Materials and Methods

Animals. Experiments were performed on 260–380 g adult male Sprague Dawley rats (Charles River Laboratories). Given the large number of experiments required to establish the role of multiple $G\alpha$s in the analgesic and side effects of different doses of multiple opioid analgesics, we elected to perform experiments in female rats in a subsequent study. Animals were housed three per cage, under a 12 h light/dark cycle, in a temperature- and humidity-controlled animal care facility at the University of California, San Francisco. Food and water were available *ad libitum*. Experimental protocols were approved by the Institutional Animal Care and Use Committee (IACUC) at the University of California, San Francisco, and adhered to the National Institutes of Health *Guide for the Care and Use of Laboratory Animals*. Effort was made to minimize the number of animals used and their suffering.

Nociceptive threshold testing. Mechanical nociceptive threshold was quantified using an analgesy-meter (Randall Selitto - paw-withdrawal device, Ugo-Basile), which applies a linearly increasing mechanical force to the dorsum of the hindpaw of a rat, as previously described (Taiwo et al., 1989; Taiwo and Levine, 1989; Araldi et al., 2015, 2017, 2018a,c, 2019). Rats were placed in cylindrical acrylic restrainers designed to provide ventilation, allow hindleg extension from lateral ports in the cylinder during the assessment of nociceptive threshold, and minimize restraint stress. To acclimatize rats to the testing procedure, they were placed in restrainers for 1 h before starting each training session, for 3 consecutive daily training sessions, and for 40 min before subsequent experimental manipulations. Nociceptive threshold was defined as the force in grams at which a rat withdrew its paw. Baseline paw-pressure nociceptive threshold was defined as the mean of the three readings taken before test agents were injected. To minimize experimenter bias, individuals conducting the behavioral experiments (D.A. and I.J.M.B.) were blinded to experimental interventions.

Drugs. The following compounds were used in this study: fentanyl citrate salt (an MOR agonist), prostaglandin-E_2 (PGE_2; a direct-acting hyperalgesic agent that sensitizes nociceptors), and morphine sulfate salt pentahydrate (an agonist at μ, δ, and κ opioid receptors), all purchased from Sigma-Aldrich. The stock solution of PGE_2 (1 $\mu g/\mu l$) was prepared in 100% ethanol, and further dilutions were made with physiological saline (0.9% NaCl), yielding a final ethanol concentration of <2%. Fentanyl and morphine were dissolved in saline.

Intradermal administration of PGE_2 was performed on the dorsum of the hindpaw, at the site of nociceptive testing, using a 30 gauge hypodermic needle adapted to a 50 μl syringe (Hamilton) by a segment of PE-10 polyethylene tubing (Becton Dickinson). Our *in vivo* control experiments have previously shown that the final concentration of ethanol (2%), used to prepare the PGE_2 solution, alone had no effect on the mechanical threshold (Ferrari et al., 2016).

Systemic administration of fentanyl (sub-AD, 0.01 mg/kg, s.c.; AD, 0.03 mg/kg, s.c.) and morphine (sub-AD, 0.03 mg/kg, s.c.; AD, 3 mg/kg, s.c.) was performed at the nape of the neck (Araldi et al., 2018a, 2019). Rats received an injection of fentanyl or morphine, and mechanical nociceptive threshold was evaluated 1 h later (Araldi et al., 2018a, 2019). Fentanyl and morphine were diluted in saline and administered (100 μl/100 g body weight, s.c.).

Oligodeoxynucleotides antisense to G-protein α-subunit mRNAs. To investigate the role of G-protein α-subunits in the analgesia, hyperalgesia, and priming, induced by systemic sub-AD and AD fentanyl and morphine, validated AS-ODNs for $G\alpha_i1$, $G\alpha_i2$, $G\alpha_i3$, and $G\alpha_o$ mRNA (Rossi et al., 1995; Standifer et al., 1996; Hadjimarkou et al., 2002; Silva et al., 2002; Wainford and Kapusta, 2012) were used.

AS-ODN sequences, directed against unique regions of the rat mRNA for G-protein α-subunits, were as follows:

$G\alpha_i1$ AS-ODN: 5′-AGACCACTGCTTTGTA-3′ (Gnai1; GenBank accession no. NM_013145.1)

$G\alpha_i2$ AS-ODN: 5′-CTTGTCGATCATCTTAGA-3′ (Gnai2; GenBank accession no. NM_031035.3)

$G\alpha_i3$ AS-ODN: 5′-AAGTTGCGGTCGATCAT-3′ (Gnai3; GenBank accession no. NM_013106.1)

$G\alpha_o$ AS-ODN: 5′-CGCCTTGCTCCGCTC-3′ (Gnao1; GenBank accession no. NM_017327.1)

As a control, the following sense (SE) ODN sequences were used:

$G\alpha_i1$ SE-ODN: 5′-TACAAAGCAGTGGTCT-3′

$G\alpha_i2$ SE-ODN: 5′-TCTAAGATGATCGACAAG-3′

$G\alpha_i3$ SE-ODN: 5′-ATGATCGACCGCAACTT-3′

$G\alpha_o$ SE-ODN: 5′-GAGCGGAGCAAGGCG-3′

ODNs were synthesized by Thermo Fisher Scientific. A nucleotide BLAST (Basic Local Alignment Search Tool) search was performed to confirm that the mRNA sequences targeted by the AS-ODN were not homologous to any other sequences in the rat database and that SE-ODN control sequences were not homologous to any sequences in the rat database as well. Before use, lyophilized ODNs were reconstituted in nuclease-free 0.9% NaCl and then administered intrathecally at a dose of 6 μg/μl in a volume of 20 μl (120 μg/20 μl). AS-ODNs or SE-ODNs were injected daily for 4 consecutive days. On the fourth day, fentanyl (AD, 0.03 mg/kg; sub-AD, 0.01 mg/kg) or morphine (AD, 3 mg/kg; or sub-AD, 0.03 mg/kg) was then injected subcutaneously on the back of the neck. Mechanical nociceptive threshold was evaluated 1 h after systemic fentanyl and morphine; 5 d later, PGE_2 (100 ng) was injected intradermally and mechanical nociceptive threshold evaluated 30 min and 4 h later. As described previously (Alessandri-Haber et al., 2003), after rats

were anesthetized with isoflurane (2.5% in O_2), ODN was injected using a 0.3 ml syringe (300 U/μl; Walgreens) with a 29 gauge hypodermic needle, inserted into the subarachnoid space between the L4 and L5 vertebrae. The intrathecal site of injection was confirmed by a sudden flick of the tail of the rat, a reflex that is evoked by accessing the subarachnoid space and bolus injection (Mestre et al., 1994). A total of 120 μg of ODN, in a volume of 20 μl, was then injected. Rats regained consciousness ~2 min after anesthesia was stopped. Use of intrathecal AS-ODN to attenuate the expression of proteins, essential for their role in nociceptor sensitization, is well supported by previous studies (Song et al., 2009; Su et al., 2011; Bogen et al., 2012; Quanhong et al., 2012; Sun et al., 2013; Oliveira-Fusaro et al., 2017; Araldi et al., 2019; Ferrari et al., 2019; Pagliusi et al., 2020).

Data analysis. All data are presented as the mean ± SEM of *n* independent observations. Statistical comparisons were made using GraphPad Prism 8.0 statistical software (GraphPad Software). A *p*-value < 0.05 was considered statistically significant. In the behavioral experiments, the dependent variable was change in the mechanical paw-withdrawal threshold, expressed as the percentage change from baseline. No significant difference in mechanical nociceptive thresholds was observed between G-protein α-subunit AS-ODN- and SE-ODN-treated groups of rats measured before the administration of fentanyl or morphine (as demonstrated in the figure legends) and immediately before the injection of PGE_2 (average mechanical nociceptive threshold before fentanyl- and morphine-induced priming, 141.5 ± 0.837 g; average mechanical nociceptive threshold before PGE_2 injection, 140.8 ± 0.839 g; $n = 192$ rats; paired Student's *t* test: $t_{(191)} = 1.87$, $p = 0.062$. As specified in figure legends, Student's *t* test or two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test was performed to compare the magnitude of the analgesia and hyperalgesia induced by systemic fentanyl or morphine, or to compare the effect produced by the G-protein α-subunit AS-ODNs on the prolongation of hyperalgesia (evaluated 4 h after injection of PGE_2) compared with the SE-ODN control groups.

Results

OIH and priming produced by subanalgesic dose fentanyl

As most opioid analgesics produce hyperalgesia at sub-ADs, we investigated whether OIH and hyperalgesic priming produced by systemic sub-AD fentanyl (0.01 mg/kg, s.c.) are dependent on $G\alpha_{i/o}$ (i.e., $G\alpha_i1$, $G\alpha_i2$, $G\alpha_i3$, and/or $G\alpha_o$). Rats were treated intrathecally with AS-ODN or SE-ODN to $G\alpha_i1$ (Fig. 1), $G\alpha_i2$ (Fig. 2), $G\alpha_i3$ (Fig. 3), or $G\alpha_o$ (Fig. 4) mRNA, daily for 4 d. On the fourth day, ~17 h after the third injection of ODN, sub-AD fentanyl (0.01 mg/kg) was administered systemically (subcutaneous, s.c.), and mechanical nociceptive threshold measured 1 h later. When compared with SE-ODN-treated rats the hyperalgesia induced by sub-AD fentanyl was markedly attenuated in rats pretreated with AS-ODN against $G\alpha_i2$ (Fig. 2*A*), $G\alpha_i3$ (Fig. 3*A*), or $G\alpha_o$ (Fig. 4*A*). Treatment with $G\alpha_i1$ AS-ODN did not affect sub-AD fentanyl-induced hyperalgesia (Fig. 1*A*). At the end of the fourth day, rats received the last intrathecal administration of G-protein α-subunit AS-ODN or SE-ODN. Five days after systemic sub-AD fentanyl administration, PGE_2 (100 ng) was injected intradermally and the mechanical nociceptive threshold evaluated 30 min and 4 h later. In the rats treated with $G\alpha_i3$ AS-ODN, the prolongation PGE_2 hyperalgesia at the fourth hour was not present (Fig. 3*C*). However, prolonged PGE_2-induced hyperalgesia was still present in $G\alpha_o$, $G\alpha_i1$, and $G\alpha_i2$ AS-ODN- and SE-ODN-treated rats (Figs. 1*C*, 2*C*, and 4*C*). Our data support the following suggestions: (1) that some side effects of fentanyl (OIH and hyperalgesic priming) are $G\alpha_{i/o}$ dependent; (2) that a side effect (OIH) may be dependent on multiple $G\alpha_{i/o}$ subunits; and (3) that the $G\alpha_{i/o}$ subunits mediating one side effect ($G\alpha_i2$, $G\alpha_i3$, and $G\alpha_o$ mediating OIH) may differ from those mediating another side effect (only $G\alpha_i3$ mediating sub-AD fentanyl-induced priming).

OIH and priming produced by subanalgesic dose morphine

We next investigated whether OIH and hyperalgesic priming induced by sub-AD of another clinical opioid analgesic, morphine (0.03 mg/kg, s.c.; Araldi et al., 2019) are also $G\alpha_{i/o}$ dependent and whether they are dependent on the same $G\alpha_{i/o}$ subunits as these effects of sub-AD fentanyl. Rats received intrathecal AS-ODN or SE-ODN to $G\alpha_i1$ (Fig. 1), $G\alpha_i2$ (Fig. 2), $G\alpha_i3$ (Fig. 3), or $G\alpha_o$ (Fig. 4) mRNA, daily for 4 d. On the fourth day, sub-AD morphine (0.03 mg/kg, s.c.) was administered and the mechanical nociceptive threshold was measured 1 h later. In rats treated with AS-ODN against $G\alpha_i1$ (Fig. 1*B*), $G\alpha_i2$ (Fig. 2*B*), and $G\alpha_i3$ (Fig. 3*B*) mRNA, sub-AD morphine did not induce hyperalgesia. However, in rats treated with $G\alpha_o$ AS-ODN, systemic sub-AD morphine still induced hyperalgesia (Fig. 4*B*). At the end of the fourth day, rats again received AS-ODN or SE-ODN. Five days after systemic sub-AD morphine administration, PGE_2 (100 ng) was injected intradermally and the mechanical nociceptive threshold was evaluated 30 min and 4 h later. The prolongation of PGE_2-induced hyperalgesia was still present in all AS-ODN-treated rats (Figs. 1*D*, 2*D*, 3*D*, and 4*D*). These findings support the following suggestions: (1) that the $G\alpha_{i/o}$ subunits mediating side effects (OIH and hyperalgesic priming) differ between the sub-AD of clinical opioid analgesics (fentanyl and morphine); and (2) that the same side effect (OIH) produced by two opioid analgesics may be dependent only partially on overlapping $G\alpha_{i/o}$ subunits (while OIH produced by sub-AD morphine is dependent on $G\alpha_i1$, $G\alpha_i2$, and $G\alpha_i3$, OIH produced by sub-AD fentanyl is $G\alpha_i2$, $G\alpha_i3$, and $G\alpha_o$ dependent). Whether opioid-induced hyperalgesic priming produced by sub-AD morphine is G-protein independent or dependent on one of the $G\alpha$ subunits not evaluated in the present experiments remains to be established.

Analgesia and hyperalgesic priming produced by analgesic dose (AD) fentanyl

Since AD opioids produce hyperalgesic priming as well as analgesia, we next determined whether analgesia and priming induced by AD fentanyl (0.03 mg/kg, s.c.; Khomula et al., 2019, 2021) are $G\alpha_{i/o}$ dependent and whether the same G-proteins mediate opioid-induced hyperalgesic priming produced by sub-AD and AD fentanyl. Groups of rats were treated with AS-ODN or SE-ODN for $G\alpha_i1$ (Fig. 5), $G\alpha_i2$ (Fig. 6), $G\alpha_i3$ (Fig. 7), or $G\alpha_o$ (Fig. 8) mRNA, daily for 4 consecutive days. On the fourth day, ~17 h after the third dose of ODN, AD fentanyl was administered (0.03 mg/kg, s.c.), and the mechanical nociceptive threshold was evaluated 1 h later. Unexpectedly, analgesia induced by AD fentanyl was increased in the $G\alpha_i1$ (Fig. 5*A*) and $G\alpha_i2$ (Fig. 6*A*) AS-ODN-treated groups of rats; while in the groups treated with either $G\alpha_i3$ (Fig. 7*A*) or $G\alpha_o$ AS-ODN (Fig. 8*A*) fentanyl-induced analgesia was not affected. At the end of the fourth day, rats again received AS-ODN or SE-ODN. Five days after AD fentanyl, PGE_2 (100 ng, i.d.) was injected and mechanical nociceptive threshold evaluated 30 min and 4 h later. In rats treated with $G\alpha_o$ (Fig. 8*C*), but not $G\alpha_i1$, $G\alpha_i2$, and $G\alpha_i3$ (Figs. 5*C*, 6*C*, and 7*C*) AS-ODN the prolongation of PGE_2-induced hyperalgesia was eliminated.

To the best of our knowledge, our data demonstrating that $G\alpha_i1$ and $G\alpha_i2$ AS-ODN enhances a $G\alpha_{i/o}$–GPCR signaling provide the first evidence that $G\alpha_{i/o}$ subunits can inhibit $G\alpha_{i/o}$ GPCR signaling. Our results in which $G\alpha_i1$ and $G\alpha_i2$ subunits

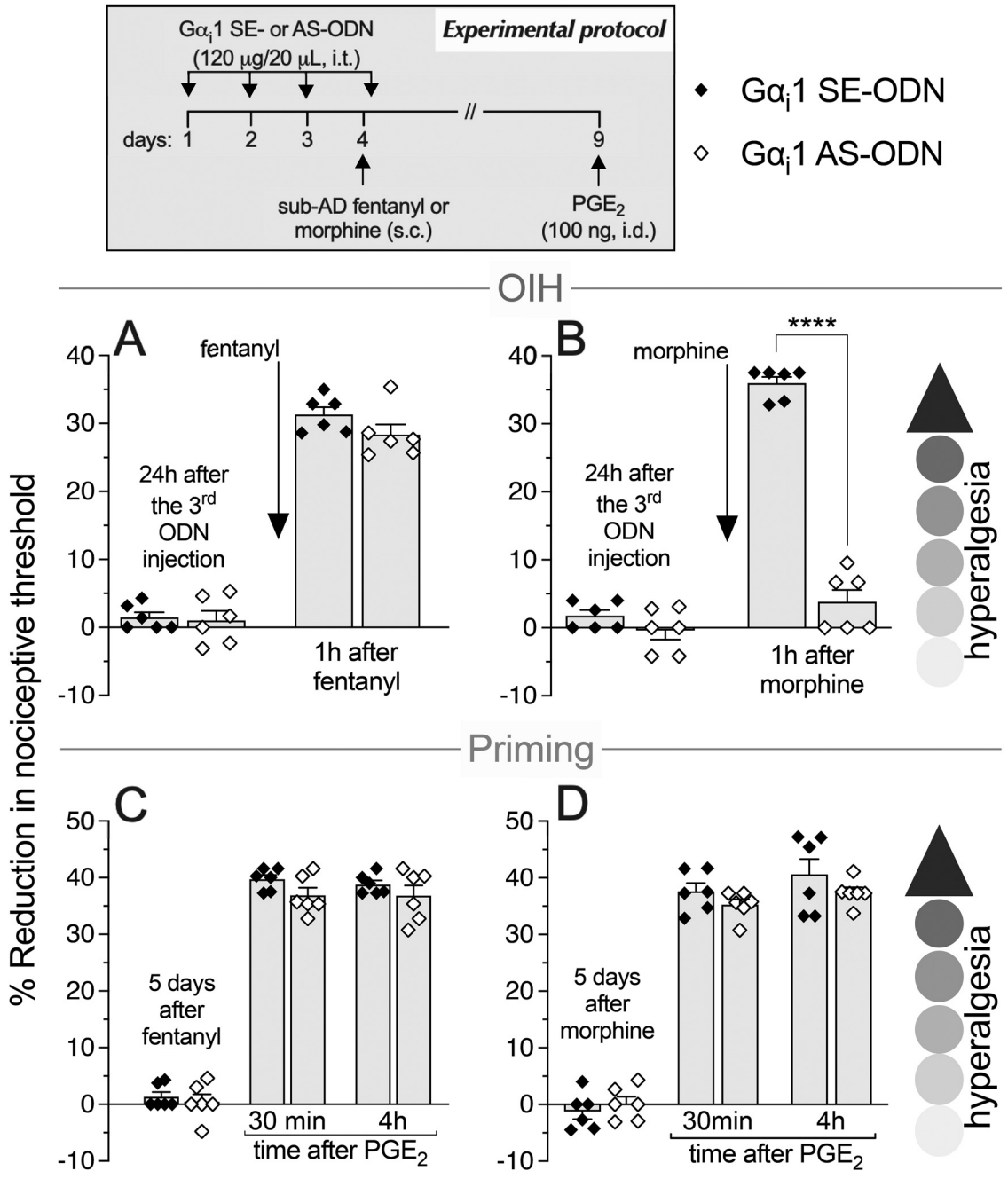

Figure 1. Role of G_i-protein α1 subunit ($G\alpha_i1$) in OIH and hyperalgesic priming produced by systemic sub-AD fentanyl and morphine. Rats received intrathecal (i.t.) injection of AS-ODN (120 μg/20 μl/d, i.t.) or SE-ODN (120 μg/20 μl/d, i.t.) to $G\alpha_i1$ mRNA for 3 consecutive days. ***A***, ***B***, On the fourth day, at which time mechanical nociceptive threshold was not different from the pre-ODN baseline (***A***: SE-ODN-treated group: $t_{(5)} = 0.5$; $p = 0.64$; AS-ODN-treated group: $t_{(5)} = 0.82$; $p = 0.45$; ***B***: SE-ODN-treated group: $t_{(5)} = 2.17$; $p = 0.08$; AS-ODN-treated group: $t_{(5)} = 0.62$; $p = 0.56$ when the mechanical nociceptive threshold is compared before and ~17 h after the third ODN injection; paired Student's *t* test), sub-AD fentanyl (***A***: 0.01 mg/kg, s.c.) or morphine (***B***: 0.03 mg/kg, s.c.) was injected and the mechanical nociceptive threshold was evaluated 1 h later. $G\alpha_i1$ AS-ODN did not affect sub-AD fentanyl-induced hyperalgesia ($F_{(1,10)} = 1.94$, $p = 0.19$, when hyperalgesia was compared between the $G\alpha_i1$ SE-ODN- and AS-ODN-treated groups 1 h after systemic sub-AD fentanyl; two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test; ***A***). However, in the $G\alpha_i1$ AS-ODN-treated group, OIH produced by sub-AD morphine was markedly attenuated ($F_{(1,10)} = 189.4$, $^{****}p < 0.0001$, when the hyperalgesia in the $G\alpha_i1$ SE-ODN- and the AS-ODN-treated groups is compared at 1 h after systemic sub-AD morphine; two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test; ***B***). At the end of the fourth day, rats again received intrathecal $G\alpha_i1$ AS- or SE-ODN. ***C***, ***D***, Five days after systemic sub-AD fentanyl and morphine, at which time mechanical nociceptive threshold was not different from preopioid baselines (***C***: SE-ODN-treated group: $t_{(5)} = 1.58$; $p = 0.18$; AS-ODN-treated group that received sub-AD fentanyl: $t_{(5)} = 0.39$; $p = 0.71$; ***D***: SE-ODN-treated group: $t_{(5)} = 0.88$; $p = 0.42$; AS-ODN-treated group that received sub-AD morphine: $t_{(5)} = 0.2$; $p = 0.85$ when the mechanical nociceptive threshold is compared before and 5 d after systemic sub-AD opioid administration; paired Student's *t* test), PGE_2 (100 ng/5 μl, i.d.) was injected and the mechanical nociceptive threshold was evaluated 30 min and 4 h later. Treatment with $G\alpha_i1$ AS-ODN did not prevent PGE_2-induced prolonged hyperalgesia in either fentanyl-treated (***C***) or morphine-treated (***D***) groups (***C***: $F_{(1,10)} = 2.12$, $p = 0.17$; ***D***: $F_{(1,10)} = 1.02$, $p = 0.34$, when the hyperalgesia in the $G\alpha_i1$ SE-ODN- and the AS-ODN-treated groups is compared at the fourth hour after intradermal PGE_2 administration; two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test). These findings support the suggestion that $G\alpha_i1$ plays a role in OIH produced by systemic sub-AD morphine, but not fentanyl, and is not involved in hyperalgesic priming produced by sub-AD fentanyl or morphine ($n = 6$ paws/6 rats/group).

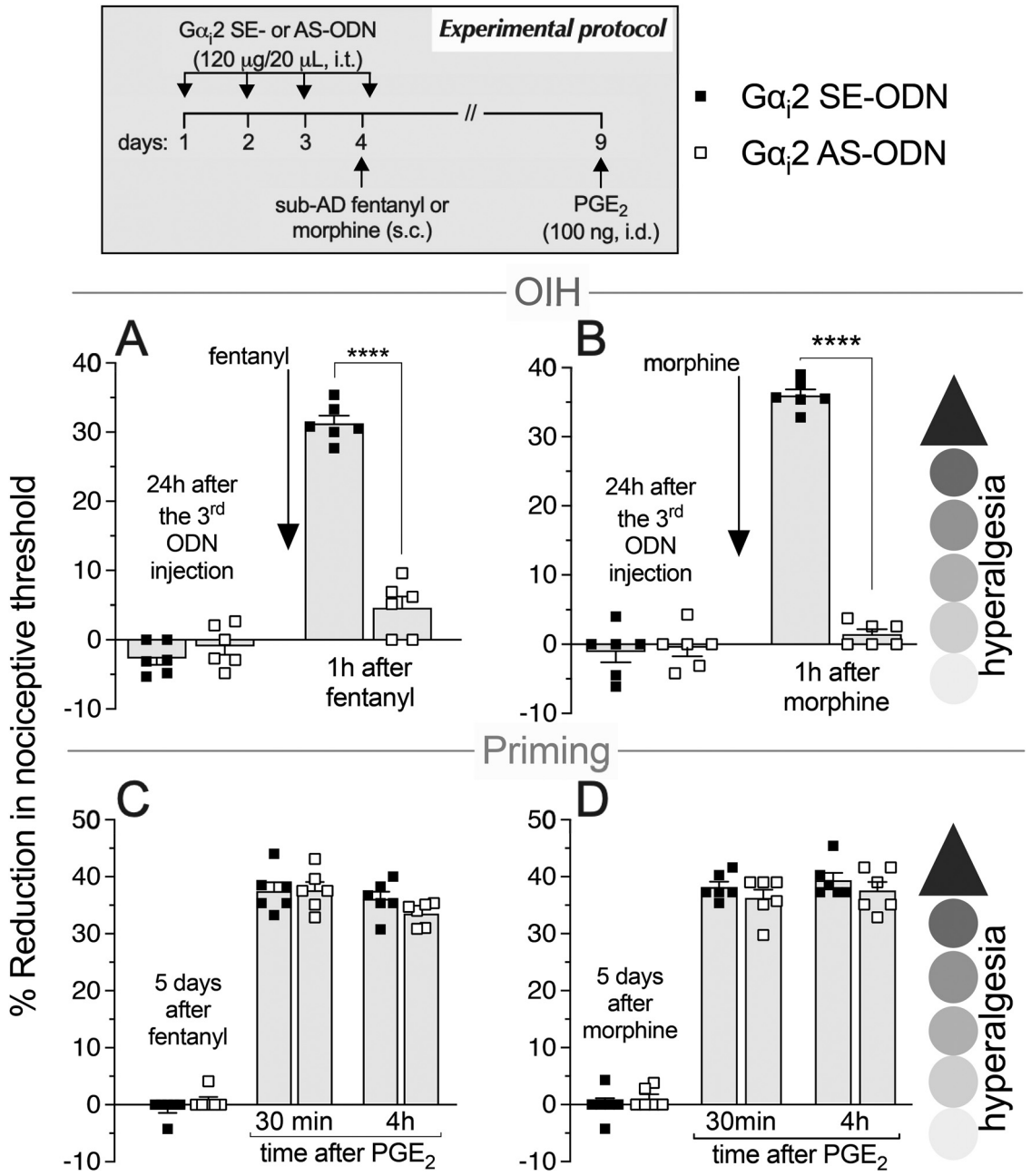

Figure 2. Role of $G\alpha_i2$ in hyperalgesia and hyperalgesic priming produced by systemic sub-AD fentanyl and morphine. Rats received injections of AS-ODN (120 μg/20 μl/d, i.t.) or SE-ODN (120 μg/20 μl/d, i.t.) against $G\alpha_i2$ mRNA, for 3 consecutive days. ***A***, ***B***, Approximately 17 h after the third injection, at which time the mechanical nociceptive threshold was not different from pre-ODN baseline levels (***A***: SE-ODN-treated group: $t_{(5)} = 0.41$, $p = 0.70$; AS-ODN-treated group: $t_{(5)} = 0.56$; $p = 0.57$; ***B***: SE-ODN-treated group: $t_{(5)} = 0.15$, $p = 0.89$; AS-ODN-treated group: $t_{(5)} = 0.13$, $p = 0.90$, when the mechanical nociceptive threshold is compared before and after the third ODN injection; paired Student's *t* test), sub-AD fentanyl (***A***: 0.01 mg/kg, s.c.) or morphine (***B***: 0.03 mg/kg, s.c.) was administered and mechanical nociceptive threshold was evaluated 1 h later. Treatment with $G\alpha_i2$ AS-ODN prevented hyperalgesia induced by both sub-AD fentanyl (***A***) and morphine (***B***), when it was compared with the SE-ODN-treated group (***A***: $F_{(1,10)} = 75.5$, $^{****}p < 0.0001$; ***B***: $F_{(1,10)} = 298.3$, $^{****}p < 0.0001$, when the hyperalgesia in the $G\alpha_i2$ SE-ODN-treated and the AS-ODN-treated groups is compared at 1 h after systemic sub-AD fentanyl and morphine; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). Rats again received intrathecal $G\alpha_i2$ AS- or SE-ODN on the fourth day. ***C***, ***D***, Five days after sub-AD opioids, at which time the mechanical nociceptive threshold was not different from preopioid baseline (***C***: SE-ODN-treated group: $t_{(5)} = 1.0$, $p = 0.36$; AS-ODN-treated group that received sub-AD fentanyl: $t_{(5)} = 0.54$, $p = 0.61$; ***D***: SE-ODN-treated group: $t_{(5)} = 0.73$, $p = 0.49$; AS-ODN-treated group that received sub-AD morphine: $t_{(5)} = 0.59$, $p = 0.57$, when the mechanical nociceptive threshold is compared before and 5 d after systemic sub-AD opioids; paired Student's *t* test), PGE_2 (100 ng/5 μl, i.d.) was injected and the mechanical nociceptive threshold was evaluated 30 min and 4 h later. In both the $G\alpha_i2$ AS-ODN-treated and SE-ODN-treated groups, the prolongation of PGE_2 hyperalgesia was present 5 d after systemic sub-AD fentanyl and morphine (***C*** and ***D***, respectively; ***C***: $F_{(1,10)} = 0.11$, $p = 0.74$; ***D***: $F_{(1,10)} = 0.50$, $p = 0.49$, when the hyperalgesia in the $G\alpha_i2$ AS-ODN-treated and the SE-ODN-treated groups is compared at the fourth hour after intradermal PGE_2; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). Our data indicate that OIH, but not priming, produced by systemic sub-AD fentanyl and morphine is $G\alpha_i2$ dependent ($n = 6$ paws/6 rats/group).

increased analgesia induced by AD fentanyl contrasts with a fentanyl saturation-binding analysis for MOR, using urea-washed MOR membranes, where fentanyl displayed high potency in activating both $G\alpha_i1$ and $G\alpha_o$ (Saidak et al., 2006).

Furthermore, our data support the suggestion that different $G\alpha_{i/o}$ subunits mediate priming induced by AD ($G\alpha_o$) and sub-AD ($G\alpha_i3$) fentanyl. While the basis for this difference is currently unknown, we have observed that sub-AD fentanyl-

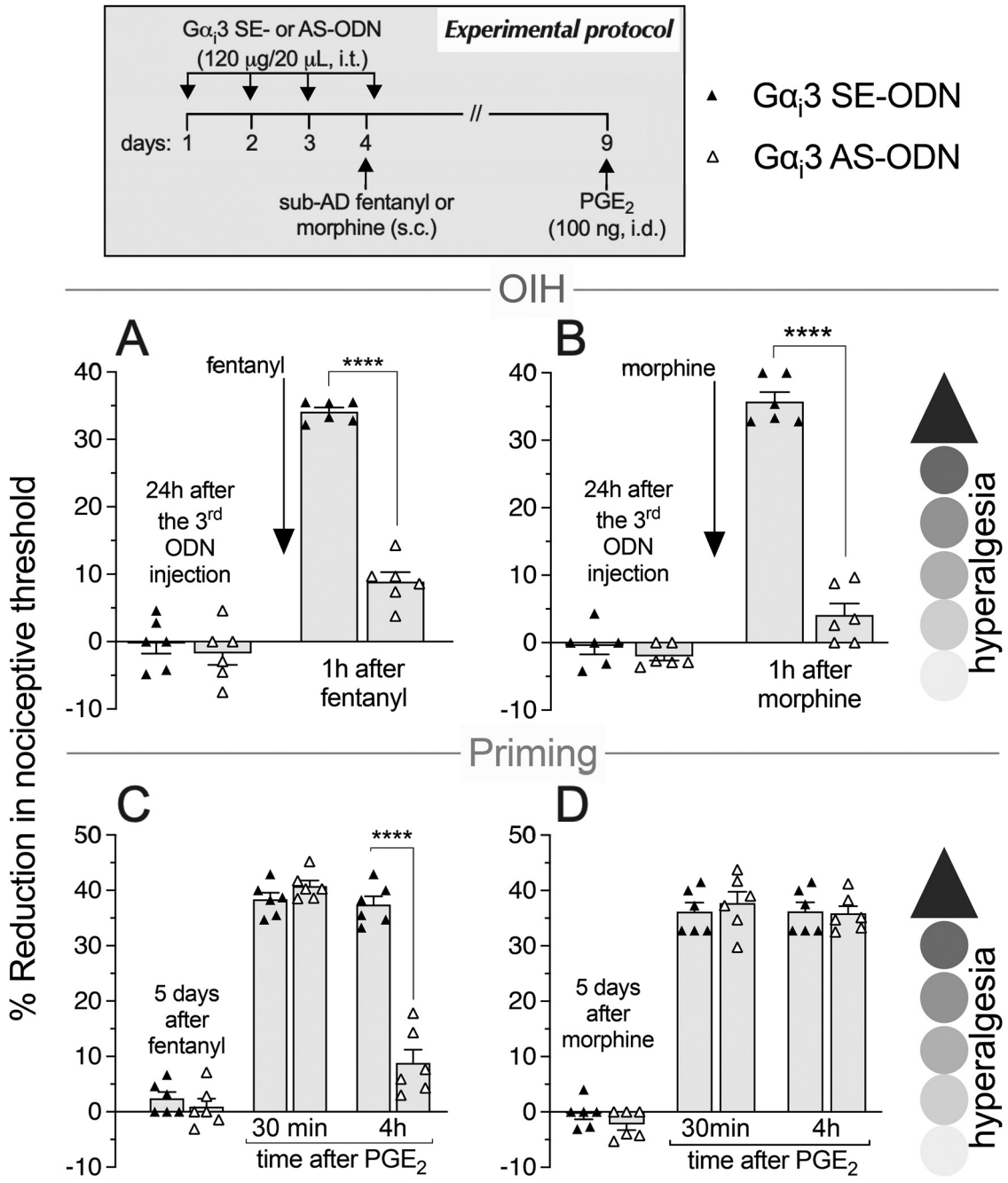

Figure 3. Role of $G\alpha_i3$ in hyperalgesia and hyperalgesic priming produced by systemic sub-AD fentanyl and morphine. Rats received injection of AS-ODN (120 μg in 20 μl/d, i.t.) or SE-ODN (120 μg in 20 μl/d, i.t.) against $G\alpha_i3$ mRNA, daily for 3 consecutive days. ***A***, ***B***, On the fourth day, ~17 h after the third intrathecal administration of ODNs, at which time mechanical nociceptive threshold was not significantly different from pre-ODN baselines (***A***: SE-ODN-treated group: $t_{(5)} = 0.67$, $p = 0.53$; AS-ODN-treated group: $t_{(5)} = 0.18$, $p = 0.86$; ***B***: SE-ODN-treated group: $t_{(5)} = 0.39$, $p = 0.71$; AS-ODN-treated group: $t_{(5)} = 0.15$, $p = 0.89$, when the mechanical nociceptive threshold is compared before and after the third $G\alpha_i3$ ODN injection; paired Student's *t* test), sub-AD fentanyl (***A***: 0.01 mg/kg, s.c.) or morphine (***B***: 0.03 mg/kg, s.c.) was administered and the mechanical nociceptive threshold was evaluated 1 h later. In the $G\alpha_i3$ AS-ODN-treated group, systemic sub-AD of neither fentanyl (***A***) nor morphine (***B***) produced hyperalgesia, measured 1 h after its administration, as is observed in the $G\alpha_i3$ SE-ODN-treated group (***A***: $F_{(1,10)} = 186.6$, ****$p < 0.0001$; *B*: $F_{(1,10)} = 193.9$, ****$p < 0.0001$, when the hyperalgesia in the $G\alpha_i3$ SE-ODN-treated and the AS-ODN-treated groups was compared at 1 h after systemic sub-AD opioids; two-way repeated-measures ANOVA followed by Bonferroni *post hoc* test). At the end of the fourth day, rats again received intrathecal $G\alpha_i3$ AS-ODN or SE-ODN. ***C***, ***D***, Five days after systemic sub-AD fentanyl and morphine, at which time mechanical nociceptive threshold was not different from preopioid baselines (***C***: SE-ODN-treated group: $t_{(5)} = 1.98$, $p = 0.11$; AS-ODN-treated group that received sub-AD fentanyl: $t_{(5)} = 1.07$, $p = 0.33$; ***D***: SE-ODN-treated group: $t_{(5)} = 0.22$; $p = 0.83$; AS-ODN-treated group that received sub-AD morphine: $t_{(5)} = 2.23$, $p = 0.07$, when the mechanical nociceptive threshold is compared before and 5 d after systemic sub-AD opioid; paired Student's *t* test), PGE_2 (100 ng/5 μl, i.d.) was injected and mechanical nociceptive threshold was evaluated 30 min and 4 h later. In the $G\alpha_i3$ AS-ODN-treated group, which received systemic sub-AD fentanyl, PGE_2-induced hyperalgesia was not present at the fourth hour (***C***: $F_{(1,10)} = 42.9$, ****$p < 0.0001$, when the hyperalgesia in the $G\alpha_i3$ AS-ODN-treated and the SE-ODN-treated groups is compared at the fourth hour after intradermal PGE_2; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). However, prolongation of PGE_2-induced hyperalgesia was not affected by the treatment with $G\alpha_i3$ AS-ODN in the systemic sub-AD morphine-treated group (***D***: $F_{(1,10)} = 0.04$, $p = 0.85$, when hyperalgesia was compared between the $G\alpha_i3$ SE-ODN-treated and AS-ODN-treated groups at the fourth hour after intradermal PGE_2; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). These findings indicate that $G\alpha_i3$ plays a role in hyperalgesia induced by both sub-AD fentanyl and morphine. However, priming induced by sub-AD fentanyl, but not sub-AD morphine, is dependent on $G\alpha_i3$ ($n = 6$ paws/6 rats/group).

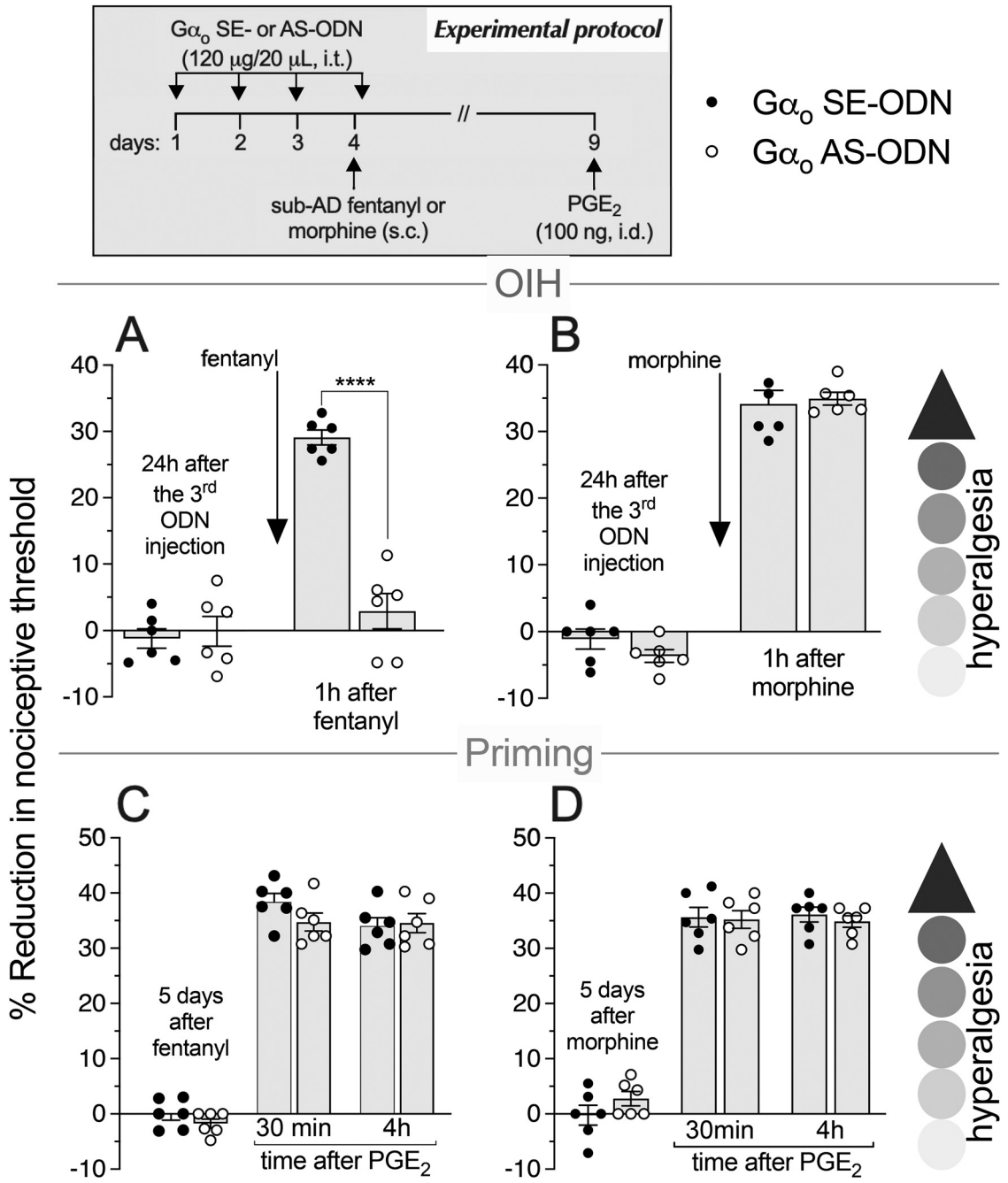

Figure 4. Role of $G\alpha_o$ in hyperalgesia and hyperalgesic priming induced by systemic sub-AD fentanyl and morphine. Rats received injection of AS-ODN (120 μg in 20 μl/d, i.t.) or SE-ODN (120 μg in 20 μl/d, i.t.) against $G\alpha_o$ mRNA daily for 3 consecutive days. On the fourth day, at which time the mechanical nociceptive threshold was not different from the pre-ODN baselines (***A***: SE-ODN-treated group: $t_{(5)} = 0.67$; $p = 0.53$; AS-ODN-treated group: $t_{(5)} = 0.65$; $p = 0.54$; ***B***: SE-ODN-treated group: $t_{(5)} = 0.74$; $p = 0.49$; AS-ODN-treated group: $t_{(5)} = 1.66$; $p = 0.15$, when the mechanical nociceptive threshold is compared before and ~17 h after the third ODN injection; paired Student's *t* test), sub-AD fentanyl (***A***, 0.01 mg/kg, s.c.) or morphine (***B***, 0.03 mg/kg, s.c.) was administered and the mechanical nociceptive threshold was evaluated 1 h later. In the group of rats treated with $G\alpha_o$ AS-ODN, systemic sub-AD fentanyl-induced hyperalgesia was prevented (***A***, $F_{(1,10)} = 53.3$, $^{****}p < 0.0001$, when the hyperalgesia in the $G\alpha_o$ SE-ODN-treated and the AS-ODN-treated groups was compared at 1 h after systemic sub-AD fentanyl; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). However, systemic sub-AD morphine-induced hyperalgesia was not affected by the treatment with $G\alpha_o$ AS-ODN (***B***, $F_{(1,10)} = 0.24$, $p = 0.63$, when the hyperalgesia in the $G\alpha_o$ SE-ODN-treated and the AS-ODN-treated groups was compared at 1 h after systemic sub-AD morphine; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). At the end of the fourth day, rats again received intrathecal $G\alpha_o$ AS-ODN or SE-ODN. Five days after systemic sub-AD fentanyl and morphine, when the mechanical nociceptive threshold was not different from preopioid baselines (***C***: SE-ODN-treated group: $t_{(5)} = 0.75$; $p = 0.48$; AS-ODN-treated group that received sub-AD fentanyl: $t_{(5)} = 2.15$; $p = 0.08$; ***D***: SE-ODN-treated group: $t_{(5)} = 0.68$; $p = 0.53$; AS-ODN-treated group that received sub-AD morphine: $t_{(5)} = 1.90$; $p = 0.11$, when the mechanical nociceptive threshold is compared before and 5 d after systemic sub-AD opioids; paired Student's *t* test), PGE_2 (100 ng/5 μl, i.d.) was administered and the mechanical nociceptive threshold was evaluated 30 min and 4 h later. Treatment with $G\alpha_o$ AS-ODN did not prevent the prolongation of PGE_2-induced hyperalgesia in both fentanyl-treated (***C***) and morphine-treated (***D***) groups of rats (***C***: $F_{(1,10)} = 2.15$, $p = 0.17$; ***D***: $F_{(1,10)} = 0.18$, $p = 0.68$, when the hyperalgesia in the $G\alpha_o$ SE-ODN-treated and the AS-ODN-treated groups is compared at the fourth hour after intradermal PGE_2; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). These findings support the suggestion that the $G\alpha_o$ subunit plays a role in OIH produced by systemic sub-AD fentanyl, but not morphine, and is not involved in hyperalgesic priming produced by sub-AD fentanyl and morphine. ($n = 6$ paws/6 rats/group).

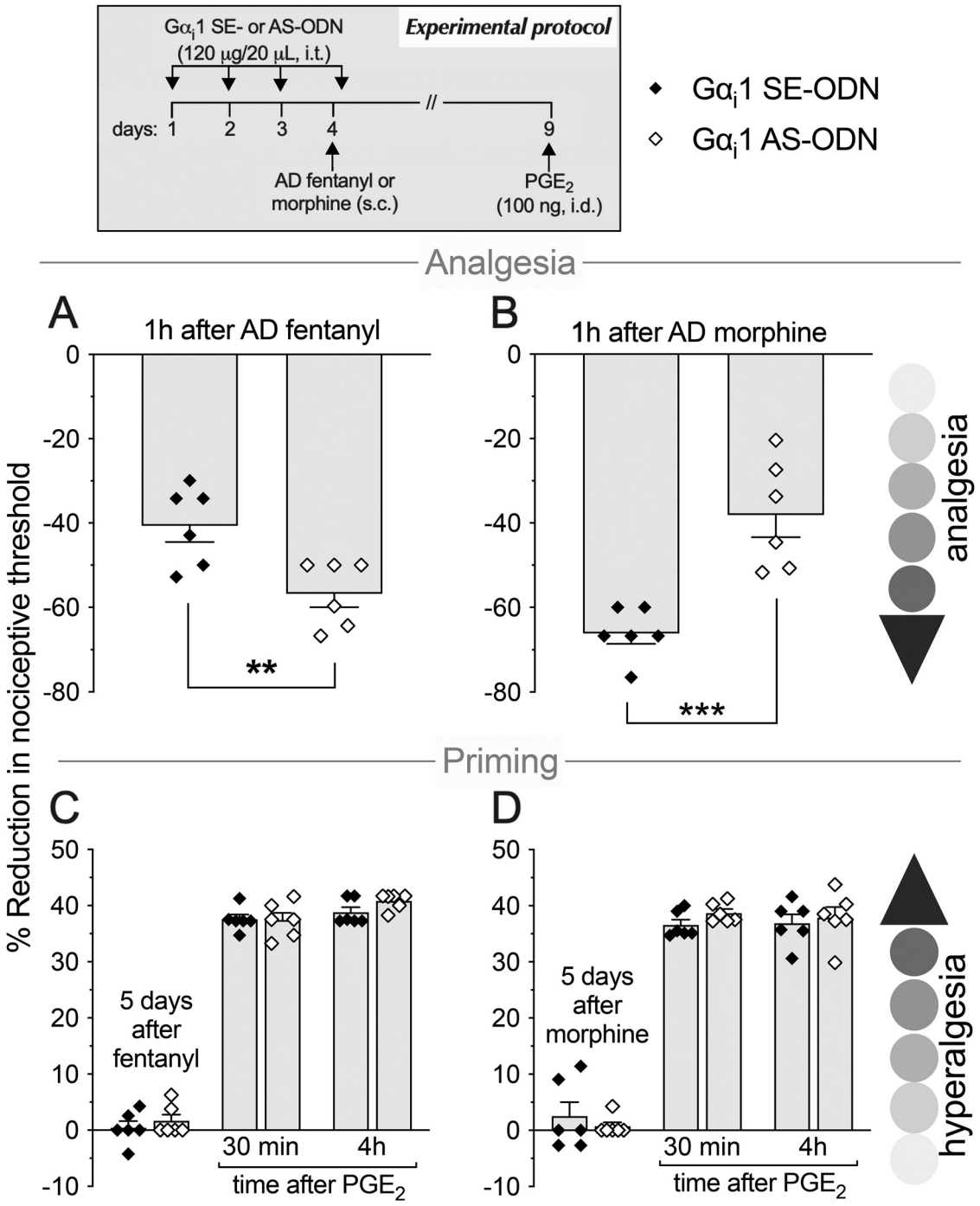

Figure 5. Role of $G\alpha_i1$ in systemic AD fentanyl- and morphine-induced analgesia and priming. Rats received intrathecal injections of AS-ODN (120 μg in 20 μl/d, i.t.) or SE-ODN (120 μg in 20 μl/d, i.t.) against $G\alpha_i1$ mRNA, daily for 3 consecutive days. On the fourth day, AD fentanyl (***A***, 0.03 mg/kg, s.c.) or morphine (***B***, 3 mg/kg, s.c.) was administered and the mechanical nociceptive threshold was evaluated 1 h later. Treatment with $G\alpha_i1$ AS-ODN increased analgesia induced by systemic AD fentanyl (***A***), while it decreased AD morphine-induced analgesia (***B***), compared with their respective $G\alpha_i1$ SE-ODN-treated groups (***A***: $t_{(10)} = 3.24$, ** $p = 0.0088$; ***B***: $t_{(10)} = 4.81$, *** $p = 0.0007$, when the analgesia in the $G\alpha_i1$ AS-ODN-treated and the SE-ODN-treated groups is compared at 1 h after systemic AD fentanyl and morphine; unpaired Student's *t* test). At the end of the fourth day, rats again received intrathecal $G\alpha_i1$ AS-ODN or SE-ODN. Five days after systemic AD fentanyl and morphine administration, at which time the mechanical nociceptive threshold was not different from preopioid baselines (***C***: SE-ODN-treated group: $t_{(5)} = 0.39$, $p = 0.71$; AS-ODN-treated group that received systemic AD fentanyl: $t_{(5)} = 1.4$, $p = 0.22$; ***D***: SE-ODN-treated group: $t_{(5)} = 1.0$, $p = 0.36$; AS-ODN-treated group that received systemic AD morphine: $t_{(5)} = 0.58$, $p = 0.59$, when the mechanical nociceptive threshold is compared before and 5 d after systemic AD opioids; paired Student's *t* test), PGE_2 (100 ng/5 μl, i.d.) was administered and the mechanical nociceptive threshold was evaluated 30 min and 4 h later. Prolongation of PGE_2-induced hyperalgesia was not affected by treatment with $G\alpha_i1$ AS-ODN in both AD fentanyl-treated (***C***) and morphine-treated (***D***) groups of rats (***C***: $F_{(1,10)} = 1.28$, $p = 0.28$; ***D***: $F_{(1,10)} = 0.13$, $p = 0.72$, when the hyperalgesia in the $G\alpha_i1$ SE-ODN-treated and the AS-ODN-treated groups is compared at the fourth hour after intradermal PGE_2 administration; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). These findings support the suggestion that $G\alpha_i1$ plays a role in analgesia, but not in hyperalgesic priming, produced by AD fentanyl and morphine ($n = 6$ paws/6 rats/group).

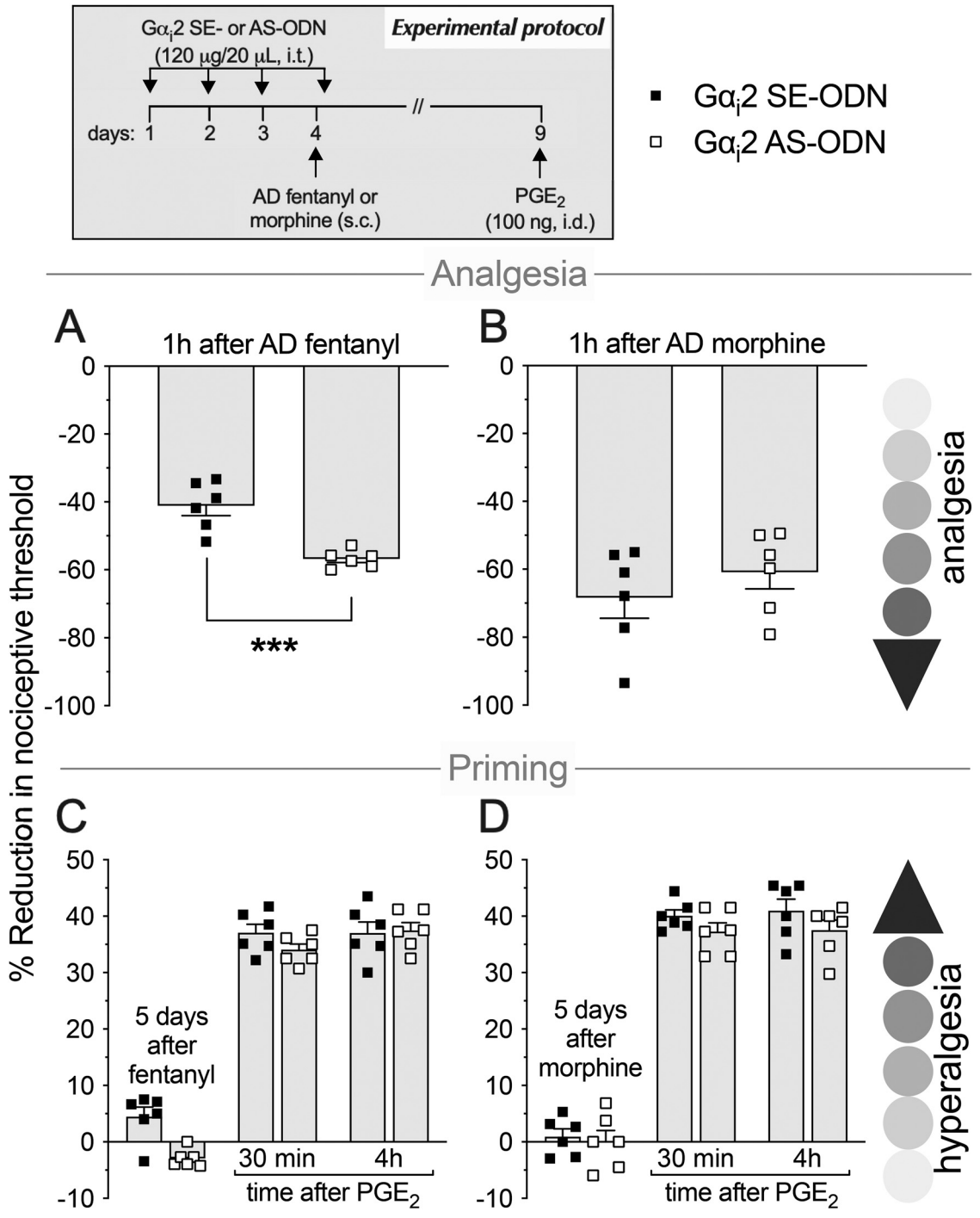

Figure 6. Role of $G\alpha_i2$ in analgesia and hyperalgesic priming induced by systemic analgesic dose fentanyl and morphine. Rats received injections of AS-ODN (120 μg/20 μl/d, i.t.) or SE-ODN (120 μg/20 μl/d, i.t.) against $G\alpha_i2$ mRNA, daily for 3 consecutive days. Approximately 17 h after the third injection of ODNs, AD fentanyl (***A***: 0.03 mg/kg, s.c.) or morphine (***B***: 3 mg/kg, s.c.) was administered and the mechanical nociceptive threshold was evaluated 1 h later. Treatment with $G\alpha_i2$ AS-ODN increased analgesia induced by AD fentanyl (***A***); however, it did not affect analgesia induced by AD morphine (***B***), when compared with the SE-ODN-treated group (***A***: $t_{(10)} = 5.07$, ***$p = 0.0005$; ***B***: $t_{(10)} = 0.96$, $p = 0.36$, when the analgesia in the $G\alpha_i2$ AS-ODN-treated and the SE-ODN-treated groups is compared at 1 h after systemic AD fentanyl and morphine, respectively; unpaired Student's *t* test). Rats again received $G\alpha_i2$ AS-ODN or SE-ODN later on the fourth day. Five days after AD opioids, at which time mechanical nociceptive threshold was not different from preopioid baselines (***C***: SE-ODN-treated group: $t_{(5)} = 2.5$, $p = 0.06$; AS-ODN-treated group that received AD fentanyl: $t_{(5)} = 0.67$; $p = 0.53$; ***D***: SE-ODN-treated group: $t_{(5)} = 1.0$, $p = 0.36$; AS-ODN-treated group that received AD morphine: $t_{(5)} = 0.12$, $p = 0.91$, when the mechanical nociceptive threshold is compared before and 5 d after systemic AD opioids; paired Student's *t* test), PGE_2 (100 ng/5 μl, i.d.) was administered and the mechanical nociceptive threshold was evaluated 30 min and 4 h later. In both the $G\alpha_i2$ AS-treated and SE-ODN-treated groups, the prolongation of PGE_2-induced hyperalgesia was present 5 d after systemic AD fentanyl and morphine (***C*** and ***D***, respectively; ***C***: $F_{(1,10)} = 0.68$, $p = 0.43$; ***D***: $F_{(1,10)} = 2.84$, $p = 0.12$, when the hyperalgesia in the $G\alpha_i2$ AS-ODN-treated and the SE-ODN-treated groups is compared at the fourth hour after intradermal PGE_2 administration; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). Our data support the suggestion that the $G\alpha_i2$ subunit plays a role in analgesia produced by AD fentanyl, but not morphine; also, hyperalgesic priming produced by AD fentanyl and morphine is not $G\alpha_i2$ dependent ($n = 6$ paws/6 rats/group).

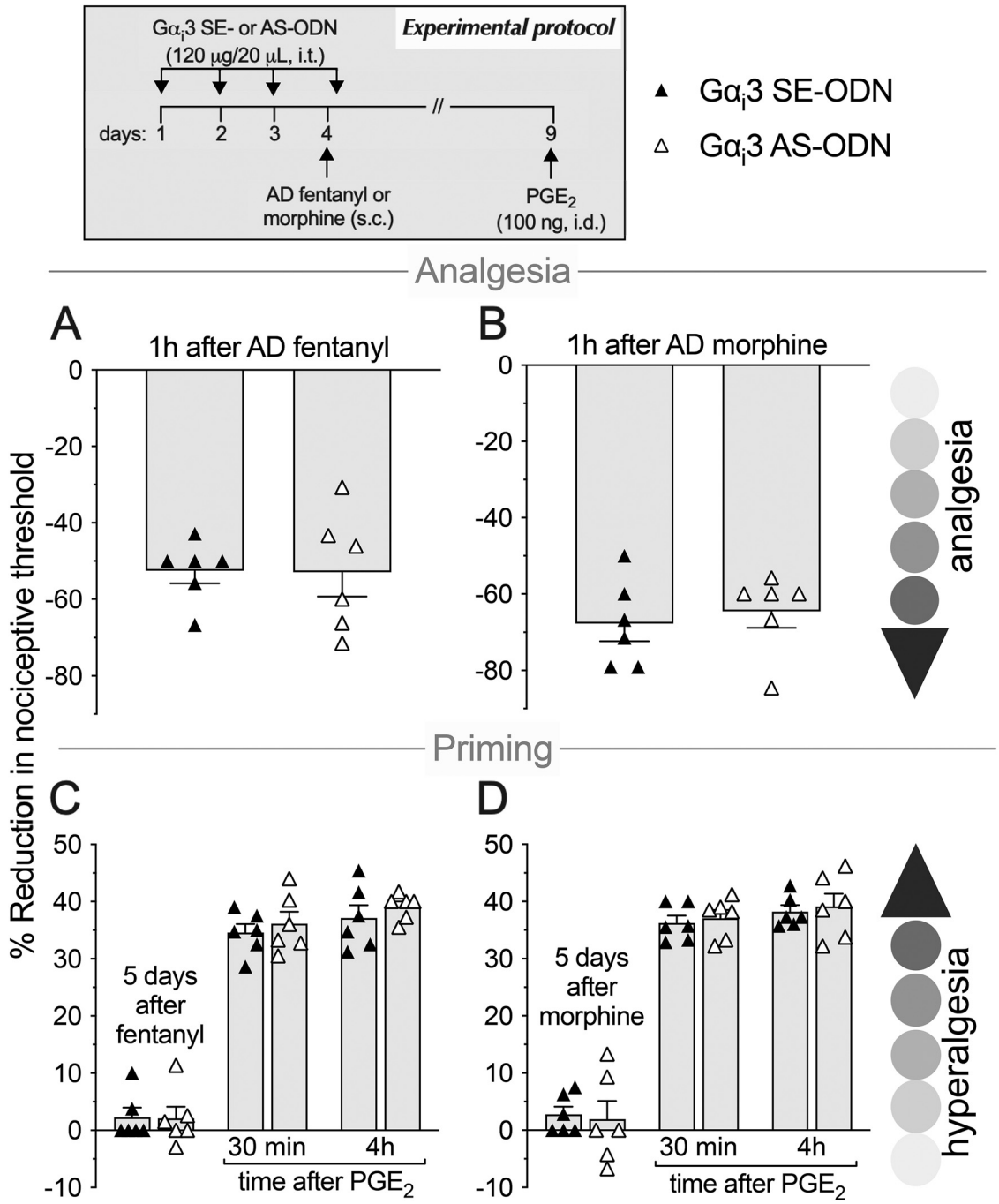

Figure 7. Role of $G\alpha_i3$ in analgesia and hyperalgesic priming induced by systemic AD fentanyl and morphine. Rats received intrathecal injections of AS-ODN (120 μg in 20 μl/d, i.t.) or SE-ODN (120 μg in 20 μl/day, i.t.) against $G\alpha_i3$ mRNA, daily for 3 consecutive days. On the fourth day, ~17 h after the third injection of ODNs, systemic AD fentanyl (***A***: 0.03 mg/kg, s.c.) or morphine (***B***: 3 mg/kg, s.c.) was injected and mechanical nociceptive threshold evaluated 1 h later. Treatment with $G\alpha_i3$ AS-ODN did not affect the analgesia produced by either AD fentanyl (***A***) or morphine (***B***), measured 1 h after their administration, as observed in the $G\alpha_i3$ SE-ODN-treated group (***A***: $t_{(10)} = 0.054$; ***$p = 0.96$; ***B***: $t_{(10)} = 0.51$, $p = 0.62$, when the analgesia in the $G\alpha_i3$ AS-ODN-treated and the SE-ODN-treated groups is compared at 1 h after systemic AD fentanyl and morphine, respectively; unpaired Student's *t* test). At the end of the fourth day, rats again received intrathecal $G\alpha_i3$ AS-ODN or SE-ODN. Five days after systemic AD fentanyl and morphine, at which time the mechanical nociceptive threshold was not different from preopioid baseline levels (***C***: SE-ODN-treated group: $t_{(5)} = 1.38$, $p = 0.23$; AS-ODN-treated group that received systemic AD fentanyl: $t_{(5)} = 1.06$, $p = 0.34$; ***D***: SE-ODN-treated group: $t_{(5)} = 2.0$, $p = 0.1$; AS-ODN-treated group that received systemic AD morphine: $t_{(5)} = 0.72$, $p = 0.5$, when the mechanical nociceptive threshold is compared before and 5 d after systemic AD opioids; paired Student's *t* test), PGE_2 (100 ng/5 μl, i.d.) was injected and the mechanical nociceptive threshold was evaluated 30 min and 4 h later. In both the $G\alpha_i3$ AS-ODN-treated and SE-ODN-treated groups, the prolongation of PGE_2-induced hyperalgesia was present 5 d after systemic AD fentanyl and morphine (***C*** and ***D***, respectively; ***C***: $F_{(1,10)} = 0.58$, $p = 0.46$; ***D***: $F_{(1,10)} = 0.1$, $p = 0.75$, when the hyperalgesia in the $G\alpha_i3$ AS-ODN-treated and the SE-ODN-treated groups is compared at the fourth hour after intradermal PGE_2; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). These findings support the suggestion that $G\alpha_i3$ did not play a role in analgesia and hyperalgesic priming induced by AD fentanyl or morphine ($n = 6$ paws/6 rats/group).

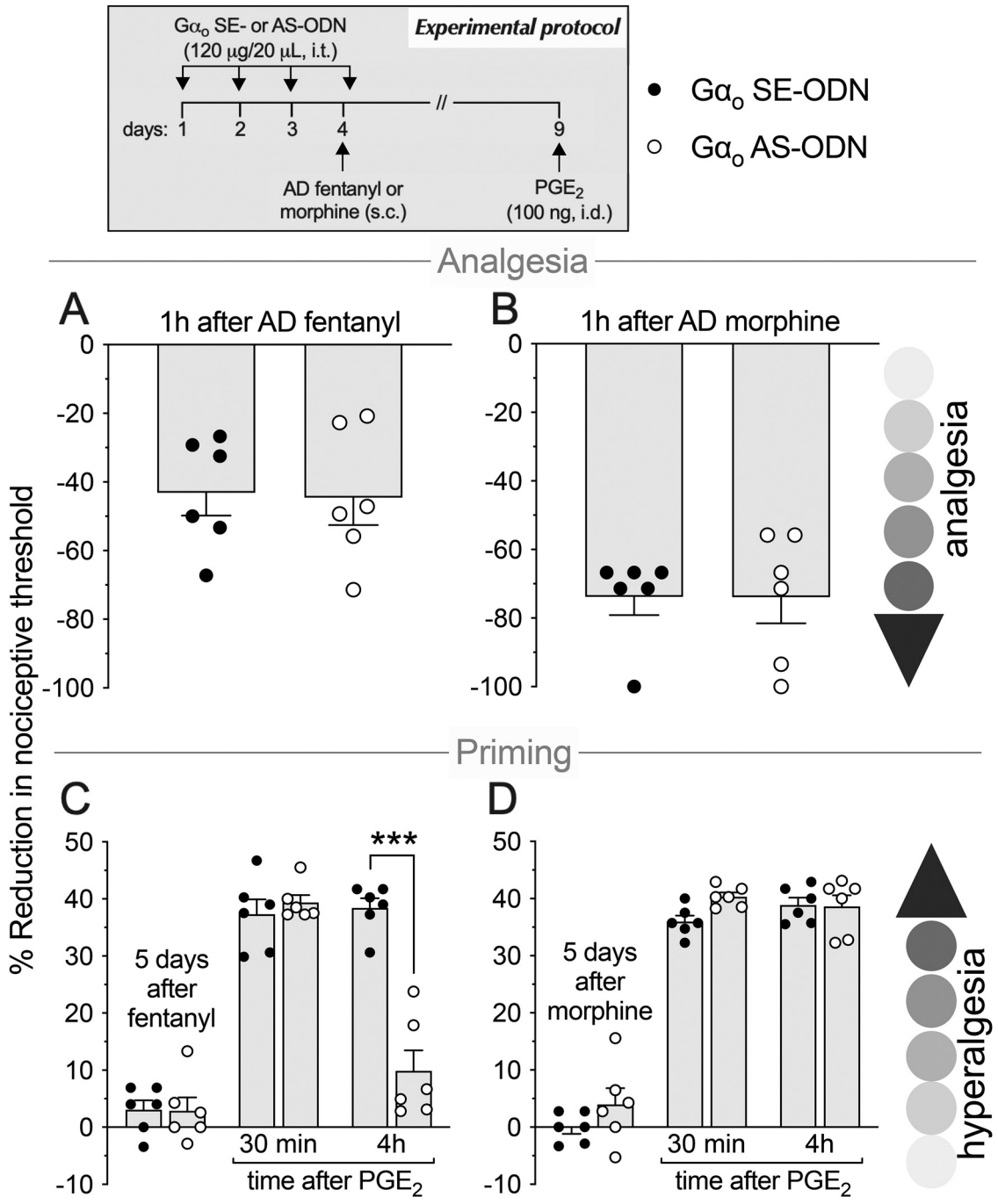

Figure 8. Role of $G\alpha_o$ in analgesia and hyperalgesic priming induced by systemic AD fentanyl and morphine. Rats received intrathecal injections of AS-ODN (120 μg in 20 μl/d, i.t.) or SE-ODN (120 μg in 20 μl/d, i.t.) against $G\alpha_o$ mRNA, daily for 3 consecutive days. On the fourth day, AD fentanyl (***A***: 0.03 mg/kg, s.c.) or morphine (***B***: 3 mg/kg, s.c.) was administered and the mechanical nociceptive threshold was evaluated 1 h later. In the groups of rats treated with $G\alpha_o$ AS-ODN, systemic AD fentanyl (***A***), and morphine (***B***) induced analgesia that was not different when compared with their respective $G\alpha_o$ SE-ODN-treated groups (***A***: $t_{(10)} = 0.13$, $p = 0.90$; ***B***: $t_{(10)} = 0.005$, $p = 0.99$, when the analgesia in the $G\alpha_o$ AS-ODN-treated and the SE-ODN-treated groups is compared at 1 h after systemic AD fentanyl and morphine, respectively; unpaired Student's *t* test). At the end of the fourth day, rats again received $G\alpha_o$ AS-ODN or SE-ODN. Five days after systemic AD fentanyl and morphine, when the mechanical nociceptive threshold was not different from the preopioids baselines (***C***: SE-ODN-treated group: $t_{(5)} = 2.03$; $p = 0.09$; AS-ODN-treated group that received AD fentanyl: $t_{(5)} = 1.26$; $p = 0.26$; ***D***: SE-ODN-treated group: $t_{(5)} = 1.1$, $p = 0.32$; AS-ODN-treated group that received AD morphine: $t_{(5)} = 1.52$, $p = 0.19$, when the mechanical nociceptive threshold is compared before and 5 d after systemic AD opioids; paired Student's *t* test), PGE_2 (100 ng/5 μl, i.d.) was injected and the mechanical nociceptive threshold was evaluated 30 min and 4 h later. Treatment with $G\alpha_o$ AS-ODN markedly attenuates PGE_2-induced prolonged hyperalgesia in rats that received AD fentanyl (***C***: $F_{(1,10)} = 23.6$, *** $p = 0.0007$, when the hyperalgesia in the $G\alpha_o$ AS-ODN-treated and the SE-ODN-treated groups is compared at the fourth hour after intradermal PGE_2; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). However, the prolongation of PGE_2-induced hyperalgesia was not affected by treatment with $G\alpha_o$ AS-ODN in the AD morphine-treated group (***D***: $F_{(1,10)} = 2.89$, $p = 0.12$, when hyperalgesia was compared between the $G\alpha_o$ SE-ODN-treated and AS-ODN-treated groups at the fourth hour after intradermal PGE_2; two-way repeated-measures ANOVA followed by Bonferroni's *post hoc* test). These findings indicate that analgesia produced by systemic AD fentanyl and morphine is not $G\alpha_o$ subunit dependent; however, the $G\alpha_o$ subunit does play a role in hyperalgesic priming induced by AD fentanyl, but not morphine ($n = 6$ paws/6 rats/group).

induced priming is attenuated by ODN antisense to Toll-like receptor 4 (TLR4) mRNA (unpublished data, Araldi D), while hyperalgesic priming induced by repeated AD fentanyl is attenuated by MOR antisense (Araldi et al., 2018a).

Analgesia and hyperalgesic priming produced by AD morphine

Finally, we investigated whether the analgesia and hyperalgesic priming induced by AD morphine (3 mg/kg, s.c.; Araldi et al., 2019) are also $G\alpha_{i/o}$ dependent. Rats received AS-ODN or SE-ODN to $G\alpha_i1$ (Fig. 5), $G\alpha_i2$ (Fig. 6), $G\alpha_i3$ (Fig. 7), or $G\alpha_o$ (Fig. 8) mRNA, intrathecally daily for 4 d. On the fourth day, AD morphine was administered systemically (3 mg/kg, s.c.), and the mechanical nociceptive threshold was measured 1 h later. AD morphine-induced analgesia was markedly reduced in the $G\alpha_i1$ AS-ODN-treated group, compared with its SE-ODN-treated group (Fig. 5*B*). On the other hand, intrathecal treatment with $G\alpha_i2$ (Fig. 6*B*), $G\alpha_i3$ (Fig. 7*B*), and $G\alpha_o$ (Fig. 8*B*) AS-ODNs did not affect morphine analgesia. Rats again received AS-ODN or SE-ODN at the end of the fourth day. Five days after AD morphine, PGE_2 (100 ng, i.d.) was injected, and the mechanical nociceptive threshold was evaluated 30 min and 4 h later. PGE_2-induced prolonged hyperalgesia was present in all four groups of AS-ODN-treated rats (Figs. 5*D*, 6*D*, 7*D*, and 8*D*). Our findings support the suggestion that, in marked contrast to the role of $G\alpha_i1$ and $G\alpha_i2$, which increased analgesia induced by AD fentanyl, AD morphine analgesia is reduced by $G\alpha_i1$ AS-ODN. Thus, the role of $G\alpha_i1$ in morphine-induced analgesia is opposite to its role in analgesia induced by AD fentanyl. Also, unexpectedly, different $G\alpha_{i/o}$ subunits mediate hyperalgesic priming induced by sub-AD and AD fentanyl, $G\alpha_i3$ versus $G\alpha_o$ dependence, respectively; and, priming induced by both sub-AD and AD morphine are $G\alpha_i1$, $G\alpha_i2$, $G\alpha_i3$, and $G\alpha_o$ independent.

Together, the experiments presented here support the following suggestions: (1) that OIH, opioid-induced hyperalgesia priming and analgesia induced by different doses of different opioid analgesics are mediated by different $G\alpha_{i/o}$ proteins; (2) that different $G\alpha_{i/o}$ subunits mediate OIH, opioid-induced hyperalgesic priming, and analgesia induced by fentanyl and morphine; (3) that OIH, opioid-induced hyperalgesic priming, and/or analgesia may be mediated by multiple $G\alpha_{i/o}$ proteins, differing for different doses and opioid analgesics; and, (4) that $G\alpha_{i/o}$ proteins may inhibit as well as mediate $G\alpha_{i/o}$–GPCR signaling.

Discussion

Although opioids remain the most effective treatment for many forms of moderate to severe pain, they produce several treatment-limiting side effects, including analgesic tolerance, addiction, OIH, induction of the transition from acute to chronic pain (modeled in the present experiments by opioid-induced hyperalgesic priming), respiratory depression, and constipation (Chu et al., 2008; Joseph et al., 2010; Lee et al., 2011; Trang et al., 2015; Roeckel et al., 2016; Araldi et al., 2015, 2017, 2018a, b, 2019; Khomula et al., 2019, 2021). Opioid analgesics, acting at MORs, produce their effects through the activation of G-proteins (Childers, 1988; Reisine and Bell, 1993; Uhl et al., 1994; Pasternak and Standifer, 1995; Standifer et al., 1996) and β-arrestins (Gainetdinov et al., 2004; Williams et al., 2013). While opioid agonists stimulate MOR phosphorylation, β-arrestin recruitment and receptor internalization, the signaling pathways involved can vary between MOR ligands. β-Arrestin-2-dependent signaling has been implicated in clinically important dose-limiting side effects of opioid analgesics (e.g., respiratory depression, constipation, and dependence), and biased MOR agonists that preferentially activate signaling via G-proteins over β-arrestins are currently being developed (DeWire et al., 2013; Manglik et al., 2016); and one biased MOR agonist, olinvyk (oliceridine), has recently been approved for use by the US Food and Drug Administration. While such biased opioids would be expected to extend the therapeutic window, increasing the safety of opioid analgesics (DeWire et al., 2013; Manglik et al., 2016), recent studies have raised the concern that they still produce substantial side effects (Kliewer et al., 2019; Ding et al., 2020; Kliewer et al., 2020), and we recently demonstrated that the biased MOR agonist PZM21 can induce OIH and hyperalgesic priming, mediated by a unique mechanism (Araldi et al., 2018c). Previous studies have suggested a potential role of $G\alpha_{i/o}$ proteins in diverse effects of opioids (Pasternak and Standifer, 1995; Rossi et al., 1995; Standifer et al., 1996; Hadjimarkou et al., 2002; Silva et al., 2002; Wainford and Kapusta, 2012). In the present experiments, we tested the hypothesis that two side effects of clinically used opioid analgesics, OIH and hyperalgesic priming, induced by systemically administered fentanyl and morphine, acting at the level of the spinal cord and dorsal root ganglion (DRG) neurons, are $G\alpha_{i/o}$ dependent.

While hyperalgesia induced by systemic sub-AD fentanyl was prevented by AS-ODN directed against three G-proteins, $G\alpha_i2$, $G\alpha_i3$, and $G\alpha_o$, antisense to only one of these G-proteins, $G\alpha_i3$, prevented hyperalgesic priming. In contrast, when sub-AD morphine was administered, a different set of $G\alpha_{i/o}$ proteins, $G\alpha_i1$, $G\alpha_i2$ and $G\alpha_i3$, mediated hyperalgesia, while hyperalgesic priming was not affected by AS-ODN to any of the tested α-subunits. Thus, while OIH produced by sub-AD fentanyl and morphine share $G\alpha_i2$ and $G\alpha_i3$ subunit dependence, $G\alpha_o$ plays a role in OIH produced only by sub-AD fentanyl, and $G\alpha_i1$ plays a role only in OIH produced by sub-AD morphine. In contrast, hyperalgesic priming produced by sub-AD fentanyl was prevented by $G\alpha_i3$ AS-ODN, while sub-AD morphine-induced priming was not affected by any of the G-protein subunit AS-ODNs. The basis of this differential contribution of G-proteins in OIH and hyperalgesic priming induced by different opioid analgesics remains to be elucidated.

We also investigated the role of $G\alpha_{i/o}$ subunits in analgesia and hyperalgesic priming induced by ADs of fentanyl and morphine. While analgesia induced by AD morphine was attenuated by $G\alpha_i1$ AS-ODN, paradoxically knockdown of $G\alpha_i1$ and $G\alpha_i2$ enhanced fentanyl analgesia. To the best of our knowledge, this is the first demonstration that G-protein α-subunits can have a negative impact on opioid-induced analgesia, and that the same $\alpha_{i/o}$ subunit can inhibit analgesia produced by one opioid analgesic (morphine) while enhancing it to another (fentanyl).

While AD fentanyl-induced priming was prevented in $G\alpha_o$ AS-ODN-treated rats, none of the $G\alpha$ subunits tested contributed to AD morphine-induced priming. While we do not currently have an explanation for the difference in the requirement of $G\alpha_i3$ versus $G\alpha_o$ for priming induced by sub-AD versus AD fentanyl, respectively, or for the lack of a role of any of the tested $G\alpha$ subunits in priming induced by sub-AD or AD morphine, it has been suggested that some effects of sub-AD doses of opioid analgesics may be mediated by their action at a different receptor, TLR4, on nociceptors (Araldi et al., 2019). The role of $G\alpha_{i/o}$ in TLR4 signaling is currently being examined.

Given the lack of effect of $G\alpha_{i/o}$ AS-ODNs on hyperalgesic priming induced by both sub-AD and AD morphine, it may be of interest to evaluate whether other $G\alpha$ proteins contribute, such as the closely related subunit $G\alpha_z$ that is thought to mediate the desensitization of MORs at supraspinal sites (Garzon et al.,

Table 1. $G\alpha_{i/o}$ subunits mediating analgesia, hyperalgesia, and priming induced by sub-ADs and ADs of systemic fentanyl and morphine

	Fentanyl		Morphine	
	Sub-AD	AD	Sub-AD	AD
OIH	↓ $G\alpha_i2$, $G\alpha_i3$ and $G\alpha_o$	NS	↓ $G\alpha_i1$, $G\alpha_i2$ and $G\alpha_i3$	NS
Analgesia	NS	↑ $G\alpha_i1$ and $G\alpha_i2$	NS	↓ $G\alpha_i1$
Priming	↓ $G\alpha_i3$	↓ $G\alpha_o$	None	None

NS, Not studied; ↑, enhances; ↓, attenuates.

2005). Otherwise, with respect to the differences between the $G\alpha$ subunits involved in hyperalgesia and analgesia induced by sub-AD and AD morphine, respectively, it has been suggested that the difference in the effect of sub-AD and AD morphine may be a result of a bimodal dose-dependent effect of opioid receptor agonists. Electrophysiology studies of the effects of opioids on mouse DRG neurons demonstrated that opioid receptors activate an excitatory signaling pathway when exposed to very low concentrations of opioid agonists, and an inhibitory pathway when exposed to higher concentrations (Shen and Crain, 1994). Alternatively, since none of the $G\alpha_{i/o}$ AS-ODNs prevented sub-AD morphine-induced priming, it is possible that it could be because of an effect of sub-AD morphine on nociceptor TLR4 (Araldi et al., 2019).

Adenylyl cyclase (AC; Sharma et al., 1977), as well as Ca^{2+} channels (Hescheler et al., 1987), G-protein-coupled inwardly rectifying K^+ channel (GIRK) activation (North et al., 1987; Henry et al., 1995), PLC stimulation (Spencer et al., 1997), and MAPK activation (Fukuda et al., 1996) are inhibited by MOR agonists. Low-dose morphine, administered intrathecally, can also couple to $G\alpha_s$ to activate protein kinase C and L-type Ca^{2+} channels to induce hyperalgesia (Esmaeili-Mahani et al., 2008); however, at higher opioid concentrations, $G\alpha_{i/o}$-coupled pathways are activated (Shen and Crain, 1990). In a recent study, we found that the application of fentanyl (0.5 nM) to DRG neurons cultured from rats pretreated with AD fentanyl induced a MOR-dependent increase in $[Ca^{2+}]_i$ and significantly decreased action potential rheobase in weakly $IB4^+$ and $IB4^-$ small-diameter DRG neurons (Khomula et al., 2019). Since MOR can also couple to $G\alpha_o$, which links to attenuation of action potential duration (Groer et al., 2007; McPherson et al., 2010), these data corroborate our findings that hyperalgesic priming induced by systemic AD fentanyl is $G\alpha_o$ dependent.

While widely accepted, the classical model of G-protein signaling, cyclical GDP–GTP exchange in response to ligand binding to seven transmembrane receptors followed by dissociation of the G-protein subunit and activation of intracellular signaling cascades, has been challenged. For example, studies have shown that $G\alpha_i2$ and $G\alpha_i3$ can display a complex interplay in GPCR signaling. $G\alpha_i2$ and $G\alpha_i3$ can be activated simultaneously by a single ligand, resulting in adenylyl cyclase-mediated effects that are dependent only on $G\alpha_i2$ (McClue et al., 1992), and $G\alpha_i3$ can have a regulatory role, its presence inhibiting $G\alpha_i2$-induced migration and GTPγS incorporation by hindering $G\alpha_i2$ binding to the receptor (Thompson et al., 2007). These studies provide evidence to support the existence of a regulatory role for $G\alpha_i$, independent of its effect on AC, and an interplay of $G\alpha_i$ proteins in transmitting G-protein-coupled receptor signals (Thompson et al., 2007). In a study of the role of T cells in "graft-versus-host" (GVH) disease, the GVH response is abolished in mice adoptively transferred with $G\alpha_i2^{-/-}$ T cells but exacerbated in mice with $G\alpha_i3$-deficient T cells (Jin et al., 2008). Many GPCRs are able to couple to multiple G-proteins, and different GPCR agonists can lead to signaling via different G-proteins, a phenomenon referred to as "agonist-directed trafficking" (Kenakin, 1995). For example, the dopamine D_2 receptor can initiate signals via $G\alpha_i1$, $G\alpha_i2$, $G\alpha_i3$, and $G\alpha_o1$ (Gazi et al., 2003); however, ligand pharmacology can be influenced greatly by the GPCR/G-protein expression ratio (Lane et al., 2007). The D_2 receptor ligand *S*-(—)−3-PPP shows partial agonism mediated by $G\alpha_o1$ and antagonism/inverse agonism mediated by $G\alpha_i1$, $G\alpha_i2$, and $G\alpha_i3$ in physiologically relevant end points (Arnt et al., 1983; Hjorth et al., 1983). Thus, an emerging signaling paradigm includes the capacity of one receptor to couple to and initiate signaling through multiple G-proteins and the capacity of one G-protein to activate multiple effectors (Woehler and Ponimaskin, 2009). Limitations of the use of AS-ODN to regulate protein levels in nociceptors include the following: (1) they do not completely eliminate their target protein; (2) their intrathecal administration affects cells in the spinal cord, so that additional approaches are required to specify the cells involved; and (3) their short half-life requires repeated administration.

In conclusion, the present data support the hypothesis that $G\alpha_i/G\alpha_o$ signaling contributes to the side effects of clinically used opioid analgesics, providing a starting point for the design of analgesics with selectivity for individual $G\alpha_i/\alpha_o$ proteins, producing a more limited range of side effects. Our current findings are summarized in Table 1.

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