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Research report

Ligand-biased activation of extracellular signal-regulated kinase 1/2 leads to differences in opioid induced antinociception and tolerance

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HIGHLIGHTS

- Opioid induced antinociception is regulated by G protein dependent and independent signaling.
- Extracellular signal-regulated kinase 1/2 is activated in a ligand-biased manner within the periaqueductal gray.
- Antinociceptive tolerance to DAMGO, but not fentanyl, is attenuated by inhibition of ERK1/2.

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ABSTRACT

Opioids produce antinociception by activation of G protein signaling linked to the mu-opioid receptor (MOPr). However, opioid binding to the MOPr also activates β-arrestin signaling. Opioids such as DAMGO and fentanyl differ in their relative efficacy for activation of these signaling cascades, but the behavioral consequences of this differential signaling are not known. The purpose of this study was to evaluate the behavioral significance of G protein and internalization dependent signaling within ventrolateral periaqueductal gray (vlPAG). Antinociception induced by microinjecting DAMGO into the vlPAG was attenuated by blocking $G\alpha_{i/o}$ protein signaling with administration of pertussis toxin (PTX), preventing internalization with administration of dynamin dominant-negative inhibitory peptide (dyn-DN) or direct inhibition of ERK1/2 with administration of the MEK inhibitor, U0126. In contrast, the antinociceptive effect of microinjecting fentanyl into the vlPAG was not altered by administration of PTX or U0126, and was enhanced by administration of dyn-DN. Microinjection of DAMGO, but not fentanyl, into the vlPAG induced phosphorylation of ERK1/2, which was blocked by inhibiting receptor internalization with administration of dyn-DN, but not by inhibition of $G\alpha_{i/o}$ proteins. ERK1/2 inhibition also prevented the development and expression of tolerance to repeated DAMGO microinjections, but had no effect on fentanyl tolerance. These data reveal that ERK1/2 activation following MOPr internalization contributes to the antinociceptive effect of some (*e.g.*, DAMGO), but not all opioids (*e.g.*, fentanyl) despite the known similarities for these agonists to induce β-arrestin recruitment and internalization.

1. Introduction

Mu opioid receptor (MOPr) agonists activate and inhibit a number of different intracellular signaling pathways. G protein signaling and the subsequent inhibition of downstream effectors, such as adenylyl cyclase, has been the most thoroughly characterized. In contrast much less is known about β-arrestin signaling following opioid binding. MOPr phosphorylation terminates G protein signaling and recruits β-arrestin to the receptor. β-arrestin binding leads to receptor internalization and activation of a distinct group of signaling proteins such as extracellular signal-regulated

Abbreviations: DAMGO, [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin; dyn-DN, dominant negative dynamin inhibitory peptide; ERK1/2, extracellular signal-regulated kinase 1 and 2; PTX, pertussis toxin; scr-dyn, scrambled control peptide; vlPAG, ventrolateral periaqueductal gray.

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kinase (ERK1/2), which is well characterized in adrenergic receptors compared to opioid receptors [24,48,14]. Recent studies have shown that some MOPr agonists such as fentanyl and [D-Ala^2, N-MePhe^4, Gly-ol]-enkephalin (DAMGO) have high efficacy to recruit β-arrestin and activate of G proteins, whereas other opioids such as morphine are biased toward G protein signaling [32,36,23,50].

Ligands with high efficacy for receptor internalization correlate inversely with susceptibility to tolerance [29] suggesting that β-arrestin signaling contributes to antinociception by preventing the development of tolerance [17]. Morphine produces limited β-arrestin recruitment and MOPr internalization compared to other opioids such as fentanyl or DAMGO [55,10,56], but maximal tolerance [19]. Although tolerance is observed following administration of morphine, fentanyl, or DAMGO, the signaling proteins underlying tolerance appear to vary. Blockade of G protein associated signaling proteins (c-Jun N-terminal kinase or protein kinase C) prevents tolerance to morphine, but not DAMGO or fentanyl. Conversely, blockade of internalization-dependent signaling pathway prevents tolerance to fentanyl and DAMGO, but not morphine [22,33,39].

Microinjection of morphine, fentanyl, or DAMGO into the ventrolateral periaqueductal gray (vlPAG) produces antinociception, and repeated administration leads to the development of tolerance [38,34,4]. Although G protein signaling is known to contribute to the antinociceptive effect of opioids, the objective of the present study was to determine whether G protein independent (*i.e.,* β-arrestin and ERK1/2) signaling following administration of these different opioids also contributes to antinociception. Despite minimal activation of ERK1/2 *in vitro* or *in vivo* following acute morphine administration, inhibition of ERK1/2 has been shown to prevent or enhance the development of morphine tolerance depending on the site of administration [25,53,26]. Given that DAMGO and fentanyl activate ERK1/2 *in vitro*, we hypothesized that ERK1/2 is activated following DAMGO and fentanyl administration into the vlPAG, and inhibition of this signaling pathway prevents the development of tolerance.

2. Materials and methods

2.1. Subjects

Male Sprague-Dawley rats ($n = 220$) weighing 220–360 g from Harlan Laboratories (Livermore, CA) were used. Rats were anesthetized with pentobarbital (60 mg/kg, i.p.) and implanted with a guide cannula (23 gauge; 9 mm long) aimed at the vlPAG using stereotaxic techniques (AP: +1.7 mm, ML: −0.6 mm, DV: −4.6 mm from lambda). Two screws were used to anchor the cannula to the skull with dental cement. A 9 mm stylet was inserted into the guide cannula following surgery. Rats were handled daily and allowed to recover for 1 week before testing. Rats were housed in groups of 2–5 until surgery and were housed individually on a reverse light cycle (lights off at 7:00 AM) after surgery. Food and water were available at all times except during experimental testing. All procedures were approved by the Washington State University Animal Care and Use Committee and conducted in accordance with the guidelines for animal use described by the International Association for the Study of Pain.

2.2. Behavioral testing

Drugs were administered through a 31-gauge injection cannula extending 2 mm beyond the guide cannula. One day prior to testing, the injector was inserted into the guide cannula without drug administration to habituate the rat to the microinjection procedure. To assess the role of $G\alpha_{i/o}$ protein signaling, receptor internalization-related signaling, or ERK1/2 activation, different groups of rats were microinjected into the vlPAG with G protein inhibitor pertussis toxin (PTX; 5 or 50 ng/0.4 μL), a myristoylated dominant negative dynamin inhibitory peptide (dyn-DN; 2 μg/0.4 μL) to block formation of the endosome, or a MEK1/2 inhibitor (U0126; 100 ng/0.5 μL) prior to administration of DAMGO or fentanyl. PTX or saline was administered one day prior to opioid administration, whereas U0126, 20% DMSO vehicle, dyn-DN, or the scrambled control peptide (dyn-scr, 2 μg/0.4 μL) were injected 20 min prior to opioid administration based on previous studies showing peak effects with microinjections into the vlPAG [6,25,27]. In preliminary studies 24 h pretreatment of 5 ng/0.5 μL was sufficient to attenuate morphine induced antinociception within the vlPAG ($F_{(1,92)} = 3.95$, $p < 0.05$). Dyn-DN was injected into the vlPAG to disrupt MOPr internalization as we have reported previously using the fluorescent opioid peptide, dermorphin-A594 [27]. In addition, a higher dose was needed to assure all internalization was blocked and preliminary data showed that dyn-DN (2 μg/0.4 μL) did not alter morphine antinociception ($F_{(1,98)} = 1.88$, $p = 0.17$).

A cumulative dosing procedure was used to assess the antinociceptive effects of DAMGO and fentanyl. Increasing doses of DAMGO were administered every 12 min resulting in third log doses of 0.046, 0.1, 0.22, 0.46, and 1 μg/0.4 μL. Nociception was assessed with the hot plate test 10 min after each injection. Fentanyl has a fast time course of action so was microinjected every 4 min resulting in third log doses of 0.46, 1, 2.2, 4.6, and 10 μg/0.4 μL. The hot plate test was conducted 2 min after each injection. Our previous data show clear dose-dependent antinociception using this procedure [5].

Tolerance was induced in a separate group of rats by twice daily microinjections of DAMGO (0.5 μg/0.4 μL) or fentanyl (3 μg/0.4 μL) for two consecutive days. On Trial 1, the hot plate test was conducted 20 min after the DAMGO microinjection and 3 min after the fentanyl microinjection. To evaluate the role of ERK1/2 on the development of tolerance, a subset of rats received U0126 or vehicle (20% DMSO in saline, 0.5 μL) 20 min prior to each opioid injection on Trials 1–4. Tolerance was assessed on Trial 5 using the cumulative dosing procedure described above. To evaluate the expression of tolerance, a subset of rats received U0126 or vehicle 20 min prior to the cumulative dosing procedure in rats previously treated with twice daily microinjections of DAMGO or fentanyl for two days. We have shown previously that tolerance develops to vlPAG microinjections of DAMGO or fentanyl using this procedure [34,4].

2.3. Histology and data analysis

Following testing, rats received a lethal dose of halothane. Brains were removed and stored in formalin (10%) and sliced coronally (100 μm) at least 2 days later to determine the injection site [43]. Only those injection sites in or adjacent to the vlPAG were included in data analysis. Dose–response curves were plotted and the half maximal antinociceptive effect (D_{50}) was calculated for each group using GraphPad (Prism 6). A unique control group was tested alongside the experimental groups for each experiment to control for variability between experiments. All comparisons were made with the control group within each experiment. Significance ($\alpha < 0.05$) was determined using ANOVA or *t*-test where appropriate. Bonferroni *post-hoc* analyses were used to compare means when necessary. Data are presented as mean ± SEM unless otherwise stated. To assess the homogeneity of variance the Brown-Forsythe test was used.

2.4. Drugs

All drugs were purchased from Tocris Bioscience except fentanyl citrate and U0126 (Sigma–Aldrich). DAMGO, fentanyl citrate, PTX,

dyn-DN, and scr-dyn, were dissolved in sterile saline. U0126 was dissolved in 20% DMSO.

2.5. ERK1/2 immunohistochemistry

A separate group of rats were deeply anesthetized with pentobarbital (150 mg/kg, i.p.) 20 min after opioid injection and then perfused transcardially through the ascending aorta with 10 mL heparinized saline followed by 400–600 mL of 4% paraformaldehyde in 0.1 M phosphate buffer (PB). Brains were postfixed in 4% paraformaldehyde for 30 min and then stored in 0.1 M PB for up to 15 h. Immunohistochemistry was performed on coronal brain slices (40 μm) containing the vlPAG. Sections were incubated in 1% sodium borohydride in 0.1 M PB for 30 min followed by another 30 min incubation with 0.3% H_2O_2 in 0.1 M PB. The blocking reagent was then used: 0.5% bovine serum albumin in 0.1 M Tris buffered saline for 30 min. The tissue was incubated in primary rabbit antibody against phospho-p44/42 MAPK (ERK 1/2) (1:400; Cell signaling, Beverly, MA) in 0.1 M Tris buffered saline containing 0.1% bovine serum albumin and 0.25% TritonX-100, for 42 h at 4 °C. Bound ERK1/2 antibody was visualized with a diaminobenzidine hydrogen peroxidase (DAB–H_2O_2) reaction. Tissue was incubated in biotinylated goat-anti rabbit IgG secondary antibody (1:400 Vector Laboratories, Burlingame, CA) for 30 min. This was followed by incubation in Avidin-Biotin (Elite Vectastain ABC kit; Vector Laboratories) for 30 min and then DAB-H_2O_2 for 3 min. Brain slices were mounted, dehydrated and then coverslipped with DPX mounting medium (Sigma–Aldrich, St. Louis, MO). Sections containing the injection site were quantified within a 300 × 300 μm^2 region. Depending on the location of the injection, up to 3 separate boxes were used to equal 90,000 μm^2 area. To avoid damaged tissue, a region 50 μm away from injection site was chosen. Images were taken with an Olympus DP71 digital camera mounted on an Olympus BX51 microscope. The number of pERK-positive cells was assessed using ImageJ particle analysis to count cell bodies that were larger than 60 $pixels^2$ (National Institutes of Health; Bethesda, MA).

3. Results

3.1. Inhibition of G proteins or internalization differentially alters DAMGO and fentanyl-induced antinociception

To investigate the mechanism of DAMGO and fentanyl induced antinociception, rats were pretreated with either G protein or internalization inhibitors prior to opioid microinjection into the vlPAG. There was no significant difference in baseline hot plate latencies prior to PTX or dyn-DN pretreatment ($F_{(4,72)} = 0.24$; $p = 0.91$) or between opioids ($F_{(1,72)} = 0.48$; $p = 0.49$). Mean baseline hot plate latencies for these groups ranged from 14.8 ± 0.9 to 18.4 ± 1.4 s. Treatment with 5 or 50 ng PTX (15.8 ± 2.5 s, 13.37 ± 0.2 s, respectively) did not alter nociception compared to saline controls (15.7 ± 0.9 s; $F_{(2,49)} = 0.54$; $p = 0.59$). Similarly hot plate latencies following dyn-DN (14.9 ± 1.0 s) did not differ from scr-dyn controls (17.3 ± 3.1 s; ($t_{(31)} = 1.07$; $p = 0.29$).

Administration of PTX significantly attenuated the antinociceptive effect of DAMGO, but not fentanyl. PTX caused a rightward shift in the DAMGO dose–response curve (Fig. 1A; $F_{(2,150)} = 9.09$; $p < 0.05$), but only the high dose (50 ng) produced a statistically significant difference from saline pretreated rats. Pretreatment with PTX caused a small non-significant rightward shift in the fentanyl dose–response curve (Fig. 1B; $F_{(2,138)} = 2.15$; $p = 0.12$).

Pretreatment with dyn-DN also had opposite effects on DAMGO and fentanyl-induced antinociception. Administration of dyn-DN reduced DAMGO potency as evident by a rightward shift in the dose–response curve (Fig. 2A; $F_{(1,85)} = 6.52$; $p < 0.05$). In contrast, administration of dyn-DN enhanced fentanyl antinociception as evident by a leftward shift in the dose–response curve (Fig. 2B; $F_{(1,98)} = 13.48$; $p < 0.05$).

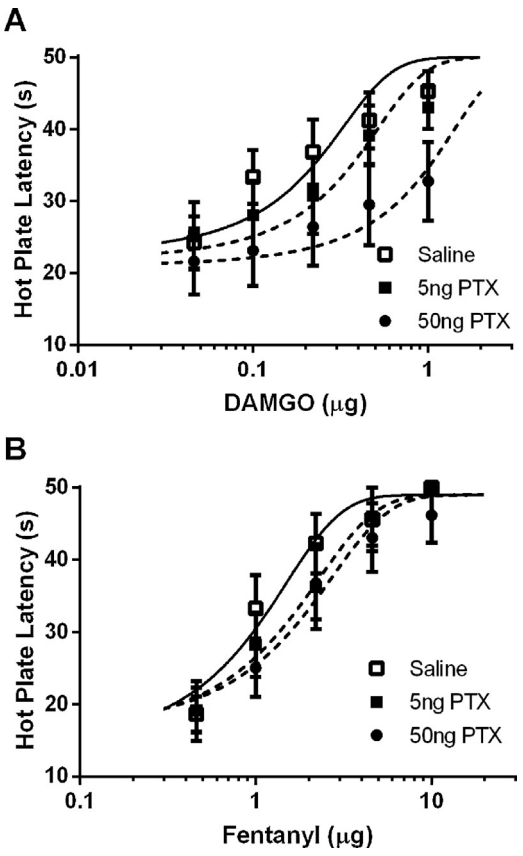

Fig. 1. Differential alteration in antinociception following G protein inhibition. Pretreatment with PTX for 24 h alters antinociception following vlPAG microinjections of DAMGO (A), but not fentanyl (B). Following pretreatment with 50 ng PTX, the DAMGO D_{50} (0.75 ± 0.45 μg; $n = 8$) was significantly different from saline treated rats (0.15 ± 0.07 μg; $n = 10$), however 5 ng PTX (0.26 ± 0.12 μg; $n = 8$) was not statistically different than saline controls. Pretreatment with PTX caused a slight rightward shift in the fentanyl D_{50} values following 5 ng (1.62 ± 0.53 μg; $n = 8$) and 50 ng (1.81 ± 0.67 μg; $n = 8$) of PTX compared to saline treated rats (1.10 ± 0.32 μg; $n = 8$), but it did not reach statistical significance.

3.2. DAMGO, but not fentanyl, activates ERK1/2 in a dynamin dependent manner

In vitro studies have shown that ERK1/2 can be activated following opioid exposure via G protein or β-arrestin signaling depending on the opioid [2,28,59]. To assess whether opioids induce ERK1/2 phosphorylation *in vivo*, DAMGO or fentanyl were microinjected into the vlPAG followed by immunohistochemical analysis of pERK1/2. DAMGO ($n = 3$) caused an increase in pERK1/2 immunoreactivity compared to saline (Fig. 3A–D: $F_{(3,11)} = 4.62$; $p < 0.05$; $n = 7$). Microinjection of PTX ($n = 2$) prior to DAMGO into the vlPAG also caused an increase in pERK positive cells compared to saline treated rats (Bonferroni, $p < 0.05$). In contrast, rats injected with dyn-DN ($n = 3$) 20 min prior to DAMGO showed a similar number of pERK1/2 positive cells as saline treated rats (Bonferroni, n.s.). The variance in each drug treated group was similar ($F_{(3,11)} = 0.81$, $p = 0.51$).

Microinjection of fentanyl ($n = 4$) into the vlPAG produced a slight increase in the number of pERK1/2 positive cells compared to saline controls ($n = 7$), but this increase failed to reach significance, and this lack of an effect was not altered by pretreatment

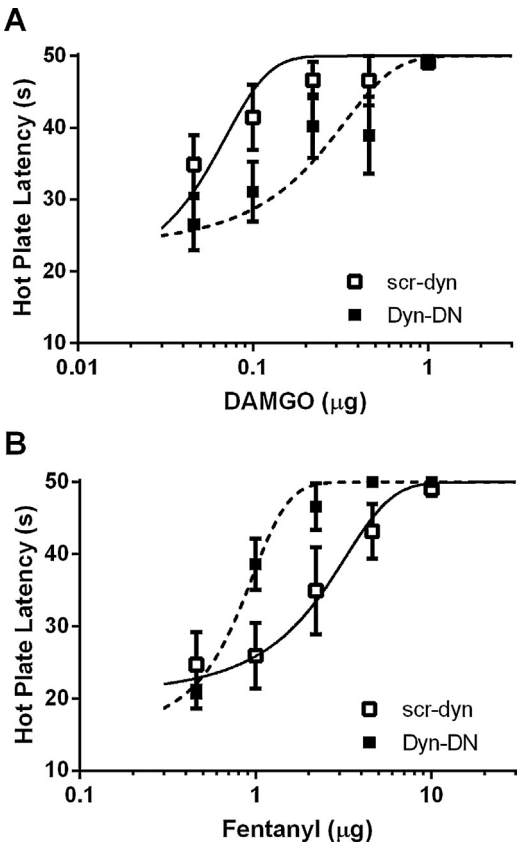

Fig. 2. Ligand-biased effects on antinociception following inhibition of internalization. Pretreatment with dyn-DN (2 μg/0.5 μL) 20 min prior to opioid dose–response decreased DAMGO-induced antinociception (A), and enhanced fentanyl-induced antinociception (B) compared to scr-dyn (2 μg/0.5 μL). Pretreatment with dyn-DN shifted the DAMGO D_{50} to 0.14 ± 0.07 μg (n = 7) compared to pretreatment with scr-dyn (0.046 ± 0.02 μg; n = 8). Conversely, pretreatment with dyn-DN caused a leftward shift in the fentanyl D_{50} (0.77 ± 0.12 μg; n = 8) compared to saline controls (1.81 ± 0.73 μg; n = 10).

with PTX or dyn-DN ($F_{(3,14)}$ = 1.24; p = 0.33, n = 3–4). The variance in each drug treated was similar ($F_{(3,14)}$ = 1.22, p = 0.34).

3.3. ERK1/2 inhibition attenuates DAMGO antinociception and tolerance

The finding above that DAMGO activation of ERK1/2 is prevented by administration of dyn-DN indicates that DAMGO activates ERK1/2 as a result of MOPr internalization. Moreover, these data raise the possibility that ERK1/2 activation also contributes to DAMGO antinociception and tolerance. This hypothesis was tested by microinjecting the ERK1/2 inhibitor U0126 (100 ng/0.5 μL) into the vlPAG. Administration of U0126 in the absence of an opioid had no effect on nociception compared to vehicle controls ($t_{(45)}$ = 0.97; p = 0.34), but attenuated the antinociceptive effect of DAMGO as indicated by a rightward shift in the DAMGO dose–response curve (Fig. 4A; $F_{(1,98)}$ = 34.10; p < 0.05). In contrast, administration of U0126 had no effect on the fentanyl dose–response curve (Fig. 4B; $F_{(1,128)}$ = 0.044; p = 0.834).

To assess the role of ERK1/2 on the expression of tolerance, U0126 was administered 20 min prior to administration of cumulative doses of DAMGO or fentanyl in rats tolerant to vlPAG microinjections of either opioid. Repeated DAMGO microinjections caused a rightward shift in the DAMGO dose–response curve compared to saline-treated controls. Tolerance to DAMGO was reversed by administration of U0126 prior to testing on Trial 5 (Fig. 5A; $F_{(2,138)}$ = 13.19; p < 0.05). Repeated microinjections of fentanyl also caused a rightward shift in the fentanyl dose response curve (*e.g.*, tolerance) compared to saline treated controls, but microinjection of U0126 prior to fentanyl administration on Trial 5 had no effect on this shift (Fig. 5B; $F_{(2,174)}$ = 8.03; p < 0.05).

Furthermore, pretreatment with U0126 20 min prior to each DAMGO injection during tolerance induction prevented the development of tolerance to DAMGO (Fig. 6A; $F_{(3,214)}$ = 10.41; p < 0.05). Similar to what was found with a single injection of U0126, four injections of U0126 caused a rightward shift in the DAMGO dose–response curve in DAMGO-naïve rats injected

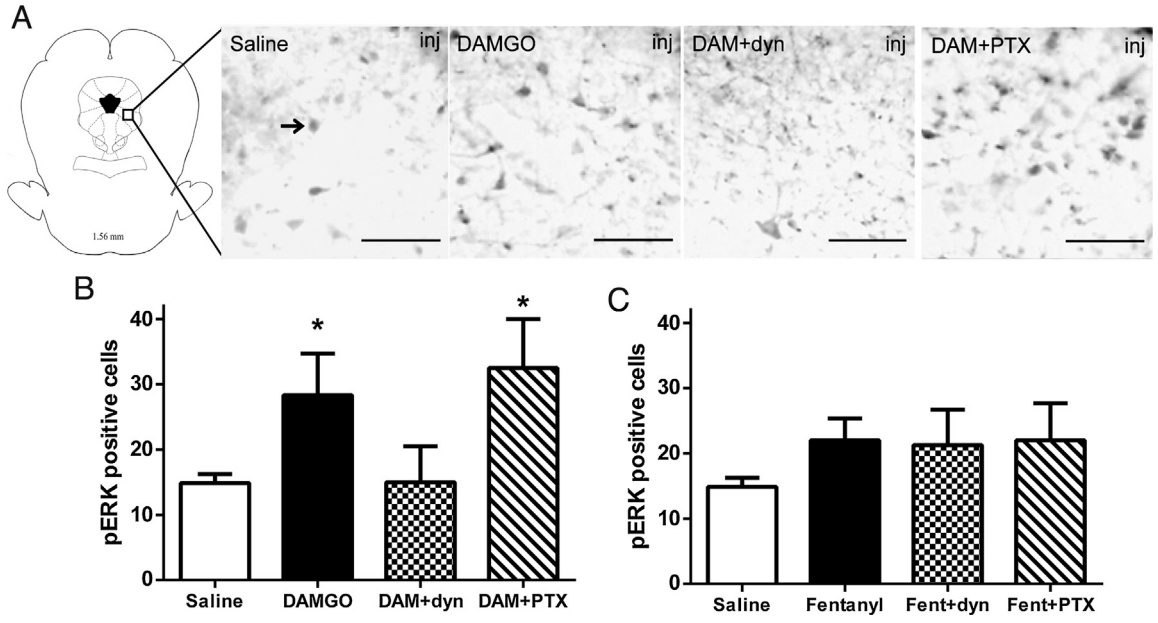

Fig. 3. ERK1/2 activation in vlPAG following opioid microinjections. Representative photomicrographs (A) of pERK1/2 immunoreactivity in vlPAG following pretreatment of saline, DAMGO, DAMGO + dyn-DN and DAMGO + PTX. Arrow designates a typical pERK1/2 positive cell. Scale bar = 100 μm. Quantification of pERK1/2 immunoreactivity 20 min following microinjection 0.5 μg/0.4 μL DAMGO (B), and 3 μg/0.4 μL fentanyl (C) into the vlPAG. A subset of rats were pretreated with dyn-DN (2 μg/0.5 μL) 20 min prior or PTX (50 ng/0.5 μL) 24 h prior to opioid pretreatment. DAMGO, but not fentanyl, caused a significant increase in pERK1/2, which was prevented by pretreatment with dyn-DN. *—Statistically different from saline.

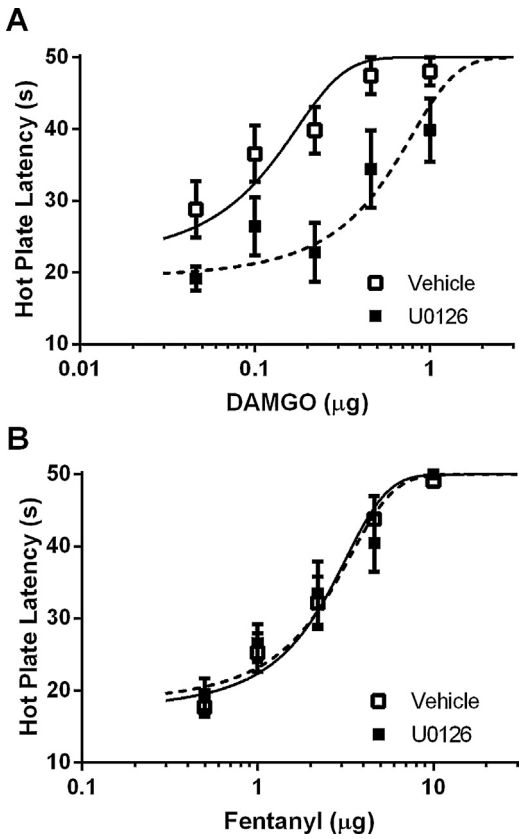

Fig. 4. ERK1/2 inhibition decreases DAMGO-induced antinociception. Rats were pretreated with 0.4 μL saline twice daily for two days. On Day 3, rats received an injection of vehicle (20% DMSO) or U0126 (100 ng/0.5 μL) 20 min prior to DAMGO (A) or fentanyl (B) dose–response. ERK inhibition caused a decrease in DAMGO-induced antinociception, but had no effect on fentanyl-induced antinociception.

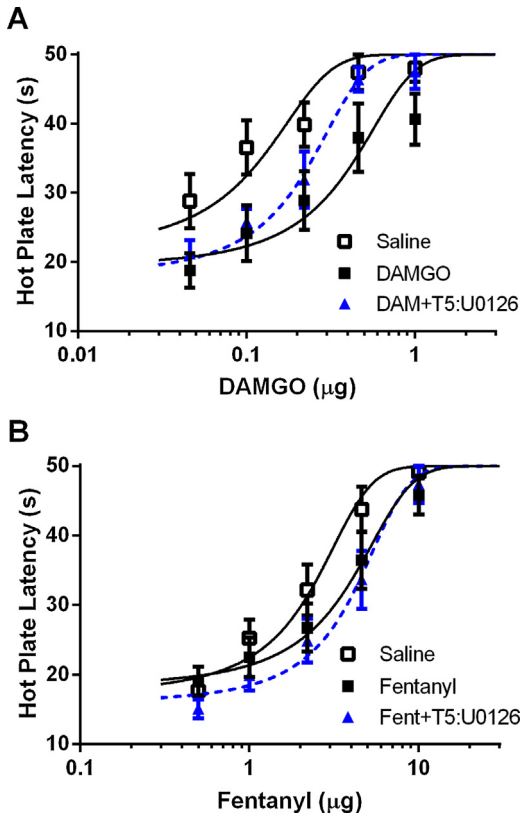

Fig. 5. ERK1/2 inhibition reverses the expression of DAMGO tolerance but does not alter fentanyl tolerance. Rats were pretreated with 0.4 μL saline, 0.5 μg/0.4 μL DAMGO, or 3 μg/0.4 μL fentanyl twice daily for two days. On Day 3, rats received an injection of vehicle (20% DMSO) or U0126 (100 ng/0.5 μL) 20 min prior to DAMGO (A) or fentanyl (B) dose–response. The expression of DAMGO, but not fentanyl, tolerance was not reversed by ERK1/2 inhibition.

repeatedly with saline as a control. The rightward shift in the fentanyl dose–response curve that occurred with repeated fentanyl microinjections (Fig. 6B; $F_{(3,226)} = 4.86$; $p < 0.05$) was attenuated by administration of U0126 prior to each injection, but this shift in the dose response curve did not reach statistical significance. D_{50} values are shown in Table 1.

4. Discussion

The current study found ligand-biased differences in opioid signaling underlying antinociception and tolerance mediated by the vlPAG. The antinociceptive effect of microinjecting DAMGO into the vlPAG was attenuated by blockade of G proteins, MOPr internalization, and ERK1/2 signaling. Blocking dynamin also blocked DAMGO-induced ERK1/2 activation suggesting that ERK1/2 signaling occurs following MOPr internalization. Furthermore, inhibition of ERK1/2 was associated with a reduction in both the expression and development of tolerance to DAMGO. In contrast, these molecular signaling pathways do not appear to contribute to fentanyl antinociception or tolerance. The antinociceptive effect of fentanyl in the vlPAG was enhanced by blocking MOPr internalization and was not dependent on G protein or ERK1/2 signaling. Likewise, ERK1/2 signaling does not appear to contribute to either the development or expression of fentanyl tolerance.

Table 1
Comparison of D_{50} values following ERK1/2 inhibition.

Pretreatment	DAMGO $D_{50} \pm$ C.I. (n)	Fentanyl $D_{50} \pm$ C.I. (n)
Saline	0.087 ± 0.033 (9)	2.16 ± 0.44 (11)
Opioid	0.340 ± 0.125 (8)*	3.42 ± 0.90 (10)*
U0126 + saline	0.530 ± 0.252 (10)*	1.79 ± 0.48 (8)
U0126 + opioid	0.152 ± 0.064 (10)**	2.62 ± 0.52 (10)
Saline + T5 U0126	0.482 ± 0.178 (8)*	2.24 ± 0.58 (11)
Opioid + T5 U0126	0.196 ± 0.044 (7)**	3.96 ± 0.82 (9)*

* Statistically different from saline ($p < 0.05$).
** Statistically different from opioid ($p < 0.05$).

The antinociceptive effect of DAMGO in the vlPAG was attenuated by administration of 50, but not 5 ng of PTX. In contrast, PTX had no significant effect on fentanyl antinociception regardless of dose. A previous study found that 5 ng of PTX was sufficient to attenuate morphine antinociception [6]. Taken together these results within the vlPAG are consistent with previous studies evaluating the role of $G\alpha_{i/o}$ proteins on antinociception using intracerebroventricular administration. Blockade of $G\alpha_{i/o}$ proteins with PTX or antisense oligodeoxynucleotides produced a ligand biased attenuation of antinociception in the order of morphine > DAMGO > sufentanil (a fentanyl analog) [44,18], which inversely correlated with agonist efficacy to induce antinociception as measured with irreversible antagonists or using a [^{35}S]GTPγS assay [35,1,51,18,32,30]. It is possible that fentanyl-induced antinociception is differentially mediated through a different MOPr splice variant, Gα protein subtype, or even heterodimers (*i.e.,* MOPr/DOPr). Fentanyl analogs have been shown to produce antinociception via $G\alpha_s$ proteins [18,46]. In addition fentanyl, but not morphine, antinociception is blocked following deletion of a particular exon on the MOPr, suggesting that certain agonists preferentially activate certain receptor variants [41,42,57]. It is unknown what variants are present in the vlPAG. It is also possible that certain agonists activate heterodimers (such as MOPr/DOPr) that preferentially signal via ERK1/2 [45,12].

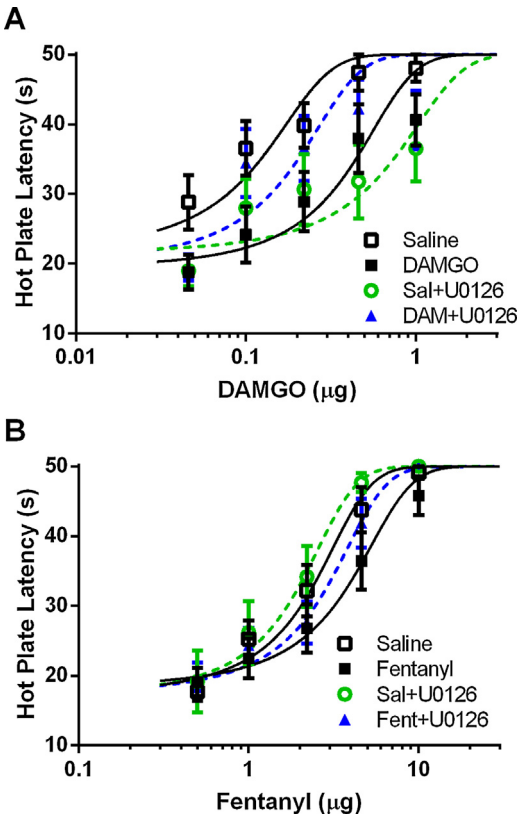

Fig. 6. ERK1/2 inhibition prevents the development of tolerance to DAMGO, but not fentanyl. Rats were pretreated with twice daily microinjections of saline (0.4 μL), DAMGO (0.5 μg/0.4 μL) or fentanyl (3 μg/0.4 μL). A subset of rats received a microinjection of U0126 20 min prior to each DAMGO or fentanyl microinjection. On Day 3, all rats recieved cumulative doses of DAMGO (A) or fentanyl (B). ERK1/2 inhibition in combination with DAMGO prevented the development of tolerance to DAMGO, but had no effect on the development of fentanyl tolerance.

It is well established that certain agonists cause robust MOPr phosphorylation and internalization. In particular, morphine is very weak at inducing MOPr internalization compared to other agonists such as DAMGO and fentanyl [54,7,9,32,33]. Given that both DAMGO and fentanyl produce rapid MOPr internalization, it is surprising that administration of dyn-DN, a GTPase that prevents the formation of endosomes [21], would have opposite effects. One explanation is that fentanyl is similar to morphine, an opioid that does not induce MOPr internalization, when microinjected into the vlPAG. The antinociceptive efficacy of fentanyl is similar to morphine when microinjected into the vlPAG [4], whereas its efficacy is much greater than morphine when administered systemically [29]. We have shown previously that dyn-DN has no effect on morphine antinociception [26], whereas in the present study administration of dyn-DN potentiated fentanyl antinociception. Fentanyl produces a rapid (3 min) and short-lived (<30 min) peak effect in comparison to morphine, DAMGO, and dermorphin, which show peak antinociception at 15–30 min and persists for more than one hour following vlPAG administration [5,27]. Blocking MOPr internalization may potentiate fentanyl antinociception by prolonging signaling from the plasma membrane. In contrast, administration of dyn-DN to block MOPr internalization had the opposite effect on DAMGO antinociception. DAMGO-induced antinociception was attenuated by inhibiting dynamin suggesting that MOPr signaling following internalization contributes to antinociception. Although this would indicate a novel signaling mechanism for the MOPr, microinjection of dyn-DN into the vlPAG also attenuated the antinociceptive effect of injecting the high efficacy MOPr agonist dermorphin [27]. Internalization of the MOPr could contribute to antinociception via β-arrestin signaling from the endosome or by rapid recycling of the receptor to the plasma membrane for additional signaling. Our findings that DAMGO activates ERK1/2 signaling in a dynamin dependent manner and inhibition of ERK1/2 activation attenuates DAMGO antinociception and tolerance indicates that MOPr signaling occurring after internalization contributes to antinociception.

Previous research has shown that ERK1/2 is activated following acute administration of fentanyl or DAMGO, but not morphine [28,58,15,26]. Our current data show that fentanyl in the vlPAG caused a small increase in ERK1/2 activation, but this increase was less than that produced by DAMGO and was not attenuated by PTX or dyn-DN administration. It is possible that fentanyl does not activate ERK1/2 in the vlPAG even though fentanyl has this effect in heterologous cell systems and cultured striatal neurons [28,59]. Morphine has been shown to induce ERK1/2 activation in a brain-region specific manner: an increase in ERK1/2 activation has been reported in the anterior cingulate and locus coeruleus, whereas a decrease occurs in the nucleus accumbens and central amygdala [16]. Prolonged, but not acute, morphine treatment showed an increase in pERK1/2 within the vlPAG, but a decrease in a heterologous cell system [3,25,26].

Although ERK1/2 is typically considered to be activated in a β-arrestin dependent manner, some agonists such as morphine may activate ERK1/2 via a different signaling mechanism. For example, morphine activates ERK1/2 in a PKC and/or calmodulin dependent mechanism, whereas DAMGO and fentanyl use a dynamin or β-arrestin dependent pathway [2,58,15]. The current study using PTX and dyn-DN confirmed that DAMGO activates ERK1/2 via a dynamin dependent mechanism within the vlPAG.

Studies investigating the role of ERK1/2 on morphine tolerance have found mixed results depending on injection site. Co-administration of intrathecal morphine with a MEK inhibitor attenuated the development of tolerance to morphine [53], whereas morphine tolerance was not altered by ERK1/2 inhibition following systemic administration [40]. Inhibition of ERK1/2 activation enhances both the expression and development of tolerance to morphine within the vlPAG [25] revealing that activation of ERK1/2 may counteract tolerance. Taken together with the present study, ERK has distinct effects on tolerance within the vlPAG depending on the opioid injected: ERK1/2 activation counteracts (morphine), enhances (DAMGO), or has no effect (fentanyl) on tolerance to MOPr agonists. The previous finding that acute tolerance to DAMGO, but not morphine or fentanyl, was prevented by GRK inhibition [22] is consistent with our data given that ERK1/2 is thought to be downstream of GRK/β-arrestin signaling [28,47]. The current study is the first to examine the role of ERK1/2 in tolerance to these other MOPr agonists.

It is unclear how phosphorylated ERK1/2 alters opioid function to contribute to DAMGO tolerance. ERK1/2 is known to alter several epigenetic markers and transcription factors including *c-fos*, brain-derived neurotrophic factor, and cAMP response element binding proteins (CREB) in several brain regions following opioid withdrawal [52,11]. The adenylyl cyclase-cAMP-CREB pathway is upregulated following prolonged opioid exposure [8]. ERK1/2 activation may contribute to this upregulation by activation of PKC, although the exact mechanism is unclear [31]. ERK1/2 also has been found to directly increase synaptic vesicle exocytosis via calcium channels [49]. Given that opioids in the vlPAG produce antinociception by inhibiting GABA release [13,37,20], an increase in GABA release via ERK1/2 inhibition would require a higher opioid dose to produce antinociception.

5. Conclusions

The present study shows that both G protein and dynamin/ERK1/2 related signaling contribute to MOPr mediated antinociception in a ligand-biased manner. DAMGO engages both G protein and β-arrestin signaling pathways within the vlPAG to facilitate antinociception and tolerance. DAMGO antinociception and tolerance is dependent on activation of ERK1/2, while fentanyl antinociception and tolerance is not. This adds to the growing body of research on ligand-biased signaling at the MOPr by revealing a distinct role for ERK1/2 using a behavioral approach.

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