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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,