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Opioid receptor internalization contributes to dermorphin-mediated antinociception

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Abstract

Microinjection of opioids into the ventrolateral periaqueductal gray (vlPAG) produces antinociception in part by binding to mu-opioid receptors (MOPrs). Although both high and low efficacy agonists produce antinociception, low efficacy agonists such as morphine produce limited MOPr internalization suggesting that MOPr internalization and signaling leading to antinociception are independent. This hypothesis was tested in awake, behaving rats using DERM-A594, a fluorescently labeled dermorphin analog, and internalization blockers. Microinjection of DERM-A594 into the vlPAG produced both antinociception and internalization of DERM-A594. Administration of the irreversible opioid receptor antagonist beta-CNA prior to DERM-A594 microinjection reduced both the antinociceptive effect and the number of DERM-A594 labeled cells demonstrating that both effects are opioid receptor-mediated. Pretreatment with the internalization blockers dynamin dominant-negative inhibitory peptide (dynamin-DN) and concanavalinA (ConA) attenuated both DERM-A594 internalization and antinociception. Microinjection of dynamin-DN and ConA also decreased the antinociceptive potency of the unlabeled opioid agonist dermorphin when microinjected into the vlPAG as demonstrated by rightward shifts in the dose-response curves. In contrast, administration of dynamin-DN had no effect on the antinociceptive effect of microinjecting the $GABA_A$ antagonist bicuculline into the vlPAG. The finding that dermorphin-induced antinociception is attenuated by blocking receptor

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internalization indicates that key parts of opioid receptor-mediated signaling depend on internalization.

Keywords

endocytosis; periaqueductal gray; opiate; analgesia; pain modulation

The antinociceptive effect of opioids is mediated primarily by mu-opioid receptors (MOPr). MOPr activity is regulated by endocytotic trafficking to and from the plasma membrane (Finn and Whistler, 2001; Tanowitz and von Zastrow, 2003; von Zastrow et al., 2003). Recently, distinct signaling from receptors located on the plasma membrane and from endosomes (Murphy et al., 2009; Sorkin and von Zastrow, 2009) suggests that internalization of MOPr could play a role in the signaling that leads to antinociception. Whether receptor trafficking contributes to the signaling that leads to antinociception is unclear. Antinociception and opioid internalization have been shown to correlate following administration of opioid agonists (Trafton and Basbaum, 2000; Pradhan et al., 2009). However, studies in cell culture find that some agonists, such as morphine, do not readily induce internalization (Alvarez et al., 2002; Borgland et al., 2003; Arttamangkul et al., 2008) suggesting that signaling precedes MOPr internalization.

Development of a fluorescent label for the MOPr agonist dermorphin (DERM-A594) has allowed *in vitro* characterization of MOPr trafficking in locus coeruleus neurons (Arttamangkul et al., 2000; Arttamangkul et al., 2006). In these studies, visualization of internalized DERM-A594 occurs rapidly upon administration and is blocked with prior administration of the internalization blocker Concanavalin A (ConA). Simultaneous electrophysiological experiments demonstrated that in the presence of ConA, DERM-induced desensitization of G protein-coupled inwardly rectifying potassium channels (GIRK) is unaffected, indicating that MOPr internalization and the signaling that leads to desensitization are separate processes.

The ventrolateral periaqueductal gray (vlPAG) is an ideal structure to study the relationship between MOPr internalization and antinociception. Microinjection of opioids into the vlPAG produces antinociception (Jacquet and Lajtha, 1976; Bodnar et al., 1991; Morgan et al., 1998), and blocking opioid action in the vlPAG attenuates antinociception produced by systemic morphine administration (Zambotti et al., 1982; Randich et al., 1992; Lane and Morgan, 2005; Lane et al., 2005). Although *in vitro* studies indicate that MOPr internalization and signaling are independent, the objective of the present study was to test this hypothesis in awake, behaving animals. The first step was to correlate DERM-A594 internalization and antinociception following microinjection of DERM-A594 into the vlPAG. The second step was to determine whether blocking receptor internalization with ConA and dynamin dominant-negative inhibitory peptide (dynamin-DN) alters dermorphin-induced antinociception.

Experimental procedures

Animals

Experiments were performed in adult male Sprague-Dawley rats (250 – 350 g; Animal Technologies, Livermore, CA). All procedures were conducted in accordance with the National Institutes of Health Guide for the Care and Use of Laboratory Animals and approved by the IACUC at Washington State University. Efforts were made to minimize the number of experimental subjects (e.g. using a within subjects design when possible).

Microinjections

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 (AP: +1.7 mm, ML: ±0.6 mm, DV: −5.0 mm from lambda) using stereotaxic techniques. The guide cannula was attached to two screws in the skull by dental cement. At the end of the surgery, a stylet was inserted to plug the guide cannula. The rat was maintained under a heat lamp until awake. Following surgery, rats were housed individually. The animal housing room was maintained on a reverse light/dark schedule (lights off at 7:00 AM) so rats could be tested during the active dark phase. Food and water were available at all times except during testing. Rats were handled daily before and after surgery. Testing began at least 7 days after surgery.

Drugs were administered directly into the vlPAG through a 31-gauge injection cannula (0.25 mm OD and 0.127 mm ID) inserted into and extending 2 mm beyond the tip of the guide cannula. One day before testing, rats received a sham injection in which an injector was inserted into the guide cannula but no drug was administered. This procedure reduces confounds resulting from mechanical stimulation of neurons on the test day and habituates the rat to the microinjection procedure. Testing with drug administration began 1 day later. Drugs were microinjected at a rate of 0.1 μl/10 s while the rat was gently restrained by hand. The injection cannula remained in place an additional 20 s to minimize backflow of the drug up the cannula track. Following the injection, the stylet was replaced and the rat was returned to its home cage.

Behavioral testing

Nociception was assessed using the hot plate test. The hot plate test consisted of measuring the latency to lick the hind paw when placed on a 52.5°C plate. The rat was removed from the hot plate if no response occurred within 50 s.

Experiment 1: Does Microinjection of DERM-A594 Produce Antinociception and Internalization?

DERM-A594 (300 ng/0.5 μl) was microinjected into the vlPAG to determine whether it produced antinociception and MOPr internalization in intact, awake rats. The irreversible opioid receptor antagonist beta-chlornaltrexamine (beta-CNA; 270 ng/0.5 μl) (Sigma-Aldrich, St. Louis, MO) or saline were microinjected into the vlPAG 6 hrs and again 30 min prior to microinjection of DERM-A594 to determine whether DERM-A594 internalization was a MOPr mediated effect. Nociception was assessed using the hot plate test 30 min following microinjection of DERM-A594.

Experiment 2: Do Internalization Blockers Prevent DERM-A594 Internalization?

If DERM-A594 enters neurons through internalization of G-protein coupled receptors (GPCRs), then blocking receptor internalization should block DERM-A594 internalization. This hypothesis was tested by microinjecting dynamin-DN (100 ng/0.4 ml; 200 mM) or the scrambled dynamin peptide (dynamin-scr) (100 ng/0.4 ml; 200 mM) (Tocris Cookson, Inc. Ellisville MO) into the vlPAG 20 min prior to DERM-A594 administration. Dynamin-DN blocks receptor internalization by competing with GTPase dynamin that moves the receptor/beta-arrestin complex from the membrane. Nociception was assessed using the hot plate test 30 min after microinjection of DERM-A594. The brain was removed 5 min after the hot plate test for confocal analysis of DERM-A594 in vlPAG neruons.

A separate group of rats was injected with ConA (800 ng/0.8 ml) (Sigma-Aldrich, St. Louis, MO) or saline 20 min prior to administration of DERM-A594 into the vlPAG. ConA is a lectin that blocks receptor internalization by preventing receptor clustering (Pippig et al., 1995). This experiment was conducted to test whether independent methods of blocking

MOPr internalization would have the same effect. Changes in hot plate latency and MOPr internalization were measured as described above.

Experiment 3: Do Internalization Blockers Prevent Dermorphin-Induced Antinociception?

The internalization blockers dynamin-DN and ConA were microinjected into the vlPAG 20 min prior to administration of the unlabeled MOPr agonist dermorphin to test the hypothesis that MOPr internalization contributes to opioid antinociception. Animals received microinjections of dynamin-DN (100 ng/0.4 ml; 200 mM) or the scrambled dynamin peptide (100 ng/0.4 ml; 200 mM) 20 minutes before microinjection of cumulative third log doses of unlabeled dermorphin (0.22, 0.46, 1.0, & 2.2 μg/0.4 μl). Dermorphin was injected every 20 min, and nociception was assessed using the hot plate test 15 min after each injection. This procedure has been shown to produce reliable dose response curves for opioids such as morphine, DAMGO, and fentanyl (Morgan et al., 2006; Bobeck et al., 2009).

A separate group of rats were injected with Con A (800 ng/0.8 μl) or saline (0.8 μl) into the vlPAG 20 min before microinjection of dermorphin (0.22, 0.46, 1.0, & 2.2 μg/0.4 μl). A large volume of ConA was used to keep the concentration low and cover a large region of the vlPAG. Rats were tested as described above. A within-subjects design was used in which half the rats were pretreated ConA or saline on one day and treated with the reverse treatment 2 days later.

The specificity of dynamin-DN for GPCRs was assessed by microinjecting dynamin-DN (100 ng/0.4 ml; 200 mM) or the scrambled dynamin peptide (100 ng/0.4 ml; 200 mM) into the vlPAG 20 minutes before microinjection of cumulative third log doses of the $GABA_A$ receptor antagonist bicuculline (4.6, 10, 22, & 46 ng/0.4 μl) (Sigma-Aldrich, St. Louis, MO). Previous research has shown that blocking tonic GABA inhibition by microinjecting bicuculline into the vlPAG produces a rapid and potent antinociception (Morgan and Clayton, 2005; Bobeck et al., 2009). Bicuculline was injected every 7 minutes, and nociception was assessed using the hot plate test 5 min after each injection. A within subjects design was used in which half the rats were pretreated with either scrambled-dynamin or dynamin-DN in the dermorphin dose-response test. On a second test, one week later, rats were pretreated with the reverse treatment, followed by bicuculline.

Histology

Rats were anesthetized with Halothane 5 – 10 min after the hot plate test and perfused with saline and then formalin (10%). The brain was removed and placed in formalin (10%) for subsequent immunohistochemistry and confocal analysis of DERM-A594 internalization (see methods below). The brain was sectioned coronally (60 μm) at least two days after being removed from the rat. The location of the injection site was identified by looking for the end of the cannula track (Paxinos and Watson, 2005). These brain sections were then prepared for confocal microscopy (see below).

Confocal microscopy

Coronal brain slices containing vlPAG were incubated in 5% goat serum (Sigma-Aldrich, St. Louis, MO), 3% BSA (Sigma-Aldrich), and 0.5% Triton X-100 (Sigma-Aldrich) in phosphate buffered saline (PBS) for 1 hr at room temperature. Sections were washed three times with PBS. All slices were then incubated in mouse anti-NeuN (1/500, Chemicon, Temecula, CA) antibody overnight, washed in PBS three times, and then incubated for 1 hr in secondary antibody (goat anti-mouse IgG 488 Alexa Fluor conjugate (1/800, Invitrogen, Carlsbad, CA)). Slices were washed three times in PBS.

Confocal analysis targeted the vlPAG adjacent to the aqueduct (Aq) and within 100–200 uM of the injection site (Figure 1B). During the image collection, the experimenter was blind to treatments. The size of the sampling region (180 μm^2) was the same in each animal. The aqueduct was centered under the 10× objective. Using the 20× objective, the aqueduct was positioned in the upper left corner of the 20× field. Without repositioning the sample, the 63× oil objective was lowered and images were collected. NeuN and DERM-A594 labeling in the vlPAG were examined in each case. NeuN labeling was used to verify that the number of cells in the different treatment groups were comparable (96–110 total NeuN cells were counted for each treatment group with the mean ranging from 13.7 to 18.3 neurons for each animal). NeuN and DERM-A594 colocalization was not determined in this study. Images of NeuN and DERM-A594 labeling in vlPAG were collected on a Zeiss LSM 510 META confocal microscope (Carl Zeiss MicroImaging, Inc., Thornwood, NY) using a single pass, multi-channel format with band pass filters to spectrally separate the NeuN and DERM-A594 labeling. We used a pinhole of 1.0 airy unit and objectives of 10× (numerical aperture (NA) 0.3), 20× (numerical aperture (NA) 0.75), and 63× oil (NA 1.4), resulting in estimated optical section thicknesses (full width at half-maximum) of 2.53, 1.01, and 0.62 μm, respectively. We determined the vertical extent of NeuN and DERM-A594 labeling through the section and then acquired a single 12-bit image of the optical section in which labeling for both NeuN and DERM-A594 was optimal using a fast scan (z-interval). Optical sections were acquired at a digital size of 1024×1024 pixels and averaged four times to reduce noise.

Data analysis

Data were analyzed and plotted using Prism 4 (GraphPad Software, San Diego, CA, USA). Differences in hot plate latency were compared using an one-way ANOVA followed by a Tukey's post hoc test or Student's t-tests between experimental and control groups. Dermorphin and bicuculline dose-response curves and D_{50} values (dose for half maximal antinociception) (Tallarida, 2001) were calculated using non-linear regression (Prism 4, GraphPad Software, San Diego, CA, USA). Only rats with injection cannula in or on the border of the vlPAG were included in data analysis (Paxinos and Watson, 2005).

DERM-A594 labeled cells were quantified using Image J (NIH, Bethesda, MD, USA). The experimenter was blind to treatments during analyses. The number of cells with DERM-A594 labeling was counted for each animal. The average number of DERM-A594 positive cells (± SEM) for animals in each condition was calculated and compared to the number of cells counted from other treatment conditions using Student's t-test.

Results

Experiment 1: Does Microinjection of DERM-A594 Produce Antinociception and Internalization?

The objective of this experiment was to determine whether microinjection of DERM-A594 into the vlPAG produces both MOPr internalization and antinociception. Microinjection of DERM-A594 (300 ng/0.5μl) into the vlPAG produced an increase in hot plate latency compared to baseline (38.1 ± 3.7 s vs. 19.0 ± 2.5 s; N = 9; $F(2, 35) = 22.13$, $p < 0.001$). Confocal analysis of vlPAG neurons located immediately adjacent to the microinjection site revealed internalization of DERM-A594. Pretreatment with the irreversible MOPr antagonist beta-CNA (N = 11) reversed both DERM-A594 internalization ($t(12) = 2.176$, $p = 0.05$) and antinociception ($p > 0.05$ compared to baseline; Figure 1). This finding demonstrates that DERM-A594 produces antinociception and internalization by binding to opioid receptors in the vlPAG.

Experiment 2: Do Internalization Blockers Prevent DERM-A594 Internalization?

The objective of this experiment was to test the hypothesis that DERM-A594 enters neurons via receptor internalization. Blockade of receptor internalization by microinjection of dynamin-DN into the vlPAG (N = 7) reduced the number of neurons showing DERM-A594 labeling compared to rats injected with the scrambled dynamin control peptide (N = 6) ($t(11) = 4.020$, $p < 0.01$, Figure 2). Microinjection of dynamin-DN also prevented the increase in hot plate latency produced by microinjection of DERM-A594 ($t(12) = 3.045$, $p<0.01$, Figure 2).

Blocking receptor internalization by microinjecting ConA into the vlPAG also attenuated both DERM-A594-mediated internalization and antinociception. Confocal analysis of DERM-A594 labeling showed a decrease in fluorescent cells in ConA pretreated rats (mean = 5.4 ± 1.0 cells per animal, N = 7) compared to saline treated animals (12.3 ± 2.9 cells per animal, N = 7; $t(12) = 2.224$, $p< .05$). Pretreatment with ConA (N = 12) also caused a significant decrease in DERM-A594 mediated antinociception from 38.1 ± 3.7 s to 24.8 ± 2.9 s ($t(19) = 2.882$, $p < 0.05$). These data show that blocking internalization, whether by dynamin-DN or ConA, prevents both DERM-A594 internalization and antinociception.

Experiment 3: Do Internalization Blockers Prevent Dermorphin-Induced Antinociception?

The hypothesis that MOPr internalization is a necessary signaling step for dermorphin-mediated antinociception was tested by measuring the antinociceptive effect of microinjecting unlabeled dermorphin into the vlPAG in rats pretreated with the receptor internalization blockers dynamin-DN and ConA. Microinjection of dermorphin into the vlPAG produced a dose-dependent increase in hot plate latency (Figure 3). This dermorphin-induced antinociception was significantly reduced in rats pretreated with dynamin-DN (D_{50} = 1.1 ± 0.3 μg, N = 7) compared to scrambled dynamin pretreated animals (D_{50} = 0.2 ± 0.05 μg, N = 7) ($F(1, 79) = 10.04$, $p < 0.01$, Figure 3A). Pretreatment with ConA also significantly decreased dermorphin-induced antinociception (D_{50} = 1.1 ± 0.15 μg) compared to vehicle-pretreated animals (D_{50} = 0.6 ± 0.1 μg) ($F(1, 51) = 12.52$; $p < 0.01$; N= 4, Figure 3B).

In contrast, there was no difference in the antinociceptive effect of microinjecting the $GABA_A$ receptor antagonist bicuculline into the vlPAG in rats pretreated with dynamin-DN (D_{50} = 16.9 ng, N = 7) or the scrambled peptide (D_{50} = 14.0 ng, N = 7; $F(1, 66) = 2.176$; n.s.). Given that bicuculline produces antinociception by blocking the $GABA_A$ receptor, this finding demonstrates that the decrease in antinociception following dynamin-DN pretreatment is specific to internalization of the MOPr and not non-specific disruption of cell signaling.

Discussion

The present study shows that microinjection of the fluorescently labeled dermorphin analog DERM-A594 into the vlPAG produces antinociception and internalization of DERM-A594. Internalized DERM-A594 was blocked with prior administration of the irreversible opioid receptor antagonist beta-CNA demonstrating that internalization of DERM-A594 is dependent on opioid receptor binding. Moreover, blocking internalization by administering dynamin-DN or ConA prevented both DERM-A594 internalization and antinociception. Although opioid-induced receptor internalization is a well-known phenomenon, this is the first report showing that blocking receptor internalization can inhibit antinociception.

Internalization of DERM-A594 following microinjection into the vlPAG is consistent with previous studies showing DERM-A594 internalization in cultured locus coeruleus cells (Arttamangkul et al., 2000; Arttamangkul et al., 2006). Although the present manuscript is

the first to show DERM-A594 internalization in a whole animal, opioid-induced MOPr internalization has been shown previously in intact animals using immunohistochemistry (Trafton and Basbaum, 2000; He et al., 2002; Trafton and Basbaum, 2004; Chen et al., 2007; Chen et al., 2008; Lao et al., 2008). The advantage of assessing MOPr internalization with a fluorescently labeled opioid agonist *in vivo* is that internalization is restricted to those receptors bound by agonist at the plasma membrane. This approach allows ligand-induced receptor internalization to be correlated to changes in behavior.

Although dermorphin is approximately 100-fold more selective for MOPr compared to delta-opioid receptors (Amiche et al., 1990), it is possible that delta opioid receptors contribute to DERM-A594 internalization. Mild antinociceptive effects have been reported following microinjection of the delta opioid receptor agonist deltorphin into the PAG (Rossi et al., 1994) (see however Ossipov et al., 1995; Morgan et al., 2009). However, MOPr receptors are primarily responsible for PAG mediated antinociception (Bodnar et al., 1988). Membrane expression of functional delta-opioid receptors primarily occurs in PAG neurons following chronic opioid administration or repeated stress (Commons, 2003; Hack et al., 2005). Kappa-opioid receptors are probably not involved in DERM-A594 internalization because microinjection of kappa receptor agonists into the PAG has no effect on nociception (Fang et al., 1989; Rossi et al., 1994).

DERM-A594 labeling was observed in vlPAG tissue taken from intact rats. DERM-A594 does not cross-link to the tissue during perfusion of the animal therefore limiting both background fluorescence and fluorescence on the plasma membrane. The observations that DERM-A594 only labels a subpopulation of cells in the vlPAG, that there is little background fluorescence, and that internalization of DERM-A594 is significantly inhibited by the irreversible opioid receptor antagonist beta-CNA support evidence that DERM-A594 is internalized following binding to opioid receptors. Much less is known about the fate of DERM-A594 following internalization, but widespread trafficking of fluorescent peptides, including to nuclear compartments, has been reported (Duchardt et al., 2007). Further experiments are needed to provide more information about the endocytotic recycling process.

The regulatory mechanisms underlying MOPr endocytosis have been studied extensively in cell culture systems, but the contribution of this process to antinociception has not been examined. The MOPr is a member of the seven transmembrane GPCR family that undergoes trafficking upon agonist binding. Activation of the receptor induces phosphorylation of the MOPr C-terminus by G-protein receptor kinases (GRKs) and subsequent recruitment of beta-arrestin and other adaptor proteins that promote the formation of clathrin-coated pits and dynamin-dependent endocytosis (for reviews see (Wolfe and Trejo, 2007; Koch and Hollt, 2008). One of the proposed functions of this internalization process is termination of the MOPr signal by uncoupling the receptor from the G-protein and removing the receptor from the membrane (Kim et al., 2008). However, GPCRs have been shown to signal from endosomes (Murphy et al., 2009; Sorkin and von Zastrow, 2009).

The present results showing attenuation of antinociception by blocking receptor internalization suggests that a key part of MOPr signaling occurs following internalization. The effect of blocking receptor internalization on antinociception was assessed using both dynamin-DN and ConA. Microinjection of dynamin-DN peptide into the vlPAG blocked the antinociceptive effect of microinjecting DERM-A594 or unlabeled dermorphin into the vlPAG. Dynamin is a GTPase that interacts with internalization machinery downstream of GRK, beta-arrestin and formation of clathrin-coated pits and functions to release the clathrin-coated pit from the plasma membrane (Wolfe and Trejo, 2007). Dynamin-DN inhibits this process. The lectin ConA binds to cell surface glycoproteins impairing their

mobility within the membrane bilayer (Luttrell et al., 1997) and has been used as an effective blocker of GPCR recycling in several cell systems (Xiang et al., 2002; Kim et al., 2004; Arttamangkul et al., 2006). ConA inhibits endocytosis of beta-adrenergic receptors without affecting the initial events in GPCR-signaling, including ligand-binding, G protein-activation, and generation of small molecule second messengers (Wang et al., 1989; Pippig et al., 1995). The effect of ConA on MOPrs appear to be similar to those for the well characterized beta-adrenergic receptors (Keith et al., 1996). Thus, it was not surprising that pretreatment with dynamin-DN or ConA decreased the number of DERM-A594 labeled cells in the vlPAG.

In contrast, the ability of both dynamin-DN and ConA to attenuate the antinociception produced by DERM-A594 and unlabeled dermorphin was surprising because the prevailing hypothesis is that MOPr signaling occurs at the membrane and internalization of GPCRs terminates signaling. According to this hypothesis, manipulations that prevent MOPr internalization should enhance antinociception by promoting additional MOPr signaling from the membrane (Bohn et al., 1999; Bohn et al., 2004). However, decreased antinociception following blockade of MOPr internalization could occur through several potential mechanisms. First, locking MOPrs to the membrane may enhance their ability to desensitize, and internalization of opioid receptors may be required to recover from desensitization (Koch et al., 2005). However, previous research has shown that opioid desensitization is unchanged in locus coeruleus neurons in the presence of ConA (Arttamangkul et al., 2006) and early processes in GPCR activation of beta-adrenergic receptors are not affected by ConA (Wang et al., 1989; Pippig et al., 1995). Second, blockade of internalization may decrease recycling of refreshed MOPrs to the membrane. Differences in opioid-induced activation, desensitization and recycling of the MOPrs indicate that these are distinct processes with varying sensitivities depending on the efficacy of the agonist (Bailey et al., 2003; Dang and Williams, 2004, 2005; Liao et al., 2007; Arttamangkul et al., 2008; Kelly et al., 2008; Virk and Williams, 2008; Ingram, 2009).

A more likely mechanism is that signaling by some opioid agonists is dependent on recruitment of internalization machinery. That is, MOPrs may signal from endosomes following internalization of the receptor (Murphy et al., 2009; Sorkin and von Zastrow, 2009). Of course, signaling may be different with high efficacy agonists like dermorphin that produce rapid receptor internalization and low efficacy agonists like morphine that produce limited internalization. Both internalization-dependent (Ignatova et al., 1999) and independent (Kramer and Simon, 2000; Zheng et al., 2008) MAPK signaling pathways have been described. For example, our previous research shows that phosphorylation of ERK1/2 occurs preferentially with high efficacy agonists both in the striatum (Macey et al., 2006) and in vlPAG after chronic morphine (Macey et al., 2009). The present results show for the first time that endosomal signaling may be necessary for opioid-induced antinociception from the vlPAG. Additional studies are needed to distinguish whether dynamin-DN and ConA block a specific internalization-dependent signaling pathway or attenuate antinociception by preventing receptor recycling back to the plasma membrane and/or resensitization.

Abbreviations

Beta-CNA	beta-chlornaltrexamine
ConA	concanavalinA
DAMGO	D-Ala2,N-Me4,Gly5-ol]-enkephalin
DERM-A594	dermorphin conjugated to Alexa Fluor 594

dynamin-DN	dynamin dominant-negative inhibitory peptide
dynamin-scr	scrambled dynamin control peptide
GIRK	G-protein-coupled inwardly rectifying potassium channels
GPCR	G-protein coupled receptors
GRK	G-protein receptor kinases
MOPr	mu-opioid receptor
PBS	Phosphate buffered saline
vlPAG	ventrolateral periaqueductal gray

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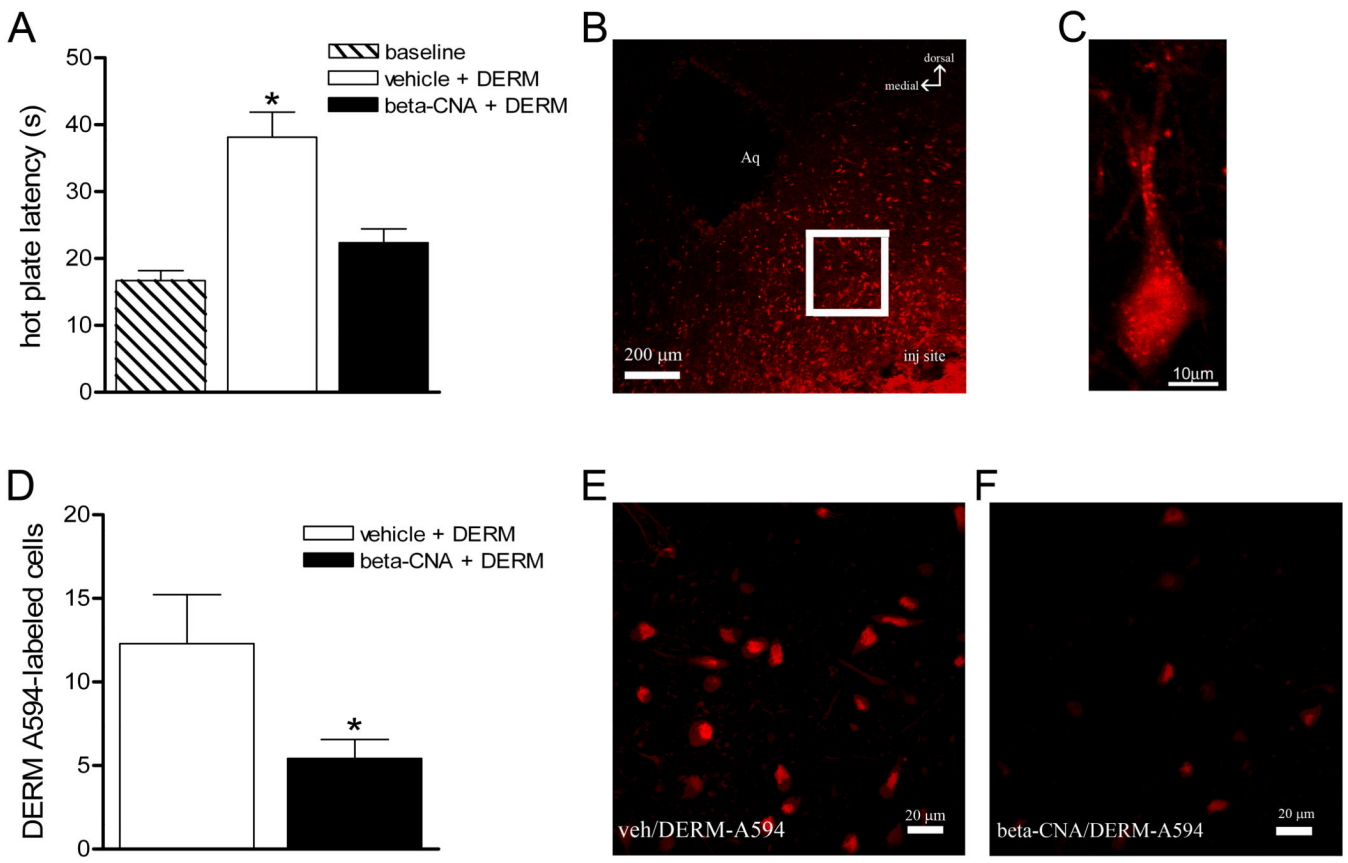

Figure 1. Microinjection of DERM-A594 into the vlPAG produces antinociception and internalization

Rats were microinjected with vehicle or beta-CNA, followed by DERM-A594 into the vlPAG. **A**: Microinjection of DERM-A594 produced an increase in hot plate latency compared to baselines and this increase was blocked by pretreating rats with beta-CNA. **B**: The number of cells labeled with DERM-A594 (red) was assessed using confocal microscopy in the vlPAG at least 100 µm from the microinjection site. **C**: View of a single vlPAG neuron with DERM-A594 labeling. **D**: The number of DERM-A594 labeled neurons was decreased in rats pretreated with beta-CNA compared to saline-pretreated animals. **E**: Labeling for DERM-A594 from a representative animal pretreated with vehicle. **F**: Labeling for DERM-A594 from a representative animal pretreated with beta-CNA.

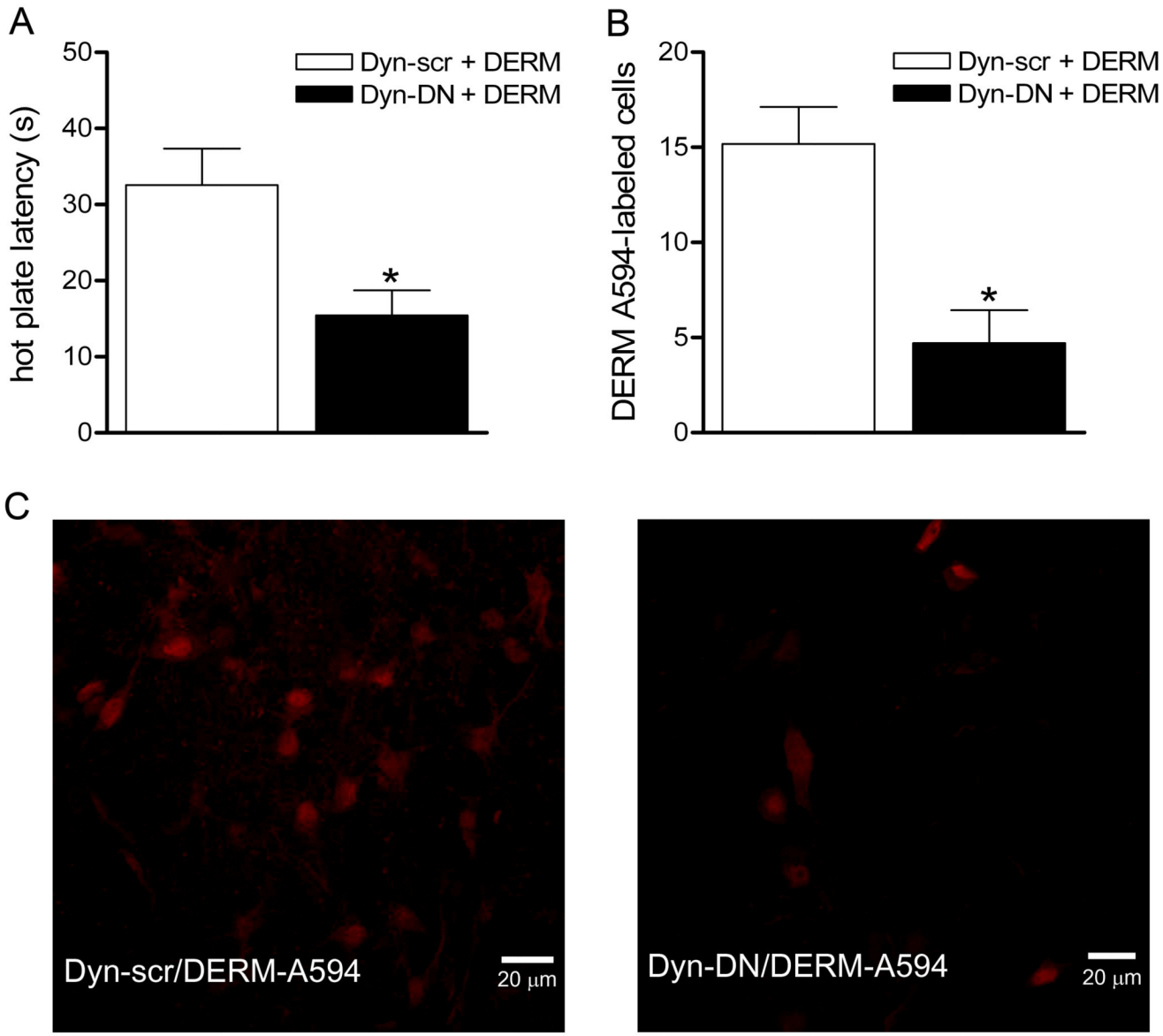

Figure 2. Microinjection of dynamin-DN into the vlPAG reduces DERM-A594 antinociception and internalization

Rats were microinjected with the dynamin inhibitory peptide (dyn-DN) or the scrambled peptide (dyn-scr) as a control prior to microinjection of DERM-A594 into the vlPAG. **A**: Pretreatment with dynamin-DN reduced the antinociceptive effect of microinjecting DERM-A594 into the vlPAG compared to pretreatment with the scrambled peptide. **B**: The number of DERM-A594 labeled cells was significantly decreased in rats pretreated with dynamin-DN compared to scrambled-dynamin pretreated animals. **C, Panel 1**, Labeling for DERM-A594 from a representative animal pretreated with scrambled-dynamin (left) compared to a representative animal pretreated with dynamin-DN (right).

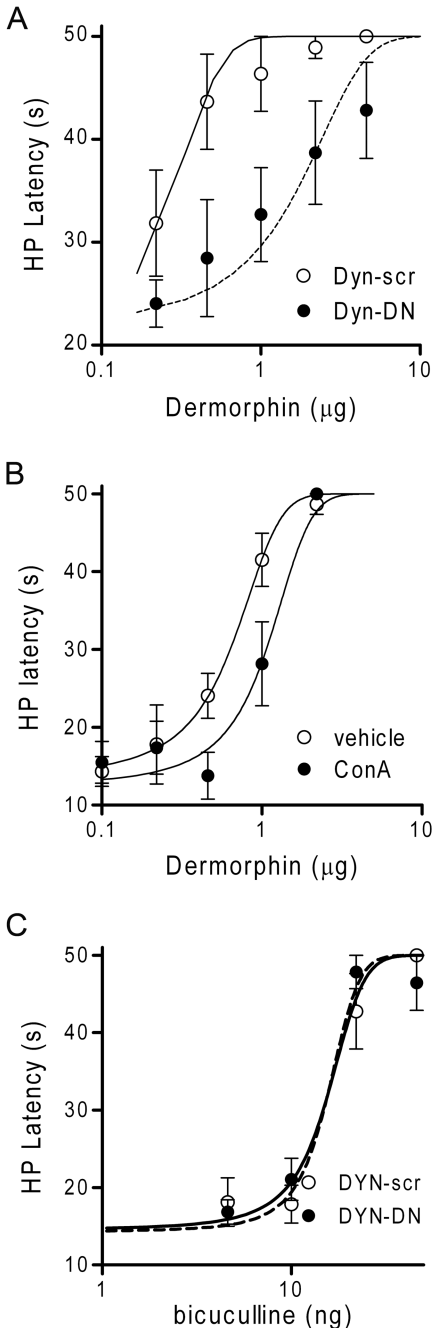

Figure 3. Blocking internalization attenuates dermorphin-induced antinociception

Rats were administered internalization blockers prior to cumulative microinjections of dermorphin. **A**: Microinjection of dermorphin produced a dose dependent increase in hot plate latency. Antinociceptive potency was decreased in rats pretreated with dynamin-DN (dyn-DN) compared to scrambled dynamin (dyn-scr) pretreated animals. **B**: The potency of dermorphin-induced antinociception also was decreased in rats pretreated with ConA compared to saline-pretreated rats. **C**: The antinociceptive potency of microinjecting the GABA antagonist bicuculline into the vlPAG was not affected by pretreatment with dynamin-DN peptide as would be expected given that blocking GABA does not produce antinociception via a GPCR.