

Contribution of dopamine receptors to periaqueductal gray-mediated antinociception

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Received: 17 September 2008 / Accepted: 29 January 2009 / Published online: 19 February 2009
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Abstract

Rationale Morphine relieves pain, in part, by acting on neurons within the periaqueductal gray (PAG). Given that the PAG contains a subpopulation of dopamine neurons, dopamine may contribute to the antinociceptive effects mediated by the PAG.

Methods This hypothesis was tested by measuring the behavioral and electrophysiological effects of administering dopamine agonists and antagonists into the ventrolateral PAG (vPAG). An initial histological experiment verified the existence of dopamine neurons within the vPAG using dopamine transporter and tyrosine hydroxylase antibodies visualized with confocal microscopy.

Results Microinjection of cumulative doses of morphine into the vPAG caused antinociception that was dose-dependently inhibited by the dopamine receptor antagonist

α -flupenthixol. α -Flupenthixol had no effect on nociception when administered alone. Injection of the dopamine receptor agonist (–) apomorphine into the vPAG caused a robust antinociception that was inhibited by the D2 antagonist eticlopride but not the D1 antagonist SCH-23390. The effects of dopamine on GABA_A-mediated evoked inhibitory post-synaptic potentials (eIPSCs) were measured in PAG slices. Administration of met-enkephalin inhibited peak eIPSCs by 20–50%. Dopamine inhibited eIPSCs by approximately 20–25%. Administration of α -flupenthixol (20 μ M) attenuated eIPSC inhibition by dopamine but had no effect on met-enkephalin-induced inhibition.

Conclusions These data indicate that PAG dopamine has a direct antinociceptive effect in addition to modulating the antinociceptive effect of morphine. The lack of an effect of α -flupenthixol on opioid-inhibition of eIPSCs indicates that this modulation occurs in parallel or subsequent to inhibition of GABA release.

This investigation was supported, in part, by funds provided for medical and biological research by the State of Washington Initiative Measure No. 171 and by NIH grant DA015498.

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Keywords Pain · Opioid receptor · Opiate · Analgesia · Catecholamine · Biogenic amine · Immunohistochemistry · Descending modulation · Patch-clamp electrophysiology · Drug addiction

Introduction

The periaqueductal gray (PAG) plays an important role in the modulation of nociception. Several studies have localized the antinociceptive effects of morphine to the vPAG and other midbrain areas (Manning et al. 1994; Pert and Yaksh 1975; Yaksh et al. 1976), and interference with neural transmission within the PAG alters the antinociceptive effects of systemically administered morphine (Lane et al.

2005). Electrophysiological experiments demonstrate that opioids activate PAG output neurons that project to the rostroventral medulla and the spinal cord (Reichling and Basbaum 1990). This activation appears to occur via inhibition of GABAergic neurons in the PAG (Vaughan and Christie 1997).

Although the mechanisms underlying opioid antinociception in the PAG have been well studied, much less is known about the role of other neurotransmitters. For example, a subpopulation of neurons within the ventrolateral PAG (vPAG) is dopaminergic. These neurons project to the central nucleus of the amygdala, bed nucleus of the stria terminalis, subnucleus of the extended amygdala, substantia innominata, and locally within the PAG (Dong and Swanson 2006; Hasue and Shammah-Lagnado 2002). Relatively little is known about the contribution of these vPAG dopamine neurons to morphine-induced antinociception, although one study reported that removal of dopaminergic input into the vPAG using 6-hydroxydopamine (6-OHDA) or dopamine antagonists attenuated the antinociceptive effect of systemically administered morphine (Flores et al. 2004).

There is substantial overlap in the neural systems containing opioid and dopamine receptors (Nestler 1996; Wood 1983; Wood et al. 1980). On the cellular level, agonist binding to opioid or dopamine receptors activates GIRK channels (Davila et al. 2003; Pillai et al. 1998), alter cAMP formation (Self 2004), and modulate GABA transmission (Cameron and Williams 1995; Vaughan and Christie 1997). These findings indicate that dopamine receptor activation within the vPAG may have opioid-like effects on nociception.

The current studies used anatomical, behavioral, and electrophysiological techniques to examine the role of PAG dopamine in nociception and morphine-induced antinociception. Specifically, the presence of dopamine transporter (DAT) and tyrosine hydroxylase (TH) immunoreactive neurons in the PAG was confirmed using confocal microscopy, and the effect of microinjecting dopamine agonists and antagonists into the PAG on nociception was measured. Finally, cellular interactions between opioids and dopamine were analyzed in PAG slices. Given the similarities between dopamine and opioid mechanisms of action, it was hypothesized that dopamine agonists would produce antinociception and dopamine antagonists would attenuate morphine antinociception.

Methods

Subjects

Adult male Sprague–Dawley rats (230–375 g) were used in all behavioral experiments. Young male and female rats (50–200 g) were used in electrophysiological experiments

because age-dependent increases in brain connective tissue makes slice recording difficult in older rats. No systematic differences between male and female rats were observed in the electrophysiological experiments. Our previous research shows that the behavioral and electrophysiological effects of opioids are similar between these ages of rats (Ingram et al. 2007). Experiments were conducted in accordance with the animal care and use guidelines outlined by the Committee for Research and Ethical Issues of the International Association for the Study of Pain and were approved by the Animal Care and Use Committee at Washington State University.

Experiment 1: Localization of dopamine neurons and fibers in the PAG

Previous experiments identified TH-positive dopamine neurons in the vPAG (Flores et al. 2004; Flores et al. 2005; Lu et al. 2006). We sought to verify this finding using anti-DAT antibodies in addition to anti-TH antibodies. Rats were deeply anesthetized using pentobarbital before they were perfused using a phosphate buffer (PB)-heparin solution followed by 4% paraformaldehyde in 0.1 M PB (pH 7.3). The brain was removed and post-fixed overnight in ice-cold (4°C) 4% paraformaldehyde in PB. The brains were rinsed extensively in PB and then stored at 4°C in PB until they were sectioned. Brain sections (100 µm) were collected in PB using a vibratome.

For immunostaining, free-floating slices containing the PAG were rinsed three times for 5 min in PB and then placed on a rotating shaker in blocking buffer (PB containing 1% Triton-X 100 and 5% normal goat serum) for 45–60 min at room temperature. The tissue was incubated overnight at 4°C in PB containing 0.1% Triton-X 100, 5% normal goat serum and either anti-mouse TH (final dilution 1:10,000; Sigma–Aldrich, St. Louis MO), anti-rat DAT (final dilution 1:400; Chemicon, a subdivision of Millipore, Billerica, MA, USA) or both. The tissue was rinsed three times (5 min) on a rotating shaker. Sections stained only for TH were incubated with anti-mouse Alexa Fluor 488 (Molecular Probes, a subdivision of Invitrogen, Carlsbad, CA, USA). Sections stained for DAT alone were incubated in anti-rat Alexa-Fluor 488. For both TH and DAT staining in the same slice, the sections were incubated in both anti-rat Alexa-Fluor 488 and anti-mouse Alexa-Fluor 568 for 30 min at room temperature. The sections were mounted onto slides, dried for 10 min and then coverslipped using Prolong (Molecular Probes).

Image capture was carried out using a TCS SP1 Laser Scanning Spectral confocal microscope (Leica Microsystems, Mannheim, Germany) equipped with four laser bands, which excite at wavelengths of 476, 488, 568, and 633 nm. Lasers were applied separately for emission at 488

(red, Alexa-Fluor 488) and 568 (green, Alexa-Fluor 568) nm. The images were digitally captured at $1,024 \times 1,024$ pixels in size. Sixteen serial sections were made through the sample down to a depth of approximately 20–40 μm . Serial reconstructions and imaging analysis was carried out using software by either Leica Microsystems (Leica Microsystems, Mannheim, Germany) or IPLabTM (Scanalytics Inc Fairfield, VA).

Experiment 2: The effect of α -flupenthixol on morphine antinociception

Each rat was anesthetized with pentobarbital (60 mg/kg). After placement in a stereotaxic frame, the skull was exposed by removal of a 15-mm diameter section of the scalp and sterilized with 70% ethanol in water using a cotton swab. Two anchor screws were then placed approximately 2.5 mm rostral and 2.0 mm to the right of lambda. A stainless steel guide cannula (21 gauge, 9 mm) was surgically placed through a hole drilled above the vPAG so that the tip was positioned above the vPAG, using the following coordinates (relative to lambda): AP +1.7 mm, ML +0.6 mm, DV -4.6 mm. A stainless steel stylette was inserted into the cannula to prevent clogging. The cannula was secured with dental acrylic that encompassed the anchor screws, and the acrylic was allowed to dry before the rat was removed from the stereotaxic stage. Rats were given 4–10 days to recover before testing. Food and water were available ad libitum except during testing.

Rats were tested in a dimly illuminated room during the dark cycle of a 12:12 h reverse light/dark schedule (lights off at 7:00 A.M.). Nociception was assessed with the hot-plate test by measuring the latency to lick the hind paw or attempt to jump from the apparatus when placed on a 52.5°C plate. The rat was removed from the apparatus if it did not respond within 50 s. Drugs were injected into the vPAG through a 23-gauge microinjector that extended 2 mm beyond the tip of the cannula. A sham microinjector was inserted into the cannula on the day before testing to habituate the rat to the microinjection procedure and prevent possible behavioral effects produced by mechanical stimulation of neurons during initial tissue penetration. No drug was injected on this day.

Rats were injected with cumulative quarter log doses of morphine (1, 1.8, 3.2, 5.6, and 10 $\mu\text{g}/0.4 \mu\text{l}$) into the vPAG. Our previous research shows that cumulative dose microinjections reveal clear shifts in morphine dose-response curves (Morgan et al. 2006a). The competitive dopamine receptor antagonist α -flupenthixol (0, 10, 18, or 32 μg) or saline vehicle was injected in combination with the first morphine injection. Separate rats were used for each α -flupenthixol dose. Following baseline hot plate testing, rats were injected every 20 min and tested on the hot-plate

15 min after each injection. The doses and interdose intervals used in this cumulative dosing procedure were adapted from previous studies (Duttaroy et al. 1997; Kalant et al. 1971; Morgan et al. 2006a; Morgan et al. 2006b). An additional control group was injected with α -flupenthixol (32 μg) in the absence of morphine and tested on the hot-plate following repeated saline injections every 20 min for five trials.

Rats were killed with an overdose of halothane following testing. Brain slices (100 μm) were cut with a vibratome and viewed under a microscope to localize the microinjector placement. Only rats with microinjector placements within or immediately adjacent to the vPAG were included in data analysis.

Experiment 3: vPAG microinjection of (-) apomorphine

Rats were surgically prepared and tested as described in Experiment 2. As dopamine may be metabolized rapidly in the brain, we used the dopamine agonist (-) apomorphine, which has more robust effects on nociception than dopamine in other brain areas (Phillips et al. 1992). Rats were weighed, and baseline hot-plate latency was assessed prior to microinjection of the dopamine D1 receptor antagonist SCH-23390 (4 μg), D2 receptor antagonist eticlopride (4 μg), or saline vehicle into the vPAG. Two minutes later, the first of four cumulative doses of (-) apomorphine (10 $\mu\text{g}/0.4 \mu\text{l}$ each) were microinjected into the vPAG. Injections were administered every 7 min, and the hot-plate test occurred 5 min after each injection. A within-group design was used in this study, in which each rat was tested with (-) apomorphine in combination with eticlopride, SCH-23390, or vehicle in a counterbalanced fashion on three separate test session. These doses were chosen from previous studies in which similar doses were microinjected into the vPAG and other brain areas (Bari and Pierce 2005; Flores et al. 2004; Sun and Rebec 2005). Each test session was separated by 1 week.

Experiment 4: In vitro analysis of α -flupenthixol/opioid interactions

Rats were deeply anesthetized with halothane (Sigma, St. Louis, MO, USA), and the brain rapidly dissected. Coronal slices (250 μm) were obtained using a vibratome and stored in external buffer (126 mM NaCl, 2.5 mM KCl, 1.4 mM NaH_2PO_4 , 1.2 mM MgCl_2 , 11 mM glucose, and 25 mM NaHCO_3) equilibrated with 95% O_2 , 5% CO_2 gas at 32°C. Slices containing the vPAG were placed in an electrophysiological recording chamber and superfused with the external buffer described above with the addition of 2,3-dihydroxy-6-nitro-7-sulphamoyl-benzo(F)quinoxaline (NBQX; 5 μM) and strychnine (1 μM) to block glutamatergic and glycinergic input, respectively. vPAG neurons

were visualized using infrared Nomarski optics. Evoked inhibitory postsynaptic currents were measured with whole-cell voltage clamp recordings using patch electrodes (2–5 M Ω) containing 140 mM CsCl, 10 mM EGTA, 5 mM HEPES, 2 mM CaCl₂, 2 mM MgATP (pH 7.3, 270–290 mosmol/l). Series resistance was compensated by 80% and the access resistance was continuously monitored for changes. The membrane was clamped at –70 mV during all experiments. To elicit IPSCs, bipolar tungsten electrodes delivered 1–10 mV stimuli at 30 mHz to the slice. Dopamine receptor ligands and met-enkephalin were administered in the superfusate.

Met-enkephalin produces antinociception by inhibiting GABAergic neurons in the PAG. The objective of this experiment was to determine whether α -flupenthixol modulates met-enkephalin signaling. Met-enkephalin, which has mixed effects at the μ and δ -opioid receptors, was chosen instead of morphine because its effects are reversible upon washout and because there are no functional δ -opioid receptors in the PAG in the naïve rat (Ma et al. 2006). PAG slices were perfused with α -flupenthixol

(20 μ M) or external buffer vehicle for at least 10 min. Slices were stimulated every 30 s for 2 min prior to superfusion of met-enkephalin (5, 10, or 20 μ M) for 2–5 min. At this point, all drugs were washed out for at least 10 min, and the experiment was repeated using the complementary α -flupenthixol/vehicle condition in a counterbalanced fashion. Only one cell was recorded per slice. For data analysis, peak eIPSCs were expressed as change from baseline, defined as the average of the four eIPSCs immediately before perfusion of met-enkephalin.

Experiment 5: Cellular effects of dopamine on GABAergic neurotransmission

PAG slices were prepared as described in Experiment 4. The direct effect of dopamine was assessed by measuring changes in eIPSCs following administration of dopamine. As in Experiment 4, slices were perfused with α -flupenthixol (20 μ M) or external buffer vehicle for at least 10 min. Slices were stimulated every 30 s for 2 min, at which point dopamine (10, 30, or 60 μ M) was perfused over the slices for

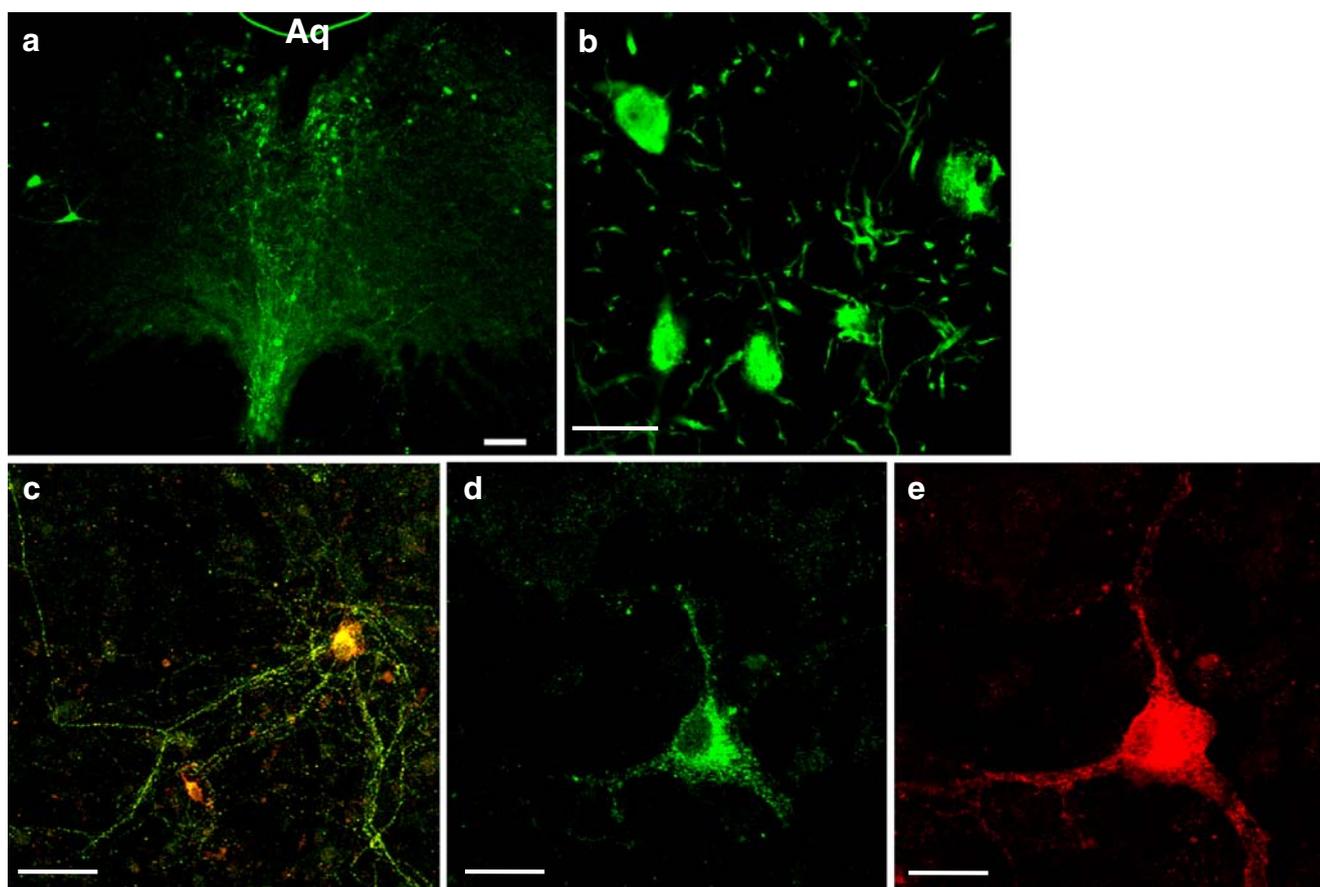


Fig. 1 Dopaminergic neurons within the vPAG. **a** Low magnification ($\times 5$) of a TH-stained brain slice of the vPAG. *Arrows* indicate examples of TH-stained cell bodies. Aq=Aqueduct. **b** Confocal magnification ($\times 40$) showing TH-stained cell bodies and processes

within the vPAG. **c** Composite image of DAT (*green*, **d**) and TH (*red*; **e**) staining of a vPAG neuron. *Scale bars* are as follows: **a** 100 μ m; **b**, **c** 40 μ m; **d**, **e** 20 μ m

an additional 2 min. Each cell received both α -flupenthixol/vehicle conditions in a counterbalanced fashion.

Statistics

Repeated-measures analysis of variance (ANOVA) was used to analyze changes in hot-plate latency. D_{50} values, defined as the dose that results in half-maximal antinociception (Tallarida 2000), were calculated to compare shifts in morphine potency. For electrophysiological experiments, changes in peak eIPSC magnitudes were analyzed. Baseline eIPSC was derived as the mean of four stimulation trials. The last four eIPSCs obtained after drug application were averaged to obtain a “post-drug” value. Repeated measures ANOVA were used to measure the effect of drug with Time (baseline/post-drug) as the repeated measure and α -flupenthixol (0, 20 μ M) as the between-groups measure. Specific differences were analyzed further using Tukey’s post-hoc tests.

Results

Experiment 1: Localization of dopamine neurons and fibers in the PAG

Previous studies have demonstrated that the PAG contains both small rounded neurons and large multipolar dopamine neurons within the PAG (Flores et al. 2004; Flores et al. 2005; Lu et al. 2006). TH and DAT immunohistochemical staining was identified within the ventral portions of the vPAG (Fig. 1). TH labeled neurons were diffusely localized throughout the ventral PAG and were concentrated near the ventral region of the aqueduct (Fig. 1a). Higher magnification of this staining identified the presence of cell bodies as well as neuronal processes (Fig. 1b). These neurons also labeled for DAT (Fig. 1c–e), demonstrating that they are dopaminergic.

Experiment 2: The effect of α -flupenthixol on morphine antinociception

Microinjection of morphine into the vPAG produced a dose-dependent increase in hot-plate latency (Fig. 2), as revealed by a main effect of morphine Dose [$F(25,125)=6.2$; $p<0.01$]. Administration of α -flupenthixol caused a dose-dependent reduction in morphine antinociception [$F(3,166)=3.4$; $p<0.05$]. Post-hoc comparisons indicate that the morphine D_{50} was significantly shifted from 2.8 μ g (in saline-pretreated rats) to 5.1 and 6.8 μ g in rats pretreatment with 18 and 32 μ g α -flupenthixol, respectively (Fig. 2b; $p_s<0.05$). Multiple injections of α -flupenthixol or saline into the vPAG had no effect on nociception (Fig. 2c). Mean hot-plate latency varied

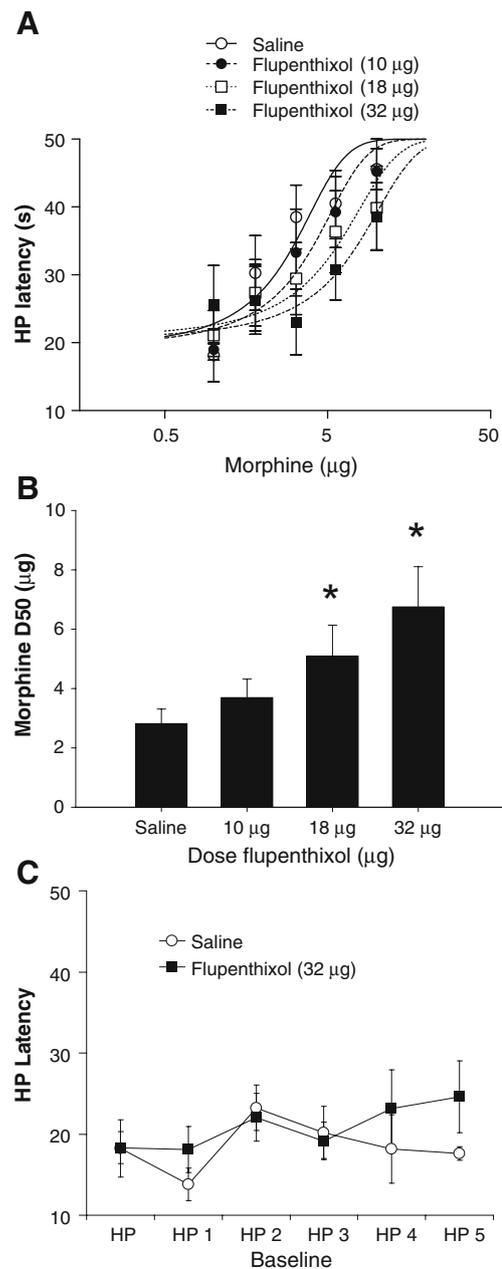


Fig. 2 The antinociceptive effect of morphine is attenuated using the nonspecific dopamine receptor antagonist α -flupenthixol. **a** Morphine dose response curves were calculated using nonlinear regression in the presence of 0, 10, 18, and 32 μ g α -flupenthixol ($n=7-8$ per dose). **b** Summary of data presented in panel **a**, showing that α -flupenthixol caused a dose-dependent reduction in morphine potency. Data are expressed as mean $D_{50} \pm$ SEM for morphine antinociception. Asterisks indicate significant D_{50} shifts from the saline treatment condition. **c** To determine whether flupenthixol altered nociception, the highest dose of flupenthixol (32 μ g) was injected into the PAG, and rats were tested for their hot-plate latencies after saline microinjections at 20-min intervals ($n=7$ per group). No consistent change in hot-plate latency was evident despite repeated microinjections of saline

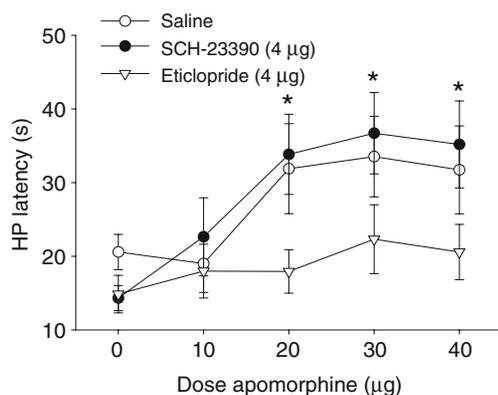


Fig. 3 Microinjection of (–) apomorphine into the vPAG produced antinociception. (–) Apomorphine was administered cumulatively in combination with vehicle, the D1 receptor antagonist SCH-23390, or the D2 receptor antagonist eticlopride ($n=9$ per treatment). Coadministration of eticlopride, but not SCH-23390, reversed the antinociceptive effect of apomorphine. Data are expressed as mean \pm SEM. Asterisks indicate doses that caused significant increases in latencies relative to baseline

between 18 and 23 s across trials despite repeated microinjection of α -flupenthixol into the vPAG.

Experiment 3: vPAG microinjection of (–) apomorphine

Microinjection of (–) apomorphine into the vPAG caused a dose-dependent increase in hot-plate latency (Fig. 3), as indicated by a main effect of Dose [$F(4,105)=5.47$; $p<0.01$]. This effect was blocked by pretreatment with eticlopride but not by SCH-23390, as indicated by a main effect of Antagonist [$F(2,105)=6.83$; $p<0.01$]. Post-hoc comparisons indicated that latencies were increased at the

20, 30, and 40 μ g doses of (–) apomorphine in the saline and SCH-23390 groups only. Infusions of apomorphine ($n=4$) outside of the vPAG did not result in significant antinociception (data not shown). These results indicate that intra-vPAG (–) apomorphine induces antinociception through a dopamine D2 receptor mechanism.

The placements of microinjectors used in the behavioral experiments are shown in Fig. 4. Only rats with placements within or immediately adjacent to the vPAG were included in the analyses. Injections of morphine outside the PAG ($n=6$) did not produce reliable antinociceptive effects.

Experiment 4: In vitro analysis of α -flupenthixol/opioid interactions

Met-enkephalin was applied to the PAG slice with or without α -flupenthixol in a counterbalanced fashion. Example traces from an experiment in which 5 μ M met-enkephalin was administered are shown in Fig. 5a. Met-enkephalin inhibited eIPSC amplitude by 20–50%; this inhibition peaked approximately 2 min following drug superfusion (Fig. 5b). As shown in summary Fig. 5c, the dose-dependent inhibition of eIPSCs by met-enkephalin [$F(1,45)=41.7$; $p<0.01$ for the effect of Time] was unaffected by co-treatment with α -flupenthixol.

Experiment 5: Cellular effects of dopamine on GABAergic neurotransmission

Example traces from an experiment in which 10 μ M dopamine was administered are shown in Fig. 6a. This dose of dopamine inhibited eIPSC amplitude by 20–25%

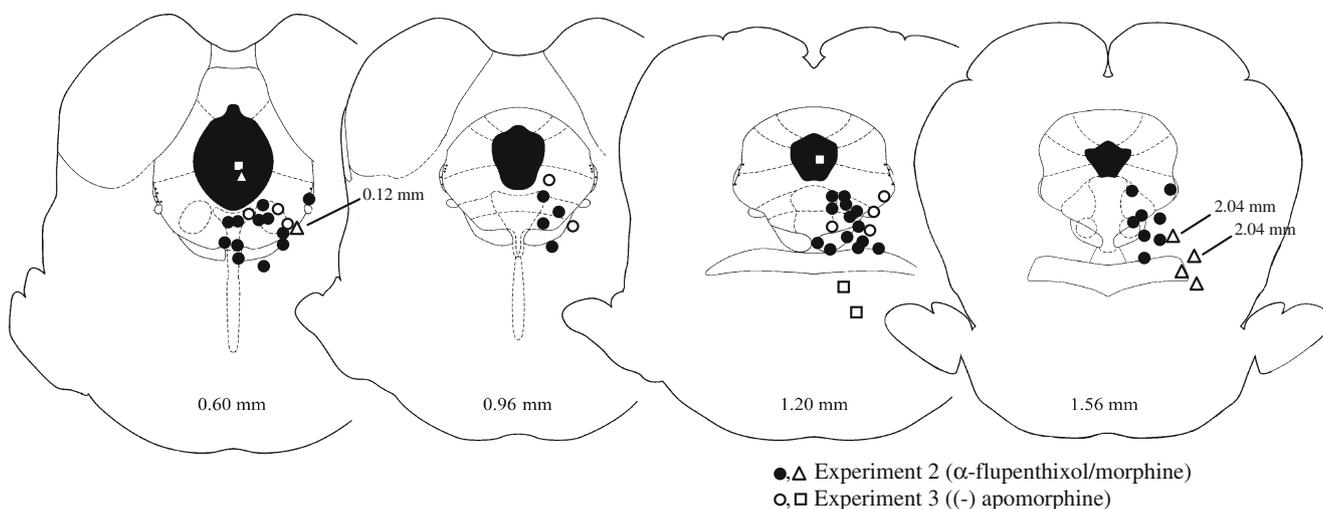


Fig. 4 Location of microinjectors for behavioral experiments. Only rats with microinjector placements within or immediately adjacent to the vPAG were included in data analyses. Numbers refer to anterior/posterior coordinates relative to the interaural line (Paxinos and Watson 2005). Closed and open circles refer to Experiments 2 and 3,

respectively. Triangles and squares refer to injections of (–) apomorphine and morphine that occurred outside of the vPAG, respectively. No difference in the location of the microinjector sites between groups was evident

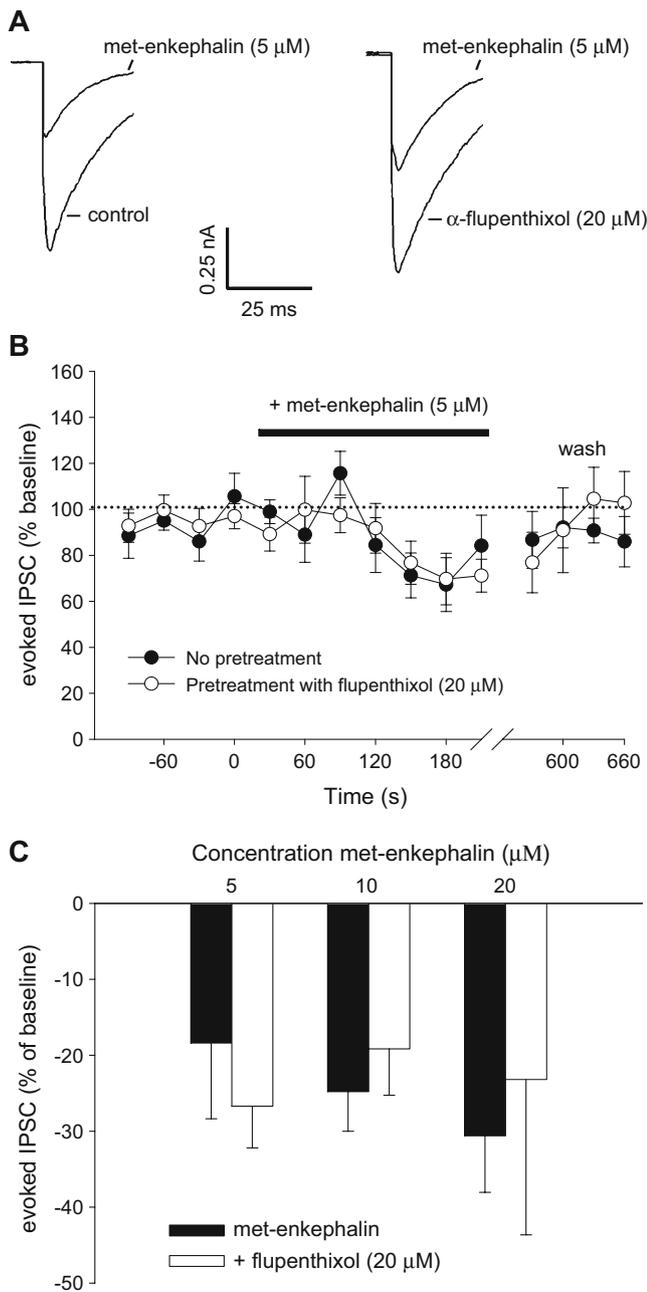


Fig. 5 α -Flupenthixol does not alter met-enkephalin induced inhibition of eIPSCs. **a** Examples of GABAergic eIPSCs and their inhibition by met-enkephalin (5 μ M) in the absence (*left*) and presence (*right*) of α -flupenthixol (20 μ M). **b** Summary of experiments ($n=8-9$ cells per treatment group) in which slices were stimulated every 30 s, and met-enkephalin (5 μ M) was superfused over the slices in the absence or presence of α -flupenthixol (20 μ M). Data are expressed as change from baseline, defined as the average of eight eIPSCs before application of met-enkephalin. Only the last four basal timepoints are shown. **c** Summary data showing the lack of effect of α -flupenthixol on increasing concentrations of met-enkephalin (5, 10, and 20 μ M). Data are expressed as mean \pm SEM

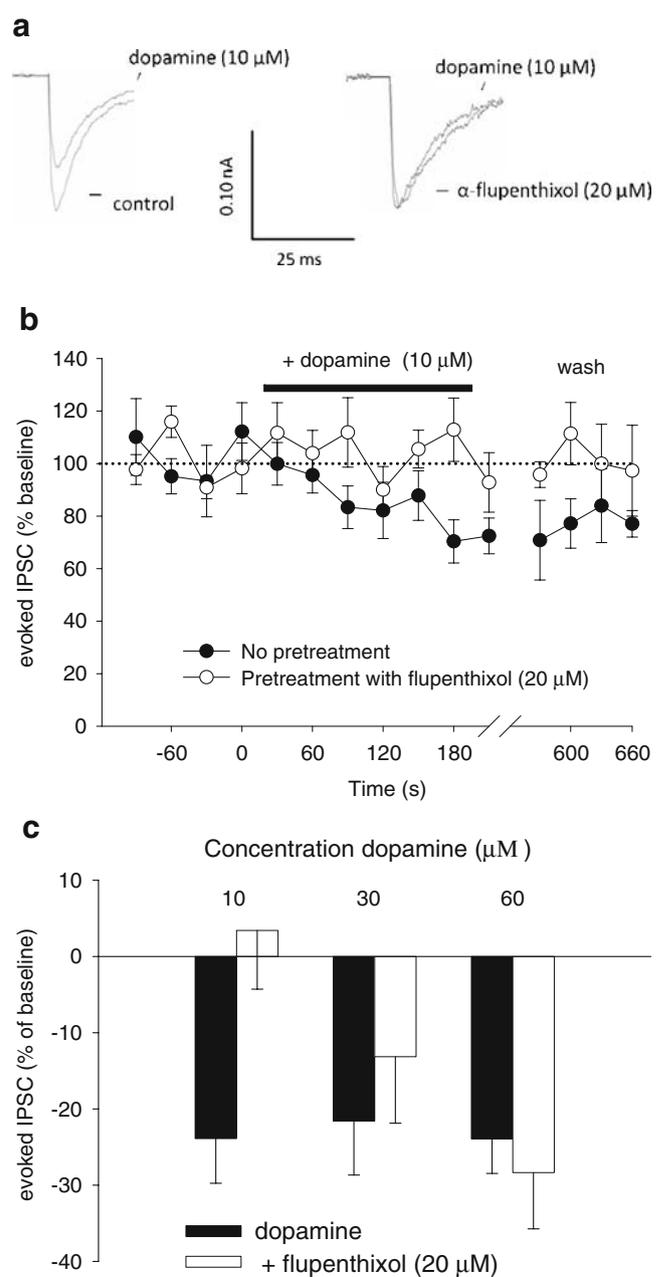


Fig. 6 Dopamine inhibits eIPSCs in the vPAG. **a** Examples of GABAergic eIPSCs and their inhibition by dopamine (10 μ M) in the absence (*left*) and presence (*right*) of α -flupenthixol (20 μ M). **b** Summary of experiments ($n=14-15$ cells per treatment group) in which slices were stimulated every 30 s, and dopamine (10 μ M) was superfused over the slices in the absence or presence of α -flupenthixol (20 μ M). Data are expressed as change from baseline, defined as the average of eight eIPSCs before application of met-enkephalin. Only the last four basal time points are shown. **c** Summary data showing the effect of α -flupenthixol on increasing concentrations of dopamine (10, 30, and 60 μ M). Note that the effect of the competitive antagonist α -flupenthixol was reversed by increasing concentrations of dopamine. Data are expressed as mean \pm SEM

(Fig. 6b). As shown in Fig. 6c, dopamine inhibited eIPSCs overall [$F(1,54)=11.8$; $p<0.01$ for the effect of Time]. α -Flupenthixol blocked the effect of 10 μ M dopamine [$F(1,27)=6.1$; $p<0.05$ for the α -Flupenthixol \times Time interaction]. However, there was no effect of α -flupenthixol on higher doses of dopamine. This is likely due to the displacement of the competitive antagonist α -flupenthixol by the high dopamine doses.

Discussion

These data provide anatomical, behavioral, and electrophysiological evidence for dopaminergic modulation of nociception in the PAG. We verified the presence of dopamine neurons within the vPAG using immunohistochemical stains for TH and DAT, finding an abundance of dopamine cell bodies and fibers within the vPAG. Administration of morphine directly into the vPAG caused marked antinociception, which is consistent with previously published research (Jensen and Yaksh 1986; Morgan et al. 1998). Recent studies (Flores et al. 2004) have found that dopamine antagonists injected directly into the vPAG attenuate the antinociceptive effects of systemically injected morphine and heroin. The present data extend these findings by demonstrating that morphine and dopamine interact within the vPAG.

Previous studies have reported antinociceptive effects of dopamine ligands when injected systemically or into specific brain areas. For example, (–) apomorphine injected into the nucleus raphe magnus (NRM) or intrathecally produced antinociception (Jensen and Smith 1982; Phillips et al. 1992). Microinjection of dopamine into the NRM had a similar although smaller and more transient effect (Phillips et al. 1992). This difference is likely caused by the rapid clearance of dopamine following microinjection. For this reason, we injected (–) apomorphine into the PAG and found substantial antinociception. Activation of dopamine receptors within the vPAG had opioid-like effects in producing antinociception and inhibiting GABA-mediated IPSCs, but there was no effect of the dopamine antagonist α -flupenthixol on opioid-induced inhibition of GABA-mediated IPSCs in vPAG slices. Together, these experiments indicate that dopamine modulation of morphine antinociception does not occur by altering opioid inhibition of GABAergic transmission within the vPAG.

Morphine has been shown to increase dopamine release in several brain areas, including the nucleus accumbens and prefrontal cortex (Di Chiara and Imperato 1988). While it is unknown whether morphine causes an increase in dopamine release within the vPAG, our behavioral data suggest that a portion of morphine's antinociceptive effects are mediated by increases in vPAG dopamine. Studies that have manipulated dopamine in other brain areas support a role

for dopamine in morphine-induced antinociception. For example, microinjection of morphine into the ventral tegmental area, which provides substantial dopaminergic inputs to many brain areas, results in antinociception (Manning et al. 1994). Depletion of dopamine and other biogenic amines with reserpine or 6-OHDA attenuates morphine analgesia (Kaneto and Kihara 1986; Nakamura et al. 1973). In addition, removal of dopaminergic input into the vPAG using 6-OHDA or dopamine antagonists attenuates the antinociceptive effect of systemically administered morphine (Flores et al. 2004). On the cellular level, however, we found that α -flupenthixol did not alter the effect of morphine on GABA eIPSCs.

Alternatively, the finding that intra-vPAG (–) apomorphine and morphine both have antinociceptive effects raises the possibility that dopamine and opioid receptors activate PAG output neurons through a similar mechanism. There is substantial evidence that both dopamine D2 and μ opioid receptors inhibit adenylyl cyclase (for review, see Missale et al. 1998; Williams et al. 2001) and activate potassium channels (Davila et al. 2003; Ingram et al. 1998), which would result in the inhibition of neuronal activity by dopamine and morphine within the vPAG. Further, it is well established that morphine and other opioids activate PAG output neurons through inhibition of GABA release within the vPAG (Ingram et al. 1998; Vaughan et al. 1997). (–) Apomorphine may activate a similar mechanism, as dopamine-mediated inhibition of GABA release has been reported in other brain areas (Nicola and Malenka 1997). In this manner, stimulation of presynaptic dopamine and opioid receptors would result in parallel disinhibition of PAG output neurons. Our current studies support this model by demonstrating that dopamine reduces GABA-mediated IPSCs, an effect blocked by α -flupenthixol. This effect was long lasting; IPSCs were reduced for at least 10 min following dopamine application (Fig. 6b). A similar long-term inhibitory effect of dopamine on GABA IPSCs has been reported in the prefrontal cortex (Seamans et al. 2001). This timecourse also matches the behavioral data: antinociception caused by intra-vPAG (–) apomorphine peaked within 5 min and lasted up to 15 min postinjection (Fig. 3).

This model, however, does not explain the finding that α -flupenthixol inhibited morphine-induced antinociception. It is possible that, in an intact animal, tonic dopamine input into the vPAG modulates opioid effects and that removal of this input reduces the antinociceptive effect of morphine and other opioids. This would suggest that morphine and dopamine interact in a more complex way than through the activation of parallel pathways as described above. However, our electrophysiological data show no evidence for an interaction. Even though the presence of intact dopamine terminals in the PAG can not be ruled out, it is possible that tonic dopaminergic input from other brain areas is removed

in our slice preparation and, therefore, the interaction between dopamine and opioid systems could not be observed. Dopaminergic connections with limbic structures (Beckstead et al. 1979; Simon et al. 1979) would explain why nociception is altered in the absence of an interaction in vPAG slices. Likewise, it is possible that feedback systems necessary for dopamine modulation of opioid antinociception are disrupted by removing the PAG for in vitro recordings.

It also is possible that dopamine modulates opioid effects within the vPAG via a mechanism other than modulation of GABA-mediated eIPSCs. For example, the rightward shift of morphine's effects could be caused by morphine releasing the inhibition of dopamine terminals. This would result in increases in dopamine within the vPAG, resulting in dopamine-mediated antinociception. We chose to study the inhibition of GABA-mediated IPSCs because other studies have suggested that antinociception induced by opioids within the vPAG is due to their effects on GABA neurotransmission (Osborne et al. 1996; Vaughan et al. 1997). However, opioids have other cellular effects within the vPAG, including the activation of voltage-gated potassium channels, calcium channels, and other currents. It is, therefore, possible that dopamine may modulate the effects of opioids on these channels. In addition, dopamine inhibits the release of glutamate within many brain regions (Manzoni and Williams 1999; Margolis et al. 2005). Future studies in our laboratory will investigate whether opioid antagonists alter the antinociceptive effects of dopamine agonists within the vPAG.

α -Flupenthixol is a nonspecific antagonist of the D1 and D2 subtypes of dopamine receptors; therefore, it is unknown which subtype mediates the effects on morphine observed in this study. Studies using systemic injections of specific dopamine receptor ligands reported antinociception produced by D2 agonists but not D1 agonists (Rooney and Sewell 1989). We have shown that the antinociceptive effects of (-) apomorphine were blocked by the D2 receptor antagonist eticlopride and not by the D1 antagonist SCH-23390. However, previous studies have suggested that the D1 receptor is critical for the attenuation seen after systemic opioid injection (Flores et al. 2004). This difference suggests that dopamine receptors modulate morphine antinociception in unique ways in different parts of the nervous system. It may be that dopamine-induced antinociception via the direct activation of D2 receptors, while opioids can indirectly modulate D1 receptors that participate in opioid-induced antinociception. Future studies are needed to fully understand the role of dopamine receptor subtypes in opioid-induced antinociception.

In conclusion, these experiments demonstrate a role for dopamine in nociception and in the modulation of opioid-mediated antinociception. Our data suggest that dopamine

inhibits the GABAergic system in the vPAG similar to opioids. The dopaminergic modulation of nociception within the PAG is likely involved in more complex behavioral phenomena, including the development of opioid tolerance and drug seeking. The vPAG is known to play an important role in tolerance to opioids (Lane et al., 2005; Meyer et al. 2007; Morgan et al. 2006a, b; Tortorici et al. 1999). In addition, the development of opioid-mediated reinforcement and sensitization has been shown to be dependent on PAG dopamine (Flores et al. 2005). Our data suggest that dopamine–opioid interactions within the vPAG have implications for the treatment of pain as well as drug addiction.

Acknowledgements The authors declare that, except for income received from our primary employers, no financial support or compensation has been received from any individual or corporate entity over the for research or professional service and there are no personal financial holdings that could be perceived as constituting a potential conflict of interest. This investigation was supported in part by funds provided for medical and biological research by the State of Washington Initiative Measure No. 171, and by NIH grant DA015498.

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