κ-Opioid Receptor Modulation of GABAergic Inputs onto Ventrolateral Periaqueductal Gray Dopamine Neurons

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Abstract
The κ-opioid receptor (KOR) system has been implicated in the regulation of many behaviors including pain. While there are numerous studies suggesting KOR regulation of pain being mediated spinally, there have been reports of pain-like behaviors regulated by central KOR signaling. In particular, oxytocin-induced analgesia appears to be mediated by KOR receptors within the ventrolateral periaqueductal gray (vlPAG). We recently found that activation of dopamine (DA) neurons within the vlPAG is antinociceptive. In this study, we sought to determine the impact of KOR signaling on GABAergic inputs onto vlPAG DA neurons, and the mechanism through which KOR impacts these inputs. We found that activation of KOR reduced GABAergic transmission onto vlPAG DA neurons. In addition, our data suggest this effect is mediated presynaptically via the G protein βγ-subunit. They raise the possibility that KOR activation disinhibits vlPAG DA neurons, which could lead to altered regulation of pain-related behaviors.

Introduction
As a member of the opioid receptor family, κ-opioid receptors (KORs) have been shown to contribute considerably to analgesia, mood, and substance use disorder [1–4]. They are ubiquitously expressed in the brain, spinal cord, and periphery [5], and are activated endogenously by the opioid peptide dynorphin [6]. Canonically, KOR activation involves the inhibition of cyclic AMP production [7], which is mediated through coupling of the inhibitory G protein G\textsubscript{αi/o} [8]. Through dissociation of the α-subunit, which in turn recruits β-arrestin, KORs activate downstream mitogen-activated protein (MAP) kinases that affect transcription factor expression, such as ERK1/2 [9] and p38 [10]. The βγ-subunit can directly bind and inhibit calcium channels, as well as increase potassium channel conductance [11]. These direct effects on ion channel conductance have been found in several

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brain regions, ranging from the hippocampus to the dor-
sal root ganglia [12]. In addition, phosphorylated KOR
activates ERK1/2, as well as phosphoinositide 3 (PI3)-
kinase and protein kinase A (PKA). Evidence suggests
that ERK signaling mediates KOR activation-induced at-
tenuation of inhibitory neurotransmission in the bed nu-
cleus of the stria terminalis (BNST) [13]. Furthermore,
studies have shown that p38 MAP kinase signaling can
regulate KOR-mediated inhibition of glutamate trans-
mis in the BNST, and is required for negative affect-
ive states, which can be blocked by KOR antagonists
[14]. The divergence in signaling pathways that mediate
the effects of KOR activation is meaningful, as it suggests
that biased agonists could be designed to selectively target
specific pathways to engender different effects.

The A10dc group dopamine (DA) neurons project
from the ventrolateral periaqueductal gray (vPAG) to the
extended amygdala – the BNST and the central amygdala,
areas known to regulate stress, anxiety, and pain-related
behaviors [15–18]. Recent studies by our group have
found that chemogenetic activation of vPAG DA neu-
rons can alter pain-related behaviors. Beyond pain, these
neurons have been implicated in arousal [19] and social
behavior [20]. Of note, antinociception induced by oxy-
tocin can be blocked by KOR antagonism in the PAG
[21]. Mechanistically, it has been shown that KOR activa-
tion can modulate DA neurons in the ventral tegmental
area (VTA) through multiple mechanisms. The postsyn-
aptic effects of KOR appear to be limited to VTA DA neu-
rons projecting to the prefrontal cortex [22], while KOR
regulation of GABAAergic plasticity appears to be presyn-
aptically mediated [23].

Although KORs have been shown to be distributed
widely in the PAG [24], their specific modulation of DA
neurons has yet to be probed due to the heterogeneity of
the PAG. Together, the existing behavioral and electro-
physiological findings lead us to hypothesize that KOR
may serve a role in modulating GABAAergic inputs onto
the vPAG DA neurons.

Materials and Methods

Animals and Husbandry

Male TH-eGFP mice on a Swiss Webster background (aged 5–9
weeks) were bred and used in accordance with an animal use pro-
tocol approved by the University of North Carolina Chapel Hill
(Institutional Animal Care and Use Committee). The mice were
bred and raised under an 11:12-h light–dark cycle, with lights on
at 7:00 a.m. daily. They were given access to rodent
chow and water ad libitum. Mating pairs of mice were created by
GENSAT and obtained from the Mutant Mouse Resource and Re-
search Center in North Carolina. In the TH-eGFP mouse line, the
genome was modified to contain multiple copies of a modified
BAC in which an eGFP reporter gene was inserted immediately
upstream of the coding sequence of the gene for tyrosine hydroxy-
lase (TH). The data presented here were obtained from the trans-
genic mice maintained in-house.

Electrophysiological Brain Slice Preparation

The mice were decapitated under isoflurane anesthesia and
their brains were rapidly removed and placed in ice-cold sucrose
artificial cerebrospinal fluid (ACSF) (in mM) 194 sucrose, 20 NaCl,
4.4 KCl, 2 CaCl2, 1 MgCl2, 1.2 NaH2PO4, 10.0 glucose, and 26.0
NaHCO3 saturated with 95% O2/5% CO2. 300-μm slices were pre-
pared using a Leica VT1200 vibratome (Wetzlar, Germany).

Slice Whole-Cell Electrophysiology

Brain slices containing PAG were obtained and stored at ap-
approximately 30 °C in a heated, oxygenated holding chamber con-
taining ACSF (in mM) 124 NaCl, 4.4 KCl, 2 CaCl2, 1.2 MgSO4, 1
NaH2PO4, 10.0 glucose, and 26.0 NaHCO3 before being trans-
ferred to a submersed recording chamber maintained at approxi-
ately 30 °C (Warner Instruments, Hamden, CT, USA). Recording
electrodes (3–5 MΩ) were pulled with a Flaming-Brown Mi-
cropipette Puller (Sutter Instruments, Novato, CA, USA), using
thin-walled borosilicate glass capillaries. During inhibitory trans-
mission experiments, recording electrodes were filled with (in mM) 70 KCl, 65 K+ gluconate, 5 NaCl, 10 4-(2-hydroxyethyl)-1-pi-
erazinediethanesulfonic acid, 2 QX-314, 0.6 EGTA, 4 ATP, and 0.4
GTP (pH 7.4, 290–295 mOsmol).

In experiments where postsynaptic GPCR signaling was
blocked, GDPβS was used to replace GTP in the internal solution.
All experiments were conducted under the voltage clamp configu-
ration, cells were held at –70 mV, and inhibitory postsynaptic cur-
rents (IPSCs) were pharmacologically isolated with 3 mM kyn-
urenate, to block AMPA (α-amino-3-hydroxy-5-methyl-4-
isoazole-propionic acid) and NMDA (N-methyl-d-aspartate)
receptor-dependent postsynaptic currents. To isolate miniature
IPSCs (mIPSCs), tetrodotoxin (0.5 μM) was added to the perfusing
ACSF solutions described above. Signals were acquired via a Mul-
tiClamp 700B amplifier (Molecular Devices, Sunnyvale, CA, USA),
digitized at 20 kHz, filtered at 3 kHz, and analyzed using Clampfit

Fig. 1. The κ-opioid receptor (KOR) agonist dynorphin A (dyn A; 300 nM) decreases miniature inhibitory postsynaptic currents
(mIPSCs) in ventrolateral periaqueductal gray dopamine neurons via KOR actions. a Representative mIPSC traces at baseline (con-
trol; i) and after 10 min of 300-nM dynorphin A wash-on (ii). b Dynorphin A significantly decreased mIPSC frequency. c Dy-
norphin A had no effect on mIPSC amplitude. d The cumulative
frequency of the mIPSC was shifted towards longer interevent in-
tervals by dynorphin A. e The cumulative probability of the mIPSC
amplitude was not affected by dynorphin A. f Dynorphin A no
longer decreased mIPSC frequency in the presence of norbinaltor-
phimine (nor-BNI; 100 nM). g Dynorphin A had no effect on
mIPSC amplitude in the presence of nor-BNI. h Nor-BNI alone
had no effect on spontaneous (s)IPSC frequency. i Nor-BNI alone
had no effect on sIPSC amplitude. * p < 0.05.

(For figure see next page.)
Dynorphin A mIPSC frequency

Nor-BNI dynorphin A mIPSC frequency

Nor-BNI sIPSC frequency

Dynorphin A mIPSC amplitude

Nor-BNI dynorphin A mIPSC amplitude

Nor-BNI sIPSC amplitude

Cumulative frequency

Interevent interval, ms

Amplitude, pA

Color version available online
Fig. 2. The postsynaptic GPCR inhibitor GDPβS (4 mM), diffused into the recorded ventrolateral periaqueductal gray dopamine neurons, did not block dynorphin A (dyn A) from attenuating miniature inhibitory postsynaptic current (mIPSC) frequency, suggesting a presynaptic mechanism. a Inhibition of postsynaptic GPCR did not hinder dynorphin A from attenuating mIPSC frequency. b Dynorphin A had no effect on mIPSC amplitude in the presence of GDPβS. c GDPβS did not block dynorphin A’s ability to shift the mIPSC cumulative frequency towards longer interevent intervals. d Dynorphin A had no effect on mIPSC amplitude cumulative probability. * p < 0.05.

Statistics
Effects of drugs during electrophysiological recordings were evaluated by comparing the magnitude of the dependent measure (mIPSC frequency and amplitude) between the baseline and washout (when the drug had reached its maximal effect at 10 min) periods using paired t tests. The effects of antagonists/blockers on the ability of the drugs to modulate synaptic transmission were compared using t tests during the washout period. All values given for drug effects throughout the article are presented as mean ± SEM.

Drugs
Dynorphin A (300 nM) and norbinaltorphimine (nor-BNI; 100 nM) were purchased from Tocris (Ellisville, MO, USA) and dissolved in distilled water. BAPTA-AM (50 µM), gallein (100 µM), and wortmannin (1 µM) were purchased from Tocris and dissolved in DMSO. 4-[4-(4-Fluorophenyl)-2-(4-[methyisulfanyl]phenyl)-1H-imidazol-5-yl]pyridine (SB203580; 20 µM) and 4-aminopyridine (4-AP; 100 µM) were purchased from Ascent and dissolved in distilled water; α-[amino([4-aminophenyl]thio)methylene]-2-(trifluoromethyl)benzeneacetonitrile (SL327; 10 µM) was purchased from Ascent and dissolved in DMSO. Tetrodotoxin citrate (500 nM) and kynurenic acid (3 mM) were purchased from Abcam and dissolved in water. GDPβS (4 mM) and RP-adenosine 3′,5′-cyclic monophosphorothioate triethylammonium salt hydrate (RP-camps; 10 µM) were purchased from Sigma-Aldrich and dissolved in water. EGTA (100 µM) was obtained from Fisher Scientific and dissolved in 1 M NaOH.

Results
Dynorphin A Attenuates GABAergic Input onto vlPAG DA Neurons via a Presynaptic Mechanism
We first examined the effects of KOR activation on GABA synaptic transmission via bath application of the
endogenous ligand dynorphin A (300 nM). A 10-min bath application of dynorphin A significantly attenuated the mIPSC in vlPAG DA neurons (Fig. 1a; \(n = 5\)). Specifically, a decrease was seen in mIPSC frequency (69.5 ± 7.4% of baseline, \(p = 0.03\); Fig. 1b, d), but not in amplitude (99.3 ± 4.8% of baseline; Fig. 1c, e), suggesting a presynaptic mechanism. To further confirm that the dynorphin effect observed was mediated through KOR activation, we incubated the slices in a selective KOR antagonist, nor-BNI (100 nM), for 40 min before and during dynorphin wash-on (\(n = 5\)). In the presence of nor-BNI, dynorphin A application failed to produce effects on either mIPSC frequency (93.3 ± 10.3% of baseline; Fig. 1f) or amplitude (89.4 ± 6.1% of baseline; Fig. 1g), indicating no tonic KOR activation in the vlPAG DA neurons. Combined, these data suggest a presynaptic effect of dynorphin on vlPAG DA neurons.

We further verified this presynaptic mechanism via blockage of postsynaptic GPCR functions by the replacement of GTP with GDP\(_{\beta}S\) in the recording pipette, disrupting the exchange of GTP and GDP, thus interfering with downstream signaling cascades upon GPCR activation. With postsynaptic GPCR function impaired, the application of dynorphin A still decreased mIPSC frequency (54.7 ± 5.8% of baseline, \(n = 6\), \(p = 0.04\); Fig. 2a, c), but not amplitude (97.8 ± 11.3% of baseline; Fig. 2b, d), providing additional support for dynorphin attenuating inhibitory input onto vlPAG DA neurons via a presynaptic mechanism.

**Effects of Dynorphin on GABA Are Not Mediated through MAP Kinase Signaling**

To identify the downstream signaling mechanisms through which KOR modulates GABAergic transmission, we examined the role of the MAP kinases ERK1/2 and p38. Brain slices were incubated in either a selective MEK inhibitor (SL327, 10 µM, \(n = 6\)) or p38 inhibitor (SB203580, 20 µM, \(n = 5\)) for 40 min before and during

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**Fig. 3.** The ERK1/2 inhibitor SL327 (10 µM) and the p38 inhibitor SB203580 (20 µM) did not hinder dynorphin A from attenuating miniature inhibitory postsynaptic current (mIPSC) frequency. **a** Dynorphin A significantly decreased mIPSC frequency in the presence of SL327. **b** Dynorphin A had no effect on mIPSC amplitude in the presence of SL327. **c** Dynorphin A significantly decreased mIPSC frequency in the presence of SB203580. **d** Dynorphin A significantly decreased mIPSC amplitude in the presence of SB203580. * \(p < 0.05\).
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Dynorphin wash-on. In the presence of the MEK inhibitor SL327, dynorphin A significantly decreased mIPSC frequency (67.2 ± 10.6% of baseline, \( p = 0.04 \); Fig. 3a), but not amplitude (97.1 ± 4.3% of baseline; Fig. 3a). In the presence of the p38 inhibitor SB203580, dynorphin A significantly decreased mIPSC frequency (46.6 ± 9.9% of baseline, \( p < 0.05 \); Fig. 3b) and amplitude (83.2 ± 3.6% of baseline, \( p = 0.02 \); Fig. 3b). Neither SL327 nor p38 altered the dynorphin-induced attenuation of GABAergic input onto vlPAG DA neurons, suggesting this observation was not mediated through MAP kinase signaling.

**Effects of Dynorphin on GABA Are Not Mediated through Calcium and Potassium Ion Channel Conductance**

Agonist-induced dissociation of the \( \beta \gamma \)-subunit from the GPCR can directly influence the conductance of ion channels. Thus, we investigated the roles of calcium and potassium channels in dynorphin’s modulation of the GABA-mediated IPSC. To test the role of calcium channels, we incubated the slices in calcium-free ACSF and the selective calcium chelators BAPTA-AM (50 \( \mu \)M) and EGTA (100 \( \mu \)M) for 1–2 h before recording, and continued to record from the slices in calcium-free ACSF in the presence of just EGTA, or EGTA plus 4-AP (100 \( \mu \)M), to block potassium channels. In the calcium-free experiments, dynorphin A significantly decreased mIPSC frequency (71.9 ± 8.6% of baseline, \( p = 0.02 \), \( n = 6 \); Fig. 4a), but not amplitude (102.3 ± 9.6% of baseline; Fig. 4a). In the calcium-free experiments where potassium channels were blocked with 4-AP, dynorphin A still caused a significant decrease in mIPSC frequency (77.8 ± 8.0% of baseline, \( p < 0.05 \), \( n = 8 \); Fig. 4b), but not in amplitude (107.5 ± 5.7% of baseline; Fig. 4b). Taken together, these data suggest that KOR inhibition of GABA release in vlPAG DA neurons was not mediated via inhibition of calcium or potassium channels.

**Fig. 4.** \( \kappa \)-Opioid receptor (KOR) inhibition does not require calcium and potassium ion channels. The GPCR \( \beta \gamma \)-subunit inhibitor hindered dynorphin A from attenuating miniature inhibitory postsynaptic current frequency. \( a \) KOR/dynorphin A inhibition persisted even when all \( Ca^{2+} \) was removed by incubating slices in 0 mM \( Ca^{2+} /4 \) mM \( Mg^{2+} \), 100 \( \mu \)M EGTA with 50 \( \mu \)M BAPTA-AM. \( b \) KOR/dynorphin A inhibition persisted even when all \( Ca^{2+} \) was removed and \( K^{+} \) channels were blocked with 4-aminopyridine (4-AP; 100 \( \mu \)M). * \( p < 0.05 \).
Effects of Dynorphin on GABA Are Mediated through \( \beta\gamma \)-Subunit-Dependent Signaling

We explored the role of the \( \beta\gamma \)-subunits in KOR inhibition of GABA transmission in vlPAG DA neurons beyond direct influence on ion channels by incubating slices in gallein (100 \( \mu \)M), an inhibitor of G protein \( \beta\gamma \)-subunit-dependent signaling. The previously observed KOR activation-induced reduction of GABA transmission was blocked in the presence of gallein (\( n = 6 \)), with no significant changes in mIPSC frequency (107.1 ± 7.1% of baseline; Fig. 5a) or amplitude (102.8 ± 7.4% of baseline; Fig. 5a). Together these data suggest that the effects

![Fig. 5. Inhibition of G protein \( \beta\gamma \)-signaling but not phosphoinositide 3 (PI3)-kinase or protein kinase A (PKA) disrupts \( \kappa \)-opioid receptor effects on GABA release in the ventrolateral periaqueductal gray. a Gallein (100 \( \mu \)M) prevented dynorphin A from inhibiting miniature inhibitory postsynaptic current (mIPSC) frequency. mIPSC amplitude remained unaffected. The PI3-kinase inhibitor wortmannin (1 \( \mu \)M) and the PKA inhibitor RP-camps (10 \( \mu \)M) did not hinder dynorphin A from attenuating mIPSC frequency. b Dynorphin A significantly decreased mIPSC frequency but had no effect on amplitude in the presence of wortmannin. c Dynorphin A decreased mIPSC frequency (\( p = 0.051 \)) but had no effect on amplitude in the presence of RP-camps.](image-url)
of KOR on GABAergic transmission were mediated via βγ-subunit signaling, but not through the change in conductance of ion channels.

Because gallein has been shown to not only inhibit the βγ-subunit but also activate PI3-kinase activity [25], we sought to clarify the role of PI3-kinase with wortmannin, a PI3-kinase inhibitor, as well as PKA signaling. We incubated slices in the PI3-kinase inhibitor wortmannin (1 µM) or the PKA inhibitor RP-camps (10 µM). Wortmannin did not block the previously observed decrease in GABA transmission frequency (72.4 ± 7.1% of baseline; Fig. 5b). RP-camps also did not block the KOR-induced decrease in GABA frequency (73.0 ± 7.0% of baseline, n = 5, p = 0.051; Fig. 5c), and there were no effects on amplitude. These data suggest that PI3-kinase and PKA do not mediate the KOR activation-induced decrease in GABA function in the vlPAG.

An overview of the mIPSC frequency raw values in vlPAG dopamine neurons under various treatments is shown in Table 1.

**Discussion**

vlPAG DA neurons have been implicated in a variety of behaviors, particularly the regulation of pain. Notably, KOR signaling in the vlPAG has been connected with oxytocin-induced analgesia. This study focused on deter-
mining the effects of KOR on inhibitory synaptic transmission onto vlPAG DA neurons – specifically, the downstream signaling mechanisms through which the effects take place. Briefly, we found that KOR can inhibit GABA release onto these neurons via a G protein βγ-subunit-dependent mechanism. The effect of KOR-induced inhibition of GABA release is consistent with findings in previous studies regarding the BNST [13], and it was abolished in the presence of a KOR antagonist (nor-BNI) in both regions, suggesting KOR-selective mediation. Although all vlPAG DA neurons recorded in this study showed a decrease in GABA-mediated IPSCs, studies on the VTA demonstrated that KOR attenuates inhibitory transmission selectively onto DA neurons that project to the basolateral amygdala [26, 27]. This raises the interesting possibility that KOR can serve to reduce the inhibitory drive onto DA neurons that project to amygdalar regions, perhaps supporting stress engagement of these pathways.

Using a pharmacological approach, we found that inhibition of G protein βγ-subunit-dependent signaling successfully prevented the dynorphin A-induced decrease in mIPSC frequency. Because gallein has been shown to not only inhibit βγ-subunit but also activate PI3-kinase activity [25], we sought to clarify the role of PI3-kinase with wortmannin, a PI3-kinase inhibitor. Our results suggested that KOR-induced reduction of synaptic inhibition lies outside of the actions of PI3-kinase. Further, our data demonstrated that in the vlPAG DA neurons, neither calcium nor potassium channels contributed to the presynaptic inhibition of GABA release. These results raised the possibility that dynorphin A could be activating KORs and directly affect the presynaptic release machinery in GABAergic inputs onto vlPAG DA neurons (Fig. 6). These data are similar to those from studies on KOR presynaptic inhibition of glutamatergic inputs in the hypothalamus, with effects persisting in the absence of presynaptic calcium channel activity and independent of cAMP signaling [28]. Although neither Iremonger and Bains [28] nor we were able to identify a precise molecular target through which dynorphin A inhibits presynaptic GABA release, the results are consistent with studies proposing a direct modulation of the exocytotic-release machinery by the βγ-subunit of the Ga1/0-coupled GPCR [29, 30].

It is currently unclear how KOR modulation of vlPAG DA neurons alters behavior. Given the prominent role that the vlPAG plays in pain and negative affect processing, as well as the correlation of KOR functions with emotional behaviors, it is tempting to speculate that KOR actions on this circuit are involved in these processes. Based on the hypothesis that KOR presynaptically inhibits GABAergic inputs, disinhibiting the vlPAG DA neurons to potentially modulate projection areas and related behaviors, further elucidation is needed regarding how KOR modulates the glutamatergic inputs, as well as the overall effect on activity of the vlPAG DA neurons. Future studies utilizing optogenetics to probe pathway-defined plasticity, as well as applying designer receptors exclusively activated by designer drugs (DREADD) to characterize pathway- and cell type-specific modulation, will likely shed light on this exciting possibility.

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**Statement of Ethics**

Animals were used and experiments were conducted in accordance with an animal use protocol approved by the University of North Carolina Chapel Hill (Institutional Animal Care and Use Committee).

**Disclosure Statement**

The authors have no conflicts of interest to declare.

| Drug            | Treatment      | mIPSC frequency (±SEM) |
|-----------------|----------------|-------------------------|
| Dynorphin A     | –              | 1.4±0.4 (n = 5)         |
| 4-AP            |                | 1.1±0.4 (n = 7)         |
| [0] Ca2+        |                | 0.4±0.1 (n = 6)         |
| [0] Ca2+ + 4-AP |                | 0.6±0.1 (n = 8)         |
| GDPβS           |                | 1.3±0.4 (n = 6)         |
| Gallein         |                | 1.6±0.4 (n = 6)         |
| Nor-BNI         |                | 1.1±0.2 (n = 5)         |
| RP-camps        |                | 1.4±0.3 (n = 5)         |
| SB203580        |                | 1.7±0.5 (n = 5)         |
| SL327           |                | 1.1±0.1 (n = 6)         |
| Wortmannin      |                | 1.0±0.1 (n = 6)         |

Nor-BNI – 1.0±0.2 (sIPSC) (n = 6)

mIPSC, miniature inhibitory postsynaptic current; sIPSC, spontaneous IPSC; 4-AP, 4-aminopyridine; nor-BNI, norbinaltorphimine.
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**Author Contributions**

Both authors conceived the studies; C.L. conducted the studies with assistance from T.L.K.; C.L. wrote the initial manuscript; T.L.K. edited the manuscript; C.L. prepared the final manuscript.

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