Cellular/Molecular

# Orexin Signaling in the VTA Gates Morphine-Induced Synaptic Plasticity

**Corey Baimel[1,2] and Stephanie L. Borgland[2]**

[1]Department of Anesthesiology, Pharmacology and Therapeutics, University of British Columbia, Vancouver, British Columbia V6T 1Z3, Canada, and [2]Hotchkiss Brain Institute, University of Calgary, Calgary, Alberta T2N 4N1, Canada

**Dopamine neurons in the ventral tegmental area (VTA) are a key target of addictive drugs, and neuroplasticity in this region may underlie some of the core features of addiction. From the very first exposure, all drugs of abuse induce synaptic plasticity in the VTA. However, it is not well understood how this diverse group of drugs brings about common synaptic change. Orexin (also known as hypocretin) is a lateral hypothalamic neuropeptide released into the VTA that promotes drug-seeking behaviors and potentiates excitatory synaptic transmission onto VTA dopamine neurons. Here we show that signaling at orexin receptor type 1 (OxR1) in the VTA is required for morphine-induced plasticity of dopamine neurons. Systemic or intra-VTA administration of the OxR1 antagonist SB 334867 in rats blocked a morphine-induced increase in the AMPAR/NMDAR ratio, an increase in presynaptic glutamate release, and a postsynaptic change in AMPAR number or function, including a switch in subunit composition. Furthermore, SB 334867 blocked a morphine-induced decrease in presynaptic GABA release, and a morphine-induced shift in the balance of excitatory and inhibitory synaptic inputs to dopamine neurons. These findings identify a novel role for orexin in morphine-induced plasticity in the VTA and provide a mechanism by which orexin can gate the output of dopamine neurons.**

*Key words:* **AMPA; dopamine; morphine; NMDA; orexin; ventral tegmental area**

## Introduction

The mesolimbic dopamine system is a well recognized target of addictive drugs, and plasticity within this neural circuitry is involved in the development and maintenance of addiction (Hyman et al., 2006; Kauer and Malenka, 2007). The activity and output of ventral tegmental area (VTA) dopamine neurons are tightly regulated by excitatory glutamate and inhibitory GABA synaptic inputs, and drug-induced plasticity at these synapses occurs with the very first drug exposure. A single *in vivo* injection of multiple addictive drugs induces a long-term potentiation (LTP) of excitatory synaptic transmission in the VTA (Ungless et al., 2001; Saal et al., 2003; Brown et al., 2010). This drug-evoked plasticity is thought to increase the incentive properties of the drug, to mediate early behavioral responses to drug exposure, and to trigger long-term neural adaptations in regions that receive dopamine input (Kauer and Malenka, 2007). Moreover, drug treatment inhibits inhibitory control mechanisms that increase GABA transmission to the VTA [GABAergic LTP ($LTP_{GABA}$); Nugent et al., 2007; Niehaus et al., 2010; Graziane et al., 2013], likely further increasing the incentive properties of the drug.

Orexin neurons have emerged as an important mediator of drug-seeking behavior and alter synaptic transmission in the VTA. Orexins are neuropeptides synthesized in the lateral hypothalamus (LH) that contribute to arousal, feeding, and reward seeking (Sakurai et al., 1998; de Lecea et al., 1998; Harris et al., 2005; Adamantidis et al., 2007). Orexin A and B signal at two G-protein-coupled receptors, OxR1 and OxR2, which are widely expressed in the brain, including in the VTA (Korotkova et al., 2003; Narita et al., 2006). Orexin neurons are activated by drug cues (Harris et al., 2005), and drug administration enhances excitatory drive at glutamate synapses onto orexin neurons (Yeoh et al., 2012; Rao et al., 2013). Importantly, orexins mediate drug seeking through their projections to the VTA (Harris et al., 2005; Borgland et al., 2006; Narita et al., 2006). Orexins increase the firing rate of dopamine neurons and can enhance dopamine release in downstream target structures (Korotkova et al., 2003; Narita et al., 2006, 2007; Vittoz and Berridge, 2006; Vittoz et al., 2008; España et al., 2010). Short-term administration of orexin A increases NMDA receptor (NMDAR) current amplitudes in dopamine neurons (Borgland et al., 2006), an effect that is enhanced by long-term drug self-administration (Borgland et al., 2009), and promotes a long-term increase in AMPAR receptor (AMPAR) signaling (Borgland et al., 2006).

Drugs of abuse differ greatly in terms of their behavioral profiles and molecular targets. Although the mechanisms by which addictive drugs increase phasic dopamine release have been well characterized (Lüscher and Ungless, 2006), it is unclear how these drugs all converge to induce synaptic plasticity in the VTA. Pre-

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Correspondence should be addressed to Stephanie L. Borgland, Hotchkiss Brain Institute, University of Calgary, 3330 Hospital Drive NW, Calgary, AB T2N 4N1, Canada. E-mail: s.borgland@ucalgary.ca.

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viously, we found that systemic administration of an OxR1 antagonist blocks both cocaine-induced synaptic plasticity at excitatory synapses in the VTA and the development of behavioral sensitization (Borgland et al., 2006). However, it is unknown whether orexin contributes to plasticity in the VTA induced by other drugs with different mechanisms of action. Here, we used whole-cell patch-clamp electrophysiology to elucidate the contribution of orexin signaling to morphine-induced synaptic plasticity of VTA dopamine neurons.

## Materials and Methods

*Animals.* All protocols were performed in accordance with the ethical guidelines established by the Canadian Council for Animal Care, and were approved by the University of British Columbia and the University of Calgary Animal Care Committees. Sprague Dawley rats were obtained from the University of British Columbia breeding facility or Charles River Laboratories and were housed in groups of two to six, except following surgery, at which time rats were single housed. Rats were maintained on a 12 h light/dark schedule, and were given food and water *ad libitum*. All experiments were performed during the animals' light cycle.

*Electrophysiology.* All electrophysiological recordings were performed in slice preparations from male Sprague Dawley rats [postnatal day 19 (P19) to P30]. Briefly, rats were anesthetized with isoflurane and decapitated, and their brains were extracted into ice-cold sucrose solution containing the following (in mM): 75 sucrose, 87 NaCl, 2.5 KCl, 1.25 $NaH_2PO_4$, 25 $NaHCO_3$, 7 $MgCl_2$, and 0.95 $CaCl_2$. Horizontal sections (250 $\mu$m) containing the VTA were cut on a vibratome (Leica), and incubated in a holding chamber for at least 45 min before being transferred to a recording chamber and superfused with bicarbonate-buffered solution (artificial CSF) containing the following (in mM): 126 NaCl, 1.6 KCl, 1.1 $NaH_2PO_4$, 1.4 $MgCl_2$, 2.4 $CaCl_2$, 26 $NaHCO_3$, and 11 glucose (32C–34C), and saturated with 95% $O_2$/5% $CO_2$. Cells were visualized using infrared differential contrast video microscopy, and whole-cell voltage-clamp recordings were made using a MultiClamp 700B Amplifier (Molecular Devices). Recording electrodes (3–5 M$\Omega$) were filled with the following (in mM): 117 cesium methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 Mg-ATP, and 0.25 Na-GTP, pH 7.2–7.3 and 270–285 mOsm to record EPSCs; and 0.1 mM spermine was added only when examining the rectification index. For IPSCs, electrodes were filled with the following (in mM): 125 KCl, 2.8 NaCl, 2 $MgCl_2$, 10 HEPES, 0.6 EGTA, 2.5 Mg-ATP, and 0.25 Na-GTP, pH 7.2–7.3 and 270–285 mOsm. Series resistance (10–25 M$\Omega$) and input resistance were monitored on-line with a 10 mV depolarizing step (400 ms) given before every afferent stimulus. Dopamine neurons, which are the most abundant cell type in the VTA, were located medial to the medial nucleus of the optic tract and were identified by the presence of a large hyperpolarizing cation current, and in a subset of cells by tyrosine hydroxylase staining according to previously published methods (Thompson and Borgland, 2013). A bipolar stimulating electrode was placed 100–300 $\mu$m rostral to the recording electrode and was used to stimulate either excitatory or inhibitory afferents at 0.1 Hz. Neurons were voltage clamped at −70 mV to record AMPAR EPSCs. EPSCs were recorded in the presence of picrotoxin (100 $\mu$M) to block $GABA_A$ receptor IPSCs; and IPSCs were recorded with strychnine (1 $\mu$M) to block potential glycine IPSCs (Zheng and Johnson, 2001); with AP5 (50 $\mu$M) and DNQX (10 $\mu$M) to block excitatory synaptic transmission; and with sulpiride (200 nM) to block dopamine $D_2$ receptor IPSCs (Gantz et al., 2013) in the bath solution.

The AMPAR/NMDAR ratio was recorded at +40 mV and was calculated according to previously published methods (Borgland et al., 2006). For the rectification index, AMPAR EPSCs were evoked at −70, 0, and +40 mV in the presence of AP5 (50 $\mu$M) and the polyamine spermine (via pipette, 0.1 mM). The rectification index was calculated by dividing the gradient of the slope at negative potentials by the gradient of the slope at positive potentials.

Miniature EPSCs (mEPSCs) and miniature IPSCs (mIPSCs) were recorded at −70 mV in the presence of tetrodotoxin (500 nM) to block action potential-driven spontaneous events. AMPAR mEPSCs were selected based on their amplitude (>12 pA), decay time (<3 ms), and rise time (<1 ms) using the MiniAnalysis program (Synaptosoft). Similarly, $GABA_A$ mIPSCs were selected for amplitude (>12 pA), rise time (<4 ms), and decay time (<10 ms).

The excitatory/inhibitory balance was recorded with an internal solution containing the following (in mM): 132 cesium methanesulfonate, 8 CsCl, 10 HEPES, 0.6 EGTA, 4 Mg-ATP, 0.3 Na-GTP, and 10 Na-phosphocreatine. EPSCs were recorded at the reversal potential for $GABA_A$ IPSCs (−67 mV), and IPSCs were recorded at the reversal potential for EPSCs (+8 mV). The $G_e/G_i$ ratio was calculated by converting the average peak current amplitude of the EPSC and the IPSC into a conductance.

*Systemic drug treatment.* Rats were removed from their home cage and placed individually in a clean cage. Rats were given either SB 334867 (10 mg/kg, i.p.; Tocris Bioscience) or vehicle (10% DMSO, 20% $\beta$-hydroxy cyclodextrin in saline), or were not given an injection (naive rats). Fifteen minutes later, rats were treated with morphine HCl (10 mg/kg, i.p.) or an equivalent volume of saline (0.9% NaCl). Following injection procedures, rats were returned to their home cage and were left for 24 h.

*Surgical procedures.* Sprague Dawley rats (50–60 g at the start of the experiment) were housed individually following surgical procedures. Animals were anesthetized with isoflurane and placed in a stereotaxic frame (Kopf) and bilateral cannulas (26 gauge; Plastics One) were lowered into the VTA (coordinates in mm: anteroposterior, −4.6 to −5.2; mediolateral, ± 0.5; dorsoventral, −7.0). As a control, some animals were implanted with cannulas that only reached dorsoventral −5.0 mm to test infusions outside of the VTA. Cannulas were anchored to the skull surface with dental cement and were occluded with a dummy cannula of the same length. Rats were treated postsurgically with ketoprofen (5 mg/kg, s.c.), and weights were monitored daily during the recovery period.

*Intra-VTA drug infusions.* During the recovery period, rats were familiarized with the infusion procedures with three habituation sessions. During these sessions, rats were transferred from their home cage to a clean cage and were left for 30 min. Afterward, rats were handled for 5–10 min and the dummy cannula was replaced with a microinjector, cut above the length of the cannula to prevent tissue damage to the VTA. The microinjector was attached to a Hamilton syringe with tubing, and a mock infusion was performed with a microinfusion pump. The microinjector was left in place for 5 min before being replaced with the dummy cannula, and rats were left in the injection cage for 15 min before being returned to their home cage. On injection day, microinfusions were conducted using 33 gauge microinjectors that protruded 0.5 mm below the base of the guide cannula to a final dorsoventral coordinate of 7.5 mm. SB 334867 (0.3 or 0.03 nmol/0.3 $\mu$l) or vehicle (50% saline, 50% DMSO) were infused bilaterally into the VTA (0.3 $\mu$l/side at 0.1 $\mu$l/min) with the microinfusion pump. Microinjectors were left in place for 5 min following the injection, at which point rats were injected systemically with morphine (10 mg/kg, i.p.) or saline. Rats were then returned to their home cage and were left for 24 h.

*Data analysis.* All values are expressed as the mean ± SEM. Statistical significance was assessed using two-way or one-way ANOVA with Bonferroni post-tests. In all experiments, the sample size is expressed as $N/n$ where $N$ refers to the number of cells recorded from $n$ animals. Prism 5 software (GraphPad Software) was used to perform the statistical analysis. Figures were generated using Illustrator CS2 software (Adobe Systems). The levels of significance are indicated as follows: $^{***}p < 0.001$, $^{**}p < 0.01$, $^{*}p < 0.05$.

## Results

### OxR1 signaling is required for morphine-induced potentiation of excitatory inputs to VTA dopamine neurons

Morphine, like other addictive drugs, induces an LTP-like potentiation of excitatory inputs to VTA dopamine neurons (Ungless et al., 2001; Saal et al., 2003; Borgland et al., 2004; Brown et al., 2010). To examine the role for orexin signaling in morphine-induced plasticity, we measured the relative contribution of AMPAR and NMDAR EPSCs 24 h after a single injection of morphine (10 mg/kg) or saline (0.9% NaCl), with or without pretreatment with the OxR1 antagonist SB 334867 (10 mg/kg).

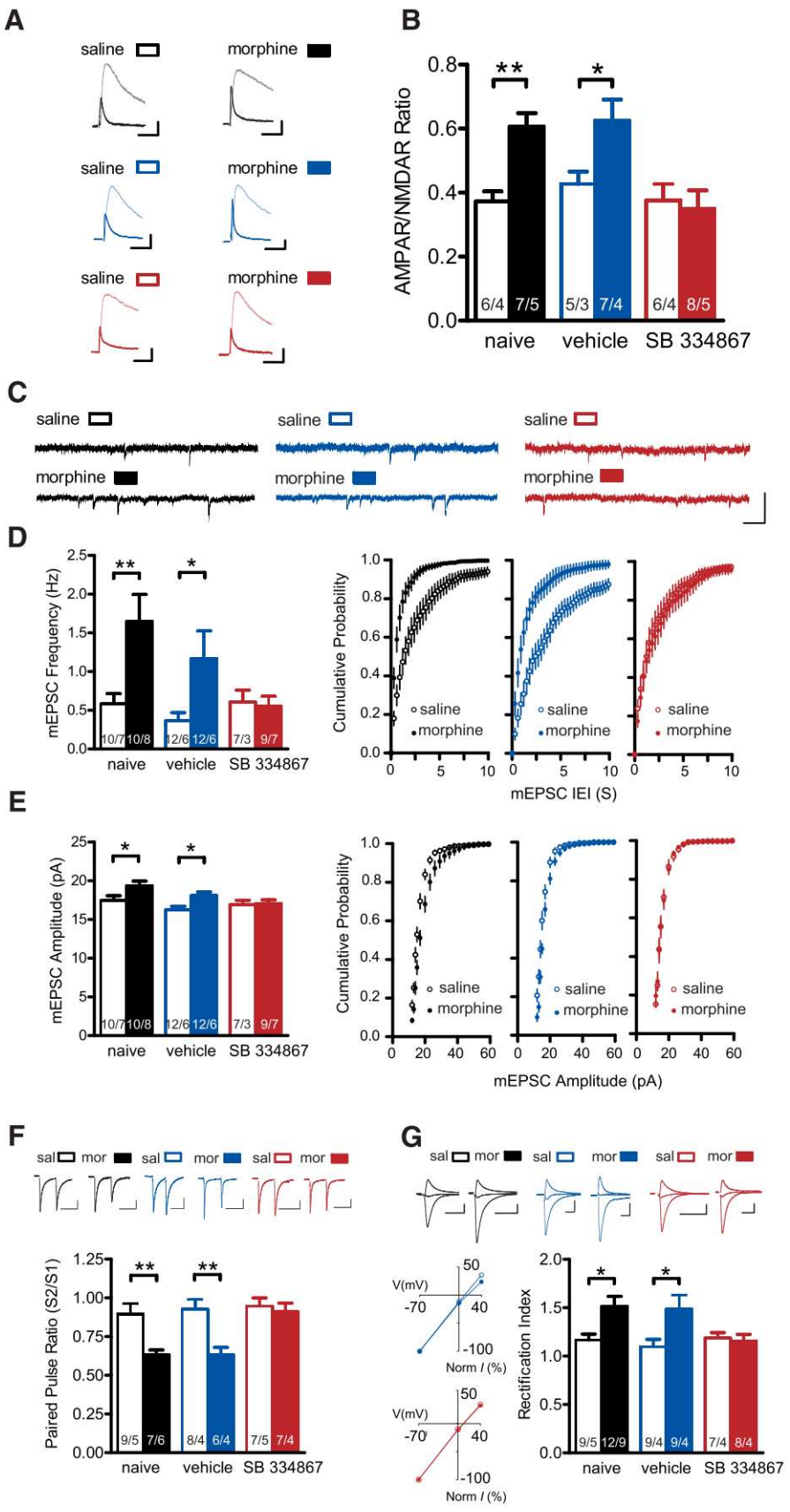


**Figure 1.** Systemic administration of the OxR1 antagonist SB 334867 blocks morphine-induced potentiation of excitatory transmission onto VTA dopamine neurons. ***A***, Example recordings of evoked NMDAR (light) and AMPAR (dark) EPSCs at +40 mV, from VTA dopamine neurons of rats 24 h after exposure to morphine or saline in naive (top), vehicle-treated (middle), and SB 334867-treated (10 mg/kg; bottom) rats. Calibration: 50 pA, 20 ms. ***B***, Treatment with morphine (10 mg/kg, filled bars), but not saline (open bars), increased the AMPAR/NMDAR ratio in naive and vehicle-treated, but not in SB 334867-treated rats ($p < 0.05$, two-way ANOVA). ***C***, Example traces of AMPAR mEPSCs recorded at −70 mV 24 h after morphine or saline treatment in naive (left), vehicle-treated (center), and SB 334867-treated (right) rats. Calibration: 50 pA, 100 ms. ***D***, Left, AMPAR mEPSC frequency was increased in morphine-treated rats compared with saline-treated rats in naive and vehicle-treated, but not in SB 334867-treated rats ($p < 0.05$, two-way ANOVA). Right, Cumulative probability plots comparing morphine or saline exposure on mEPSCs for naive, vehicle-treated, and SB 334867-treated animals. ***E***, Left, Morphine increased AMPAR mEPSC amplitude compared with saline in naive and vehicle-treated, but not SB 334867-treated rats ($p < 0.05$, two-way ANOVA). Right, Cumulative probability plots comparing morphine or saline exposure on mEPSC amplitude for naive, vehicle-treated, and SB 334867-treated rats. ***F***, Morphine (filled bars) induced a paired-pulse depression of evoked AMPAR EPSCs in naive and vehicle-treated rats, but not in SB 334867-treated rats ($p < 0.05$, two-way ANOVA). Inset, Sample traces of evoked AMPAR EPSC paired pulses recorded at −70 mV. Calibration: 50 pA, 20 ms. ***G***, Pretreatment with SB 334867 blocked a morphine-induced increase in the rectification index ($p < 0.05$, two-way ANOVA). Inset, Sample traces of AMPAR EPSCs recorded at −70, 0, and +40 mV with spermine in the pipette solution. Current–voltage relationship of AMPAR EPSCs for morphine and saline in vehicle-treated and SB 334867-treated rats. Calibration: 50 pA, 10 ms. $n/N$ = cells/rats. Bars represent the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$.

Consistent with previous observations (Saal et al., 2003), a single injection of morphine significantly potentiated the AMPAR/NMDAR ratio in both naive (saline, 0.4 ± 0.03; morphine, 0.6 ± 0.04; $p < 0.01$) and vehicle-treated rats [saline, 0.4 ± 0.04; morphine, 0.6 ± 0.06; $p < 0.05$; Fig. 1*A*,*B*; two-way-ANOVA: drug (morphine vs saline) × pretreatment (naive, vehicle, SB 334867) interaction, $F_{(2,33)} = 3.870$, $p = 0.031$; drug, $F_{(1,33)} = 10.30$, $p = 0.003$; pretreatment, $F_{(2,33)} = 5.558$, $p = 0.008$]. This potentiation was blocked in rats pretreated with SB 334867 15 min before morphine exposure (saline, 0.4 ± 0.05; morphine, 0.4 ± 0.06; $p > 0.05$; Fig. 1*A*,*B*).

To further investigate the effect of morphine on AMPARs at excitatory synapses of dopamine neurons, we recorded AMPAR mEPSCs to determine the locus of synaptic change. Morphine induced a long-lasting increase in the frequency of AMPAR mEPSCs in naive rats (saline, 0.6 ± 0.1 Hz; morphine, 1.6 ± 0.3 Hz; $p < 0.01$) and vehicle-treated rats (saline, 0.4 ± 0.1 Hz; morphine, 1.2 ± 0.3; $p < 0.05$), but not in SB 334867-treated rats (saline, 0.6 ± 0.2 Hz; morphine, 0.6 ± 0.1 Hz; $p > 0.05$; Fig. 1*C*,*D*; two-way ANOVA: interaction, $F_{(2,54)} = 2.488$, $p = 0.093$; drug, $F_{(1,54)} = 8.861$, $p = 0.004$; pretreatment, $F_{(2,54)} = 2.252$, $p = 0.115$). This increase in the probability of presynaptic glutamate release was confirmed

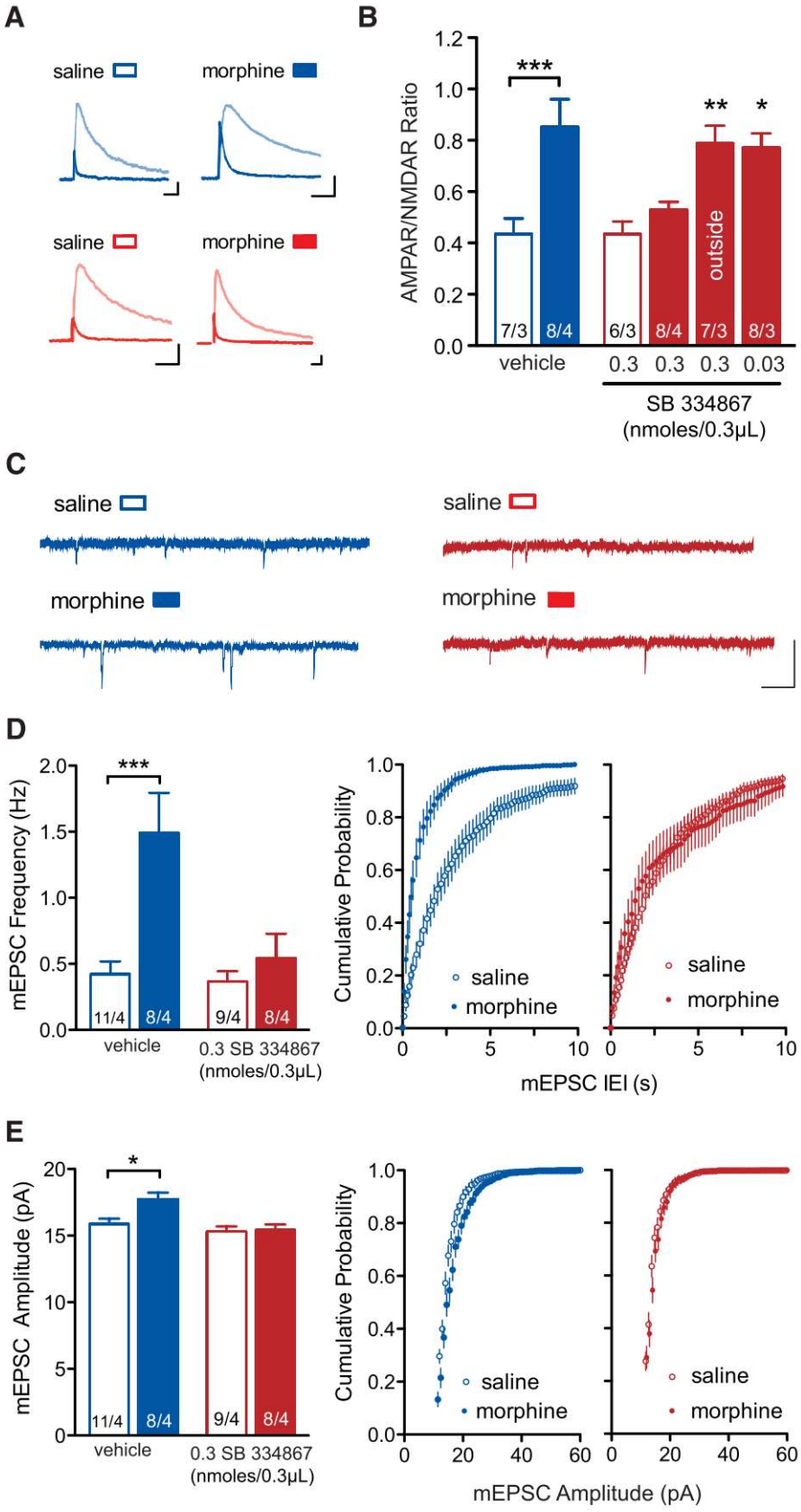


**Figure 2.** OxR1 signaling in the VTA is required for morphine-induced plasticity at glutamatergic synapses. ***A***, Sample traces of evoked AMPAR (dark) and NMDAR (light) EPSCs recorded at +40 mV, 24 h after exposure to morphine or saline in rats microinfused with intra-VTA vehicle or SB 334867 rats. Calibration: 50 pA, 20 ms. ***B***, Morphine (filled bars) potentiated the AMPAR/NMDAR ratio compared with saline (open bars) in intra-VTA vehicle-treated animals, but not intra-VTA SB 334867-treated animals ($p < 0.05$, two-way ANOVA). SB 334867 was ineffective when infused outside the VTA or when the dose was lowered to 0.03 nmol/0.3 $\mu$l. ***C***, Sample traces of AMPAR mEPSCs recorded at −70 mV, 24 h after morphine or saline exposure in intra-VTA vehicle-treated and intra-VTA SB 334867-treated rats. Calibration: 50 pA, 100 ms. ***D***, Left, Morphine increased the frequency of AMPAR mEPSC relative to saline in intra-VTA vehicle-treated rats, but not intra-VTA SB-334867-treated rats ($p < 0.05$, two-way ANOVA). Right, Cumulative probability plot comparing morphine and saline exposure on mEPSCs for intra-VTA vehicle- or SB 334867-treated rats. ***E***, Left, Intra-VTA SB 334867 inhibited a morphine-induced increase in the amplitude of AMPAR mEPSCs in VTA dopamine neurons ($p < 0.05$, two-way ANOVA). Right, Cumulative probability plot comparing morphine and saline exposure on mEPSC amplitude for intra-VTA vehicle- or SB 334867-treated rats. *n/N* = cells/rats. Bars represent the mean ± SEM. $^*p < 0.05$, $^{**}p < 0.01$, $^{***}p < 0.001$.

with the paired-pulse ratio. Morphine-induced a paired-pulse depression in naive (saline: 0.9 ± 0.07; morphine: 0.6 ± 0.03, $p < 0.01$) and vehicle-treated rats (saline: 0.9 ± 0.06; morphine: 0.6 ± 0.05, $p < 0.01$; Fig. 1*F*). Morphine-induced paired-pulse depression was inhibited by SB 334867 (saline, 0.9 ± 0.05; morphine, 0.9 ± 0.06; $p > 0.05$; Fig. 1*F*; two-way ANOVA: interaction, $F_{(2,38)} = 3.371$, $p = 0.045$; drug, $F_{(1,38)} = 20.37$, $p < 0.0001$; pretreatment, $F_{(2,38)} = 4.468$, $p = 0.018$).

Orexin signaling also modulated postsynaptic AMPAR effects induced by morphine exposure. Morphine treatment increased the amplitude of AMPAR mEPSCs in VTA neurons of control rats (naive: saline, 17.4 ± 0.6 pA; morphine, 19.4 ± 0.6 pA; $p < 0.05$; vehicle: saline, 16.2 ± 0.5 pA; morphine, 18.1 ± 0.5 pA; $p < 0.05$), an effect that was blocked by pretreatment with SB 334867 (saline, 16.9 ± 0.6 pA; morphine, 17.1 ± 0.5 pA; $p > 0.05$; Fig. 1*C*,*E*; two-way ANOVA: interaction, $F_{(2,54)} = 1.583$, $p = 0.214$; drug, $F_{(1,54)} = 9.102$, $p = 0.004$; pretreatment, $F_{(2,54)} = 4193$, $p = 0.020$). Because morphine treatment is associated with a change in the subunit composition of AMPARs in the VTA (Brown et al., 2010), we measured the rectification index following morphine treatment. Morphine increased the rectification index in naive rats (saline, 1.1 ± 0.06; morphine, 1.5 ± 0.1; $p < 0.05$) and vehicle-treated rats (saline, 1.1 ± 0.08; morphine, 1.5 ± 0.1; $p < 0.05$), but not in those that were treated with SB 334867 (saline, 1.2 ± 0.06; morphine, 1.2 ± 0.07; $p > 0.05$; Fig. 1*G*; two-way ANOVA: interaction, $F_{(2,28)} = 2.549$, $p = 0.088$; drug, $F_{(1,54)} = 8.876$, $p = 0.005$; pretreatment, [$F_{(2,48)} = 1.520$, $p = 0.230$). Together, these data suggest that orexin signaling is necessary for morphine-induced potentiation of presynaptic and postsynaptic efficacy of glutamatergic synapses.

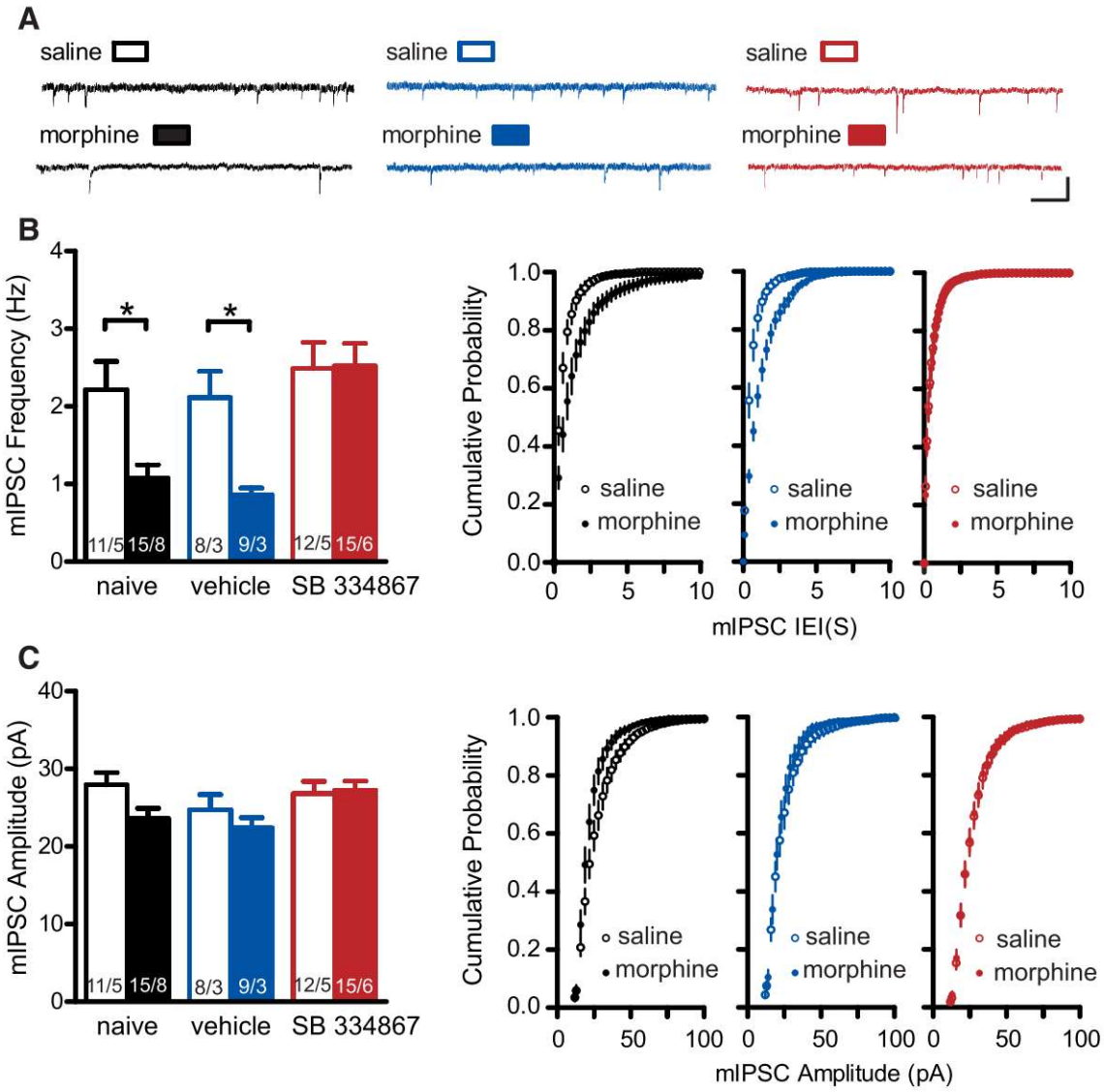


**Figure 3.** Morphine decreases the probability of presynaptic GABA release in an OxR1-dependent manner. ***A***, Example recordings of $GABA_A$ mIPSCs recorded at −70 mV, 24 h after exposure to morphine or saline in naive, vehicle-treated, or SB 334867-treated rats. Calibration: 50 pA, 200 ms. ***B***, Left, Morphine (filled bars) decreased the frequency of $GABA_A$ mIPSCs relative to saline (open bars) in naive and vehicle-treated rats, but not in SB 334867-treated rats ($p < 0.05$, two-way ANOVA). Right, Cumulative probability plots comparing morphine or saline exposure on mIPSC for naive, vehicle-treated, and SB 334867-treated animals. ***C***, Left, Morphine exposure did not alter $GABA_A$ mIPSC amplitude compared with saline exposure in naive, vehicle-treated, or SB 334867-treated animals ($p > 0.05$, two-way ANOVA). Right, Cumulative probability plots comparing morphine or saline exposure on mIPSC amplitude for naive, vehicle-treated, and SB 334867-treated animals. *n/N* = cells/rats. Bars represent mean ± SEM. *$p < 0.05$.

**Orexin signals locally in the VTA to mediate morphine-induced potentiation of excitatory synaptic transmission**

OxR1 is expressed widely throughout the brain (Trivedi et al., 1998; Marcus et al., 2001). Therefore, systemic administration of SB 334867 precluded identification of the site of action of orexin in modulating morphine-induced plasticity. Because microinfusion of SB 334867 into the VTA inhibits the reinstatement of morphine-induced conditioned place preference (CPP; Narita et al., 2006), we hypothesized that orexin was signaling locally in the VTA to mediate morphine-induced potentiation of glutamatergic synaptic transmission. To test this, we implanted rats with a bilateral cannula aimed at the VTA and microinfused SB 334867 locally. In rats that received vehicle infusions into the VTA, systemic morphine administration increased the AMPAR/NMDAR ratio compared with saline (saline, 0.4 ± 0.06; morphine, 0.8 ± 0.1; $p < 0.01$; Fig. 2*A*,*B*). This effect of morphine was blocked by an intra-VTA infusion of SB 334867 (0.3 nmol/0.3 μl; saline, 0.4 ± 0.05; morphine, 0.5 ± 0.03; $p > 0.05$; Fig. 2*A*,*B*; two-way ANOVA: interaction, $F_{(1,25)} = 5.122$, $p = 0.033$; drug, $F_{(1,25)} = 12.89$, $p = 0.001$; pretreatment, $F_{(1,25)} = 5.103$, $p = 0.033$). However, SB 334867 did not block a morphine-induced increase in the AMPAR/NMDAR ratio when infused outside the VTA (morphine, 0.8 ± 0.07) or when the dose was 10-fold lower (0.03 nmol/0.3 μl; morphine, 0.8 ± 0.05; one-way ANOVA: $F_{(3,25)} = 10.85$, $p < 0.0001$).

To test whether intra-VTA orexin signaling modulates morphine-induced presynaptic or postsynaptic plasticity at excitatory synapses, we recorded AMPAR mEPSCs. Morphine increased the frequency of AMPAR mEPSCs compared with saline in intra-VTA vehicle-treated (saline, 0.4 ± 0.1 Hz; morphine, 1.5 ± 0.3 Hz; $p < 0.001$), but not SB 334867-treated rats (0.3 nmol/0.3 μl; saline, 0.4 ± 0.08 Hz; morphine, 0.5 ± 0.2 Hz; $p > 0.05$; Fig. 2*C*,*D*; two-way ANOVA: interaction, $F_{(1,32)} = 6.551$, $p = 0.015$; drug, $F_{(1,32)} = 12.84$, $p = 0.001$; pretreatment, $F_{(1,32)} =$

8.289, $p = 0.007$). Similarly, morphine increased AMPAR mEPSC amplitude in an orexin-dependent manner (two-way ANOVA: interaction, $F_{(1,32)} = 3.685$, $p = 0.064$; drug, $F_{(1,32)} = 4.894$, $p = 0.034$; pretreatment, $F_{(1,32)} = 10.26$, $p = 0.003$). In morphine-exposed rats, mEPSC amplitude was increased in intra-VTA vehicle-treated (saline, 15.9 ± 0.4 pA; morphine, 17.7 ± 0.5 pA; $p < 0.05$), but not in intra-VTA SB 334867-treated rats (0.3 nmol/0.3 μl; saline, 15.3 ± 0.4 pA; morphine, 15.4 ± 0.4 pA; $p > 0.05$; Fig. 2*C*,*E*). Together, these results suggest that orexin signaling in the VTA is necessary for morphine-induced presynaptic and postsynaptic potentiation at excitatory synapses onto dopamine neurons.

### OxR1 signaling is required for a morphine-induced decrease in presynaptic GABA release

Short-term morphine administration increases VTA dopamine neuronal activity by way of disinhibition resulting from μ-opioid receptor activation on GABAergic inputs to dopamine neurons (Di Chiara and Imperato, 1988; Johnson and North, 1992; Jalabert et al., 2011). Moreover, morphine has long-term effects on GABA transmission in the VTA (Nugent et al., 2007; Dacher and Nugent, 2011). Therefore, we recorded $GABA_A$ mIPSCs in VTA dopamine neurons 24 h after a single morphine exposure to assess whether orexin signaling had a role in morphine-induced plasticity at GABAergic synapses in the VTA. Morphine treatment decreased $GABA_A$ mIPSC frequency on to dopamine neurons of control rats (naive rats: saline, 2.2 ± 0.4 Hz; morphine, 1.1 ± 0.2 Hz; $p < 0.05$; vehicle-treated rats: saline, 2.1 ± 0.3 Hz; morphine, 0.9 ± 0.08 Hz; $p < 0.05$; Fig. 3*A*—*C*; two-way ANOVA: interaction, $F_{(2,62)} = 3.262$, $p = 0.045$; drug, $F_{(1,62)} = 11.19$, $p = 0.001$]; pretreatment, [$F_{(2,62)} = 7.566$, $p = 0.001$). However, SB 334867 inhibited morphine-induced suppression of mIPSC frequency (saline, 2.5 ± 0.3 Hz; morphine, 2.5 ± 0.3 Hz; $p > 0.05$; Fig. 3*A*–*C*). There was no effect of morphine on $GABA_A$ mIPSC amplitude (two-way ANOVA: interaction, [$F_{(2,62)} = 1.493$, $p = 0.232$; drug, $F_{(1,62)} = 3.011$, $p = 0.088$; pretreatment, $F_{(2,62)} = 2.537$, $p = 0.088$; Fig. 3*A*,*D*,*E*).

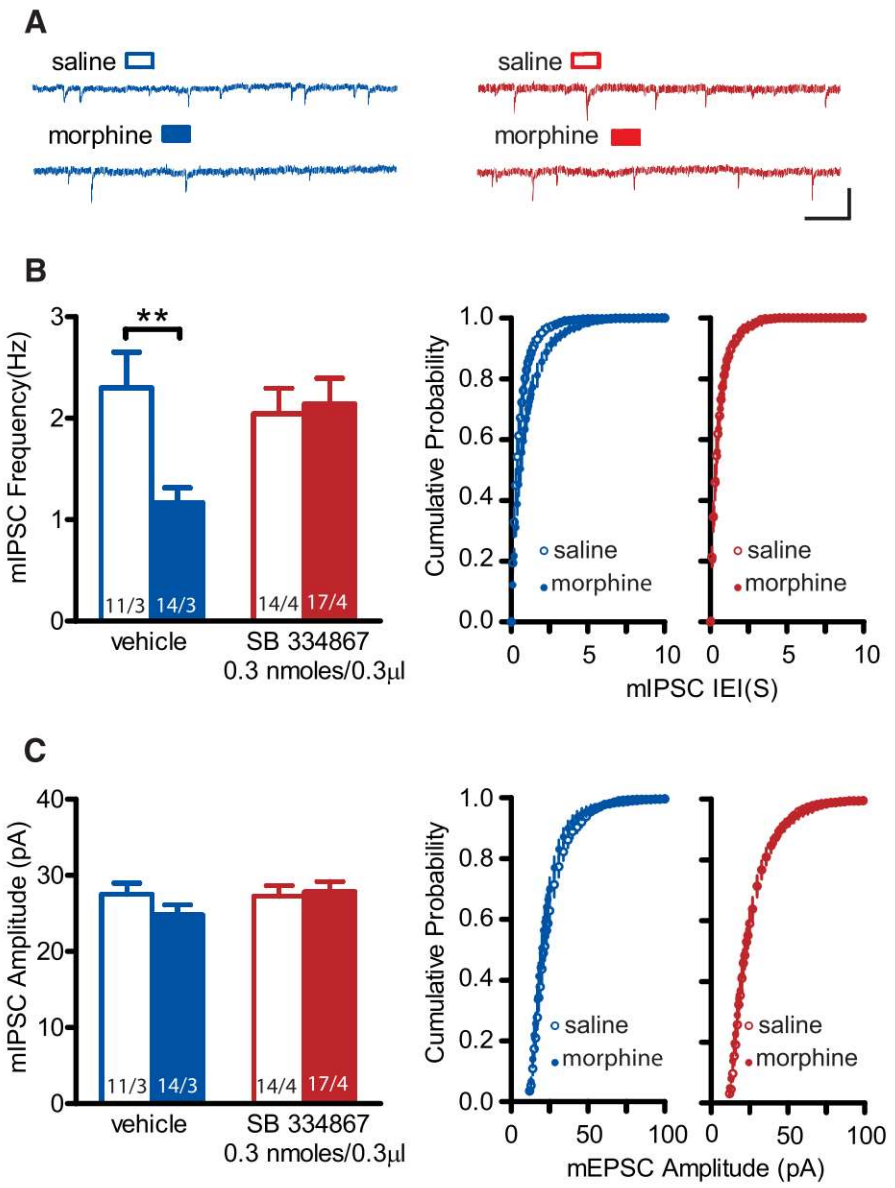


**Figure 4.** OxR1 signaling in the VTA is necessary for a morphine-induced suppression of presynaptic GABA release. ***A***, Sample recordings of $GABA_A$ mIPSCs at −70 mV, from rats that were exposed to morphine or saline after pretreatment with intra-VTA vehicle or intra-VTA SB 334867. Calibration: 50 pA, 200 ms. ***B***, Left, Morphine (filled bars) decreased $GABA_A$ mIPSC frequency relative to saline (open bars) in intra-VTA vehicle-treated rats, but not intra-VTA SB 334867-treated rats ($p < 0.05$, two-way ANOVA). Right, Cumulative probability plot comparing morphine and saline exposure on mIPSC frequency for intra-VTA vehicle-treated rats or SB 334867-treated rats. ***C***, Left, There was no effect of morphine on the amplitude of $GABA_A$ mIPSCs ($p > 0.05$, two-way ANOVA). Right, Cumulative probability plots comparing morphine and saline exposure on mIPSC amplitude for intra-VTA vehicle-treated rats or SB 334867-treated rats. *n*/*N* = cells/rats. Bars represent the mean ± SEM. $^{**}p < 0.01$.

### OxR1 activation in the VTA is required for a morphine-induced decrease in the probability of presynaptic GABA release

We next examined whether the morphine-induced decrease in the probability of GABA release was mediated by OxR1 activation within the VTA. Morphine decreased $GABA_A$ mIPSC frequency in intra-VTA vehicle-treated rats (saline, 2.3 ± 0.4 Hz; morphine, 1.2 ± 0.1; $p < 0.05$), but not in those that received intra-VTA SB 334867 (0.3 nmol/0.3 μl; saline, 2.0 ± 0.3 Hz; morphine, 2.1 ± 0.3; $p > 0.05$; Fig. 4*A*,*B*; two-way ANOVA: interaction, $F_{(1,52)} = 5.845$, $p = 0.019$; drug, $F_{(1,52)} = 4.118$, $p = 0.047$; pretreatment, $F_{(1,52)} = 1.951$, $p = 0.169$). There was no effect of morphine treatment on $GABA_A$ mIPSC amplitude in either group (two-way ANOVA: interaction, $F_{(1,52)} = 1.316$, $p = 0.256$; drug, $F_{(1,52)} = 0.588$, $p = 0.447$; pretreatment, $F_{(1,52)} = 0.955$, $p = 0.333$; Fig. 4*A*,*C*).

### Morphine alters the synaptic excitation/inhibition balance in an OxR1-dependent manner

Our results demonstrate that a single morphine exposure induces a simultaneous OxR1-dependent increase in glutamate transmission and a decrease in GABA transmission on to VTA dopamine

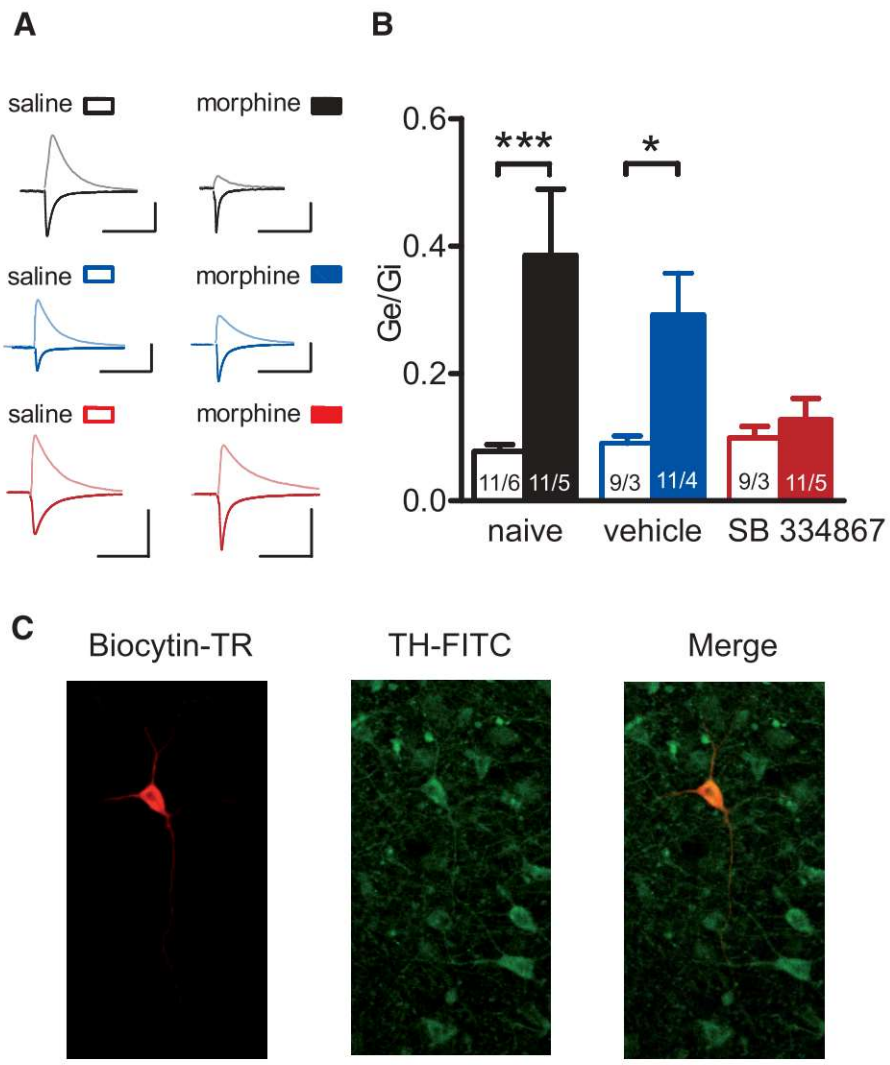


**Figure 5.** OxR1 signaling is required for a morphine-induced shift in the balance of excitatory and inhibitory synaptic transmission onto dopamine neurons. ***A***, Example recordings of EPSCs (dark) at −67 mV, and IPSCs (light) at +8 mV from VTA dopamine neurons in naive, vehicle-treated, and SB 334867-treated animals. Calibration: 200 pA, 20 ms. ***B***, Morphine (filled), compared with saline (open), induced a switch in the ratio of excitatory and inhibitory conductances onto dopamine neurons from naive or vehicle-treated animals. This shift was inhibited by systemic SB 334867 ($p < 0.05$, two-way ANOVA). ***C***, SB 334867 inhibited the morphine-induced increased $G_e/G_i$ ratio in seven of nine identified tyrosine hydroxylase (TH)-positive neurons. TH was labeled with anti-TH antibodies and FITC. Biocytin was labeled with streptavidin-conjugated Texas Red. *n/N* = cells/rats. Bars represent the mean ± SEM. *$p < 0.05$, ***$p < 0.001$.

neurons. By acting at both excitatory and inhibitory synapses, orexin may drive a morphine-induced shift in the excitatory/inhibitory ($G_e/G_i$) balance of synaptic inputs on to dopamine neurons. To verify this, we recorded locally evoked EPSCs and IPSCS onto the same cell. EPSCs were recorded at the IPSC reversal potential (−67 mV), and IPSCs were recorded at the EPSC reversal potential (+8 mV). Morphine induced a shift in the $G_e/G_i$ balance onto dopamine neurons from naive rats (saline, 0.08 ± 0.01; morphine, 0.4 ± 0.1; $p < 0.05$) and vehicle-treated rats (saline, 0.09 ± 0.01; morphine, 0.3 ± 0.07; $p < 0.05$; Fig. 5*A*,*B*), but not in those that were treated with SB 334867 (saline, 0.1 ± 0.02; morphine, 0.1 ± 0.03; $p > 0.05$; Fig. 5*A*,*B*; two-way ANOVA: interaction, $F_{(2,56)} = 3.184$, $p = 0.049$; drug, $F_{(1,56)} = 15.37$, $p = 0.0002$; pretreatment, $F_{(2,56)} = 2.338$, $p = 0.106$). Together, these data suggest that orexin signaling is necessary for a morphine-induced shift in the balance of inhibitory and excitatory control of dopamine neurons.

## Discussion

Here, we establish a critical role for orexin signaling in morphine-induced synaptic plasticity in the VTA. Both systemic and intra-VTA administration of the OxR1 antagonist SB 334867 inhibited morphine-induced potentiation of excitatory synaptic transmission. Additionally, SB 334867 blocked a long-lasting decrease in the probability of presynaptic GABA release at inhibitory synapses onto dopamine neurons. Last, OxR1 signaling was required for a morphine-induced shift in the balance of excitatory and inhibitory inputs to dopamine neurons. These data provide further evidence that orexin signaling in the VTA plays a critical role in drug-induced plasticity of dopamine neurons.

Previous work (Saal et al., 2003; Brown et al., 2010) has demonstrated that a single morphine exposure increases the AMPAR/NMDAR ratio and promotes the insertion of GluA2-lacking AMPARs to the postsynaptic site, as revealed by an increase in the rectification index. Not only have we replicated these results, but we have demonstrated that morphine also increases the probability of presynaptic glutamate release, and that both presynaptic and postsynaptic changes induced by morphine to enhance synaptic efficacy onto dopamine neurons require OxR1 signaling in the VTA. Because overexpression of GluA1 subunits in the VTA potentiates morphine reward in a CPP task (Carlezon et al., 1997), orexin-dependent morphine-induced plasticity at excitatory synapses likely contributes to learning the association between context and the drug experience. Consistent with this, intra-VTA administration of an orexin receptor antagonist inhibits morphine CPP (Narita et al., 2006; Harris et al., 2007). Drug-induced plasticity and changes in AMPAR subunit composition have previously been attributed to activation of dopamine $D_5$ receptors and the cAMP-dependent protein kinase A signaling pathway (Argilli et al., 2008; Brown et al., 2010). Because the effects of orexin in the VTA are dependent on protein kinase C signaling (Borgland et al., 2006; Narita et al., 2007), OxR1 likely represents an alternative mechanism for drug-induced synaptic plasticity. Interestingly, both OxR1 and $D_5$ receptor signaling can potentiate NMDAR receptor currents in the VTA, albeit through different mechanisms (Borgland et al., 2006, 2009; Schilström et al., 2006). Because NMDAR activation is required for drug-induced plasticity (Ungless et al., 2001; Argilli et al., 2008; Engblom et al., 2008), OxR1 and $D_5$ receptors may act cooperatively to enhance NMDAR signaling and to promote drug-induced synaptic changes in the VTA. Exposure to morphine also induced an OxR1-dependent increase in the probability of presynaptic glutamate release. Orexin-induced increases in glutamate release have previously been observed in multiple brain regions, includ-

ing the VTA (van den Pol et al., 1998; Burlet et al., 2002; Li et al., 2002; Smith et al., 2002; Lambe and Aghajanian, 2003; Borgland et al., 2006, 2009). Increased glutamate release probability may reflect a mechanism additional to postsynaptic trafficking of AMPARs to further enhance synaptic efficacy *cis*- or *trans*-synaptically.

In addition to morphine action at excitatory synapses, we observed that morphine induces an OxR1-dependent decrease in the probability of GABA release. Previous work has demonstrated that morphine modulates GABAergic synapses onto VTA dopamine neurons. A single exposure to morphine blocks the induction of $LTP_{GABA}$, and likely occludes GABAergic long-term depression ($LTD_{GABA}$) onto dopamine neurons (Nugent et al., 2007; Dacher and Nugent, 2011; Graziane et al., 2013). $LTD_{GABA}$ requires the activation of postsynaptic dopamine $D_2$ receptors (Dacher and Nugent, 2011). Here, we propose a novel mechanism by which orexin signaling in the VTA facilitates a morphine-induced inhibition of GABA release. Future work will need to determine the mechanism by which orexin signaling can modulate GABAergic synapses, although preliminary studies (Baimel et al., 2015) have indicated that orexin induces an endocannabinoid-mediated inhibition of GABA release onto dopamine neurons.

Rapid modulation of the $G_e/G_i$ is important for optimal information processing (Sarti et al., 2013). Here we demonstrate that orexin facilitates the morphine-induced shift from inhibitory to excitatory influence on dopaminergic neurons. Because the balance of excitatory and inhibitory inputs of dopamine neurons is one of the key regulators of dopamine neuron activity (Floresco et al., 2003), this shift likely reflects a mechanism by which orexin can modulate the output of dopamine neurons in response to reward-predictive cues (Harris et al., 2005). The ability of orexin to increase glutamate transmission in parallel with a decrease in GABA transmission suggests an important role for orexin in gating the output of dopamine neurons.

How morphine exposure induces orexin release remains to be determined. Orexin neurons express $\mu$-opioid receptors (Georgescu et al., 2003) and are c-Fos activated with morphine administration (Harris et al., 2005, 2007; Richardson and Aston-Jones, 2012) or withdrawal (Georgescu et al., 2003). Orexin neurons that project to the VTA, but not those that project to the locus ceruleus, are activated by *in vivo* exposure to morphine in proportion to the level of CPP (Richardson and Aston-Jones, 2012). Furthermore, OxR1 antagonists reduce heroin self-administration and cue-induced reinstatement of opioid seeking (Smith and Aston-Jones, 2012), suggesting that increased orexin in the VTA is required for opioid seeking. Consistent with this, orexin A in the VTA is sufficient to reinstate drug seeking (Harris et al., 2005).

In contrast to these studies, a short-term (30 s) morphine application to LH slices has direct inhibitory effects on orexin neuronal activity (Li and van den Pol, 2008). Because orexin neurons are a heterogeneous cell population, and only ~50% of orexin cells express high levels of $\mu$-opioid receptor immunoreactivity (Georgescu et al., 2003), opioids like morphine may differentially regulate orexin neurons that project to different brain areas. For example, opioid-induced inhibition of orexin neurons may be restricted to those neurons that project to arousal-related brain areas and thus mediate the sedating effects of morphine exposure. Alternatively, orexin neurons may be activated upon removal of morphine inhibition, potentially leading to increased orexin release in the VTA. Further research is required to test these possibilities.

In summary, we propose a novel role for orexin signaling in morphine-induced plasticity at both glutamate and GABA synapses in the VTA. Morphine enhances excitatory synaptic efficacy onto VTA dopamine neurons by way of an orexin-dependent increase in presynaptic glutamate release, and a postsynaptic increase in AMPAR function. Moreover, orexin facilitated a long-term decrease in presynaptic GABA release to dopamine neurons. Finally, orexin signaling was required for a shift in the balance of excitatory and inhibitory control of dopamine neurons. Together, these findings provide novel insights into how orexin gates drug-induced plasticity in the VTA.

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