NEUROSYSTEMS

Orexin B/hypocretin 2 increases glutamatergic transmission to ventral tegmental area neurons

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Abstract

The orexins (hypocretins) play a crucial role in arousal, feeding and reward. Highly relevant to these functions, orexin-containing neurons from the lateral hypothalamus project densely to the ventral tegmental area (VTA), which is the origin of dopamine projections implicated in motivation and reward. Orexin A/hypocretin 1 (oxA/hcrt-1) can enable long-term changes associated with drugs of abuse; however, the effects of orexin B/hypocretin 2 (oxB/hcrt-2) on excitatory synaptic transmission in the VTA are unknown. We used whole-cell patch-clamp electrophysiology in rat horizontal midbrain slices to examine the effects of oxB/hcrt-2 on excitatory synaptic transmission. We observed that oxB/hcrt-2 has distinct effects from oxA/hcrt-1 in the VTA. oxB/hcrt-2 (100 nM) increased presynaptic glutamate release in addition to a postsynaptic potentiation of NMDA receptors (NMDARs). The oxB/hcrt-2-mediated postsynaptic potentiation of NMDARs was mediated via activation of orexin/hypocretin 2 (OX2/Hcrt-2) receptors and protein kinase C (PKC). Furthermore, the increase in transmitter release probability was also PKC-dependent, but not through activation of orexin/hypocretin 1 (OX1/Hcrt-1) or OX2/Hcrt-2 receptors. Finally, oxB/hcrt-2 or the selective OX2/Hcrt-2 receptor agonist ala¹¹-D-leu¹⁵-orexin B, significantly reduced spike-timing-induced long-term potentiation. Taken together, these results support a dual role for oxB/hcrt-2 in mediating enhanced glutamatergic transmission in the VTA, and suggest that oxA/hcrt-1 and oxB/hcrt-2 exert different functional roles in modulating the enhancement of the motivational components of arousal and feeding.

Introduction

Ventral tegmental area (VTA) neurons are critically involved in reward, arousal and appetitive behaviors (Grace *et al.*, 1998; Carlezon & Nestler, 2002; Berridge, 2007). VTA neurons are the main source of dopamine for the ventral striatum and prefrontal cortex, critical forebrain regions that mediate incentive learning and reinforcement mechanisms associated with positive reward (Berridge, 2007). Excitatory synaptic transmission onto VTA dopamine neurons increases locomotor activity (Dunn *et al.*, 2005) and is an important locus of neural plasticity induced by psychostimulant administration (Fitzgerald *et al.*, 1996; Ungless *et al.*, 2001; Saal *et al.*, 2003; Borgland *et al.*, 2004).

Two neuropeptides, orexin A/hypocretin 1 (oxA/hcrt-1) and orexin B/hypocretin 2 (oxB/hcrt-2), have also been implicated in arousal, feeding and reward (DeLecea *et al.*, 1998; Sakurai *et al.*, 1998; Georgescu *et al.*, 2003; Yamanaka *et al.*, 2003; Boutrel *et al.*, 2005; Harris *et al.*, 2005; Borgland *et al.*, 2006; Narita *et al.*, 2006). These peptides are synthesized in neurons exclusively localized to the lateral

hypothalamic area and project widely through the brain (Peyron *et al.*, 1998; Sakurai *et al.*, 1998). The VTA receives an input from orexin-containing neurons (Fadel & Deutch, 2002; Baldo *et al.*, 2003) composed of both synaptic terminals and en-passant fibers (Balcita-Pedicino & Sesack, 2007), and this input is a critical element in motivation and reward circuits activated by drugs of misuse (Wise, 1996; Harris & Aston-Jones, 2006).

The actions of orexins are mediated by two known G-protein-coupled receptors termed orexin/hypocretin receptor 1 (OX1/hcrt-1) and orexin/hypocretin receptor 2 (OX2/hcrt-2; Sakurai *et al.*, 1998). oxA/hcrt-1 has high affinity for both receptors whereas oxB/hcrt-2 has much higher affinity for OX2/hcrt-2 than OX1/hcrt-1 receptors (Sakurai *et al.*, 1998). Both receptors are expressed on VTA dopamine and GABAergic neurons (Korotkova *et al.*, 2003; Narita *et al.*, 2006). Furthermore, both oxA/hcrt-1 and oxB/hcrt-2 increased the firing rate of VTA dopamine or GABAergic neurons *in vitro* (Korotkova *et al.*, 2003). oxA/hcrt-1 or oxB/hcrt-2 also activated phospholipase C- and protein kinase C (PKC)-mediated Ca² signaling in dopamine neurons (Uramura *et al.*, 2001; Narita *et al.*, 2007), and we have previously reported that oxA/hcrt-1 potentiates NMDA receptor (NMDAR)-mediated synaptic transmission in the VTA via a PKC-dependent translocation of the receptors to the synapse (Borgland *et al.*, 2006).

A role for oxA/hcrt-1 and OX1/Hcrt-1 receptors in mediating some of the effects of drugs of abuse has recently been defined (Boutrel *et al.*, 2005; Harris *et al.*, 2005; Borgland *et al.*, 2006;

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Received 12 November 2007, revised 11 June 2008, accepted 4 July 2008

Lawrence *et al.*, 2006; Narita *et al.*, 2006, 2007). OX2/Hcrt-2 receptors have been thought to play a primary role in mediating wakefulness and arousal, as OX2/Hcrt-2 knockout mice experience narcolepsy (Chemelli *et al.*, 1999; Akanmu & Honda, 2005). Although intracerebroventricular administration of oxB/hcrt-2 has been shown to increase locomotor activity (Narita *et al.*, 2006) and intra-VTA administration increases preference for morphine (Narita *et al.*, 2007), it is unknown if these effects of oxB/hcrt-2 are mediated through activation of OX2/Hcrt-2 receptors. Enhancement of glutamatergic synaptic transmission in VTA dopamine neurons has been proposed to underlie some of the long-term changes associated with chronic drug use (Borgland *et al.*, 2004; Kauer, 2004; Wolf *et al.*, 2004), but the effects of oxB/hcrt-2, which is presumably co-released with oxA/hcrt-1, on synaptic plasticity in the VTA are unknown. Therefore, using whole-cell patch clamp, we examined the role of oxB/hcrt-2 on excitatory synaptic transmission in VTA neurons.

Methods and materials

Electrophysiology

All electrophysiological recordings were performed in male Sprague–Dawley rats ranging from postnatal day (P)21 to P30. All experiments were performed according to NIH and UCSF guidelines on animal use. Briefly, rats were anesthetized with halothane and decapitated. Horizontal sections of the VTA (230 μ m) were prepared with a vibratome (Leica, Nussloch, Germany). Slices were placed in a holding chamber and allowed to recover for at least 1 h before being placed in the recording chamber and superfused with bicarbonate-buffered solution (artificial cerebrospinal fluid; aCSF) saturated with 95% O₂/5% CO₂ and containing (in mM): 119 NaCl, 1.6 KCl, 1.0 NaH₂PO₄, 1.3 MgCl₂, 2.5 CaCl₂, 26.2 NaHCO₃ and 11 glucose (at 32–34 °C). For all experiments, picrotoxin (100 μ M) was added to block GABA_A receptor-mediated inhibitory postsynaptic currents. Cells were visualized using infrared differential interference contrast video microscopy. Whole-cell voltage-clamp recordings were made using an Axopatch 1D amplifier (Axon Instruments, Union City, CA, USA). Electrodes (3–4.0 M Ω) contained (in mM): 120 cesium methanesulfonate, 20 HEPES, 0.4 EGTA, 2.8 NaCl, 5 TEA-Cl, 2.5 MgATP, and 0.25 NaGTP, pH 7.2–7.3 (270–285 mOsm). Series resistance (10–40 M Ω) and input resistance were monitored on-line with a 4-mV depolarizing step (50 ms) given just after every afferent stimulus. Neurons were visualized with an upright microscope using infrared differential interference contrast illumination. Putative dopaminergic VTA neurons were identified by the presence of a large hyperpolarization-activated, cyclic nucleotide-regulated cation (*I_h*) current (Grace & Onn, 1989; Lacey *et al.*, 1990; Johnson & North, 1992; Cameron *et al.*, 1997; Ford *et al.*, 2006) and were selected from the population of neurons surrounding the medial side of the medial terminal nucleus of the accessory optic tract, a region with a substantial population of *I_h*-positive dopamine neurons (Margolis *et al.*, 2007). We recognize that *I_h* is present in both principal and tertiary VTA neurons and that the presence of *I_h* does not unequivocally identify dopamine neurons from other non-GABAergic neurons in midbrain slices (Margolis *et al.*, 2007). In some cases, neurons were identified by biocytin (0.2%) filling and *post hoc* staining for tyrosine hydroxylase (TH; see below). A bipolar stimulating electrode was placed 100–300 μ m rostral to the recording electrode and was used to stimulate excitatory afferents at 0.1 Hz. Neurons were voltage-clamped at –70 and +40 mV to record AMPAR- and NMDAR-mediated evoked excitatory post-synaptic currents (eEPSCs). eEPSCs were filtered at 2 kHz, digitized at 5–10 kHz and collected on-line

using Igor Pro software (Wavemetrics, Lake Oswego, OR, USA). Paired-pulse ratio was elicited by evoking two EPSCs with an interval of 50 ms. Miniature EPSCs (mEPSCs) in the presence tetrodotoxin (500 nM) to block action potentials driven by spontaneous events were recorded using PCLAMP 8 software (Axon Instruments). For these experiments, mEPSCs were recorded before and 10 min after a 5-min application of oxB/hcrt-2 (100 nM, 5 min). We did not observe a time effect of mEPSC event size or frequency in control neurons (data not shown). mEPSCs were selected based on their amplitude (> 8 pA), decay time (< 3 ms) and rise time (< 1 ms) using the MINIANALYSIS program (SYNAPTOSOFT). Average noise levels were 2 ± 0.2 pA.

Amplitudes of currents evoked by bath application of NMDA (20 μ M, 30 s) before and 10 min after a 5-min oxB/hcrt-2 application were determined by subtracting the baseline (average of 2 min before NMDA application) from the NMDA-induced current.

Spike timing-dependent LTP

Neurons were patch clamped in current-clamp mode with electrodes containing: 0.95% KOH (v/v), 0.76% methanesulfonic acid (v/v), 0.18% hydrochloric acid (v/v), 20 mM HEPES, 0.2 mM EGTA, 2.8 mM NaCl, 2.5 mg/mL MgATP, 0.25 mg/mL GTP and 0.2% biocytin, pH 7.2–7.4 (275–285 mOsm). To induce LTP, the spike-timing protocol consisted of 20 bursts of excitatory post-synaptic potential (EPSP)–spike pairs, with each burst consisting of five paired stimuli delivered at 100-ms intervals (10 Hz) and an inter-burst interval of 5 s. The postsynaptic spikes were evoked by injection of depolarizing current pulses (1–2 nA, 2–3 ms), with the onset of EPSPs preceding the peak of the postsynaptic spikes by 5 ms (Liu *et al.*, 2005). eEPSPs were sampled at 0.1 Hz before and after LTP induction. For quantifying LTP, we averaged the mean of 30 sweeps 5 min before drug treatment (baseline) and averaged 30 sweeps 25 min after the pairing protocol (peak LTP).

TH immunocytochemistry

Brain slices from patch-clamp recording were fixed overnight in cold 4% paraformaldehyde, rinsed in PBS, blocked in 10% normal donkey serum, incubated with monoclonal mouse anti-TH antibody (1 : 3000; Sigma) and Avidin-Texas Red (1 : 500; Vector Labs) for 48 h at 4 °C. Secondary donkey anti-mouse Alexa 488 antibody (1 : 500) was applied overnight. Slices were mounted using Vectashield (Vector Labs).

Data analysis

Mean potentiation of NMDAR or AMPAR currents by oxB/hcrt-2 represents the mean EPSCs over a period of 2 min, 25 min after application of oxB/hcrt-2. All values are expressed as mean $\pm$ SEM. Statistical significance was assessed using two-tailed Student's *t*-tests or a one-way ANOVA for multiple group comparisons. A Bonferroni *post-hoc* test following an ANOVA was used to test for significant differences between multiple groups.

Results

oXB/Hcrt-2 enhances excitatory synaptic transmission in VTA neurons

eEPSCs were recorded from VTA neurons in rat midbrain slices. NMDAR EPSCs were evoked while holding neurons in voltage clamp at +40 mV. Measurements were taken 20 ms after the stimulus

FIG. 1. oxB/hcrt-2 increases excitatory synaptic transmission in VTA neurons. (A) oxB/hcrt-2 (100 nM) increased NMDAR eEPSCs ($n = 8$) evoked at +40 mV. (B) An example recording of NMDAR eEPSCs before, during and after oxB/hcrt-2 application. Inset is an example trace before (1) and after (2) oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (C) oxB/hcrt-2 (100 nM) increases AMPAR eEPSCs evoked at -70 mV ($n = 10$). (D) An example recording of oxB/hcrt-2 effects on AMPAR-mediated eEPSCs. Inset is an example trace before (1) and after (2) oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (E) oxB/hcrt-2 applied with oxA/hcrt-1 (100 nM, 5 min) has additive effects on NMDAR currents ($n = 8$). (F) An example recording of NMDAR eEPSCs before, during and after a 5-min application of oxB/hcrt-2 and oxA/hcrt-1 application. Inset is an example trace before (1) and after (2) oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (G) Maximal potentiation of NMDAR eEPSCs by 10 nM oxB/hcrt-2, 100 nM oxB/hcrt-2, 300 nM oxB/hcrt-2 or 100 nM oxB/hcrt-2 with oxA/hcrt-1. All treatments were significantly greater than the baseline ($P < 0.05$; one-way ANOVA) and both peptides caused significantly greater potentiation of NMDAR eEPSCs than oxB/hcrt-2 alone ($P < 0.05$, one-way ANOVA, Bonferroni post-test). (H) oxB/hcrt-2 at 100 nM significantly potentiated AMPAR eEPSCs ($P < 0.05$), but not at 10 nM ($P < 0.05$; one-way ANOVA). Bars represent mean and SEM. * $P < 0.05$, ** $P < 0.01$. Stimulus artifact on example traces has been removed for clarity.

artifact, a time point at which the glutamatergic eEPSC is mediated purely by NMDARs. Bath application of oxB/hcrt-2 (5 min) resulted in a concentration-dependent potentiation of NMDAR-mediated eEPSCs (10 nM: $120 \pm 6\%$, $n = 7$; 100 nM: $138 \pm 8\%$, $n = 8$; 300 nM $140 \pm 7\%$, $n = 7$; 19/22 neurons responded to oxB/hcrt-2; Fig. 1A, B and G). To measure AMPAR eEPSCs, VTA neurons were voltage-clamped at -70 mV. oxB/hcrt-2 (100 nM for 5 min) also potentiated AMPAR eEPSCs $128 \pm 9\%$ (Fig. 1C and D, $n = 10$, $P < 0.01$, 8/10 cells responded to oxB/hcrt-2). However, a lower concentration of oxB/hcrt-2 (10 nM for 5 min) did not potentiate AMPAR eEPSCs ($n = 7$, $P > 0.05$; Fig. 1H). Taken together, these results demonstrate that oxB/hcrt-2 potentiated AMPAR and NMDAR currents in VTA neurons.

In contrast to oxB/hcrt-2, oxA/hcrt-1 potentiated only NMDAR currents in VTA neurons (Borgland *et al.*, 2006). Therefore, we tested whether oxA/hcrt-1 and oxB/hcrt-2, when bath applied together, had additive effects on NMDAR eEPSCs. We found that when a saturating concentration of oxA/hcrt-1 (Uramura *et al.*, 2001; Narita *et al.*, 2007; Fig. 1G) was added with oxB/hcrt-2, the effects were significantly greater than when oxB/hcrt-2 (100 nM) was applied alone ($165 \pm 9\%$, $n = 8$, $P < 0.05$; Fig. 1E and F), suggesting that oxA/hcrt-1 and oxB/hcrt-2 exert their actions through distinct receptors, signal transduction pathways and/or cellular targets.

We previously found that oxA/hcrt-1 potentiated NMDAR currents through activation of phospholipase C (PLC) and PKC (Borgland *et al.*, 2006). Therefore, we next tested if oxB/hcrt-2 potentiated NMDAR eEPSCs through a similar signal transduction mechanism. Inhibitors of PKC were applied intracellularly through the patch pipette. Bisindolylmaleimide (bis; $1 \mu\text{M}$) or chelerythrin ($1 \mu\text{M}$) significantly reduced but did not completely block oxB/hcrt-2-mediated potentiation of NMDAR eEPSCs compared with untreated neurons (Fig. 2A and B, $P < 0.05$). oxB/hcrt-2 significantly potentiated NMDAR currents 120 ± 9 and $120 \pm 13\%$ in the presence of bis ($1 \mu\text{M}$; $n = 7$, $P < 0.05$) or chelerythrin ($1 \mu\text{M}$; $n = 7$, $P < 0.05$), respectively. To test if oxB/hcrt-2-mediated potentiation of NMDARs was mediated by protein kinase A (PKA), we applied PKI ($20 \mu\text{M}$) through the patch pipette. In the presence of PKI, oxB/hcrt-2 potentiation of NMDARs was not significantly different from that of untreated VTA neurons ($138 \pm 14\%$, $n = 6$, $P > 0.05$, Fig. 2C and D). To determine whether potentiation of AMPAR eEPSCs by oxB/hcrt-2 occurred through activation of PKC, we recorded AMPAR eEPSCs with bis ($1 \mu\text{M}$) or chelerythrin ($1 \mu\text{M}$) in the patch pipette. Neither bis nor chelerythrin had effects on oxB/hcrt-2-mediated potentiation of AMPAR eEPSCs when applied intracellularly directly to the postsynaptic neuron (bis: 128 ± 9 , $n = 5$; chelerythrin: $128 \pm 11\%$, $n = 8$; Fig. 2E and F). Because oxB/hcrt-2 potentiation of NMDAR currents was partially mediated by PKC signaling and oxB/hcrt-2 potentiation of AMPAR currents was not, we hypothesized that oxB/hcrt-2 may be exerting both a presynaptic increase of glutamate release and a post-synaptic PKC-dependent potentiation of NMDARs. To determine if oxB/hcrt-2 was signaling via PKC presynaptically to increase glutamate release, we soaked slices in the PKC inhibitor bis ($10 \mu\text{M}$) for 45 min prior to the application of oxB/hcrt-2 (100 nM). oxB/hcrt-2 potentiation of NMDARs ($n = 7$; Fig. 2G) or AMPARs was abolished in the presence of externally applied bis ($n = 7$; Fig. 2H). These results suggest that oxB/hcrt-2 signals presynaptically through a PKC-dependent mechanism.

To verify further if oxB/hcrt-2-mediated potentiation of AMPARs was mediated pre- or post-synaptically, we examined the effects of oxB/hcrt-2 on the probability of transmitter release, comparing the response to paired pulses, a measure that changes in a highly predictable fashion with release probability (Zucker & Regehr, 2002).

We recorded the effect of oxB/hcrt-2 (100 nM, 5 min) on AMPAR eEPSCs at -70 mV using a paired-pulse stimulation protocol with a 50-ms interval. Fifteen minutes after bath application, oxB/hcrt-2 increased the amplitude of the first eEPSC in the paired-pulse protocol ($126 \pm 12\%$ of basal, $P < 0.05$, data not shown), but did not significantly alter the second eEPSC. Application of oxB/hcrt-2 significantly decreased the ratio between the two pulses (Fig. 3A and B; $n = 8$, $P < 0.01$), suggesting that oxB/hcrt-2 increases the probability of transmitter release.

Next, we further examined the locus of synaptic change by measuring the frequency and amplitude of AMPAR mEPSCs recorded when neurons were voltage clamped at -70 mV. Although oxB/hcrt-2 had no effect on the amplitude of mEPSCs (control: 13.0 ± 0.8 , $n = 6$; oxB/hcrt-2: 12.6 ± 0.6 , $n = 6$, $P > 0.05$; Fig. 3D, E and G), the frequency of mEPSCs was significantly greater than in controls (control: 1.0 ± 0.2 Hz, $n = 6$, oxB/hcrt-2: 1.4 ± 0.2 , $n = 6$, $P < 0.05$; Fig. 3C, E and F). Taken together, these results indicate that oxB/hcrt-2 can increase the probability of glutamate release at VTA synapses.

Activation of OX2/hcrt-2 receptors potentiates NMDAR but not AMPAR currents

oXB/hcrt-2 interacts mainly with OX2/Hcrt-2 receptors, although it also has low affinity for OX1/Hcrt-1 receptors (Sakurai *et al.*, 1998). In the next series of experiments, we attempted to determine through which receptor oxB/hcrt-2 acted to increase glutamatergic synaptic transmission. To determine if oxB/hcrt-2 potentiated NMDAR EPSCs via activation of OX1/Hcrt-1 receptors, we bath applied oxB/hcrt-2 (100 nM) in the presence of the OX1/Hcrt-1 receptor antagonist SB 334867 ($1 \mu\text{M}$; Fig. 4A). oxB/hcrt-2 potentiated NMDAR eEPSCs by $128 \pm 7\%$ ($n = 7$, Fig. 4A). This effect was not significantly different from NMDAR potentiation by oxB/hcrt-2 alone ($P > 0.05$, Fig. 1G). We tested if oxB/hcrt-2 could potentiate NMDAR eEPSCs in the presence of a putative OX2/hcrt-2 receptor antagonist, JNJ-10397049, which has 600-fold selectivity for OX2/Hcrt-2 over OX1/Hcrt-1 (McAtee *et al.*, 2004). The putative OX2/Hcrt-2 receptor antagonist significantly inhibited oxB/hcrt-2-mediated potentiation of NMDAR eEPSCs (117 ± 5 , $n = 7$, $P < 0.05$; Fig. 4B). Additionally, we found that NMDAR-mediated potentiation by 10 nM oxB/hcrt-2, a concentration that does not potentiate AMPARs, could be completely blocked by JNJ-10397049 ($1 \mu\text{M}$; $n = 6$; Fig. 4C), suggesting that the postsynaptic effects of oxB/hcrt-2 on NMDAR currents occur through activation of OX2/Hcrt-2 receptors. To confirm that NMDAR eEPSCs potentiated via activation of OX2/Hcrt-2 receptors, we applied the highly selective OX2/Hcrt-2 agonist Ala¹¹-D-Leu¹⁵-orexin B to VTA slices. Bath-applied Ala¹¹-D-Leu¹⁵-orexin B (10 nM) significantly increased NMDAR eEPSCs in VTA neurons (Fig. 4D, $166 \pm 20\%$, $n = 7$, $P < 0.01$, 6/7 neurons responded). This effect was completely blocked by the putative OX2/Hcrt-2 receptor antagonist JNJ-10397049 (Fig. 4E; $n = 5$). To determine if a purely post-synaptic effect of oxB/hcrt-2 could be blocked by the OX2 antagonist, we measured the response of post-synaptic NMDARs to a submaximal concentration of NMDA ($20 \mu\text{M}$, 30 s) before and 10 min after a 5-min bath application of oxB/hcrt-2 in the presence or absence of JNJ-10397049 ($1 \mu\text{M}$). oxB/hcrt-2 significantly potentiated the response to NMDA (before: 88 ± 10 pA, after: 139 ± 18 pA, Fig. 4F, $n = 7$, $P < 0.05$). In the presence of JNJ-10397049, the NMDA-mediated change in holding current was not significantly different after oxB/hcrt-2 application (before: 90 ± 1 pA, after: 91 ± 12 pA, Fig. 4H, $n = 7$, $P > 0.05$). To determine which receptor was responsible for the presynaptic effects of oxB/hcrt-2 on AMPAR

FIG. 2. oxB/hcrt-2-mediated potentiation of glutamatergic synaptic transmission is dependent on PKC signaling. (A) PKC inhibitors, either bisindolylmaleimide (bis, 1 μ M, $n = 7$) or chelerythrin (1 μ M, $n = 7$) reduced oxB/hcrt-2-mediated potentiation of NMDAR eEPSCs in VTA neurons. (B) An example recording of NMDAR eEPSCs before, during and after a 5-min application of oxB/hcrt-2 in the presence of intracellularly administered bis (1 μ M). Inset is an example trace before (1) and after (2) oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (C) oxB/hcrt-2-mediated potentiation of NMDARs is not altered by intracellularly applied PKA inhibitor, PKI (20 μ M; $n = 6$). (D) An example recording of oxB/hcrt-2 potentiation of NMDAR eEPSCs in the presence of PKI. Inset is an example trace before (1) and after (2) oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (E) AMPAR eEPSCs were potentiated by oxB/hcrt-2 in the presence of intracellularly applied bis (1 μ M, $n = 5$) or chelerythrin (1 μ M, $n = 8$). (F) An example recording of AMPAR currents evoked at -70 mV in the presence of bis (1 μ M) before, during and after oxB/hcrt-2 (100 nM, 5 min) application. Inset is an example trace before (1) and after (2) oxB/hcrt-2 application. Scale bars, 50 pA, 20 ms. (G) oxB/hcrt-2 (100 nM) did not significantly potentiate NMDARs in the presence of externally applied bis (10 μ M, $n = 7$). Inset is an example trace before and after oxB/hcrt-2 application. Scale bars, 50 pA, 20 ms. (H) oxB/hcrt-2 (100 nM) did not significantly potentiate AMPARs in the presence of externally applied bis (10 μ M, $n = 7$). Inset is an example trace before and after oxB/hcrt-2 application. Scale bars, 50 pA, 20 ms. Stimulus artifact on example traces has been removed for clarity.

FIG. 3. oxB/hcrt-2 causes presynaptic glutamate release. (A) A paired-pulse protocol using a 50-ms inter-stimulus interval showed depressed ratio after application of oxB/hcrt-2 (100 nM; $n = 9$; $P < 0.01$). (B) An example trace of a neuron recorded before (upper panel) and 10 min after a 5-min oxB/hcrt-2 application (lower panel). Stimulus artifact has been removed for clarity. (C) oxB/hcrt-2 (100 nM; $n = 7$) induced a significant increase in mEPSC frequency ($n = 7$; $P < 0.05$, paired t -test). (D) mEPSC amplitude was unchanged after oxB/hcrt-2 application (100 nM; $n = 7$; $P > 0.05$, paired t -test). Bars represent mean and SEM (** $P < 0.01$). (E) Example of mEPSCs before (left) and 10 min after (right) oxB/hcrt-2 (100 nM, 5 min) treatment. (F) Frequency histogram of amplitudes from all mEPSCs from before ($n = 7$) or 10 min after oxB/hcrt-2 treatment ($n = 7$). (G) Frequency histogram of inter-event intervals of all mEPSCs from before ($n = 7$) or 10 min after oxB/hcrt-2 treatment ($n = 7$).

eEPSCs, we tested whether oxB/hcrt-2 potentiation of AMPARs could be blocked by OX1/Hcrt-1 or OX2/Hcrt-2 receptor antagonists. Surprisingly, neither SB 334867 (1 μ M, $123 \pm 7\%$, $n = 6$;

Fig. 5A) nor JNJ-10397049 (1 μ M, $125 \pm 7\%$, $n = 7$; Fig. 5B) significantly blocked oxB/hcrt-2-mediated potentiation of AMPAR currents. Furthermore, bath application of the selective OX2/Hcrt-2

FIG. 4. Potentiation of post-synaptic NMDAR-mediated synaptic transmission by OX2/Hcrt-2 receptors. (A) oxB/hcrt-2 (100 nM, 5 min) potentiated NMDAR eEPSCs in the presence of the selective OX1/Hcrt-1 receptor antagonist SB 334867 (1 μ M, 10 min, $n = 7$). Inset shows an example trace before and after oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (B) The OX2/Hcrt-2 receptor antagonist JNJ-10397049 (1 μ M) partially blocked NMDAR-mediated potentiation by 100 nM oxB/hcrt-2 ($n = 7$). Inset shows an example trace before and after oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (C) oxB/hcrt-2 at 10 nM did not potentiate NMDAR eEPSCs in the presence of JNJ-10397049 (1 μ M). Open squares reflect NMDAR potentiation by 10 nM oxB/hcrt-2 ($n = 7$). Filled squares represent oxB/hcrt-2 application in the presence of 1 μ M JNJ-10397049 ($n = 6$). Inset shows an example trace before and after oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (D) The selective OX2/Hcrt-2 receptor agonist Ala¹¹-D-Leu¹⁵-orexin B (10 nM) increased NMDAR eEPSCs recorded at +40 mV ($n = 7$, 6/7 cells responded). Inset shows an example trace before and after oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (E) JNJ-10397049 (1 μ M) blocked Ala¹¹-D-Leu¹⁵-orexin B- (10 nM) mediated potentiation of NMDARs ($n = 5$). Inset shows an example trace before and after oxB/hcrt-2 application. Scale bars, 50 pA, 50 ms. (F) Response of NMDARs to NMDA (20 μ M, 30 s) was increased 10 min after oxB/hcrt-2 (100 nM, 5 min) bath application. Inset shows an example recording of NMDA-mediated change in current before and after oxB/hcrt-2 (100 nM). (G) In the presence of JNJ-10397049 (1 μ M), the response of NMDARs to NMDA (20 μ M, 30 s) was not potentiated 10 min after oxB/hcrt-2 (100 nM, 5 min) application. Inset shows an example recording of NMDA-mediated change in current before and after oxB/hcrt-2 (100 nM) in the presence of JNJ-10397049 (1 μ M).

FIG. 5. orexin B/hcrt-2 potentiation of AMPARs is not blocked by OX1/Hcrt-1 or OX2/Hcrt-2 antagonists. (A) orexin B/hcrt-2 (100 nM) potentiated AMPAR eEPSCs in the presence of the OX1/Hcrt-1 receptor antagonist SB 334867 (1 μM, $n = 6$). Inset shows an example trace before and after orexin B/hcrt-2 application. Scale bars, 50 pA, 20 ms. (B) In the presence of the OX2/Hcrt-2 receptor antagonist JNJ-10397049 (1 μM, $n = 7$), orexin B/hcrt-2 (100 nM) potentiated AMPAR eEPSCs. Inset shows an example trace before and after orexin B/hcrt-2 application. Scale bars, 50 pA, 20 ms. (C) Ala¹¹-D-Leu¹⁵-orexin B (10 nM) did not increase AMPAR eEPSCs ($n = 7$). Inset shows an example trace before and after orexin B/hcrt-2 application. Scale bars, 50 pA, 20 ms.

receptor agonist Ala¹¹-D-Leu¹⁵-orexin B (10 nM) did not potentiate AMPAR currents (Fig. 5C, $n = 7$). Taken together, these results suggest that orexin B/hcrt-2 can potentiate NMDAR, but not AMPAR currents via activation of OX2/Hcrt-2 receptors, and the receptor

responsible for the orexin B/hcrt-2-mediated presynaptic effect remains unknown.

orexin B/hcrt-2 potentiation of NMDARs inhibits LTP

We have demonstrated that orexin B/hcrt-2 can increase transmitter release and cause a postsynaptic potentiation of NMDARs. LTP in the VTA is dependent on activation of NMDARs (Bonci & Malenka, 1999; Overton *et al.*, 1999). Therefore, we wanted to determine if orexin B/hcrt-2 activation of NMDARs or orexin B/hcrt-2-mediated increases in intracellular Ca²⁺ release in the VTA (Uramura *et al.*, 2001) could alter LTP in VTA slices. To induce LTP, we used a spike-timing protocol consisting of bursts of EPSP–spike pairs, where the onset of EPSPs precedes the postsynaptic spike by 5 ms (Liu *et al.*, 2005). This pairing protocol reliably induces an NMDAR-dependent LTP in VTA dopamine neurons (Liu *et al.*, 2005). EPSP–spike pairing induced robust LTP in untreated rat VTA neurons reaching a maximum of $141 \pm 7\%$ ($n = 11$; Fig. 6A and D). Pre-treatment of orexin B/hcrt-2 (100 nM for 5 min) induced a potentiation of AMPAR EPSPs (Fig. 6B). Interestingly, only a small, non-significant potentiation could be induced with the pairing protocol after orexin B/hcrt-2 potentiated AMPARs ($120 \pm 7\%$, $n = 11$; Fig. 6B and D). We tested if Ala¹¹-D-Leu¹⁵-orexin B, which only potentiates NMDARs but not AMPARs, could occlude LTP. We bath applied Ala¹¹-D-Leu¹⁵-orexin B (10 nM) for 5 min and then induced LTP with the pairing protocol 10 min after washout. EPSP–spike pairing-induced LTP was significantly less than in untreated VTA neurons (117 ± 4 , $n = 7$, $P < 0.05$; Fig. 6C and D). Taken together, these results indicate that significantly less LTP was induced after orexin B/hcrt-2 activation of NMDAR currents in VTA neurons.

To verify if orexin B/hcrt-2 inhibition of LTP occurred in dopaminergic neurons of the VTA, we filled neurons with biocytin while recording EPSPs and subsequently processed slices for TH immunocytochemistry, a marker for dopamine neurons. Of 11 neurons recorded, nine were stained for TH (Fig. 6E).

Discussion

orexin B/hcrt-2 increases presynaptic glutamate release in the VTA

Our findings demonstrate that orexin B/hcrt-2 enhances glutamatergic synaptic transmission in the VTA through action at both pre- and postsynaptic sites. Two peptide products are released from the cleavage of pre-pro-orexin/hypocretin: orexin A/hcrt-1 and orexin B/hcrt-2. orexin A/hcrt-1 is more stable in blood and cerebrospinal fluid (Kastin & Akerstrom, 1999). However, central nervous system orexin B/hcrt-2 levels are consistently 2–5 times higher than orexin A/hcrt-2 levels (Mondal *et al.*, 1999a,b; Date *et al.*, 2000a,b). Both peptides have action in the VTA in that both increase firing in dopaminergic neurons (Korotkova *et al.*, 2003) and, when micro-injected in the VTA, increase dopamine release in the nucleus accumbens (Narita *et al.*, 2006, 2007). Additionally, in a previous study, we found that orexin A/hcrt-1 potentiated NMDARs but not AMPARs in VTA dopamine neurons via activation of OX1/Hcrt-1 receptors and PKC (Borgland *et al.*, 2006). Here, orexin B/hcrt-2 played a distinct role from orexin A/hcrt-1, as orexin B/hcrt-1 potentiated both AMPAR and NMDAR currents in VTA slices. orexin B/hcrt-2 increased mEPSC frequency, but not amplitude, and caused paired-pulse depression of AMPAR currents, two measures that are highly indicative of presynaptic glutamate release. Furthermore, orexin B/hcrt-2-mediated potentiation of AMPAR currents was blocked only by external application of the PKC inhibitor bis, but not intracellularly applied bis. These data suggest that

FIG. 6. oxB/hcrt-2 partially occludes LTP in VTA dopamine neurons. The protocol for LTP induction consisted of 20 bursts of EPSP–spike pairs. (A) Paired stimulation induced LTP in VTA neurons ($n = 11$). (B) Pretreatment of oxB/hcrt-2 (100 nM, 5 min) potentiated AMPAR EPSPs. Further potentiation could not be induced after EPSP–spike pairing ($n = 11$). (C) LTP was reduced after application of Ala^{11} -D-Leu 15 -orexin B (10 nM; $n = 7$). (D) Summary of LTP induction in untreated VTA neurons ($n = 11$), neurons treated with oxB/hcrt-2 ($n = 11$) or Ala^{11} -D-Leu 15 -orexin B-treated neurons ($n = 7$). AMPAR EPSPs were significantly elevated after the pairing protocol in naïve and Ala^{11} -D-Leu 15 -orexin B-treated neurons ($P < 0.001$), but not oxB/hcrt-2-treated neurons ($P > 0.05$). Bars represent mean and SEM. One-way ANOVA with a Bonferroni post test ($**P < 0.01$). (E) Slices from LTP experiments were fixed and processed for the presence or absence of TH and biocytin. oxB/hcrt-2 partially occluded LTP in TH-positive neurons (9/11 neurons were TH positive). TH was labeled with anti-TH antibodies and FITC. Biocytin was labeled with streptavidin-conjugated Texas Red.

oxB/hcrt-2 potentiation of AMPAR currents is due to a PKC-mediated increase in pre-synaptic glutamate release. PKC-mediated increases in presynaptic excitatory synaptic transmission were initially observed in the hippocampus (Malenka *et al.*, 1986; Terrian *et al.*, 1991); however, this is the first observation of PKC-mediated increase in glutamate release in the VTA.

Surprisingly, the oxB/hcrt-2-mediated increase in presynaptic glutamate release was not due to activation of OX1/Hcrt-1 or OX2/Hcrt-2 receptors, as neither antagonists SB334867 nor JNJ-10397049 blocked oxB/hcrt-2-mediated increase in AMPA currents. Furthermore, the selective OX2/hcrt-2 agonist did not potentiate AMPAR currents. Enhanced firing of VTA neurons by oxB/hcrt-2 (Korotkova *et al.*, 2003) could result in increased somatodendritic release of unknown peptides or neuromodulators that may act

presynaptically to increase the probability of transmitter release. However, this is unlikely, as oxB/hcrt-2 enhanced AMPAR mEPSC frequency in the presence of lidocaine, which blocks action potentials. The enhanced presynaptic effect could be due to oxB/hcrt-2 or a metabolite acting at an unidentified PKC-coupled receptor. Further experiments with other antagonists or receptor knockout mice are required to determine the exact pharmacology of oxB/hcrt-2-mediated potentiation of glutamate release.

A presynaptic effect of oxB/hcrt-2 has been observed in other brain regions. In mouse laterodorsal tegmental neurons, oxB/hcrt-2 elevated mEPSC frequency (Burllet *et al.*, 2002). Furthermore, in rat embryonic hypothalamic neurons in culture, oxB/hcrt-2 increased inhibitory post-synaptic current and EPSC frequency in a tetrodotoxin-insensitive manner (Van den pol *et al.*, 1998).

oxB/hcrt-2 potentiates NMDARs in VTA neurons

In addition to the oxB/hcrt-2-mediated increase in transmitter release probability, oxB/hcrt-2 also potentiated postsynaptic NMDAR currents. This effect was independent of the presynaptic action of oxB/hcrt-2, as evidenced by the reduction of NMDAR potentiation with intracellular application of PKC inhibitors, whereas the oxB/hcrt-2-mediated increase in AMPAR synaptic transmission was unaffected. OX2/Hcrt-2 receptors expressed on the VTA dopamine neurons can couple to $G\alpha_q$ or $G\alpha_i$ proteins and enhance intracellular calcium via $G\alpha_q$ or $G\beta\gamma$ activation of phospholipase C and PKC (Uramura *et al.*, 2001; Narita *et al.*, 2007). Furthermore, the post-synaptic current induced by NMDA application was increased after oxB/hcrt-2 application, albeit that the increase induced by oxB/hcrt-2 was smaller than that observed with evoked NMDAR EPSCs. This difference may be attributed to the fact that the evoked recordings examine the effects of both oxB/hcrt-2-induced presynaptic glutamate release on NMDARs in addition to oxB/hcrt-2-mediated post-synaptic receptor potentiation, whereas the bath application of NMDA experiment measures only the latter. Moreover, post-synaptic effects of oxB/hcrt-2 may result in potentiation of selective subtypes of post-synaptic NMDARs, similar to that observed with oxA/hcrt-1 (Borgland *et al.*, 2006), in contrast to the presynaptic enhancement of glutamate release, which would likely affect all NMDAR subtypes.

In contrast to the effects of oxA/hcrt-1-mediated potentiation of NMDARs via both OX2/Hcrt-2 and OX1/Hcrt-1 receptors, oxB/hcrt-2-mediated potentiation of NMDARs was likely mediated solely by activation of OX2/Hcrt-2 receptors because (1) the OX1/Hcrt-1 receptor antagonist SB 334867 did not reduce oxB/hcrt-2 potentiation of NMDARs or AMPARs; (2) the putative OX2/Hcrt-2 receptor antagonist JNJ-10397049 blocked potentiation of NMDARs mediated by 10 nM oxB/hcrt-2, a concentration of oxB/hcrt-2 that potentiates NMDARs but not AMPARs; and (3) the selective OX2/Hcrt-2 receptor agonist Ala¹¹-D-Leu¹⁵-orexin B potentiated NMDAR eEPSCs; this is consistent with previous reports that both OX1/Hcrt-1 and OX2/Hcrt-2 receptors are present on VTA dopamine neurons (Korotkova *et al.*, 2003; Narita *et al.*, 2006). OX1/Hcrt-1 and OX2/Hcrt-2 receptors have been proposed to play distinct roles within the central nervous system, in that activation of OX2/Hcrt-2 receptors exhibited a more pronounced functional effect in serotonergic neurons and OX1/Hcrt-1 exhibited a more pronounced functional effect in noradrenergic neurons (Soffin *et al.*, 2004). Additionally, OX2/Hcrt-2 receptor gene mutation or deletion appears to be the primary cause of narcolepsy in dogs or mice, respectively (Chemelli *et al.*, 1999; Lin *et al.*, 1999), and selective stimulation of OX2/Hcrt-2 receptors promotes wakefulness (Akanmu & Honda, 2005). Therefore, oxB/hcrt-2 enhancement of glutamatergic synaptic transmission via OX2/Hcrt-2 receptors in the VTA may underlie arousal-related effects.

oxB/hcrt-2 inhibits LTP in the VTA

An enhanced AMPAR/NMDAR ratio in VTA dopamine neurons has been associated with the occlusion of LTP, suggesting that excitatory synapses in the VTA are saturated and no further plasticity can be induced (Ungless *et al.*, 2001). Therefore, the size of induced LTP could be inversely related to the degree to which AMPARs can be trafficked or stabilized at the synapse by LTP stimulation paradigms. For example, if there is an increased AMPAR/NMDAR ratio due to enhanced AMPAR-mediated synaptic transmission, there would be less LTP induced as the synapses are already saturated with AMPARs (Ungless *et al.*, 2001). Previously, we found that oxA/hcrt-1 can potentiate the AMPAR/NMDAR ratio when measured hours after application (Borgland *et al.*, 2006).

Spike-timing-dependent LTP pairs a preceding presynaptic spike with a postsynaptic depolarization (Dan & Poo, 2004). In the VTA, this plasticity is NMDAR-dependent (Liu *et al.*, 2005). NMDAR activation is thought to be a coincidence detector and primary source of calcium for inducing spike-timing-dependent LTD (Dan & Poo, 2004). We observed that after application of oxB/hcrt-2 or Ala¹¹-D-Leu¹⁵-orexin B, LTP induced by spike pairing was less than in untreated cells. OX2/Hcrt-2 receptor activation could initiate a signaling pathway that results in increased intracellular calcium influx through NMDAR activation or other mechanisms (Narita *et al.*, 2007) that facilitate AMPAR trafficking. Once this pathway is engaged, the stimulation protocol for spike-timing-dependent LTP is less effective at trafficking AMPARs to the synapse due to the already activated signal transduction pathway or because the synapses are already partially saturated with AMPARs. Presumably, if we induced LTP at a much later time point after application of oxB/hcrt-2, synapses would be completely saturated and we would be unable to induce LTP. Further experiments are required to determine the exact mechanism of oxB/hcrt-2-mediated reduction in LTP at dopamine neuron synapses.

In conclusion, we have observed that oxB/hcrt-2 caused both pre- and post-synaptic strengthening of excitatory synaptic transmission in the VTA. Both pre- and post-synaptic effects were mediated by activation of PKC. A recent study suggested that microinjection of a PKC inhibitor directly in the VTA significantly decreased time spent in a context associated with preference to morphine (Narita *et al.*, 2007). Furthermore, the PKC inhibitor blocked an intra-VTA oxB/hcrt-2-mediated increase in dopamine release in the nucleus accumbens (Narita *et al.*, 2007). Therefore, our results demonstrating a PKC-dependent increase in pre- and post-synaptic excitatory synaptic transmission by oxB/hcrt-2 in the VTA may reflect a strengthening of the association between drugs of abuse and the context in which they are taken.

Acknowledgements

We thank Drs G. Hjelmstad, F. W. Hopf and R. C. Malenka for helpful comments on the manuscript. We also thank Johnson & Johnson Pharmaceutical Research & Development, L.L.C. for its supply of JNJ-10397049. This work was supported by the State of California for medical research on alcohol and substance misuse through the University of California, San Francisco and the National Institutes of Health 1RO1DA15096-01. S.L.B. is supported by an NARSAD young investigators grant.

Abbreviations

bis, bisindolylmaleimide; EPSC, excitatory post-synaptic current; EPSP, excitatory post-synaptic potential; LTP, long-term potentiation; NMDAR, NMDA receptor; OX1/hcrt-1, orexin/hypocretin receptor 1; OX2/hcrt-2, orexin/hypocretin receptor 2; oxA/hcrt-1, orexin A/hypocretin 1; oxB/hcrt-2, orexin B/hypocretin 2; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C; TH, tyrosine hydroxylase; VTA, ventral tegmental area.

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