Orexin Neurons Are Directly and Indirectly Regulated by Catecholamines in a Complex Manner

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Yamanaka, Akihiro, Yo Muraki, Kanako Ichiki, Natsuko Tsujino, Thomas S. Kilduff, Katsutoshi Goto, and Takeshi Sakurai. Orexin neurons are directly and indirectly regulated by catecholamines in a complex manner. J Neurophysiol 96: 284–298, 2006. First published April 12, 2006; doi:10.1152/jn.01361.2005. We reported elsewhere that orexin neurons are directly hyperpolarized by noradrenaline (NA) and dopamine. In the present study, we show that NA, dopamine, and adrenaline all directly hyperpolarize orexin neurons. This response was inhibited by the α2 adrenergic receptor (α2-AR) antagonist, idazoxan or BRL44408, and was mimicked by the α2-AR-selective agonist, UK14304. A low concentration of Ba2+ inhibited NA-induced hyperpolarization, which suggests that activation of G protein coupled inward rectifier potassium channels is involved in the response. In the presence of a high concentration of idazoxan, NA induced depolarization or inward current. This response was inhibited by α1-AR antagonist, prazosin, which suggests the existence of α1-ARs on the orexin neurons along with α2-AR. We also examined the effects of NA on glutamatergic and GABAergic synaptic transmission. NA application dramatically increased the frequency and amplitude of spontaneous inhibitory synaptic currents (sIPSCs) and inhibited excitatory synaptic currents (sEPSCs) in orexin neurons; however, NA decreased the frequency of miniature EPSCs (mEPSCs) and IPSCs and the amplitude of evoked EPSCs and IPSCs through the α2-AR, because the NA response on mPSCs was inhibited by idazoxan. These results suggest that the NA-induced increase in sIPSC frequency and amplitude is mediated via α1-ARs on the somata of GABAergic neurons that innervate the orexin neurons. Calcium imaging using orexin/VC2.1 transgenic mouse brain revealed that NA-induced inhibition of orexin neurons is not altered by sleep deprivation or circadian time in mice. The evidence presented here revealed that orexin neurons are regulated by catecholamines in a complex manner.

INTRODUCTION

Orexin A and orexin B (also called hypocretin-1 and hypocretin-2) are a pair of neuropeptides implicated in the regulation of sleep/wakefulness and energy homeostasis (de Lecea et al. 1999; Sakurai 2005; Sakurai et al. 1998). Orexin neurons are exclusively located in the lateral hypothalamic area (LHA) and project to almost all parts of the brain (Nambu et al. 1999; Takahashi et al. 2002; Yamanaka et al. 2003b). Conversely, serotonin (5-HT) and catecholamines, including noradrenaline (NA) and dopamine (DA), inhibit orexin neurons, whereas histamine has little effect on these cells (Yamanaka et al., 2003b). These observations suggest the possibility that orexin neurons receive direct inhibitory inputs from serotonergic and catecholaminergic neurons. Elsewhere we showed that activation of 5-HT1A receptors and G protein-activated inward rectifier potassium (GIRK) channels are involved in 5-HT-induced hyperpolariz-
tion of orexin neurons (Muraki et al., 2004). Li and van den Pol (2005) reported that orexin neurons are inhibited by noradrenaline through $\alpha_2$ adrenergic receptors ($\alpha_2$-AR) and GIRK channels in mice. Recent reports using rats, however, showed an intriguing result that orexin neurons are activated by NA but are inhibited when rats were sleep deprived for a few hours (Bayer et al. 2005; Grivel et al. 2005). Thus, the effects of catecholamines on the orexin neurons is still controversial. Here we report the effect of catecholamines on orexin neurons in detail by slice patch clamp and calcium imaging of orexin neurons using transgenic mice in which orexin neurons specifically express enhanced green fluorescent protein (EGFP) or a calcium-sensing protein (YC2.1). Electrophysiological experiments revealed that NA activates nonselective cation channels (NSCCs) and GIRK channels on the orexin neurons through $\alpha_1$-AR and $\alpha_2$-AR, respectively. The direct effects of NA on orexin neurons, however, are a balance between an $\alpha_1$-AR-mediated depolarization and $\alpha_2$-AR-mediated hyperpolarization. In addition, NA indirectly inhibited orexin neurons through an $\alpha_1$-AR-mediated increase in GABAergic inhibitory inputs and through an $\alpha_2$-AR-mediated decrease in glutamatergic excitatory inputs. On the other hand, NA inhibited both miniature EPSCs (mEPSCs) or IPSCs through the $\alpha_2$-AR, which is located on the presynaptic membrane of these glutamatergic or GABA-ergic interneuron. Calcium imaging experiments revealed that the NA effect on the orexin neurons was not altered by either sleep deprivation (SD) or circadian time in mice.

**METHODS**

**Animal usage**

All experimental procedures involving animals were approved by the University of Tsukuba Animal Resource Center and were in accordance with National Institutes of Health guidelines. All efforts were made to minimize animal suffering or discomfort and to reduce the number of animals used.

**Slice preparation**

Male and female orexin/EGFP mice (3–4 wk old) in which the human prepro-orexin promoter drives expression of EGFP (lines E2 and E7; Muraki et al. 2004; Yamanaka et al. 2003a; Yamanaka et al. 2003b), were used for experiments. The mice were deeply anesthetized with isoflurane (Takeda, Osaka, Japan) and then decapitated. The brains were isolated in ice-cold bubbled (100% O2) physiological solution containing (mM): NaCl 140, KCl 2, CaCl2 1, MgCl2 1, NaHPO4 1.25; glucose 10. For electrophysiological recording, the physiological solution containing (mM): NaCl 125; KCl 2.0; CaCl2 1; MgCl2 1; NaHCO3 26; NaHPO4 1.25; glucose 10. For electrophysiological recording, the slices were transferred to a recording chamber (RC-26G, Warner Instrument, Hamden, CT) at a controlled temperature of 34°C on a fluorescence microscope stage (BX51WI, Olympus, Tokyo). The slices were superfused with physiological solution that was warmed by an in-line heater (Warner Instrument) to 34°C before entering the recording chamber at a rate of 3 ml/min using a peristaltic pump (Dynamax, Rainin Instruments, Oakland, CA). The fluorescence microscope was equipped with an infrared camera (C2741-79, Hamamatsu Photonics, Hamamatsu, Japan) for infrared differential interference contrast (IR-DIC) imaging and a charge-coupled device (CCD) camera (IK-TU51CU, Olympus) for fluorescent imaging. Each image was displayed separately on a monitor (Gawin, EIZO, Tokyo) and was saved on a Power Macintosh G4 computer (Apple, Cupertino, CA) through a graphic converter (PIX-MPTV, Pixcela, Osaka, Japan).

**Electrophysiological recordings**

Patch pipettes were prepared from borosilicate glass capillaries (GC150-10, Harvard Apparatus, Holliston, MA) with a micropipette puller (P-97, Sutter Instruments, Pangerbrough, UK). The pipettes were filled with an internal solution containing (mM): KCl 145; MgCl2 1; EGTA-Na3 1.1; HEPES 10; MgATP 2; NaGTP 0.5; pH 7.2 with KOH. Osmolarity of the solution was checked by a vapor pressure osmometer (model 5520, Wescor, Logan, UT). Pipette resistance was 4–10 MΩ. The series resistance during recording was 10–25 MΩ and was not compensated. The osmolarities of the internal and external solution were 280–290 and 320–330 mOsm/l, respectively. The trace was low-pass filtered at 5 kHz and digitized at 10 kHz. Data were recorded on a computer through a Digidata 1322A A/D converter using pClamp software version 8.2 (Axon Instruments). The trace was processed for presentation using Origin 6.1 (Origin Lab Corporation, Northampton, MA) and Canvas 9.0 (ACD Systems, Miami, FL) software. Miniature PSCs (mPSCs) were recorded in the presence of TTX (1 μM) in the extracellular solution. Spontaneous PSCs (sPSCs) were recorded in the presence of TTX. Spontaneous EPSCs (sEPSCs) were recorded in the absence of TTX. Spontaneous EPSCs (sEPSCs) and miniature PSCs (mPSCs) were recorded using KCl-based pipette solution containing the sodium channel blocker QX-314 (1 mM) to inhibit action potentials in the recording neuron and in the presence of picrotoxin (100 μM) in the bath to block GABA_A receptor-mediated neurotransmission. To block NMDA and AMPA ionotropic glutamate receptor–mediated neurotransmission, sIPSCs and miniature IPSCs (mIPSCs) were recorded using the same pipette solution but in the presence of AP-5 (50 μM) and CNQX (20 μM), respectively. The frequency and amplitude of PSCs or IPSCs was analyzed by mini analysis software (Synaptosoft, Fort Lee, NJ); only those events with amplitudes >10 pA were used.

**Immunohistochemistry**

Adult male mice C57BL/6J (20–25 g, Charles-River, Kanagawa, Japan) were anesthetized with sodium pentobarbital (50 mg/kg, ip) and perfused sequentially with 20 ml chilled saline and 20 ml chilled 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, trimmed, and immersed in the same fixative solution for 12 h and were then immersed in 30% sucrose solution for 2 days at 4°C. The brains were quickly frozen in optimum cutting temperature (O.C.T.) compound (Sakura Finetechical, Tokyo). Cryostat sections (40 μm) were stained by the avidin-biotin-peroxidase method. Brain
sections were incubated for 40 min in Tris-buffered saline containing 0.3% H2O2 to inactivate endogenous peroxidase. Sections were transferred into Tris-buffered saline containing 0.25% Triton X-100 and 1% bovine serum albumin fraction V (TBS-BX) for 30 min and then incubated with monoclonal anti-tyrosine hydroxylase (TH) antibody (CHEMICON Temecula, CA) diluted 1/400 in TBS-BX overnight at 4°C. Sections were then incubated with biotin labeled anti-mouse IgG goat antibody (Vector) for 1 h at RT followed by incubation with avidin and biotinylated peroxidase complex solution for 30 min at RT. Bound peroxidase was visualized by incubating sections with 0.01 M imidazole acetate buffer containing 0.05% 3,3'-diaminobenzidine tetrahydrochloride, 0.005% hydrogen peroxide and 2.5% nickel ammonium sulfate, which resulted in a black reaction product. For double-labeling, sections were incubated overnight at 4°C with rabbit anti-orexin antibody (Nambu et al. 1999) diluted 1/2000 in TBS-BX. Sections were then incubated with biotin labeled anti-rabbit IgG goat antibody (Vector) for 1 h at RT. Bound peroxidase was visualized as described above without nickel sulfate, resulting in a golden-brown reaction product. The sections were mounted and examined with a microscope (AX-70, Olympus). To confirm the specificity of antibodies, incubations without primary antibody were conducted as a negative control in each experiment and no signal was observed.

Calcium imaging of orexin neurons

Male orexin/YC2.1 mice (8 wk old, 20–24 g) in which orexin neurons specifically express calcium sensing protein (Tsujino et al. 2005), were housed under controlled lighting (12 h light-dark cycle; light-on 8:00 A.M.–8:00 P.M.) and temperature (22°C) conditions. Orexin/YC2.1 mice were sleep deprived for 2 or 4 h in a slow motor-driven drum (60 cm diameter) rotating at a rate of 3 rpm. During SD, the mice were constantly observed and received a gentle nudge when they stopped moving. SD was initiated at Zeitgeber Time 1 (ZT1) and ended at ZT5 for 4 h SD and was initiated at ZT3 and ended at ZT5 for 2 h SD. Brain slices (350 µm thickness) were made at 13:00 (ZT5) by the same method as described in the electrophysiological studies. To evaluate possible circadian effects, brain slices were made at ZT5 (light period) and ZT14 (dark period) without SD. Optical recordings were performed on a fluorescence microscope (BX51WI, Olympus) equipped with a cooled CCD camera (Cascade 550, Roper Scientific, Tucson, AZ) controlled by MetaFluo 5.0.7 software (Universal Imaging, West Chester, PA). YC2.1 was excited through a 440DF20 filter and its fluorescent image was subjected to dual emission ratio imaging through two emission filters (480DF30 for ECFP and 535DF26 for EYFP) controlled by a filter changer (Lambda 10–2, Sutter Instruments, Novato, CA). Images were captured at a rate of 1 Hz (300–500 ms exposure time) with 2 × 2 binning through a 20 × UMPlanFI water immersion objective (Olympus).

Drugs and drug application

The drugs used were tetrodotoxin, barium chloride (Wako, Osaka, Japan), noradrenaline, dopamine, adrenaline (±-arterenol), idazoxan, prazosin, QX-314, 6-cyano-7-nitroquinoline-2,3-dione (CNQX), 2-aminopyridine-5-pentonic acid (AP-5), picROTOxin, UK14304 (Sigma, St Louis, MO) and BRL44408 (TOCRIS, Northport, UK). In the electrophysiological experiments, drugs were dissolved in HEPES-buffered solution and applied either by bath application or local application by gravity flow through a thin polyethylene tube (diameter 100 µm) positioned near the cells being recorded. Agonists were dissolved in the extracellular solution. The solution was switched by a valve perfusion control system (VC-6, Warner Instrument). Selective receptor antagonists were applied by bath application using a peristaltic pump (Dynamax, Rainin Instruments) at a rate of 3 ml/min. In the calcium imaging experiments, both agonists and antagonists were applied by bath application at a rate of 3 ml/min. In both experiments, the same recording chamber (RC-26G, Warner Instrument Corp.; 180 µl volume) was used.

Statistical analyses

Data were analyzed by two-way ANOVA followed by Fisher’s protected least significant difference test using the Stat View 4.5 software package (Abacus Concepts, Berkeley, CA). Probability (p) values <0.05 were considered statistically significant.

RESULTS

Orexin-ir neurons are in apposition to TH-ir nerve endings

Orexin neurons have been well established to be localized to the LHA. To determine whether these cells receive catecholaminergic input, coronal sections through the region containing these neurons were studied using double immunostaining for orexin (brown) and for TH (black). Figure 1 demonstrates TH immunoreactivity (TH-ir) in the region of the orexin neurons. The majority of orexin-ir neurons were located within a field of dense TH-ir axons (Fig. 1A). TH-ir varicosities were closely apposed to orexin-ir cell bodies (Fig. 1, B and C, arrowhead).

Catecholamines hyperpolarize orexin neurons in the presence or absence of TTX

To study the effect of catecholamines on orexin neurons, whole-cell current clamp and voltage clamp recordings were made on acute slice preparations of orexin/EGFP transgenic mice. In current clamp mode, NA application hyperpolarized

FIG. 1. TH-ir nerve endings are in close apposition to orexin-ir neurons. Coronal sections through the hypothalamus containing orexin neurons were studied using double immunostaining. A: immunoreactivity for orexin (brown) is localized in the LHA. Axon fibers and terminals which show immunoreactivity for TH (black) are localized in the same area. TH-ir varicosities are closely apposed to an orexin-ir cell body (B and C, red arrowheads). Scale bars 20 µm in B and C.
the membrane potential of all EGFP-positive neurons (orexin neurons) tested in the presence or absence of TTX (Fig. 2A, top and middle, n = 80). In the presence of TTX, NA (30 μM) application significantly decreased membrane resistance to 47% of control values; membrane resistance of orexin neurons before and after NA application was 613.1 ± 24.8 and 289.6 ± 16.9 MΩ (n = 5), respectively. At a holding potential of −60 mV under voltage clamp, NA (30 μM) induced an outward current in orexin neurons in the presence of TTX (19.0 ± 3.8 pA, n = 4; Fig. 2A, bottom). Figure 2C demonstrates that NA hyperpolarized orexin neurons in a concentration-dependent manner: E\text{max} was 17.3 ± 0.5 mV at 100 μM; IC\text{50} was 6.7 ± 0.7 μM (n = 4–6). Adrenaline also induced hyperpolarization of orexin neurons in a concentration-dependent manner (Fig. 2C); the effect was more potent than that of NA (E\text{max} was 24.2 ± 0.4 mV at 30 μM; IC\text{50} was 2.4 ± 0.2 μM, n = 4–6).

DA also induced hyperpolarization of orexin neurons but was much less potent than either NA or adrenaline: although the efficacy of the DA-induced response was similar to that of NA (E\text{max} = 17.5 ± 0.6 mV), the potency was much lower (IC\text{50} = 141.5 ± 21.9 μM, n = 4–6; Fig. 2C). In current clamp mode, DA hyperpolarized orexin neurons in the presence of 1 μM TTX (Fig. 2B, top trace). In the presence of TTX, DA (300 μM) application decreased membrane resistance to 67.0% of control values; membrane resistance of orexin neurons before and after DA application was 749.1 ± 118.4 and 502.3 ± 49.1 MΩ (n = 4), respectively. At a holding potential of −60 mV in voltage clamp, DA (300 μM) induced an outward current in the presence of TTX (5.8 ± 1.7 pA, n = 4; Fig. 2B, bottom trace).

Activation of the α\text{2}-adrenergic receptor (α\text{2}-AR) is involved in NA-induced hyperpolarization

To identify the subtype of the adrenergic receptor involved in NA-induced hyperpolarization of orexin neurons, preferential adrenergic receptor antagonists were used. Idazoxan, a selective α\text{2}-AR antagonist, inhibited the hyperpolarization induced by 30 μM NA in a concentration-dependent manner (Fig. 3). NA-induced hyperpolarization was partially blocked by bath application of 0.1 μM idazoxan and completely blocked by 1 μM idazoxan (Fig. 3A–B). Pretreatment of slices with 0.01 and 0.1 μM idazoxan for 1.5 min inhibited 30-μM NA-induced hyperpolarization to 65.5 ± 10.7% (n = 4) and 27.0 ± 15.8% (n = 6), respectively, compared with before antagonist treatment. Involvement of the α\text{2}-AR in this NA-induced hyperpolarization was further confirmed by use of the α\text{2}-AR selective agonist, UK14304 (Fig. 3C). UK14304 appli-

![Graph](image-url)
cation induced hyperpolarization in the orexin neurons in a concentration-dependent manner: 1 μM and 10 μM of UK14304 hyperpolarized orexin neurons by 38.3 ± 8.0% (n = 4) and 72.1 ± 6.3% (n = 4), respectively, compared with prior application of NA (30 μM). To determine which subtype of the α2-AR is involved in this response, the α2A receptor selective antagonist, BRL44408 (Young et al., 1989), was tested. BRL44408 (3 μM) almost completely inhibited NA-induced hyperpolarization to 6.3 ± 2.5% of control values (n = 3), which suggests that the α2A receptor subtype may mediate this response. We also found that NA induces a slight depolarization of orexin neurons in the presence of 1 μM idazoxan. The depolarization became more prominent at membrane potentials more negative than −50 mV; 1, 10, and 30 μM NA induced 1.9 ± 0.9, 12.0 ± 2.5, and 17.5 ± 3.0 mV (n = 6) depolarization, respectively, when membrane potential was adjusted at −70 mV before the experiment (Fig. 3, D and E). NA-induced depolarization was eliminated by coapplication of the selective α1-AR antagonist prazosin (n = 6, Fig. 3B). Isoproterenol (100 μM, n = 6), a β-adrenergic receptor agonist, had little effect on orexin neurons (Fig. 3C). In addition, propranolol (20 μM, n = 6), a β-adrenergic receptor antagonist, did not influence the NA-induced response (Fig. 3C), suggesting that the β-adrenergic receptor is not involved in the NA-induced response in orexin neurons.

DA-induced hyperpolarization was also inhibited by idazoxan; 0.1, 1, and 10 μM idazoxan suppressed the DA (300 μM) response to 78.2 ± 8.6%, 63.0 ± 1.5% and 38.4 ± 4.4% (n = 4) of the pretreatment level, respectively, which suggests that this response is also mediated by α2-ARs. This is consistent with our observation that potency of DA-induced hyperpolarization was much lower than that of NA or adrenaline.

NA-induced hyperpolarization of orexin neurons is mediated by activation of GIRK potassium channels

Figure 4 presents evidence that the NA-induced decrease in input resistance occurs through an increase in potassium conductance. The reversal potential estimated from the I-V relationship was −106 mV (n = 6) in normal external solution containing 2 mM K+ (Fig. 4, A and B). This value is similar to the theoretical K+ equilibrium potential (−116 mV) calculated
from the Nernst equation (dotted line in Fig. 4C) by using the K⁺ concentration of the external and pipette solutions. Similar results were obtained when recording in the physiological bicarbonate buffer; reversal potential of the NA-induced response was –110.2 ± 5.9 mV (n = 5) in this condition. As the extracellular K⁺ concentration ([K⁺]o) was increased to 10 mM, the reversal potential (E_{rev}) shifted to –66.6 ± 4.9 mV (n = 5; Fig. 4C). The slope of the E_{rev} values for a 10-fold change in [K⁺]o was 43.6 mV. E_{rev} of the DA-induced response (110.3 ± 14.4 mV; n = 5) was also similar to the theoretical K⁺ equilibrium potential.

We used the GIRK channel inhibitor Ba²⁺ to evaluate the possible involvement of the GIRK channel in NA-induced hyperpolarization. Pretreatment with Ba²⁺ for 2 min inhibited NA-induced hyperpolarization in a concentration-dependent manner (Fig. 4, D and E). Incubation with 30 μM and 300 μM Ba²⁺ inhibited 30-μM NA-induced hyperpolarization to 55.2 ± 8.8% (n = 5) and 26.4 ± 7.2% (n = 5), respectively, compared with the response before Ba²⁺ treatment (Fig. 4E). The inhibition by Ba²⁺ was reversible; NA-induced hyperpolarization recovered after washout for 10 min (data not shown).

In the presence of idazoxan, NA-induced inward current through α₁-ARs

Although NA 30 μM induced 22.5 ± 5.7 pA (n = 9) of outward current in voltage clamp experiments (Figs. 2A and 5A), in the presence of idazoxan, NA induced 13.7 ± 2.5 pA (n = 15) of inward current (Fig. 5A). Therefore we studied the NA-induced inward current in the presence of idazoxan in detail. This inward current was robustly enhanced in the extracellular calcium ion–free (Ca²⁺-free) solution (see Fig. 6, A and B). Thus the concentration dependency of this inward current was tested in the Ca²⁺-free solution. NA induced an inward current in a concentration-dependent manner in the presence of idazoxan and in the Ca²⁺-free solution (Fig. 5B). E_{max} and EC_{50} were 165.6 ± 5.2 pA and 10.7 ± 0.7 μM (n = 6), respectively. This inward current was inhibited by prazosin, a selective α₁-AR antagonist, in a concentration-dependent manner (Fig. 5, C and D). Prazosin inhibited NA-induced inward current to 45.2 ± 9.0% (0.01 μM prazosin, n = 6) and 4.8 ± 1.9% (0.1 μM prazosin, n = 6) of control levels (Fig. 5D). The α₁-AR selective agonist, phenylephrine, mimicked an inward current (Fig. 5E), which supports the results obtained with the α₁-AR selective antagonist. Phenylephrine-induced inward current was 16.0 ± 2.2% (10 μM, n = 7) and 46.6 ± 6.1% (100 μM, n = 7) of the 30-μM NA-induced inward current in the presence of idazoxan (Fig. 5F).

An activation of NSCCs is involved in the NA-induced inward current

NA-induced inward current in the presence of idazoxan was robustly potentiated by removing extracellular calcium ions. This inward current increased approximately 14-fold in calcium-free solution, which suggests that the NA-induced inward current was suppressed by extracellular calcium ions. Thirty-micrometer NA-induced current in calcium-free extra-
cellular solution was 195.8 ± 73.3 pA (n = 8; Fig. 6A). This value is larger than the E\textsubscript{max} value, which was obtained from the concentration-response curve (Fig. 5B), because this potentiated inward current in the calcium-free solution has the tendency to dampen by repeated activation. To examine what types of channels are involved in the NA-induced inward current, the reversal potential was determined by means of a ramp protocol. Reversal potential of the NA-induced current in the presence of idazoxan (1\,\mu{M}) was near 0 mV (4.5 ± 5.8 mV, n = 7) when measured using a CsCl pipette solution containing the sodium channel blocker QX-314 (1 mM) in the bath solution. The IPSC is recorded as an inward current because the high intracellular chloride concentration used results in a reversal potential, which is more negative Na\textsuperscript{+}, which suggests the involvement of NSCCs. NA-induced inward current is inhibited by a NSCC blocker, SKF96365, in a concentration-dependent manner (Fig. 5, A and B). NA (30\,\mu{M}) induced an inward current in a concentration-dependent manner. The experiments were performed in Ca\textsuperscript{2+}-free solution in the presence of idazoxan (n = 7). NA-induced inward current in the presence of 1\,\mu{M} idazoxan was inhibited by \alpha\textsubscript{1}-AR antagonist, prazosin, in a concentration-dependent manner (n = 6). NA (30\,\mu{M}) was applied during the period indicated by solid bars in the presence of idazoxan (1\,\mu{M}; in voltage clamp mode held at -60 mV, NA (30 \mu{M}) normally induced an outward current, but under the presence of \alpha\textsubscript{2}-AR antagonist idazoxan (1 \mu{M}) induced an inward current. B: NA induced an inward current in a concentration-dependent manner. The experiments were performed in Ca\textsuperscript{2+}-free solution in the presence of idazoxan (n = 7). C and D: NA-induced inward current in the presence of 1 \mu{M} idazoxan was inhibited by \alpha\textsubscript{1}-AR antagonist, prazosin, in a concentration-dependent manner (n = 6). NA (30 \mu{M}) was applied during the period indicated by solid bars in the presence of idazoxan (1 \mu{M}). E and F: \alpha\textsubscript{1}-AR agonist, phenylephrine, mimicked NA-induced an inward current in a concentration-dependent manner (n = 7). Responses were normalized to the peak current induced by 30 \mu{M} NA, which was applied before each experiment. Values are means ± SE.

NA influences EPSCs and IPSCs in orexin neurons

To examine the possibility that NA also affects synaptic inputs to orexin neurons, EPSCs and IPSCs were recorded in orexin neurons under whole-cell voltage clamp mode at a holding potential of -60 mV. sEPSCs or sIPSCs were recorded in the absence of TTX. sEPSCs were recorded using KCl-based pipette solution containing calcium channel blocker QX-314 (1 mM) to inhibit action potentials in the recording neuron and in the presence of the GABA\textsubscript{A} receptor antagonist, picrotoxin (100 \mu{M}), in the bath solution. NA (30 \mu{M}) significantly decreased sEPSC frequency to 38.2 ± 6.7% of control values (n = 6, P < 0.0001, ANOVA) (Fig. 5A, B), whereas sIPSC frequency recovered partially after NA washout. sEPSC amplitude was not altered by NA application. Mean amplitude of before and after NA application was 24.3 ± 2.9 pA and 26.5 ± 3.1 pA, respectively (n = 6, P = 0.31, not significantly different). When the ionotropic glutamate receptor antagonists AP-5 (50 \mu{M}) and CNQX (20 \mu{M}) were added to the bath solution, sEPSCs were completely abolished, suggesting that they were due to the activation of ionotropic glutamate receptors (data not shown). The 30-\mu{M} NA-induced decrease in sEPSC frequency was inhibited in the presence of the \alpha\textsubscript{2}-AR antagonist, idazoxan (1 \mu{M}, n = 6, P < 0.05) (Fig. 7B).

We also examined the effect of NA on the sIPSCs. sIPSCs were recorded using KCl-based pipette solution containing QX-314 (1 mM) in the presence of AP-5 (50 \mu{M}) and CNQX (20 \mu{M}) in the bath solution. The IPSC is recorded as an inward current because the high intracellular chloride concentration used results in a reversal potential, which is more

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FIG. 5. Orexin neurons express both \alpha\textsubscript{1}-ARs and \alpha\textsubscript{2}-ARs. A: in voltage clamp mode held at -60 mV, NA (30 \mu{M}) normally induced an outward current, but under the presence of \alpha\textsubscript{2}-AR antagonist idazoxan (1 \mu{M}) induced an inward current. B: NA induced an inward current in a concentration-dependent manner. The experiments were performed in Ca\textsuperscript{2+}-free solution in the presence of idazoxan (n = 7). C and D: NA-induced inward current in the presence of 1 \mu{M} idazoxan was inhibited by \alpha\textsubscript{1}-AR antagonist, prazosin, in a concentration-dependent manner (n = 6). NA (30 \mu{M}) was applied during the period indicated by solid bars in the presence of idazoxan (1 \mu{M}). E and F: \alpha\textsubscript{1}-AR agonist, phenylephrine, mimicked NA-induced an inward current in a concentration-dependent manner (n = 7). Responses were normalized to the peak current induced by 30 \mu{M} NA, which was applied before each experiment. Values are means ± SE.
positive than the holding potential used. NA (30 μM) application dramatically increased IPSC frequency by 398.1 ± 78.6% (n = 6, P < 0.0001) (Fig. 7, B and D). sIPSC amplitude was also increased by 340 ± 100% (n = 6) of control value. sIPSCs returned to basal levels after NA washout and were abolished by coapplication of picrotoxin (100 μM) (data not shown). The 30-μM NA-induced increase in sIPSCs was abolished in the presence of the α1-AR antagonist, prazosin (1 μM, n = 6, P < 0.0001, ANOVA) (Fig. 7D). The α1-AR agonist, phenylephrine (100 μM), significantly increased sIPSC frequency to 531.6 ± 173.3% (n = 5, P < 0.05) of control levels. On the other hand, the α2-AR agonist UK14304 (10 μM) significantly decreased sIPSC frequency to 24.9 ± 6.8% (n = 5, P = 0.0002) of control values. NA did not induce further increase in sIPSC frequency in the presence of idazoxan. In the presence of idazoxan, NA application increased sIPSC frequency to 408.7 ± 92.7% of control levels (n = 6).

To reveal whether these NA effects on the PSCs are mediated via a presynaptic mechanism or via neuronal somata, the effect of NA on the mEPSCs and mIPSCs were studied in the presence of TTX (1 μM). NA application decreased mEPSCs frequency to 38.5 ± 8.9% of control levels (n = 6, P < 0.001, ANOVA) (Fig. 8, A-C); however, in contrast to the NA effect on the sIPSCs, NA induced a decrease in mIPSC frequency: mIPSCs decreased to 32.6 ± 4.1% of control levels (n = 7, P < 0.0001, ANOVA) (Fig. 8, B-D). mEPSC and mIPSC frequency were recovered by NA washout. The amplitude of both mEPSCs and IPSCs was not altered by NA (30 μM) application. mEPSC and mIPSC amplitude during NA application was 89.1 ± 5.5% (n = 5) and 99.8 ± 4.9% (n = 5), respectively, compared with basal amplitude. Although the frequency of mEPSCs or mIPSCs was not significantly altered in the presence of idazoxan (1 μM), it abolished the NA-induced decrease in mEPSCs and mIPSCs. This observation suggests an involvement of the α2-AR in these responses (Fig. 8, C and D). These data suggest a possibility that the NA-induced increase in sIPSCs is mediated through the α2-AR localized on the somata of GABAergic interneurons, which innervate orexin neurons. On the other hand, α2-ARs might exist on both GABAergic and glutamatergic terminals that synapse onto orexin neurons. To confirm this hypothesis, the effects of NA on the electrically evoked EPSCs (eEPSCs) or IPSCs (eIPSCs) were also examined. Electrical stimuli (100–200 μA, 0.1 ms, 0.1 Hz) were generated using bipolar stimulation electrodes placed within the LHA. eEPSCs or eIPSCs were recorded from orexin neurons voltage clamped at −60 mV by use of a KCl-based pipette solution. In the presence of picrotoxin (100 μM), eEPSCs with an amplitude of 226.0 ± 22.6 pA (n = 5) were recorded. NA (30 μM) application rapidly reduced the eEPSC
Effect of NA on the calcium current

To examine the possibility that NA also affects calcium current in orexin neurons, calcium current was recorded under whole-cell voltage clamp. AP-5 (50 μM) and CNQX (20 μM), picrotoxin (100 μM), and TTX (1 μM) were added to the bath solution to block synaptic activity. BaCl$_2$ was substituted for CaCl$_2$ in the bath solution to increase the conductance of the calcium channels. Voltage ramps from −60 mV to 40 mV for 2 s induced −258.9 ± 26.0 pA (n = 6) inward current, which was inhibited by the calcium channel inhibitor, Cd$_{2}^{2+}$ (200 μM) and Ni$_{2}^{2+}$ (100 μM) (Fig. 10). NA (100 μM) decreased Ba$_{2}^{2+}$ current by 197 ± 19.0 pA (n = 6), (P < 0.001, ANOVA). Ba$_{2}^{2+}$ current was completely recovered after NA washout. This result suggests that NA inhibits calcium channels on the orexin neurons.

Effect of SD on the NA responsiveness of orexin neurons

Recent reports using rats showed that orexin neurons are activated by NA but are inhibited when the rats were sleep deprived for a few hours before the experiments (Bayer et al. 2005; Grivel et al. 2005). In contrast, we and Li and van den Pol (2005) observed that mouse orexin neurons showed NA-induced hyperpolarization. To examine whether SD alters the response of mouse orexin neurons to NA, we performed calcium imaging using hypothalamic slices from orexin/YC2.1 transgenic mice in which orexin neurons specifically express calcium-sensing protein. We previously reported that this system detects both increases and decreases in intracellular calcium concentration (Tsujino et al. 2005). In addition, this system allows us to examine the effect of biologically active substances on orexin neurons in adult mice for more than several hours, whereas only very young mice are usable for patch clamp recording. First, we confirmed that this system can detect NA-induced inhibition or activation of orexin neurons.

Orexin/YC2.1 mice of 6 wk of age were sleep deprived for 2 or 4 h before experiments in a motor-driven drum (60-cm diam) rotating at a rate of 3 rpm. To control for circadian effects, all slices were prepared at the circadian time 13:00 (ZT 5). NA (30 μM) application decreased the YFP/CFP ratio in orexin neurons in both control and sleep-deprived mice (Fig. 11, A–C). eEPSCs were completely blocked by coapplication of picrotoxin (100 μM) (Fig. 11, D–E). These results suggest that this system detects both increases and decreases in intracellular calcium concentration and increases GABAergic synaptic transmission to orexin neurons. Stimulation of α$_{1}$-AR antagonist, prazosin (1 μM), significantly increased eEPSC frequency and amplitude (Fig. 11, B and D). These results suggest that this system detects both increases and decreases in intracellular calcium concentration and increases GABAergic synaptic transmission to orexin neurons.
No NA-induced activation of orexin neurons was observed in the sleep-deprived mice. The mean YFP/CFP ratio alteration induced by NA application in control mice and those sleep deprived for 2 h and 4 h was $0.069 \pm 0.006 (n = 19), 0.063 \pm 0.008 (n = 22, P = 0.52, ANOVA)$ and $0.065 \pm 0.007 (n = 23, P = 0.64, ANOVA)$, respectively. Neither the amplitude nor the time course of the NA-induced response was distinguishable between control and sleep-deprived mice (Fig. 11E).

We also examined the effect of circadian timing on NA-induced response of orexin neurons by preparing slices in the dark period at 22:00 (ZT 14). NA induced a decrease in the YFP/CFP ratio of the same magnitude in the orexin neurons: the NA-induced ratio was $0.065 \pm 0.007 (n = 9, P = 0.75, ANOVA)$. These results indicate that NA-induced inhibition of orexin neurons was altered by neither SD nor circadian time in mice.

**Discussion**

Previous studies have demonstrated that NA (Li et al. 2002; Li and van den Pol 2005; Yamanaka et al. 2003b) and DA (Yamanaka et al. 2003b) show inhibitory effects on orexin neurons. The present study confirms these results, extends these observations to adrenaline and establishes that the hyperpolarization induced by catecholamines involves $\alpha_{2A}$-ARs and, subsequently, an activation of GIRK channels. Furthermore, we demonstrate that orexin neurons express $\alpha_1$-ARs as well as $\alpha_{2A}$-ARs and that NA activates NSCCs through an $\alpha_1$-AR-mediated mechanism. Last, catecholamines were found to indirectly influence the activity of orexin neurons by modulating both glutamatergic and GABAergic neurotransmission onto these cells.

**Catecholamines directly inhibit orexin neurons through $\alpha_{2A}$-AR-mediated activation of GIRK channels**

Multiple lines of evidence suggest that the direct catecholaminergic inhibition of orexin neurons is mediated through activation of $\alpha_{2A}$-AR. As indicated in Fig. 2, the potency order for hyperpolarization of the orexin neurons by the three catecholamines tested was adrenaline $> \text{NA} \gg \text{DA}$ (IC$_{50}$ values $2.4 \pm 0.2 \mu M, 6.7 \pm 0.7 \mu M$ and $141.5 \pm 21.9 \mu M$, respectively). Of these three catecholamines, adrenaline is known to have the highest affinity for the $\alpha_2$-ARs. NA has a higher affinity for $\alpha_{1A}$-AR than adrenaline, which might cause the differences of maximal response (Watson and Abbott 1991). NA-induced hyperpolarization was blocked by the $\alpha_2$-AR antagonist idazoxan and was mimicked by the $\alpha_2$-AR agonist UK14304 (Fig. 3, B and C). BRL44408, a selective $\alpha_{2A}$ receptor antagonist (Young et al., 1989), inhibited the NA-induced hyperpolarization. Together, these observations suggest involvement of $\alpha_{2A}$-ARs in the catecholamine-induced hyperpolarization of orexin neurons. On the other hand, at the highest concentration of idazoxan used (1 $\mu M$), NA (30 $\mu M$) did not induce a decrease in mEPSC or mIPSC frequency. PSC frequency was normalized to basal PSC frequency obtained before each experiment. Data are means $\pm$ SE. *, $P < 0.05$, ANOVA. Wash, washout.

**FIG. 8.** NA decreased frequency of both mEPSCs and mIPSCs. mEPSCs and mIPSCs were recorded using whole cell voltage clamp at a holding potential of $-60$ mV in the presence of TTX (1 $\mu M$). mEPSCs were recorded in the presence of picrotoxin (100 $\mu M$), whereas mIPSCs were recorded in the presence of AP-5 (50 $\mu M$) and CNQX (20 $\mu M$). A: NA (30 $\mu M$) application decreased mEPSC frequency. B: NA (30 $\mu M$) application decreased mIPSC frequency. C and D: summaries of the data in A and B, respectively. In the presence of idazoxan (1 $\mu M$), NA (30 $\mu M$) did not induce a decrease in mEPSC or mIPSC frequency. PSC frequency was normalized to basal PSC frequency obtained before each experiment. Data are means $\pm$ SE. *, $P < 0.05$, ANOVA. Wash, washout.
selective α₁-AR antagonist prazosin (Fig. 3B), which suggests that the response of orexin neurons to catecholamines may involve both α₁-AR-mediated depolarization and α₂-AR-mediated hyperpolarization. This means that NA-induced hyperpolarization is a balance between α₁-AR-mediated depolarization and α₂-AR-mediated hyperpolarization. As a result, NA

**FIG. 9.** NA decreases the amplitudes of both eEPSCs and eIPSCs in orexin neurons. eEPSCs and eIPSCs were recorded using whole cell voltage clamp at a holding potential of −60 mV in the absence of TTX. eEPSCs were recorded in the presence of picROTOX (100 μM), whereas eIPSCs were recorded in the presence of AP-5 (50 μM) and CNQX (20 μM).

**A:** typical traces showing the NA effect on the amplitude of eEPSCs that was evoked by electrical stimulation (100–200 μA, 0.1 ms, 0.1 Hz) using bipolar stimulation electrodes placed within the LHA. Traces represent the mean of 10 recordings. **B:** graph showing the mean effect of NA on eEPSC amplitude. **C:** time course of eEPSC inhibition by NA (30 μM). NA application quickly depressed the amplitude of eEPSCs. eEPSCs recovered after NA washout and were completely abolished by application of AP-5 (50 μM) and CNQX (20 μM), which suggests that they were due to activation of ionotropic glutamate receptors. eEPSC amplitude is plotted as the mean of five eEPSC amplitude determinations. NA or AP-5 and CNQX were applied to the bath during the period indicated by the bars. **D:** typical traces showing the NA effect on the amplitude of eIPSCs that was evoked by electrical stimulation (100–200 μA, 0.1 ms, 0.1 Hz) using bipolar stimulation electrodes placed within the LHA. Traces represent the mean of five recordings. **E:** graph showing the mean effect of NA on eIPSC amplitude. Data are means ± SE, * vs. control, P < 0.05. Wash, washout.

**FIG. 10.** NA decreased calcium currents. **A:** NA (100 μM) application inhibited Ba²⁺ current. **B:** summary of the data obtained from experiments such as those illustrated in A. BaCl₂ was substituted for CaCl₂ in the bath solution to increase the conductance of the calcium channel. Calcium currents were recorded by voltage ramp from −60 mV to 40 mV for 2 s. AP-5 (50 μM), CNQX (20 μM), picrotoxin (100 μM), and TTX (1 μM) were added in the bath solution to block synaptic activity. Ba²⁺ currents were blocked by calcium channel blocker Cd²⁺ (200 μM) and Ni²⁺ (100 μM). Data are by means ± SE, * P < 0.05, ANOVA.
acts on α2-AR like a partial agonist. Although we could not completely exclude the presence of DA receptors on the orexin neurons, it seems likely that the DA-induced hyperpolarization is mostly mediated by α2-ARs rather than by DA receptors since a very high concentration of DA is necessary to show hyperpolarization. The fact that DA-induced hyperpolarization is inhibited by the α2-AR antagonist idazoxan supports this idea. These results obtained by electrophysiological experiments are in agreement with recent immunohistochemical studies using adult rats by Modirrousta et al. (2005) showing that orexin neurons express both α1A- and α2A-ARs.

The data in Fig. 4 strongly suggest that the membrane conductance change induced by NA involves an α2-AR-mediated activation of GIRK currents. NA has been reported to increase potassium conductance through GIRK channels via activation of α2-ARs in LC neurons (Arima et al. 1998; Williams et al. 1985). Although three α2-AR subtypes are well known, the concentration-response curves for NA-induced GIRK current activation do not allow distinction among these receptor subtypes. However, BRL44408, a selective α2A-receptor antagonist (Young et al. 1989), almost completely inhibited the NA-induced hyperpolarization. This suggests that an α2A receptor is involved in this NA-induced hyperpolarization of orexin neurons. GIRK current activation has also been proposed to underlie inhibitory effects on orexin neurons that are mediated by Gi-coupled receptors, including the 5-HT1A receptor (Muraki et al. 2004) and the neuropeptide Y1 receptor (Fu et al. 2004).

Both α1-AR and α2-AR are localized on orexin neurons

In the presence of an α2-AR antagonist, NA induced depolarization or inward current in orexin neurons. This NA-induced depolarization or inward current was inhibited by an α1-AR antagonist, which suggests that α1-ARs are involved in this response. Electrophysiological studies revealed an in-
volvement of NSCCs in NA-induced inward current. Elsewhere, we reported that cholecystokinin activates orexin neurons through extracellular calcium ion sensitive NSCCs (Tsujino et al. 2005). NA-induced inward current was also enhanced by removing extracellular calcium ions. In addition, both inward currents are inhibited by SKF96365, a NSCC blocker, which suggests that same NSCC might be involved. Why is hyperpolarization a dominant response to NA on orexin neurons? One possibility is that the hyperpolarization mediated through $\alpha_2$-AR and GIRK channel is faster than the depolarization mediated through $\alpha_1$-AR and NSCC because the former response does not need an intracellular signal cascade. In addition, we reported that NSCC on the orexin neurons activated by CCK through the CCK$_A$ receptor showed voltage dependency (Tsujino et al. 2005). NSCC would be inactivated by the hyperpolarization. The physiological significance of $\alpha_1$-AR mediated activation of NSCC in the orexin neurons is not clear because $\alpha_1$-AR-mediated activation of orexin neurons is only evident in the presence of idazoxan.

Bayer et al. (2005) reported completely opposite effects of NA on orexin neurons using immature rat hypothalamic slice preparation: they observed NA-induced depolarization in orexin neurons. Furthermore, Grivel et al. (2005) recently reported using immature rats that the action of NA on orexin neurons changes from excitation to inhibition after a short 2-h period of total SD. In the current study, we performed electrophysiological analyses using orexin/EGFP mice (immature mice: 2–3 wk old) and concluded that all orexin neurons are inhibited by NA in mice (Fig. 11). It is possible, however, that the type of response to NA on orexin neurons is altered by circadian time or SD, because all electrophysiological experiments were performed during the light period without SD. To address this issue, we performed calcium imaging experiments with orexin/YC2.1 transgenic mice. This system enabled us to analyze several orexin neurons simultaneously and study both young and adult mice. Calcium imaging experiments revealed that the type of response to NA is not dependent on circadian time. This inhibitory response was not altered by SD for 2 or 4 h. It seems likely that catecholamines inhibit orexin neurons in adult mice regardless of circadian time or SD. We and Modirrousta et al. (2005) found that orexin neurons express both $\alpha_1$-AR as well as $\alpha_2$-AR. It is possible that the populations

![Diagram of neural network](image-url)
of orexin neurons that express these adrenergic receptors differ between mice and rats.

**Indirect effects of catecholaminergic systems on orexin neurons**

Catecholamines such as NA not only elicited direct inhibition of orexin neurons through $\alpha_2$-ARs, but also showed an indirect influence on orexin neurons by modulating both IPSCs and EPSCs. NA application resulted in an increase in sIPSC amplitude and an increase in SPSC frequency. The $\alpha_1$-AR antagonist prazosin inhibited the NA-induced increase in sIPSC frequency and $\alpha_1$-AR agonist phenylephrine mimicked NA response (increased sIPSC frequency), which suggests that the $\alpha_1$-AR is involved in this response. This idea is consistent with a report that showed NA increases sIPSCs in GABAergic neurons in the hypothalamic paraventricular nucleus through $\alpha_1$-ARs (Chong et al., 2004). Li and van den Pol (2005) have also reported that orexin neurons were directly inhibited by NA through $\alpha_2$-ARs and that NA indirectly inhibited orexin neurons by facilitating GABAergic transmission at presynaptic sites through $\alpha_1$-ARs. Our results, however, suggest that GABAergic neurons, which innervate orexin neurons, separately express $\alpha_1$-ARs and $\alpha_2$-ARs in somata and presynaptic terminals, respectively. In addition, the NA-induced increase in sIPSCs was mediated through $\alpha_1$-ARs located on somatodendritic membrane, not by a presynaptic mechanism, because mIPSCs frequency and mIPSCs amplitude were both inhibited by NA. On the other hand, the NA-induced decrease in mEPSCs is inhibited by idazoxan, which suggests an involvement of $\alpha_2$-ARs in this response. Both mEPSC frequency and mEPSC amplitude were also decreased by NA, which suggests that a presynaptic inhibitory mechanism through $\alpha_2$-ARs is involved in this response. Li et al. (2002) showed the existence of glutamatergic local circuitry that positively modulates the activity of orexin neurons in the hypothalamus. Furthermore, we recently reported that orexin neurons are innervated by the local interneurons located in the LHA (Sakurai et al., 2005). Thus, it is possible that catecholamines influence the activity of orexin neurons through glutamatergic or GABAergic interneurons in the LHA, as well as through direct action. In the presence of idazoxan (to block presynaptic inhibition through $\alpha_2$-AR), NA did not induce a further increase in sIPSC frequency (408.7 ± 92.7%) compared with NA alone (398.1 ± 78.6%). This result might suggest that presynaptic $\alpha_2$-ARs are expressed on GABAergic neurons, which innervate orexin neurons from outside the hypothalamic slice rather than the interneurons located near the hypothalamic slice.

**Physiological significance of catecholamine-mediated inhibition of orexin neurons**

Although it may appear surprising that waking-active NA neurons would inhibit orexin neurons, which are likely to also be waking-active (Lee et al. 2005; Mileykovskiy et al. 2005), we found that catecholamines also indirectly modulate both GABAergic and glutamatergic inputs to orexin neurons, which suggests a complex physiological role for catecholaminergic influences on regulation of the orexin system. Monoaminergic cell groups such as the NA-containing cells of the LC are generally known to be waking-active neurons (Chu and Bloom 1974; Hobson et al. 1975). Recent studies, however, suggest that orexin neurons do not receive direct synaptic input from the LC in mice or in rats (Sakurai et al. 2005; Yoshida et al. 2006). Rather, orexin neurons might receive noradrenergic input from neurons outside the LC, such as the C1/A1 region, whose discharge rate in relation to behavioral states is currently unknown; however, because these tracing studies are not likely to label secondary or higher-order afferents, indirect effects of NA on orexin neurons might exist. Because NA appears to both directly and indirectly inhibit orexin neurons by modulating local interneurons, the net effect of the noradrenergic system on orexin neurons is very complex.

What are the consequences of the inhibitory actions of NA for orexin neuron activity? The orexin neurons appear to lack the property of spike frequency adaptation and can follow stimulus frequencies as fast as 333 Hz (Li et al. 2002; Yamakaka et al. 2003b). Hyperpolarization of the orexin neurons by catecholamines and 5-HT during wakefulness may have important functional consequences to balance excitatory drive onto these cells. The inhibitory action of NA on orexin neurons might work as a negative feedback system that maintains orexin neuronal activity within appropriate ranges during each behavioral state. Alternatively, this might play an important role in the presynaptic inhibition of orexin neurons at projection sites such as the LC because NA concentration in the LC increases in proportion to the neuronal activity of noradrenergic LC neurons. This idea is supported by the fact that NA also inhibited calcium current in the orexin neurons (Fig. 10), which is involved in the release of neurotransmitters.

**In conclusion,** catecholamines directly and indirectly inhibit orexin neurons. The mechanism of direct inhibition is an $\alpha_2$-AR-mediated activation of GIRK channels in the orexin neurons. The indirect inhibitory mechanism involves both an increase in IPSCs and a decrease in EPSCs in these cells. These direct and indirect influences on orexin neurons by catecholaminergic neurons, summarized in Fig. 12, likely have an important role in both the physiological regulation of orexin neuronal activity and the regulation of sleep and wakefulness.

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