Title: Orexin neurons are directly and indirectly regulated by catecholamines in a complex manner

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Running head: Catecholamines inhibit orexin neurons

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Keywords: orexin, noradrenaline, patch clamp, EPSC, IPSC, calcium imaging
Abstract

We previously reported that orexin neurons are directly hyperpolarized by noradrenaline (NA) and dopamine. In the present study, we show that NA, dopamine and adrenaline all directly hyperpolarized orexin neurons. This response was inhibited by the $\alpha_2$ adrenergic receptor ($\alpha_2$-AR) antagonist, idazoxan or BRL44408, and was mimicked by the $\alpha_2$-AR selective agonist, UK14304. A low concentration of $\text{Ba}^{2+}$ inhibited NA-induced hyperpolarization, suggesting 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 $\alpha_1$-AR antagonist, prazosin, suggesting the existence of $\alpha_1$-ARs on the orexin neurons along with $\alpha_2$-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 $\alpha_2$-AR since 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 $\alpha_1$-ARs on the somata of GABAergic neurons that innervate the orexin neurons. Calcium imaging using orexin/YC2.1 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. 1998; 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; Peyron et al. 1998). Especially dense projections are observed in monoaminergic nuclei such as the noradrenergic locus coeruleus (LC), serotonergic raphe nuclei, histaminergic tuberomammillary nucleus (TMN) and the dopaminergic ventral tegmental area (VTA). The activities of monoaminergic neurons in the brain stem and hypothalamus are reportedly synchronized and strongly associated with behavioral states: they fire tonically during wakefulness, less during non-REM sleep, and not at all during REM sleep. This regulation might be influenced by orexin neurons (which are also wake-active (Lee et al. 2005; Mileykovskiy et al. 2005)) because orexin neurons project to and excite histaminergic neurons in the TMN, noradrenergic neurons in the LC, and serotonergic neurons in the dorsal raphe (DR). The presence of orexin receptor 1 (OX1R) in the LC and OX2R in the TMN and both receptors in the DR have been confirmed. Furthermore, orexins activate isolated cells from these nuclei in vitro (Brown et al. 2002; Eggermann et al. 2001; Hagan et al. 1999; Horvath et al. 1999; Nakamura et al. 2000; Takahashi et al. 2002; Yamanaka et al. 2002).

Mice lacking either the orexin gene (prepro-orexin knockout mice) or orexin neurons (orexin/ataxin-3 transgenic mice) have phenotypes remarkably similar to the human sleep disorder narcolepsy (Chemelli et al. 1999; Hara et al. 2001). Consistent with these findings, human narcolepsy is accompanied by a loss of orexin neuropeptide production and specific destruction of orexin neurons (Nishino et al. 2000; Peyron et al. 2000; Thannickal et al. 2000). The involvement of orexin neurons in narcolepsy supports the idea that these neurons have important roles in the normal regulation of sleep/wakefulness states (Hungs and Mignot 2001;
Electrophysiological and histological studies have shown that orexin neurons excite monoaminergic neurons (Hagan et al., 1999; Horvath et al., 1999; Bourgin et al., 2000; Nakamura et al., 2000; Brown et al., 2001; Eriksson et al., 2001; Brown et al., 2002; (Yamanaka et al. 2003b). Conversely, serotonin (5-HT) and catecholamines, including noradrenaline (NA) and dopamine (DA), inhibit orexin neurons while 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. We showed that activation of 5-HT$_{1A}$ receptors and G-protein-activated inward rectifier potassium (GIRK) channels are involved in 5-HT-induced hyperpolarization 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. However, recent reports using rats 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 non-selective cation channels and GIRK channels on the orexin neurons through $\alpha_1$-AR and $\alpha_2$-AR, respectively. However, the direct effects of NA on orexin neurons are a balance between an $\alpha_1$-AR-mediated depolarization and $\alpha_2$-AR-mediated hyperpolarization. Additionally, 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 or IPSCs through the α2-AR which is located on the presynaptic membrane of these glutamatergic or GABAergic interneuron. Calcium imaging experiments revealed that the NA effect on the orexin neurons was not altered by either sleep deprivation or circadian time in mice.
Methods

Animal usage

All experimental procedures involving animals were approved by the University of Tsukuba Animal Resource Center and the Animal Care and were in accordance with NIH 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 weeks 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 fluothane (Takeda, Osaka, Japan) and then decapitated. The brains were isolated in ice-cold bubbled (100% O2) physiological solution containing (mM): sucrose 280, KCl 2, MgCl2 10, CaCl2 0.5, HEPES 10, glucose 10, pH 7.4 with NaOH. Brains were cut coronally into 300 µm slices with a microtome (VTA-1000S, Leica, Germany). Slices containing the hypothalamus were transferred for at least 1 hr to an incubation chamber at room temperature (RT; 24-26°C) filled with physiological solution containing (mM): NaCl 140, KCl 2, CaCl2 1, MgCl2 1, HEPES 10, glucose 10, pH 7.4 with NaOH. Some experiments were also conducted in physiological bicarbonate buffer 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 Corp., CT, USA) at a controlled temperature of 34°C on a fluorescence microscope stage (BX51WI, Olympus, Tokyo, Japan). The slices were superfused with physiological solution that was warmed by an in-line heater (Warner Instruments, USA) to 34°C before entering the recording chamber at a rate of 3 ml/min using a peristaltic pump (Dynamax, Rainin, CA, USA). The fluorescence microscope was equipped with an infrared camera (C2741-79, Hamamatsu Photonics,
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Hamamatsu, Japan) for infra-red 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, Japan) and was saved on a Power Macintosh G4 computer (Apple, Cupertino, CA, USA) through a graphic converter (PIX-MPTV, Pixcela, Osaka, Japan).

**Electrophysiological recordings**

Patch pipettes were prepared from borosilicate glass capillaries (GC150-10, Harvard Apparatus, MA, USA) with a micropipette puller (P-97, Sutter Instruments, Pangbourne, 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 Inc., Logan, UT, USA). Pipette resistance was 4-10 MΩ. The series resistance during recording was 10-25 MΩ and was not compensated. The osmolarity of the internal and external solution was 280-290 and 320-330 mOsm/L, respectively. The liquid junction potential of the patch pipette solution and perfused HEPES solution was estimated to be 3.9 mV and was applied to the data. Recording pipettes were advanced towards individual cells in the slice while under positive pressure. On contact, tight seals on the order of 0.5-1.0 GΩ were made by negative pressure. The membrane patch was then ruptured by suction and membrane current and potential were monitored using an Axopatch 200B patch clamp amplifier (Axon Instruments, Foster City, CA, USA). Depolarizing and hyperpolarizing current pulses were applied to cells at durations of 200 msec at 20 pA steps at 2-sec intervals from the resting membrane potential (-60 mV) set by varying the intensity of a constantly injected current. The reference electrode was an Ag-AgCl pellet immersed in bath solution. All current clamp recordings were made in Axopatch 200B fast mode. The membrane capacitance was calculated by dividing the time constant by the input resistance. Input resistance was calculated from the slope of the current-voltage
relationship. The output signal was low pass filtered at 5 kHz and digitized at 10 kHz. Data were recorded on a computer through a Digidata 1322 A/D converter using p-Clamp software version 8.2 (Axon Instruments, Union City, CA, USA). The trace was processed for presentation using Origin 6.1 (Origin Lab Corporation, Northampton, MA, USA) and Canvas 9.0 (ACD Systems, Miami, FL, USA) software. Miniature PSCs (mPSCs) were recorded in the presence of TTX (1 µM) in the extracellular solution. Spontaneous PSCs (sPSCs) were recorded in the absence of TTX. Spontaneous EPSCs and miniature EPSCs 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 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 EPSCs or IPSCs was analyzed by mini analysis software (Synaptosoft, Fort Lee, NJ, USA); 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, i.p.) and perfused sequentially with 20 ml chilled saline and 20 ml of chilled 4% paraformaldehyde in 0.1 M phosphate buffer. The brains were removed, trimmed, and immersed in the same fixative solution for 12 hr and were then immersed in 30% sucrose solution for 2 days at 4°C. The brains were quickly frozen in O.C.T. compound (Sakura Finetechnical Co. Ltd., Tokyo, Japan). 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% H_2O_2 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) diluted 1/400 in TBS-BX overnight at 4°C. Sections were then incubated with biotin labeled anti-mouse IgG goat antibody (Vector Laboratories, Burlingame, CA, USA) for 1 hr 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, resulting 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 hr 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 Co., Ltd., Tokyo, Japan). 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 (6 weeks old, 20-24 g) in which orexin neurons specifically express calcium sensing protein (Tsujino et al. 2005), were housed under controlled lighting (12 hr 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 hr in a slow motor driven drum (60 cm diameter) rotating at a rate of 3 rpm. During sleep deprivation (SD), the mice were constantly observed and received a gentle nudge when they stopped moving. SD was initiated at Zeitgeber Time (ZT)1 and ended at ZT5 for 4 hr SD, and was initiated at ZT3 and ended at ZT5 for 2 hr 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 charge-coupled device (CCD) camera (Cascade 650, Roper Scientific, Tucson, AZ) controlled by Meta Fluor 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 x 2 binning through a 20x 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-7nitroquinoxaline-2,3-dione (CNQX), DL-2-amino-5-phosphono-pentanoic acid (AP-5), picrotoxin, UK14304 (Sigma, St Louis, MO, USA) and BRL44408 (TOCRIS, Northpoint, 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 Corp., CT, USA). Selective receptor antagonists were applied by bath application using a peristaltic pump (Dynamax, Rainin Instruments, Oakland, CA, USA) 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, USA). Probability ($p$)-values less than 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 (Figure 1A). TH-ir varicosities were closely apposed to orexin-ir cell bodies (arrowhead in Figure 1B and 1C).

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 the membrane potential of all EGFP-positive neurons (orexin neurons) tested in the presence or absence of TTX (Figure 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; Figure 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$_{50}$ was 6.7 ± 0.7 µM (n=4-6). Adrenaline also induced hyperpolarization of orexin neurons in a concentration-dependent manner (Figure 2C); the effect was more potent than that of NA ($E_{\text{max}}$ was 24.2 ± 0.4 mV at 30 µM; IC$_{50}$ 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 ($E_{\text{max}} = 17.5 \pm 0.6$ mV), the potency was much lower ($IC_{50} = 141.5 \pm 21.9$ µM, n=4-6) (Figure 2C). In current clamp mode, DA hyperpolarized orexin neurons in the presence of 1 µM TTX (Figure 2B, upper 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; Figure 2B, lower trace).

Activation of the $\alpha_2$ adrenergic receptor ($\alpha_2$-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 $\alpha_2$-AR antagonist, inhibited the hyperpolarization induced by 30 µM NA in a concentration-dependent manner (Figure 3). NA-induced hyperpolarization was partially blocked by bath application of 0.1 µM idazoxan and completely blocked by 1 µM idazoxan (Figure 3A and 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 $\alpha_2$-AR in this NA-induced hyperpolarization was further confirmed by use of the $\alpha_2$-AR selective agonist, UK14304 (Figure 3C). UK14304 application 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 $\alpha_2$-AR is involved in this
response, the $\alpha_{2A}$ receptor selective antagonist, BRL44408 (Young et al., 1989), was tested. BRL44408 (3 µM) almost completely inhibited NA-induced hyperpolarization to $6.3 \pm 2.5\%$ of control values ($n=3$), suggesting that the $\alpha_{2A}$ 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 (Figure 3D and E). NA-induced depolarization was eliminated by co-application of the selective $\alpha_1$-AR antagonist prazosin ($n=6$, Figure 3B). Isoproterenol (100 µM, $n=6$), a $\beta$-adrenergic receptor agonist, had little effect on orexin neurons (Figure 3C). In addition, propranolol (20 µM, $n=6$), a $\beta$-adrenergic receptor antagonist, did not influence the NA-induced response (Figure 3C), suggesting that the $\beta$-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, suggesting that this response is also mediated by $\alpha_2$-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$^+$ (Figure 4A and B). This value is similar to the theoretical K$^+$ equilibrium potential (-116 mV) calculated
from the Nernst equation (dotted line in Figure 4C) 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) (Figure 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 Ba\(^{2+}\) to evaluate the possible involvement of the GIRK channel in NA-induced hyperpolarization. Pretreatment with Ba\(^{2+}\) for 2 min inhibited NA-induced hyperpolarization in a concentration-dependent manner (Figure 4D and E). Incubation with 30 and 300 µM Ba\(^{2+}\) 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 Ba\(^{2+}\) treatment (Figure 4E). The inhibition by Ba\(^{2+}\) was reversible; NA-induced hyperpolarization recovered after washout for 10 min (data not shown).

In the presence of idazoxan, NA induced activation of orexin neurons through \(\alpha_1\)-ARs

Although NA (30 µM) induced 22.5 ± 5.7 pA (n=9) of outward current in voltage clamp experiments (Figures 2A and 5A), in the presence of idazoxan, NA induced 13.7 ± 2.5 pA (n=15) of inward current (Figure 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 (Ca\(^{2+}\)-free) solution (see Figure 6A and B). Thus, the concentration dependency of this inward current was tested in the Ca\(^{2+}\)-free solution. NA induced an inward current in a concentration-dependent manner in the presence of idazoxan and in the Ca\(^{2+}\)-free solution (Figure 5B). E\(_{max}\) and EC\(_{50}\) was 165.6 ± 5.2 pA and 10.7 ± 0.7 µM (n=6), respectively. This inward current was inhibited by prazosin, a selective \(\alpha_1\)-AR
antagonist, in a concentration-dependent manner (Figure 5C 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 (Figure 5D). The α₁-AR selective agonist, phenylephrine, mimicked an inward current (Figure 5E), supporting 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 NA (30 µM)-induced inward current in the presence of idazoxan (Figure 5F).

An activation of non-selective cation channels 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, suggesting that the NA-induced inward current was suppressed by extracellular calcium ions. NA (30 µM)-induced current in calcium-free extracellular solution was 195.8 ± 73.3 pA (n=8) (Figure 6A). This value is larger than the E_{max} value which was obtained from the concentration-response curve (Figure 5B) since 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 in calcium-free extracellular solution (mM: 140 NaCl, 2 CsCl, 1 MgCl₂, 1 EGTA, 10 HEPES and 10 glucose) was near 0 mV (4.5 ± 1.4 mV, n=5) when measured using a CsCl pipette solution (mM: 145 CsCl, 1 MgCl₂, 10 HEPES, 1.1 EGTA and 0.5 Na₂GTP) (Figure 6C and D). This reversal potential is midway between negative Cs⁺ and positive Na⁺, suggesting the involvement of non-selective cation channels. NA-induced inward current is inhibited by a non-selective cation channel blocker, SKF96365, in a
concentration-dependent manner (Figure 6E and F). NA (30 µM)-induced inward current was inhibited to 74.4 ± 5.8% (3 µM SKF96365) and 28.6 ± 5.3% (30 µM SKF96365) of control levels. These results suggest that an activation of non-selective cation channels through \( \alpha_{1}\)-AR is involved in the NA-induced inward current observed in the presence of idazoxan.

**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. Spontaneous EPSCs (sEPSCs) or IPSCs (sIPSCs) were recorded in the absence of TTX. sEPSCs 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 the GABA\(_A\) receptor antagonist, picrotoxin (100 µM), in the bath solution. NA (30 µM) application significantly decreased sEPSC frequency to 38.2 ± 6.7% of control values (\( n=6, \ p<0.0001, \) ANOVA) (Figure 7A and C). sEPSC 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 µM) and CNQX (20 µM) 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 NA (30 µM)-induced decrease in sEPSC frequency was inhibited in the presence of the \( \alpha_{2}\)-AR antagonist, idazoxan (1 µM, \( n=6, \ p<0.05\)) (Figure 7C).

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 µM) and CNQX (20 µM) 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
positive than the holding potential used. NA (30 µM) application dramatically increased IPSC frequency by 398.1 ± 78.6% (n=6, p<0.0001) (Figure 7B 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 co-application of picrotoxin (100 µM) (data not shown). The NA (30 µM)-induced increase in sIPSCs was abolished in the presence of the α1-AR antagonist, prazosin (1 µM, n=6, p<0.0001, ANOVA) (Figure 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 miniature EPSCs (mEPSCs) and IPSCs (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) (Figure 8A and 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) (Figure 8B and 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 (Figure 8C and D). These data suggest a possibility that the NA-induced increase in sIPSCs is mediated through the α1-AR localized on the somata of GABAergic
interneurons which innervate orexin neurons. On the other hand, $\alpha_2$-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 msec, 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 using a KCl-based pipette solution. In the presence of picrotoxin (100 µM), eEPSCs with an amplitude of $226.0 \pm 22.6$ pA (n=5) were recorded. NA (30 µM) application rapidly decreased the eEPSC amplitude to $41.3 \pm 8.0\%$ (n=5, $p<0.0001$, ANOVA) as compared with before NA application (Figure 9A, B and C). eEPSCs recovered within 10-18 min after NA washout. Recovered eEPSCs were completely abolished by co-application of AP-5 (50 µM) and CNQX (20 µM) in the bath solution, suggesting that they were attributable to the activation of ionotropic glutamate receptors (Figure 9C).

In the presence of AP-5 (50 µM) and CNQX (20 µM), we observed an eIPSC with an amplitude of $589.8 \pm 277.7$ pA (n=5). NA application decreased the eIPSCs to $27.8 \pm 10.9\%$ (n=5) as compared with before application (Figure 9D and E). eIPSCs were partially recovered after NA washout. Recovered eIPSCs were completely blocked by co-application of picrotoxin (100 µM, data not shown). These observations suggest that NA inhibits both glutamatergic and GABAergic inputs to orexin neurons through a presynaptic mechanism.

The 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), 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 sec induced -258.9 ± 26.0 pA (n=6) inward current, which was inhibited by the calcium channel inhibitor, Cd²⁺ (200 µM) and Ni²⁺ (100 µM) (Figure 10). NA (100 µM) decreased Ba²⁺ current by 197 ± 19.0 pA (n=6), (p<0.001, ANOVA). Ba²⁺ current was completely recovered after NA washout. This result suggests that NA inhibits calcium channels on the orexin neurons.

The effect of sleep deprivation 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). Additionally, 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. Sequential application of NA (30 µM) induced the same amplitude of decrease in YFP/CFP ratio (Figure 11A and B). The ratio alteration induced by the first and the second NA application was 0.091 ± 0.013 (n=7) and 0.094 ± 0.013 (n=7), respectively. In the presence of idazoxan, NA (30 µM) induced an increase in YFP/CFP ratio (0.053 ± 0.007, n=5, Figure 11C and D). These results suggest that this system detects both NA-induced inhibition and activation.

Orexin/YC2.1 mice of 6 weeks of age were sleep deprived for 2 or 4 hr before
experiments in a motor driven drum (60 cm diameter) 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 (Figure 11E). 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 hr and 4 hr was 0.069 ± 0.006 (n=19), 0.063 ± 0.008 (n=22, \( p=0.52, \) ANOVA) and 0.065 ± 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 (Figure 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 ± 0.007 (n=9, \( p=0.75, \) ANOVA). These results indicate that NA-induced inhibition of orexin neurons was neither altered by sleep deprivation nor by 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 α_{2A}-ARs and, subsequently, an activation of GIRK channels. Furthermore, we demonstrate that orexin neurons express α_{1}-ARs as well as α_{2A}-ARs and that NA activates non-selective cation channels through an α_{1}-AR-mediated mechanism. Lastly, 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 α_{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 α_{2A}-AR. As indicated in Figure 2, the potency order for hyperpolarization of the orexin neurons by the three catecholamines tested was adrenaline > NA >> DA (IC_{50} values 2.4 ± 0.2 µM, 6.7 ± 0.7 µM and 141.5 ± 21.9 µM, respectively). Of these three catecholamines, adrenaline is known to have the highest affinity for the α_{2}-ARs. NA has a higher affinity for α_{1A}-AR than adrenaline, which might cause the differences of maximal response (Watson and Abbott, 1991). NA-induced hyperpolarization was blocked by the α_{2}-AR antagonist idazoxan and was mimicked by the α_{2}-AR agonist UK14304 (Figure 3B and 3C). BRL44408, a selective α_{2A} receptor antagonist (Young et al., 1989), inhibited the NA-induced hyperpolarization. Together, these observations suggest involvement of α_{2A}-ARs in the catecholamine-induced hyperpolarization of orexin neurons. On the other hand, at the highest concentration of idazoxan used (1 µM), NA induced a slight depolarization of orexin...
neurons. This depolarization became more prominent in membrane potentials more negative than -50 mV and was eliminated by co-application of the selective $\alpha_1$-AR antagonist prazosin (Figure 3B), suggesting that the response of orexin neurons to catecholamines may involve both $\alpha_1$-AR-mediated depolarization and $\alpha_2$-AR-mediated hyperpolarization. This means that NA-induced hyperpolarization is a balance between $\alpha_1$-AR-mediated depolarization and $\alpha_2$-AR-mediated hyperpolarization. As a result, NA acts on $\alpha_2$-AR like a partial agonist.

Though 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 $\alpha_2$-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 $\alpha_2$-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 $\alpha_{1A}$- and $\alpha_{2A}$-ARs.

The data in Figure 4 strongly suggest that the membrane conductance change induced by NA involves an $\alpha_2$-AR-mediated activation of GIRK currents. NA has been reported to increase potassium conductance through GIRK channels via activation of $\alpha_2$-ARs in LC neurons (Arima et al. 1998; Williams et al. 1985). Although three $\alpha_2$-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 $\alpha_{2A}$ receptor antagonist (Young et al., 1989), almost completely inhibited the NA-induced hyperpolarization. This suggests that an $\alpha_{2A}$ 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-HT$_{1A}$ receptor (Muraki et al. 2004) and the neuropeptide Y1 receptor (Fu et al. 2004).
Both $\alpha_1$-AR and $\alpha_2$-AR are localized on orexin neurons

In the presence of an $\alpha_2$-AR antagonist, NA induced depolarization or inward current in orexin neurons. This NA-induced depolarization or inward current was inhibited by an $\alpha_1$-AR antagonist, suggesting that $\alpha_1$-ARs are involved in this response. Electrophysiological studies revealed an involvement of non-selective cation channels (NSCCs) in NA-induced inward current. Previously, 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, suggesting 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 since the former response does not need an intracellular signal cascade. Additionally, 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 hr period of total sleep deprivation. In the current study, we performed electrophysiological analyses using orexin/EGFP mice (immature mice: 2-3 weeks old) and concluded that all orexin neurons are inhibited by NA in mice (Figure 11). However, it is possible that the type of response to NA on
orexin neurons is altered by circadian time or sleep deprivation since all electrophysiological experiments were done during the light period without sleep deprivation. To address this issue, we performed calcium imaging experiments using orexin/YC2.1 transgenic mice. This system allows us to analyze several orexin neurons simultaneously and both young and also adult mice can be studied. Calcium imaging experiments revealed that the type of response to NA is not dependent on circadian time. This inhibitory response was not altered by sleep deprivation for 2 or 4 hr. It seems likely that catecholamines inhibit orexin neurons in adult mice regardless of circadian time or sleep deprivation. 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 of orexin neurons which 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 $\alpha_{2A}$-AR, 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 sIPSC 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), suggesting 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. However, our results 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 somata membrane, not by a presynaptic mechanism since mIPSCs frequency and eIPSCs amplitude were both inhibited by NA. On the other hand, the NA-induced decrease in mEPSCs is inhibited by idazoxan, suggesting an involvement of α₂-ARs in this response. Both mEPSC frequency and eEPSC amplitude were also decreased by NA, suggesting that a presynaptic inhibitory mechanism through α₂-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 α₂-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 α₂-ARs are expressed on GABAergic neurons which innervate orexin neurons from outside the hypothalamic slice rather than the interneurons located near the orexin neurons.

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, suggesting a complex physiological role for catecholaminergic influences on regulation of the orexin neurons. 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). However, recent studies suggest that orexin neurons do not receive direct synaptic input from the LC in either mice or rats (Sakurai et al. 2005; Yoshida et
Rather, orexin neurons might receive noradrenergic input from neurons outside the LC, such as the C1/A region, whose discharge rate in relation to behavioral states is currently unknown. However, since these tracing studies are not likely to label secondary or higher order afferents, indirect effects of NA on orexin neurons might exist. Since 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(Yamanaka 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 (Figure 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 α2A-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 Figure 12, likely have an important role in both the physiological regulation of orexin neuronal activity and in the regulation of sleep and wakefulness.
Acknowledgements

This study was supported by a grant-in-aid for scientific research (S), (B) and Grant-in-Aid for Scientific Research on Priority Areas <Elucidation of neural network function in the brain> from the Ministry of Education, Culture, Sports, Science and Technology of Japan (17023007), Kanae Foundation, a grant for anorexia nervosa research from the Japanese Ministry of Health, Labor and Welfare and by NIH RO1MH61755 and RO1AG020584. Thanks to Charles Jones for proofreading the manuscript.
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Legends

Figure 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 lateral hypothalamic area. 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 (shown by red arrowheads in B and C). Scale bars 20 µm in B and C.

Figure 2

NA hyperpolarizes orexin neurons. A, In current clamp mode, NA (30 µM) was applied onto orexin neurons in the absence (top) or presence (middle) of TTX (1 µM). Before the experiments, the membrane potential was set at -60 mV by current injection. TTX was applied by bath application and NA was applied locally during the period indicated by the bars. Input resistance was monitored by the amplitude of electrotonic potentials generated by injection of a rectangular wave current pulse (-20 pA, 500 msec, 0.1 Hz). In voltage clamp mode, NA induced an outward current (A, bottom). Membrane potential was held at -60 mV. B, DA hyperpolarizes orexin neurons. In current clamp mode, DA (300 µM) was applied onto orexin neurons in the presence of TTX (1 µM) (upper trace). In voltage clamp, DA induced an outward current (B, lower trace). C, The concentration dependence of the responses to NA, adrenaline and DA. The IC_{50} and E_{max} of NA-, adrenaline- and DA-induced response were 6.7 ± 0.7 µM and 17.3 ± 0.5 mV, 2.4 ± 0.2 µM and 24.2 ± 0.4 mV, and 141.5 ± 21.9 µM and 17.5 ± 0.6 mV, respectively. As shown in Figure 3, the effect of NA on orexin neurons is balance between α_{2}-AR-mediated hyperpolarization and α_{1}-AR-mediated depolarization. The data were fitted by a normal sigmoid fitting since NA response can be thought of as a response to a
partial agonist acting on the $\alpha_2$-AR. Values are mean ± S.E.M. (n=4-12). CC, current clamp; VC, voltage clamp.

**Figure 3**

NA hyperpolarizes orexin neurons via the $\alpha_{2A}$ receptor. A, Idazoxan, a selective $\alpha_2$ receptor antagonist, inhibited NA (30 µM)-induced hyperpolarization of orexin neurons in a concentration-dependent manner. B, The effect of $\alpha$- or $\beta$-AR antagonists on the NA (30 µM)-induced hyperpolarization. Idazoxan inhibited NA-induced hyperpolarization in a concentration-dependent manner. Prazosin, a selective $\alpha_1$-AR antagonist, inhibited the weak NA-induced depolarization that was observed in the presence of a high concentration of idazoxan (1 µM). The $\beta$-AR selective antagonist, propranolol, had no effect on the NA-induced hyperpolarization of orexin neurons. The $\alpha$- or $\beta$-AR antagonists were applied by bath application, whereas NA was applied locally through a fine polyethylene tube located near the recording neurons. C, The effect of $\alpha$- or $\beta$-AR receptor agonists on the membrane potential of orexin neurons. UK14304, a selective $\alpha_2$-AR agonist, induced hyperpolarization in a concentration-dependent manner, while a high concentration of the $\beta$-AR agonist, isoproterenol (100 µM), had little effect on the orexin neurons. The $\alpha$- or $\beta$-AR agonists were applied locally through a fine polyethylene tube located near the recording neurons. All responses were normalized to the peak response induced by 30 µM NA which was applied before each experiment. D, Typical trace showing that NA induced significant depolarization in the presence of 1 µM idazoxan when membrane potential was held at -70 mV before the experiment. E, NA induced depolarization in the presence of 1 µM idazoxan in a concentration dependent manner (n=6). Membrane potential was adjusted by current injection at -70 mV before the experiment. NA, noradrenaline; UK, UK14304; Iso, Isoproterenol. Values are mean ± S.E.M.
Figure 4

NA decreases input resistance through an increase in a potassium conductance. A, Records of membrane potential in response to a series of 100 ms current steps (in 20 pA increments, -200 pA to 0 pA) from resting potential (-60 mV) in the absence (left) or presence (right) of NA (30 µM). B, Current-voltage relationship derived from the data in A. The potential at the end of current injection was plotted; control (open circle) and 30 µM NA (filled circle). Estimated reversal potential ($E_{rev}$) was -110 mV (n=6). C, Reversal potential was shifted by changing potassium concentration in the extracellular solution from 2 mM to 10 mM (n=5). The dotted line shows the potassium reversal potential calculated from each external and internal potassium concentration by means of the Nernst equation. D, The trace showing that the GIRK inhibitor Ba$^{2+}$ concentration-dependently inhibited NA-induced hyperpolarization. Ba$^{2+}$ (30 µM and 300 µM) was applied by bath application. E, Bar graph summarizing the data in D (n=4, *, vs control, $p<0.05$, ANOVA). Values are mean ± S.E.M.

Figure 5

Orexin neurons express both $\alpha_1$-ARs and $\alpha_2$-ARs. A, In voltage clamp mode held at -60 mV, NA (30 µM) normally induced an outward current, but under the presence of $\alpha_2$-AR antagonist idazoxan (1 µM) induced an inward current. B, NA induced an inward current in a concentration-dependent manner. The experiments were performed in Ca$^{2+}$-free solution in the presence of idazoxan (n=7). C and D, NA-induced inward current in the presence of 1 µM idazoxan was inhibited by $\alpha_1$-AR antagonist, prazosin, in a concentration-dependent manner (n=6). NA (30 µM) was applied during the period indicated by solid bars in the presence of idazoxan (1 µM). E and F, $\alpha_1$-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 µM NA which was applied before each experiment. Values are mean ± S.E.M.

**Figure 6**

An opening of non-selective cation channels is involved in the NA-induced inward current in the presence of α2-AR antagonist, idazoxan. A, The effect of extracellular Ca\(^{2+}\) on the NA-induced inward current. NA induced an inward current in the presence of idazoxan that was dramatically increased by removing extracellular Ca\(^{2+}\) ions (n=8). This character is consistent with opening of non-selective cation channels. B, Graph summarizing the data in A. C and D shows the current-voltage relationship obtained by the voltage ramp protocol indicated in C using a CsCl pipette in the Ca\(^{2+}\)-free extracellular solution. The I-V curve shows that the reversal potential of the NA (30 µM)-induced current was 4.5 ± 1.4 mV (n=6). Neurons were voltage-clamped at +40 mV for 2 sec, and the membrane potential was gradually lowered from +40 mV to -60 mV at a duration of 2 sec. E and F, NA-induced inward current in the presence of idazoxan was inhibited by the non-selective cation channel inhibitor, SKF96365, in a concentration-dependent manner (n=7). F, Graph summarizing the data in E. Responses were normalized to the peak current induced by 30 µM NA which was applied before each experiment. Values are mean ± S.E.M.

**Figure 7**

NA decreases glutamatergic synaptic transmission and increases GABAergic synaptic transmission to orexin neurons. sEPSCs and sIPSCs were recorded using whole cell voltage clamp at a holding potential of -60 mV. sEPSCs were recorded in the presence of picrotoxin (100 µM), while sIPSCs were recorded in the presence of AP-5 (50 µM) and CNQX (20 µM). NA (30 µM) application decreased sEPSC frequency (A) and increased sIPSC frequency and
amplitude (B). Mean effect of NA on sEPSC (C) or sIPSC (D) frequency. sEPSCs partially recovered and sIPSCs returned to basal levels after NA washout. The NA-induced decrease in sEPSC frequency is inhibited by the $\alpha_2$-AR antagonist, idazoxan (1 µM). The NA-induced increase in sIPSC frequency is inhibited by the $\alpha_1$-AR antagonist, prazosin (1 µM). PSC frequency was normalized to basal PSC frequency obtained before each experiment. Data shown are mean ± S.E.M. (*, $p<0.05$, ANOVA). Wash, washout.

**Figure 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 µM). mEPSCs were recorded in the presence of picrotoxin (100 µM), while mIPSCs were recorded in the presence of AP-5 (50 µM) and CNQX (20 µM). A, NA (30 µM) application decreased mEPSC frequency. B, NA (30 µM) application decreased mIPSC frequency. C and D summarize the data in A and B, respectively. In the presence of idazoxan (1 µM), NA (30 µM) did not induce a decrease in mEPSC or mIPSC frequency. PSC frequency was normalized to basal PSC frequency obtained before each experiment. Data shown are mean ± S.E.M. (*, $p<0.05$, ANOVA). Wash, washout.

**Figure 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 picrotoxin (100 µM), while 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 msec, 0.1 Hz) using bipolar stimulation electrodes placed within the LHA. Traces
represent the mean of ten 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), suggesting 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 msec, 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 shown are mean ± S.E.M. *, vs control, p<0.05. Wash, washout.

**Figure 10**

NA decreased calcium currents. A, NA (100 µM) application inhibited Ba\(^{2+}\) current. B summarizes the data obtained from experiments such as those illustrated in A. BaCl\(_2\) was substituted for CaCl\(_2\) 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 sec. AP-5 (50 µM), CNQX (20 µM), picrotoxin (100 µM) and TTX (1 µM) were added in the bath solution to block synaptic activity. Ba\(^{2+}\) currents were blocked by calcium channel blocker Cd\(^{2+}\) (200 µM) and Ni\(^{2+}\) (100 µM). Data are shown by mean ± S.E.M. (*, p<0.05, ANOVA).

**Figure 11**

Sleep deprivation did not affect the NA-induced response in mouse orexin neurons. The response to NA in orexin neurons was determined by calcium imaging using a hypothalamic slice preparation of orexin/YC2.1 transgenic mice. A and B, Sequentially applied NA (30 µM)
induced almost the same amplitude of YFP/CFP ratio alteration. C and D, NA (30 μM) increased YFP/CFP ratio in the presence of idazoxan (1 μM). E, Typical traces showing that NA (30 μM) application decreased YFP/CFP ratio in orexin neurons in control (upper trace) and mice sleep deprived for 4 hr (lower trace). Orexin/YC2.1 transgenic mice were sleep deprived for 2 or 4 hr during the light period in a motor driven rotating drum at a rate of 3 rpm. The experiments (control, 2 hr and 4 hr sleep deprivation) were performed at the same circadian time (mice were sacrificed at 13:00 (ZT5)). F, Bar graph showing the mean effect of NA on the YFP/CFP ratio. “Dark phase” are the slices that were prepared during the dark period (22:00 (ZT14)). Data shown are mean ± S.E.M. SD, sleep deprivation.

**Figure 12**

Schematic illustration of the electrophysiologically-demonstrated neural network between orexin neurons and catecholaminergic neurons. Catecholaminergic neurons negatively regulate orexin neurons both directly and indirectly. Direct inhibition is mediated by α2-ARs and GIRK channels on the orexin neurons. Orexin neurons express α1-ARs along with α2-ARs. Catecholamines activate non-selective cation channels (NSCCs) through α1-ARs. Indirect inhibition is mediated by modulation of both glutamatergic and GABAergic neurotransmission. GABAergic neurons are activated through α1-ARs. Glutamatergic neurons are inhibited through α2-ARs. Both glutamatergic and GABAergic neurons express α2-ARs on the presynaptic membrane to inhibit release of neurotransmitters.
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 lateral hypothalamic area. 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 (shown by red arrowheads in B and C). Scale bars 20 μm in B and C.
NA hyperpolarizes orexin neurons. A, In current clamp mode, NA (30 µM) was applied onto orexin neurons in the absence (top) or presence (middle) of TTX (1 µM). Before the experiments, the membrane potential was set at -60 mV by current injection. TTX was applied by bath application and NA was applied locally during the period indicated by the bars. Input resistance was monitored by the amplitude of electrotonic potentials generated by injection of a rectangular wave current pulse (-20 pA, 500 msec, 0.1 Hz). In voltage clamp mode, NA induced an outward current (A, bottom). Membrane potential was held at -60 mV. B, DA hyperpolarizes orexin neurons. In current clamp mode, DA (300 µM) was applied onto orexin neurons in the presence of TTX (1 µM) (upper trace). In voltage clamp, DA induced an outward current (B, lower trace). C, The concentration...
dependence of the responses to NA, adrenaline and DA. The IC50 and Emax of NA-, adrenaline- and DA-induced response were 6.7 ± 0.7 μM and 17.3 ± 0.5 mV, 2.4 ± 0.2 μM and 24.2 ± 0.4 mV, and 141.5 ± 21.9 μM and 17.5 ± 0.6 mV, respectively. Values are mean ± S.E.M. (n=4-12). CC, current clamp; VC, voltage clamp.
NA hyperpolarizes orexin neurons via the ±2A receptor. A, Idazoxan, a selective ±2 receptor antagonist, inhibited NA (30 μM)-induced hyperpolarization of orexin neurons in a concentration-dependent manner. B, The effect of ±- or ²-AR antagonists on the NA (30 μM)-induced hyperpolarization. Idazoxan inhibited NA-induced hyperpolarization in a concentration-dependent manner. Prazosin, a selective ±1-AR antagonist, inhibited the weak NA-induced depolarization that was observed in the presence of a high concentration of idazoxan (1 μM). The ²-AR selective antagonist, propranolol, had no effect on the NA-induced hyperpolarization of orexin neurons. The ±- or ²-AR antagonists were applied by bath application, whereas NA was applied locally through a fine polyethylene tube located near the recording neurons. C, The effect of ±- or ²-AR receptor
agonists on the membrane potential of orexin neurons. UK14304, a selective ±2-AR agonist, induced hyperpolarization in a concentration-dependent manner, while a high concentration of the ²-AR agonist, isoproterenol (100 µM), had little effect on the orexin neurons. The ±- or ²-AR agonists were applied locally through a fine polyethylene tube located near the recording neurons. All responses were normalized to the peak response induced by 30 µM NA which was applied before each experiment. D, Typical trace showing that NA induced significant depolarization in the presence of 1 µM idazoxan when membrane potential was held at -70 mV before the experiment. E, NA induced depolarization in the presence of 1 µM idazoxan in a concentration dependent manner (n=6). Membrane potential was adjusted by current injection at -70 mV before the experiment. NA, noradrenaline; UK, UK14304; Iso, Isoproterenol. Values are mean ± S.E.M.
NA decreases input resistance through an increase in a potassium conductance. A, Records of membrane potential in response to a series of 100 ms current steps (in 20 pA increments, -200 pA to 0 pA) from resting potential (-60 mV) in the absence (left) or presence (right) of NA (30 μM). B, Current-voltage relationship derived from the data in A. The potential at the end of current injection was plotted; control (open circle) and 30 μM NA (filled circle). Estimated reversal potential (Erev) was -110 mV (n=6). C, Reversal potential was shifted by changing potassium concentration in the extracellular solution from 2 mM to 10 mM (n=5). D, The trace showing that the GIRK inhibitor Ba2+ concentration-dependently inhibited NA-induced hyperpolarization. Ba2+ (30 μM and 300 μM) was applied by bath application. E, bar graph summarizing the data in D (n=4, *, vs control, p<0.05, ANOVA). Values are mean ± S.E.M.
Orexin neurons express both ±1-ARs and ±2-ARs. A, In voltage clamp mode held at -60 mV, NA (30 μM) normally induced an outward current, but under the presence of ±2-AR antagonist idazoxan (1 μM) induced an inward current. B, NA induced an inward current in a concentration-dependent manner. The experiments were performed in Ca2+-free solution in the presence of idazoxan (n=7). C and D, NA-induced inward current in the presence of 1 μM idazoxan was inhibited by ±1-AR antagonist, prazosin, in a concentration-dependent manner (n=6). NA (30 μM) was applied during the period indicated by solid bars in the presence of idazoxan (1 μM). E and F, ±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 μM NA which was applied before each experiment. Values are mean ± S.E.M.
An opening of non-selective cation channels is involved in the NA-induced inward current in the presence of ±2-AR antagonist, idazoxan. A, The effect of extracellular Ca2+ on the NA-induced inward current. NA induced a weak inward current in the presence of idazoxan that was dramatically increased by removing extracellular Ca2+ ions (n=8). This character is consistent with opening of non-selective cation channels. B, Graph summarizing the data in A. C and D shows the current-voltage relationship obtained by the voltage ramp protocol indicated in C using a CsCl pipette in the Ca2+-free extracellular solution. The I-V curve shows that the reversal potential of the NA (30 μM)-induced current was 4.5 ± 1.4 mV (n=6). Neurons were voltage-clamped at +40 mV for 2 sec, and the membrane potential was gradually lowered from +40 mV to -60 mV at a
duration of 2 sec. E and F, NA-induced inward current in the presence of idazoxan was inhibited by the non-selective cation channel inhibitor, SKF96365, in a concentration-dependent manner (n=7). F, Graph summarizing the data in E. Responses were normalized to the peak current induced by 30 µM NA which was applied before each experiment. Values are mean ± S.E.M.
NA decreases glutamatergic synaptic transmission and increases GABAergic synaptic transmission to orexin neurons. sEPSCs and sIPSCs were recorded using whole cell voltage clamp at a holding potential of -60 mV. sEPSCs were recorded in the presence of picrotoxin (100 μM), while sIPSCs were recorded in the presence of AP-5 (50 μM) and CNQX (20 μM). NA (30 μM) application decreased sEPSC frequency (A) and increased sIPSC frequency and amplitude (B). Mean effect of NA on sEPSC (C) or sIPSC (D) frequency. sEPSCs partially recovered and sIPSCs returned to basal levels after NA washout. The NA-induced decrease in sEPSC frequency is inhibited by the ±2-AR antagonist, idazoxan (1 μM). The NA-induced increase in sIPSC frequency is inhibited by the ±1-AR antagonist, prazosin (1 μM). PSC frequency was normalized to basal PSC frequency obtained before each experiment. Data shown are mean ± S.E.M. (*, p<0.05, ANOVA). Wash, washout.
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 μM). mEPSCs were recorded in the presence of picrotoxin (100 μM), while mIPSCs were recorded in the presence of AP-5 (50 μM) and CNQX (20 μM). A, NA (30 μM) application decreased mEPSC frequency. B, NA (30 μM) application decreased mIPSC frequency. C and D summarize the data in A and B, respectively. In the presence of idazoxan (1 μM), NA (30 μM) did not induce a decrease in mEPSC or mIPSC frequency. PSC frequency was normalized to basal PSC frequency obtained before each experiment. Data shown are mean ± S.E.M. (*, p<0.05, ANOVA). Wash, washout.
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 picrotoxin (100 μM), while 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 msec, 0.1 Hz) using bipolar stimulation electrodes placed within the LHA. Traces represent the mean of ten 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).
µM), suggesting 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 msec, 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 shown are mean ± S.E.M. *, vs control, p<0.05. Wash, washout.
NA decreased calcium currents. A, NA (100 µM) application inhibited Ba2+ current. B summarizes 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 sec. 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 shown by mean ± S.E.M. (*, p<0.05, ANOVA).
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(ZT5)). F, Bar graph showing the mean effect of NA on the YFP/CFP ratio. ‘Dark phase’ are the slices that were prepared during the dark period (22:00 (ZT14)). Data shown are mean ± S.E.M. SD, sleep deprivation.
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