Behavioral/Systems/Cognitive

Serotonergic Regulation of the Orexin/Hypocretin Neurons through the 5-HT_{1A} Receptor

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Both orexin and serotonin (5-HT) have important roles in the regulation of sleep–wakefulness, as well as in feeding behavior. We examined the effects of 5-HT on orexin/hypocretin neurons, using hypothalamic slices prepared from orexin/enhanced green fluorescent protein (EGFP) transgenic mice in which EGFP is expressed exclusively in orexin neurons. Patch-clamp recording from EGFP-expressing cells showed that 5-HT hyperpolarized all orexin neurons in a concentration-dependent manner. The response was inhibited by the 5-HT_{1A} receptor antagonist WAY100635. A 5-HT_{1A} receptor agonist, 8-hydroxy-2-(dl-*N*-propyl-amino)tetralin, also evoked hyperpolarization on orexin neurons with potency comparable with 5-HT. A low concentration of Ba^{2+} (30 μM) inhibited 5-HT-induced hyperpolarization. Single-channel recording revealed that the conductance of 5-HT-induced channel activity was 33.8 pS, which is in good agreement with that of the G-protein-coupled inward rectifier potassium channel (GIRK). Moreover, 5-HT_{1A} receptor-like immunoreactivity was observed on orexin neurons, and 5-HT transporter immunoreactive nerve endings are in close apposition to orexin neurons. Intracerebroventricular injection of the 5-HT_{1A} receptor-selective antagonist WAY100635 (100 ng) increased locomotor activity during the latter half of dark phase in wild-type mice but not in *orexin/ataxin-3* mice in which orexin neurons are specifically ablated, suggesting that activation of orexin neurons is necessary for the WAY100635-induced increase in locomotor activity. These results indicate that 5-HT hyperpolarizes orexin neurons through the 5-HT_{1A} receptor and subsequent activation of the GIRK and that this inhibitory serotonergic input to the orexin neurons is likely to be important for the physiological regulation of this neuropeptide system.

Key words: **orexin; hypocretin; serotonin; patch clamp; transgenic mouse; slice**

Introduction

The orexins, also known as hypocretins, are a pair of neuropeptides implicated in regulation of sleep–wakefulness, as well as energy homeostasis (de Lecea et al., 1998; Sakurai et al., 1998). Orexin neurons are located specifically in the lateral hypothalamic area (LHA) and project to almost all parts of the brain (Peyron et al., 1998; Nambu et al., 1999). Especially dense projections are observed in monoaminergic nuclei, such as noradrenergic locus ceruleus, serotonergic raphe nuclei, histaminergic tuberomammillary nucleus, and dopaminergic ventral tegmental area. These monoaminergic nuclei have been implicated in sleep–wakefulness regulation, express orexin receptors (OX_1R and/or OX_2R), and are activated by orexin (Hagan et al., 1999; Horvath et al., 1999; Nakamura et al., 2000; Brown et al., 2002; Yamanaka et al., 2002). These observations suggest that orexin neurons regulate and synchronize these monoaminergic nuclei to regulate sleep–wakefulness. Mice lacking either the orexin gene (*prepro-orexin* knock-out 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, recent reports suggest that 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). The implication of orexin neurons in narcolepsy suggests that these neurons have important roles in regulating normal sleep–wakefulness states. Until recently, little was known about the factors that influence the activity of these neurons, because it has been difficult to apply electrophysiological techniques to these cells. To facilitate identification of orexin neurons in living tissue, we made transgenic mice (*orexin/EGFP* mice), in which orexin neurons express enhanced green fluorescent protein (EGFP) (Yamanaka et al., 2003a,b). We reported that orexin neurons are directly hyperpolarized by serotonin (5-HT) using slice preparations from these mice (Yamanaka et al., 2003b).

5-HT was initially thought to be a mediator of sleep because the destruction of 5-HT neurons of the raphe nuclei or the inhibition of 5-HT synthesis with *p*-chlorophenylalanine (pCPA) induces a severe insomnia, which is reversed by restoring 5-HT synthesis (Jouvet, 1999; Adrien, 2002). However, in contradiction to this hypothesis, the activity of the raphe neurons is highest

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in waking, low in slow-wave sleep (SWS), and almost quiescent in rapid eye movement (REM) sleep (McGinty and Harper, 1976). The dorsal raphe (DR) nucleus sends inhibitory projections to the sleep-active neurons in the ventrolateral preoptic area (Chou et al., 2002). Also, DR is thought to project the cerebral cortex to induce cortical arousal. These observations suggest the roles of 5-HT in maintenance of wakefulness. Here, we report that 5-HT hyperpolarizes orexin neurons by an activation of a 5-HT_{1A} receptor and by an activation of the G-protein-coupled inward rectifier potassium channel (GIRK). Intracerebroventricular injection of a 5-HT_{1A} antagonist resulted in increased locomotor activity in wild-type mice but not in *orexin/ataxin-3* mice. These results suggest that an inhibitory input from serotonergic neurons to orexin neurons is one of the critical pathways for physiological regulation of orexin neuronal activity and highly important for sleep–wakefulness regulation.

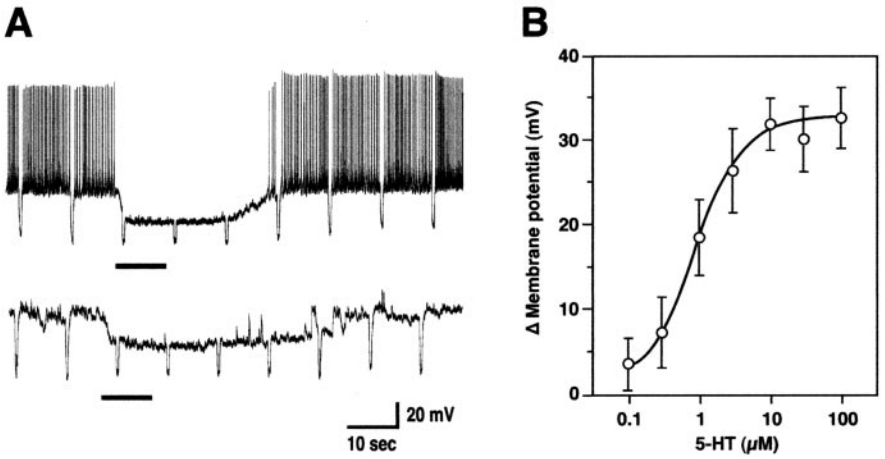

Figure 1. 5-HT hyperpolarizes orexin neurons in the presence or absence of TTX. *A*, In current-clamp mode, 5-HT (10 μM) was applied onto orexin neurons in the absence (top) or presence (bottom) of TTX (1 μM). Before the experiments, the membrane potential was set at −60 mV by current injection. TTX was applied by bath application, and 5-HT was applied locally through a fine polyethylene tube 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, 0.16 Hz). 5-HT-induced hyperpolarization is associated with a decrease in input resistance. *B*, The concentration dependence of the 5-HT response. The IC_{50} value was 0.87 ± 0.18 μM. Values are mean ± SEM ($n = 4–7$).

Materials and 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 weeks old, in which human prepro-orexin promoter drives expression of EGFP (lines E2 and E7) (Yamanaka et al., 2003a,b), 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% O_2) physiological solution containing the following (in mM): 140 choline Cl, 2 KCl, 0.1 $CaCl_2$, 1.9 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4, with NaOH or in sucrose solution (in mM: 234 sucrose, 2.5 KCl, 1.25 $NaHPO_4$, 10 $MgSO_4$, 0.5 $CaCl_2$, 26 $NaHCO_3$, and 10 glucose. Brains were cut coronally into 300 μm slices with a microtome (VTA-1000S; Leica, Nussloch, Germany). Slices containing the LHA were transferred to an incubation chamber filled with physiological solution containing the following (in mM): 140 NaCl, 2 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 10 HEPES, and 10 glucose, pH 7.4, with NaOH at room temperature (24–26°C) for at least for 1 hr. Some experiments were also conducted in physiological bicarbonate buffer containing the following (in mM): 125 NaCl, 2.0 KCl, 1 $CaCl_2$, 1 $MgCl_2$, 26 $NaHCO_3$, 1.25 $NaHPO_4$, and 10 glucose. For electrophysiological recording, the slices were transferred to a recording chamber (RC-27L; Warner Instruments, Hamden, CT) at a controlled temperature of 34°C on a fluorescence microscope stage (BX51WI; Olympus Optical, Tokyo, Japan). The slices were superfused with physiological solution that was warmed by an in-line heater (Warner Instruments) to 34°C before entering the recording chamber at a rate of 2 ml/min using a peristaltic pump (Dynamax; Rainin, Oakland, CA). The fluorescence microscope was equipped with an infrared camera (C2741-79; Hamamatsu Photonics, Hamamatsu, Japan) for infrared differential interference contrast imaging and a charge-coupled device camera (IK-TU51CU; Olympus Optical) for fluorescent imaging. Each image was displayed separately on a monitor (Gawin; EIZO, Tokyo, Japan) and was saved on a Power Macintosh G4 (Apple Computers, Cupertino, CA) computer 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, Pangbourne, UK). The pipettes were filled with an internal solution containing the following (in mM): 145 KCl, 1 $MgCl_2$, 1.1 EGTA-Na_3, 10 HEPES, 2 MgATP, 0.5 NaGTP, and 2 Lucifer yellow, pH 7.2, with KOH. Osmolarity of the solution was checked by a vapor pressure osmometer (model 5520; Wescor, Logan, UT). The tip of the pipette was polished by using a heat polisher just before use (MF-83; Narishige, Tokyo, Japan). Pipette resistance was 4–10 MΩ after being heat polished. The series resistance during recording was 10–25 MΩ and was not compensated. The osmolarity of the internal and external solutions 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 toward 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). 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. In the cell-attached and inside-out single-channel recording, the recorded signals were filtered at 2 kHz and digitized at a sampling rate of 10 kHz. Data were recorded on a computer through a Digidata 1322A analog-to-digital converter using pClamp 8.0.1 software (Axon Instruments). The trace was processed for presentation using Origin 6.1 (Microcal Software, Northampton, MA) and Canvas 8.0 (Deneba Systems, Miami, FL) software.

Immunohistochemistry. Adult male mice C57BL/6J (20–25 gm; Charles River Laboratories, Kanagawa, Japan) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and perfused sequentially with 10 ml of saline and 20 ml of 4% paraformaldehyde in 0.1 M phosphate buffer for 20 min. 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 d at 4°C. The brains were quickly frozen in embedding solution with OCT compound (Sakura Finetechnical, Tokyo, Japan). The frozen brains were cut into 40-μm-thick coronal sections on a cry-

ostat (MICROM HM 500; MICROM International, Walldorf, Germany). These slices were washed in Tris-buffered saline (TBS) containing 0.25% Triton X-100 (TBS-TX) and incubated in 1% bovine serum albumin fraction V in TBS-TX for 30 min. For orexin and 5-HT transporter double staining, sections were incubated with rabbit anti-orexin antiserum (1:2000) (Nambu et al., 1999) and goat anti-5-HT transporter antiserum (1:100) (Santa Cruz Biotechnology, Santa Cruz, CA) for 24 hr at 4°C. Sections were then incubated with Cy3-labeled donkey anti-goat IgG antibody (1:800; Molecular Probes, Eugene, OR) for 1 hr at room temperature and with fluorescein isothiocyanate-labeled donkey anti-rabbit IgG (1:800; Molecular Probes) for 1 hr at room temperature. For orexin and 5-HT_{1A} receptor double staining, sections were incubated with rabbit anti-orexin antiserum (1:2000) (Nambu et al., 1999) and guinea pig anti-5-HT_{1A} receptor antiserum (1:1000) (Kia et al., 1996) (Chemicon, Temecula, CA) for 48 hr at 4°C. These sections were simultaneously incubated with Alexa 488-labeled goat anti-rabbit IgG antibody (1:800; Molecular Probes) and with Alexa 594-labeled goat anti-guinea pig IgG (1:800; Molecular Probes) for 1 hr at room temperature. The sections were mounted and examined with a fluorescence microscope (AX-70; Olympus Optical). To confirm the specificity of antibodies, incubations without primary antibody were conducted as a negative control in each experiment, and no signal was observed.

Intracerebroventricular administration. Male C57BL/6J wild-type mice (20–25 gm; Charles River Laboratories) or *orexin/ataxin-3* hemizygous mice (25–27 gm, N7 backcross to C57BL/6J) were housed under controlled lighting (12 hr light/dark cycle; lights on from 8:00 A.M. to 8:00 P.M.) and temperature (22°C) conditions. Food and water were available *ad libitum*. Mice were anesthetized with pentobarbital (50 mg/kg, i.p.) and positioned in a stereotaxic frame (David Kopf Instruments, Tujunga, CA), and a guide cannula was implanted into the third ventricle under sterile conditions. Mice were then housed separately for a recovery period of at least 7 d. The position of the cannula was verified by central administration of human neuropeptide Y (0.3 nmol in sterile water) to test for a positive response. Mice that ate at least 0.5 gm of food over a 1 hr period after injection were used for experiments. WAY100635, a 5-HT_{1A} receptor antagonist, was delivered in saline at a volume of 3 μl over 60 sec, and the injector was left in position for an additional 60 sec to ensure complete dispersal of the drug. Saline alone was injected in the vehicle control experiment. Intracerebroventricular injection was initiated at 7:30 P.M. and was completed by the end of the light period (8:00 P.M.). The locomotor activity of individual mice during the dark period after intracebroventricular injection was assessed with an infrared activity monitor (Supermex; Muromachi Kikai, Tokyo, Japan) in Plexiglas cages to which mice had been well habituated. The chambers were light controlled (12 hr light/dark cycle; lights on from 8:00 A.M. to 8:00 P.M.) and were sound attenuated. The infrared activity monitor is a sensor mounted above the cage to detect changes in heat across multiple zones of the cage through an array of Fresnel lenses.

Statistical analysis. Data were analyzed by two-way ANOVA followed by *post hoc* analysis of significance by Fisher's protected least significant difference test using the Stat View 4.5 software package (Abacus Concepts, Berkeley, CA). p values <0.05 were considered statistically significant.

Drugs. The drugs used were tetrodotoxin (TTX), barium chloride (Wako, Osaka, Japan), 5-HT, Lucifer yellow dipotassium salt, WAY100635, and (±)8-hydroxy-2-(dl-*N*-propyl-amino)tetralin (8-OH-DPAT) (Sigma, St. Louis, MO). In the electrophysiological experiments, drugs were dissolved in HEPES-buffered solution and applied by either bath application or local application through a thin polyethylene tube positioned near the cells being recorded.

Results

5-HT hyperpolarizes orexin neurons in the presence or absence of TTX

To study the effect of 5-HT on orexin neurons, whole-cell current-clamp recordings were made on acute slice preparations of *orexin/EGFP* transgenic mice. Mean resting potential (−63.2 ± 4.6 mV) of EGFP-positive neurons was similar to our previous reports (Yamanaka et al., 2003a,b). 5-HT application hyperpolarized the membrane potential of orexin neurons in the presence or absence of TTX (Fig. 1*A*). All EGFP-positive neurons (orexin neurons) tested were hyperpolarized by 5-HT ($n = 81$), whereas EGFP-negative neurons in the same area (nonorexin neurons and probably a few orexin neurons, because EGFP fluorescence of 20% of orexin neurons is under the detection limit) showed a variety of responses to 5-HT: 33% of the neurons (23 of 70) showed hyperpolarization, 20% (14 of 70) showed depolarization, and 47% (33 of 70) showed no effect. 5-HT hyperpolarized orexin neurons in a concentration-dependent manner (Fig. 1*B*); E_{max} was 32.0 ± 1.0 mV at 100 μM, and the IC_{50} value was 0.87 μM ($n = 4–6$). Similar results were obtained when recording

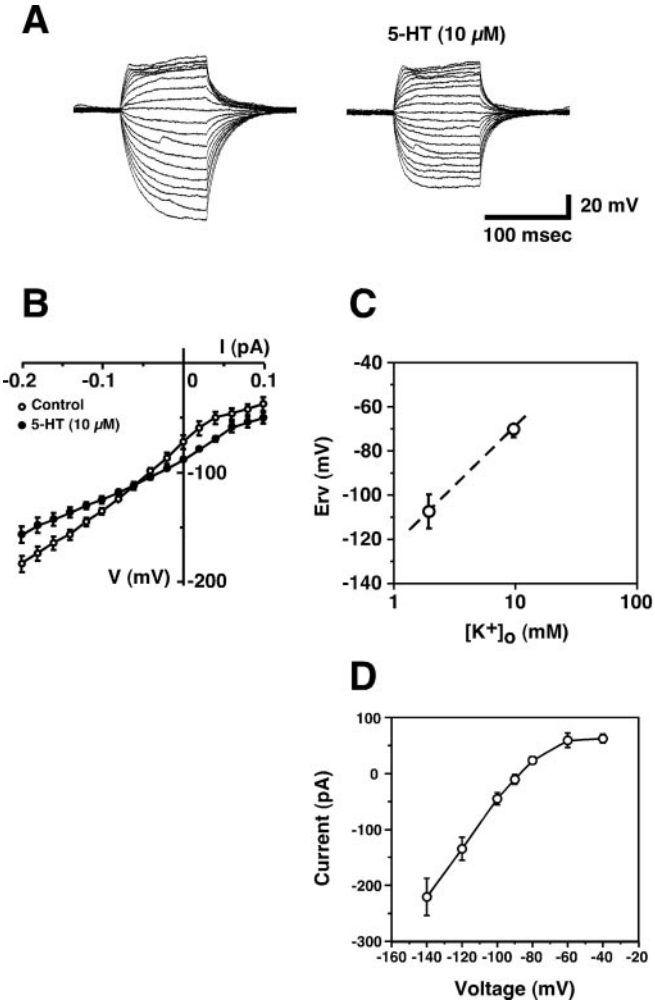

Figure 2. 5-HT decreases input resistance through an increase in a potassium conductance. *A*, Records of membrane potential in response to a series of 100 msec current steps (in 20 pA increments, −200 to 180 pA) from resting potential (−60 mV) in the absence (left) or presence (right) of 5-HT (10 μM). *B*, Current–voltage relationship derived from the data in *A*. The potential at the end of current injection was plotted; control (open circles) and 10 μM 5-HT (filled circles). Estimated reversal potential was −111.2 mV ($n = 7$). *C*, Reversal potential was shifted by changing potassium concentration in the extracellular solution from 2 to 10 mM ($n = 6–7$). Values are mean ± SEM. Erv, Estimated reversal potential. *D*, 5-HT-induced current shows an inward rectification. Orexin neurons were clamped at various voltages (−140, −120, −100, −90, −80, −60, and −40 mV), and then 5-HT (10 μM) was applied at each voltage. The maximal 5-HT-induced current was used to plot the *I–V* relationship. Slices were superfused by bicarbonate-buffered solution containing the following (in mM): 125 NaCl, 5.0 KCl, 1$CaCl_2$, 1$MgCl_2$, 26 $NaHCO_3$, 1.25 $NaHPO_4$, and 10 glucose. In this condition, potassium reversal potential was calculated to be −91.5 mV. 5-HT-induced current is markedly suppressed at holding potentials greater than the reversal potential (−60 and −40 mV; $n = 4$). Values are mean ± SEM.

in the physiological bicarbonate buffer. At a holding potential of −60 mV under voltage clamp, 5-HT (10 μM) induced an outward current in orexin neurons in the presence of TTX (32.0 ± 2.8 pA; $n = 3$). The hyperpolarization was accompanied by a decrease in membrane resistance. In the presence of TTX, 5-HT (10 μM) application significantly decreased membrane resistance to 60%; membrane resistance of orexin neurons before and after 5-HT application was 551 ± 44 and 328 ± 32 MΩ ($n = 7$), respectively (Fig. 2*A*). The reversal potential estimated from the *I–V* relationship was −111.2 ± 7.7 mV ($n = 7$) in normal external solution containing 2 mM K^+ (Fig. 2*B*). This value is similar to the theoretical K^+ equilibrium potential (−116 mV) calculated from the Nernst equation (Fig. 2*C*, dotted line) at the given K^+ concentration of the external and pipette solutions. Similar results were obtained when recording in the physiological bicarbonate buffer: reversal potential of the 5-HT-induced response was −111.1 ± 7.5 mV ($n = 4$) in this condition. As the extracellular K^+ concentration ($[K^+]_o$) was increased to 10 mM, the E_{rev} shifted to −74.7 ± 1.6 mV ($n = 6$). The slope of the E_{rev} values for a 10-fold change in $[K^+]_o$ was 53 mV.

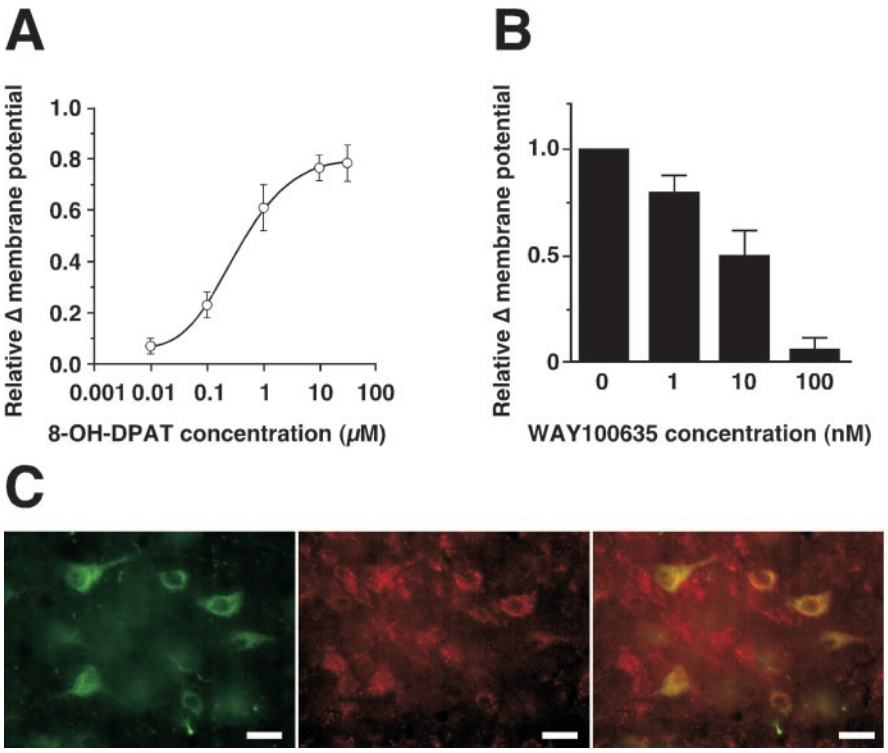

Figure 3. 5-HT hyperpolarizes orexin neurons via an activation of a 5-HT$_{1A}$ receptor. *A*, 8-OH-DPAT, a 5-HT$_{1A}$ receptor preferential agonist, hyperpolarized orexin neurons in a concentration-dependent manner (0.01–30 μM). All responses were normalized to the peak response induced by 10 μM 5-HT, which was applied before each experiment. The IC_{50} value was 0.30 ± 0.002 μM ($n = 4$–7). *B*, WAY100635, a 5-HT$_{1A}$ receptor-preferential antagonist, concentration dependently inhibited 10 μM 5-HT-induced hyperpolarization (1–100 nM; $n = 5$–14). Values are mean ± SEM. *C*, Double-label immunofluorescence analysis showing that orexin neurons express the 5-HT$_{1A}$ receptor. Immunoreactivity for orexin (green, Alexa 488) and 5-HT$_{1A}$ receptor (red, Alexa 594) overlaps in the merged image, resulting in a yellowish color. Scale bars, 20 μm.

Effect of 5-HT receptor agonists and antagonists on orexin neurons

To identify which subtype of 5-HT receptor is involved in 5-HT-induced hyperpolarization, 5-HT receptor agonist and antagonists were used. 8-OH-DPAT, a 5-HT$_{1A}$ receptor-preferential agonist, hyperpolarized orexin neurons concentration dependently (Fig. 3*A*). Potency of 8-OH-DPAT was comparable with 5-HT; the IC_{50} value was 0.3 μM ($n = 4$–7). α-Methyl 5-HT, a wide-range 5-HT receptor agonist, showed a moderate effect (1, 10, and 100 μM α-methyl 5-HT hyperpolarized 1.2 ± 0.6, 14.4 ± 2.4, and 19.4 ± 3.5 mV, respectively; IC_{50} 5.4 μM; $n = 5$). On the other hand, WAY100635, a selective antagonist for the 5-HT$_{1A}$ receptor, inhibited 5-HT-induced hyperpolarization in a concentration-dependent manner (Fig. 3*B*). Pretreatment of slices with 1, 10, and 100 nM WAY100635 for 2 min inhibited 10 μM 5-HT-induced hyperpolarization to 80 ± 8, 60 ± 10, and 4 ± 4%, respectively, compared with before antagonist treatment. This inhibition was often irreversible; 5-HT-induced hyperpolarization did not recover completely after washout for 15 min.

Expression of 5-HT$_{1A}$ receptor immunoreactivity on orexin neurons

To confirm expression of 5-HT$_{1A}$ receptor in orexin neurons, we performed double-label immunofluorescence analysis. 5-HT$_{1A}$ receptor immunostaining colocalized with orexin-like immunoreactivity in the LHA (Fig. 3*C*). The merged picture shows that almost all orexin-immunoreactive (IR) neurons showed 5-HT$_{1A}$ receptor immunoreactivity. Other neurons in the hypothalamus expressed 5-HT$_{1A}$ receptors as well. This 5-HT$_{1A}$ receptor antibody recognized mouse and rat 5-HT$_{1A}$ receptor and showed a strong immunoreactivity to serotonergic neurons in the raphe nuclei, which are known to densely express 5-HT$_{1A}$ receptor (data not shown).

Characterization of 5-HT-induced current by single-channel recording

To examine the properties of 5-HT-induced current in more detail, a cell-attached single-channel recording technique was performed. The tip of the pipette was filled with 140 mM KCl solution, and then the same solution containing 5-HT (1 μM) was backfilled. A cell-attached recording made at a pipette potential of 0 mV showed that current increased over time (Fig. 4*B*, 8 and 13 min) ($n = 4$). The amplitude histogram showed that the peak of 5-HT-induced current was 3.4 pA (Fig. 4*C*). In contrast, a pipette solution without 5-HT did not induce such current. 5-HT-induced single-channel activity was also observed in inside-out patches ($n = 4$), suggesting that this hyperpolarization is not dependent on intracellular second messengers. The channel conductance was characterized by a linear current–voltage relationship (−20, 0, and 20 mV), in isosmotic 140 mM KCl with slope conductance of 33.8 ± 4.3 pS, which is similar to that observed for the GIRK in cerebellar granule neurons (Han et al., 2003). An inward rectification of 5-HT-induced current was observed in a whole-cell voltage-clamp experiment (Fig. 2*D*).

We used the GIRK channel inhibitor Ba^{2+} to clarify the involvement of GIRK channel in 5-HT-induced hyperpolarization. Pretreatment with Ba^{2+} for 2 min inhibited 5-HT-induced hyperpolarization in a concentration-dependent manner (Fig. 5*A*). Incubation with 30 and 300 μM Ba^{2+} inhibited 10 μM 5-HT-induced hyperpolarization to 78 and 56%, respectively, com-

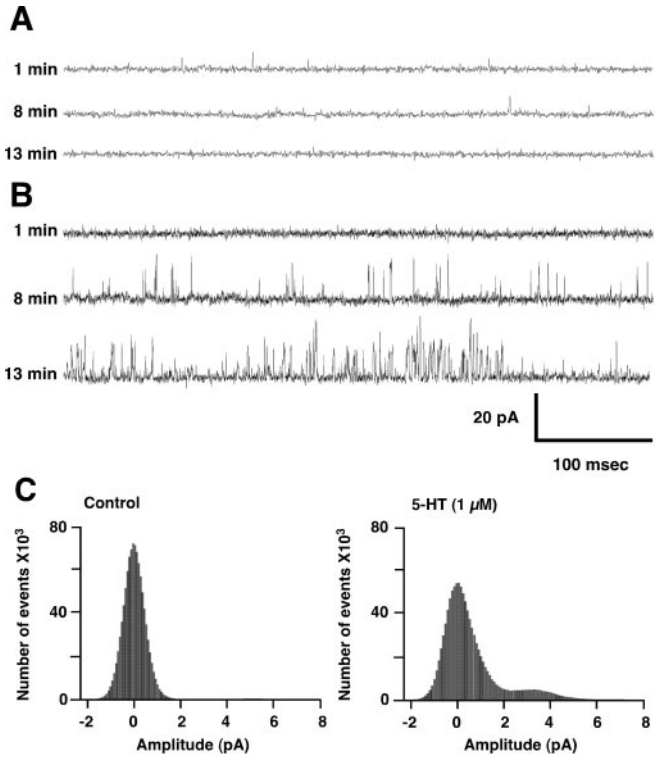

Figure 4. Single-channel characteristics of the channel activated by 5-HT. *A*, Cell-attached recording made at a pipette potential of 0 mV under voltage-clamp conditions. The pipette that was filled with 140 mM potassium solution did not contain 5-HT (control). *B*, The tip of the pipette was filled with the solution without 5-HT. Pipette was then backfilled with a recording solution containing 1 μM 5-HT. 5-HT diffused and reached the patch membrane within a few minutes. Initially, no channel openings occurred, but, after 8–13 min, channel activity is observed. *C*, Histograms of single-channel current amplitude in the absence (left) and presence (right) of 1 μM 5-HT. Values are mean ± SEM.

pared with before Ba^{2+} treatment ($n = 5–8$) (Fig. 5*B*). The inhibition by Ba^{2+} was reversible; 5-HT-induced hyperpolarization recovered completely after washout for 10 min (data not shown).

Orexin-IR neurons are in apposition to 5-HT transporter-IR nerve endings

5-HT neurons are distributed in the midbrain, pons, and medulla oblongata and innervate almost all parts of brain. To determine whether serotonergic neurons innervate orexin neurons, sections of hypothalamus were double-stained immunohistochemically for the 5-HT transporter and orexin. The 5-HT transporter is located on serotonergic presynaptic membranes to recycle 5-HT; therefore, 5-HT transporter immunoreactivity indicates serotonergic nerve endings. Many 5-HT transporter-IR nerve endings (red) were observed in the LHA (Fig. 6*A*). These nerve endings were distributed around the somata and dendrites of orexin-IR neurons (green). Arrows in Figure 6*D* show 5-HT transporter-IR nerve endings distributed along the shape of a soma. All orexin-IR neurons were surrounded by 5-HT transporter-IR nerve endings.

A 5-HT antagonist increases locomotor activity in wild-type but not in orexin-neuron-deficient mice

To investigate the physiological importance of the serotonergic inhibition of orexin neurons in mice, WAY100635, a 5-HT$_{1A}$ receptor antagonist, was injected into the third ventricle of wild-type and *orexin/ataxin-3* transgenic mice. At this age (10 weeks), almost all orexin neurons are ablated in *orexin/ataxin-3* mice (Hara et al., 2001). WAY100635 was injected at the end of the light phase (7:30 P.M.), and locomotor activity was then monitored during the dark phase. Wild-type mice injected with WAY100635 (100 ng) did not show an increase in locomotor activity in the first half of the dark phase, although they showed hyperactivity in the latter half of the dark phase compared with saline-injected control mice ($n = 4–6$) (Fig. 7*A*,*B*). This hyperactivity continued into the light phase. Cumulative locomotor activity for the first 6 hr (8:00 P.M. to 2:00 A.M.) showed no difference, but, for the latter 6 hr (2:00–8:00 A.M.), it was significantly increased in WAY100635-injected wild-type mice (Fig. 7*A*). In contrast, *orexin/ataxin-3* transgenic mice failed to increase locomotor activity in either time period ($n = 6$) (Fig. 7*B*).

Discussion

In the present study, we investigated the effects of 5-HT on orexin neurons using the patch-clamp technique on hypothalamic slices prepared from transgenic mice in which EGFP is specifically expressed in orexin neurons. In the whole-cell current-clamp mode, all orexin neurons tested in this experiment ($n = 81$) were hyperpolarized by 5-HT in a concentration-dependent manner. We showed that the hyperpolarization was mediated by an activation of 5-HT$_{1A}$ receptor using a 5-HT$_{1A}$-preferential antagonist and an agonist. This response is likely mediated through increase in potassium conductance via a GIRK channel. To reveal the physiological importance of serotonergic inhibitory input to orexin neurons, *in vivo* experiments were performed. Intracerebroventricular injection of a 5-HT$_{1A}$-selective antagonist to wild-type mice resulted in increased locomotor activity, whereas *orexin/ataxin-3* mice failed to increase locomotor activity when injected with this 5-HT$_{1A}$-selective antagonist.

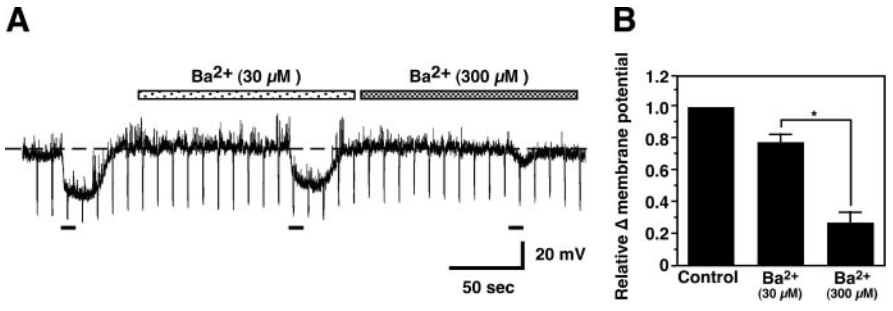

Figure 5. 5-HT activates a GIRK channel. *A*, The GIRK inhibitor Ba^{2+} concentration dependently inhibited 5-HT-induced hyperpolarization. Ba^{2+} (30 and 300 μM) was applied by bath application as indicated by the bars above the trace. 5-HT (10 μM) was applied locally through a fine polyethylene tube during the period indicated by the bars below the trace. 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). The data are summarized in *B*. Values are mean ± SEM. *$p < 0.05$.

The mechanism of 5-HT-induced hyperpolarization

The raphe nuclei are the areas of the brain most enriched with 5-HT$_{1A}$ receptor (Chalmers and Watson, 1991; Kia et al., 1996). 5-HT$_{1A}$ receptor is distributed in the plasma membrane of perikarya and dendrites in 5-HT-containing neurons and act as an inhibitory autoreceptor (Sinton and Fallon, 1988; Riad et al., 2000). However, the 5-HT$_{1A}$ receptor is

expressed not only in serotonergic neurons but also in many other types of neurons (Yamada et al., 2000; Jeong et al., 2001). It has been reported that the 5-HT$_{1A}$ receptor couples with the pertussis toxin-sensitive G-protein ($G_{i/o}$ protein), and activation of this receptor increases the conductance of the inward-rectifying potassium current (Katayama et al., 1997).

In the present experiments, we observed that the 5-HT$_{1A}$ receptor-preferential agonist 8-OH-DPAT hyperpolarizes orexin neurons, whereas 5-HT-induced hyperpolarization of orexin neurons was inhibited by the 5-HT$_{1A}$ receptor-selective antagonist WAY100635. 5-HT-induced hyperpolarization was blocked by Ba^{2+} ion, and single-channel analysis showed that the conductance of 5-HT-induced single-channel activity (33.8 pS) was consistent with previously reported GIRK conductance (32–39 pS) (Bajic et al., 2002; Han et al., 2003). These results suggest that 5-HT-induced hyperpolarization of orexin neurons is mediated by the 5-HT$_{1A}$ receptor and by subsequent activation of the GIRK channel. 5-HT induced single-channel activity in both cell-attached and inside-out patch-clamp modes. This result is consistent with recent reports that neuronal GIRK channels are activated by direct binding of $\beta\gamma$ subunits of G-proteins to the channel without the involvement of any second messengers (Wickman et al., 1994; Kofuji et al., 1995).

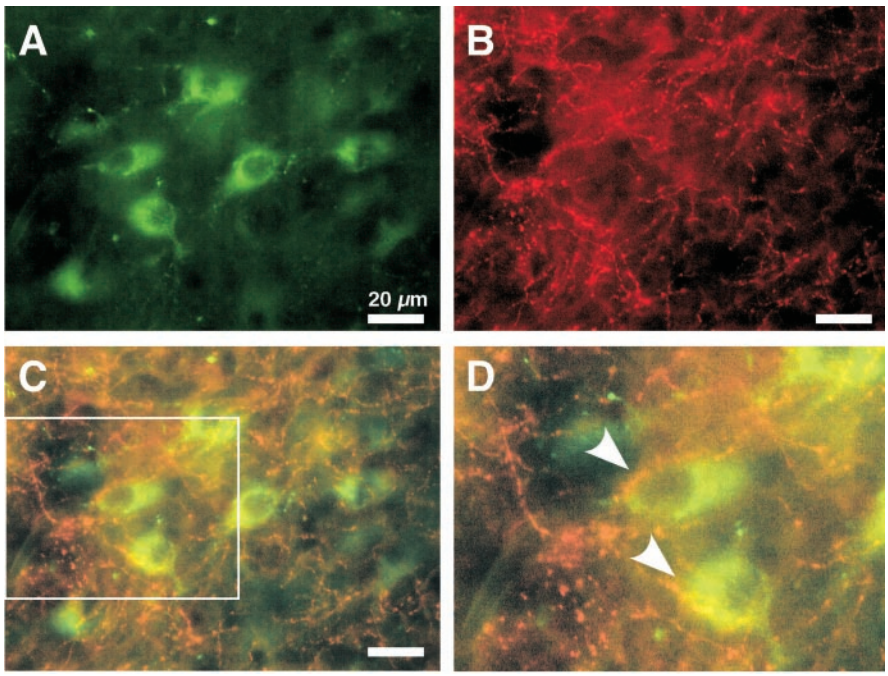

Figure 6. 5-HT transporter-IR nerve endings are in close apposition to orexin-IR neurons. Coronal section through the hypothalamus containing orexin neurons was studied using double-immunofluorescent staining. *A*, Immunoreactivity for orexin (green, fluorescein isothiocyanate) is localized in the lateral hypothalamic area. *B*, Axon fibers and terminals, which show immunoreactivity for 5-HT transporter (red, Cy3), are localized in the same area. *C*, *D*, The merged image shows that 5-HT transporter-IR varicosities are closely apposed to an orexin-IR cell body (*D*, arrows). *D* shows a magnified view marked in *C*. Scale bars, 20 μm.

Previously, we reported that not only 5-HT, but also noradrenaline, hyperpolarized orexin neurons (Li et al., 2002; Yamanaka et al., 2003b). Noradrenaline-induced hyperpolarization was also accompanied by a decrease in membrane conductance (our unpublished data). In locus ceruleus neurons, it has been reported that noradrenaline increased potassium conductance through GIRK via an activation of α_2-adrenergic receptor (Arima et al., 1998). Thus, a similar mechanism might be involved in noradrenaline-induced hyperpolarization of orexin neurons.

Serotonergic nerve endings in the hypothalamus mainly originate from the rostral group of raphe nuclei in the midbrain, the dorsal and median raphe nuclei (Leander et al., 1998). Retrograde tracing studies have revealed that serotonergic neurons in the dorsal and median raphe nuclei project to the hypothalamus (Petrov et al., 1992). Although there is little information about the functional differences between dorsal and median raphe, it is likely that these two nuclei are functionally connected (Glass et al., 2003; Tischler and Morin, 2003). Immunohistochemical studies showed that all orexin neurons are surrounded by dense serotonergic nerve endings, and all orexin neurons examined responded to 5-HT by hyperpolarization. If the serotonergic neurons in the raphe nuclei innervate orexin neurons, this serotonergic inhibitory input might form a negative feedback loop, because it has been reported that orexin directly or indirectly activates 5-HT neurons in the raphe nuclei (Brown et al., 2002; Liu et al., 2002).

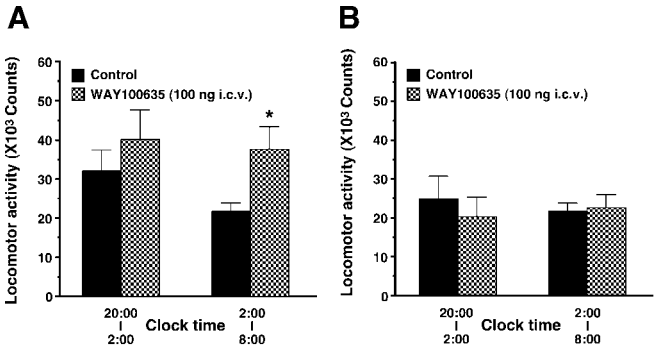

Figure 7. The 5-HT$_{1A}$ receptor antagonist WAY100635, administered intracerebroventricularly, induces hyperactivity in the latter half of the dark period. Mice received a third ventricular injection at the end of the light period (7:30 – 8:00 P.M.). WAY100635 was dissolved in saline and injected at a volume of 3 μl. Saline alone was used in control experiments. Locomotor activity was measured by an infrared sensor. Intracerebroventricular administration of WAY100635 induced hyperactivity at the end of the dark period in wild-type mice (*A*), whereas *orexin/ataxin-3* mice did not show hyperactivity (*B*). Values are mean ± SEM.

Physiological importance of serotonergic inhibitory input to orexin neurons

Although orexin neurons project widely throughout the brain, some monoaminergic nuclei receive strong innervation, and the serotonergic DR is one of the densest projection sites (Nambu et al., 1999). Serotonergic neurons in the DR express both OX_1R and OX_2R (Marcus et al., 2001) and are activated by orexin (Brown et al., 2002; Liu et al., 2002). Involvement of a serotonergic pathway in orexin-induced behavioral alteration has also been reported (Matsuzaki et al., 2002). Because depletion of 5-HT by lesion of the raphe nuclei or pCPA (an inhibitor of 5-HT formation) treatment greatly reduced sleep and induced hyperactivity

in animals paralleled by a reduction of brain 5-HT, serotonin was originally proposed to be a sleep neurotransmitter (Jouvet, 1972). In support of this idea, 5-HT precursor treatment induces drowsiness and reduces sleep latency (Ursin, 2002). On the other hand, the serotonergic DR neurons have their highest firing rate in waking, and this activity slows during SWS to almost complete cessation of firing during REM sleep (McGinty and Harper, 1976; Trulson and Jacobs, 1979; Wu et al., 2004). Neural activity of the DR is also closely linked to motor activity (Trulson et al., 1981; Lydic et al., 1984). Because of these and other observations, 5-HT has also been proposed to be a waking neurotransmitter.

The resolution of these conflicting proposals for the role of 5-HT in behavioral state regulation lies in understanding the anatomical projections of the 5-HT neurons and specific actions of different 5-HT receptor subtypes. The DR has primarily ascending projections that, along with the activity of histamine cells, probably makes a contribution to cortical arousal in normal waking (Jacobs and Azmitia, 1992), whereas the major source of serotonin release seen in brainstem motoneuron pools likely arises from pontine and medullary 5-HT nuclei. In regard to the present study, 5-HT_{1A} receptors are located on the soma and dendrites of 5-HT neurons (somatodentritic autoreceptors), as well as postsynaptically on 5-HT target neurons (Kia et al., 1996; Riad et al., 2000). It has been proposed that 5-HT_{1A} receptors are involved in the regulation of REM sleep and wakefulness (Dzoljic et al., 1992; Tissier et al., 1993; Portas et al., 1996; Thakkar et al., 1998), whereas 5-HT_{2A} receptors participate in the control of SWS (Idzikowski et al., 1986; Dugovic et al., 1989). The observation that 5-HT_{1A} null mutant mice have higher amounts of REM sleep than wild-type mice during both the light and dark phases (Boutrel et al., 2002) is consistent with this idea. In the present study, 5-HT hyperpolarized all orexin neurons through a 5-HT_{1A} receptor. Consequently, we propose that high levels of motor activity facilitates 5-HT release that leads to hyperpolarization of orexin cells. Because orexin cells have a critical role in the maintenance of arousal, a decrease in wakefulness might be predicted. However, it is likely that 5-HT works together with other inhibitory and excitatory inputs (Li et al., 2002; Yamanaka et al., 2003) to regulate the net activity of orexin neurons in a given behavioral state. The cessation of DR activity in REM sleep should result in a reduction of 5-HT_{1A}-mediated hyperpolarization of orexin neurons, and, indeed, orexin-A levels have been shown to increase during paradoxical sleep relative to SWS (Kiyashchenko et al., 2002).

Inhibition of the 5-HT_{1A} receptor also induced hyperactivity in wild-type mice. Interestingly, this hyperactivity occurred at the end of the dark phase, although the antagonist was injected before the dark phase. This delayed effect might suggest that a cumulative serotonergic inhibitory input is needed for suppression of orexin neuronal activity. On the other hand, *orexin/ataxin-3* mice did not show hyperactivity by WAY100635 treatment, suggesting that the hyperactivity was mediated by an activation of orexin neurons. In addition, pCPA treatment (100 $mg \cdot kg^{-1} \cdot d^{-1}$, i.p.) for 2 d did not induce hyperactivity in *orexin/ataxin-3* mice (data not shown). It has been reported previously that intracerebroventricular injection of orexin induced not only an arousal response but also an increase in locomotor activity (Matsuzaki et al., 2002). Thus, it is possible that the blocking of serotonergic inhibitory input to orexin neurons increases orexin neural activity, resulting in hyperactivity. However, we also cannot completely rule out the possibility that the loss of orexin neurons is the cause of the reduced responsiveness to WAY100365 or pCPA administration in *orexin/ataxin-3* mice.

The effects of 5-HT on sleep–wakefulness are complicated. Blockade of the 5-HT transporter by selective 5-HT reuptake inhibitors (SSRIs) increases extracellular 5-HT, but SSRIs reduce REM sleep and sometimes improve insomnia in depressed patients. Sleep analysis using 5-HT receptor knock-out mice showed that not only the 5-HT_{1A} receptor but also other subtypes of receptors, such as the 5-HT_{1B} and 5-HT_{2C} receptors, are also involved in the regulation of sleep (Boutrel et al., 1999, 2002; Frank et al., 2002). Additional studies are needed to clarify the interactions between the 5-HT and orexin systems *in vivo* and the effects on sleep–wakefulness regulation.

In conclusion, serotonergic input hyperpolarized orexin neurons via the 5-HT_{1A} receptor. This hyperpolarization was achieved by an increase in potassium conductance through GIRK. *In vivo* experiments showed that serotonergic inhibitory input is important to regulate firing of orexin neurons. This work gives important insight into the roles of 5-HT in the regulation of sleep–wakefulness states.

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