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Regulation of orexin neurons by the monoaminergic and cholinergic systems

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Abstract

Orexins are a pair of neuropeptides implicated in energy homeostasis and arousal. Here we characterize the electrophysiological properties of orexin neurons using slice preparations from transgenic mice in which orexin neurons specifically express green fluorescent protein. Orexin neurons showed high frequency firing with little adaptation by injecting a positive current. The hyperpolarization-activated current was observed in orexin neurons by a negative current injection. The neurotransmitters, which were implicated in sleep/wake regulation, affected the activity of orexin neurons; noradrenaline and serotonin hyperpolarized, while carbachol depolarized orexin neurons in either the presence or absence of tetrodotoxin. It has been reported that orexins directly or indirectly activate the nuclei that are the origin of the neurons containing these neurotransmitters. Our data suggest that orexin neurons have reciprocal neural circuitries between these nuclei for either a positive or negative feedback loop and orchestrate the activity of these neurons to regulate the vigilance states.

Keywords: Orexin; Patch clamp; Action potential; Hyperpolarization-activated current; Serotonin; Noradrenaline; Acetylcholine

Orexins, also called hypocretins, are a recently described pair of neuropeptides orexin-A and -B [1,2]. Orexin-containing neurons are located in the perifornical, dorsomedial, lateral, and posterior hypothalamus, from where they innervate almost all regions of the brain, except the cerebellum. Especially dense projections are observed in specific regions of the thalamus, limbic system, and monoaminergic centers, such as the noradrenergic locus coeruleus (LC), serotonergic raphe nucleus, dopaminergic ventral tegmental area (VTA), and histaminergic tuberomammillary nucleus (TMN) [3,4]. These monoaminergic nuclei are thought to play a crucial rule in the regulation of vigilance states [5–7]. Recently it has been reported that orexins directly or indirectly activate all these aminergic nuclei [8–16].

Orexins were initially implicated in energy homeostasis; however, recent reports suggest the importance of these peptides in the regulation of vigilance states. Especially, the importance of orexin in promoting arousal is highlighted by the discovery that mice lacking either the orexin gene (prepro-orexin knockout mice) or orexin neurons (orexin/ataxin-3 transgenic mice), as well as dogs with null mutations in the orexin receptor-2 gene, have phenotypes remarkably similar to the human sleep disorder narcolepsy [17–19]. Narcolepsy is the only neurological disorder characterized by a primary disorganization of sleep and wakefulness. Patients with narcolepsy suffer from excessive daytime sleepiness, cataplexy (a sudden weakening of posture muscle tone usually triggered by emotion), and an alteration in the amount of and entry into rapid eye movement (REM) sleep [20]. Recently, postmortem studies in six narcoleptic subjects indicated undetectable orexin peptides in projection sites such as cortex and pons and an 80–100% reduction in the number of orexin-containing neurons in the hypothalamus as determined by in situ hybridization [21]. Thannickal et al. [22] independently reported a global loss of orexin-containing neurons and residual gliosis in four brains examined in the perifornical area of

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brains of narcolepsy patients by immunohistochemistry. These results strongly suggest that orexins and orexin neurons have a crucial role in the regulation of vigilance states.

Orexin neurons have been shown to regulate directly or indirectly the activity of aminergic nuclei. However, little is known about how the orexin neuronal activity is regulated in the brain. Despite the importance to study orexin neurons, it has been difficult to identify and directly examine their electrophysiological properties, because orexin neurons of the LHA are scarce, diffusely distributed, and lack distinct morphological features. To facilitate finding orexin neurons, we made transgenic mice in which orexin neurons specifically express enhanced green fluorescent protein (EGFP) [23,24]. We prepared brain slices from the hypothalamus of these transgenic mice and subjected them to whole cell patch-clamp recordings. Fluorescence imaging of EGFP was strong enough to identify neurons in freshly prepared slice preparations. First, we determined the basic membrane properties (membrane capacitance, input resistance, firing frequency, etc.) of orexin neurons. Then, we tested the effects of several neurotransmitters, which are implicated in sleep/wake regulation, on orexin neuronal activity. These include serotonin, noradrenaline, histamine, and acetylcholine. Among these neurotransmitters, noradrenaline and serotonin hyperpolarized, while acetylcholine depolarized, the membrane potential of the orexin neurons.

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 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, 3–6 weeks old in which human prepro-orexin promoter drives expression of EGFP (line E2 and E7) [24,25], 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) Hepes-buffered physiological solution containing (mM): NaCl 135, KCl 5, $CaCl_2$ 1, $MgCl_2$ 1, Hepes 10, glucose 10, and mannitol 20, pH 7.4, with NaOH; then they were cut coronally into 250 μm slices with a microtome (VTA-1000S, Leica, German). The slices, containing the lateral hypothalamic area, were transferred to an incubation chamber at least for 1 h at room temperature (24–26 °C). For electrophysiological recording, the slices were transferred to a recording chamber (RC-27L, Warner Instrument, USA) at a controlled temperature of 34 °C on a fluorescence microscope stage (BX51WI, Olympus, Tokyo, Japan). The slices were superfused with Hepes-buffered physiological solution which was pre-heated before entering a recording chamber by an in-line heater (Warner) at 34 °C 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, Hamamatsu, Japan) for infrared differential interference contrast (IR-DIC) imaging and a 3 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 G3 computer 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): potassium-gluconate 130, KCl 10, $MgCl_2$ 0.1, $CaCl_2$ 0.05, EGTA-Na_3 0.5, Hepes 10, Na_2ATP 2, and lucifer yellow 2, pH 7.4, with KOH. Osmolarity of the solution was checked by a vapor pressure osmometer (Model 5520, Wescor, USA). The tip of the pipette was polished by using a heat polisher just before use (MF-83, Narishige, Japan). Pipette resistance was 4–10 MΩ after heat polishing. The series resistance during recording was 10–25 MΩ and was not compensated. The osmolarities of the internal and external solutions were 280–290 and 320–330 mOsm/L, respectively. The liquid junction potential of the patch pipette solution and superfused Hepes solution was estimated to be +16 mV and was applied to the data. Recording pipettes were advanced while under positive pressure towards individual cells in the slice. On contact, tight seals, of 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 a duration of 100, 200 or 300 ms at 20 or 40 pA steps at 2-s 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. All voltage clamp recordings were made in Axopatch 200B voltage clamp mode held at −60, −45 or −30 mV dependent on the type of experiment. QX-314 (5 mM) was added to pipette solution to record EPSCs and IPSCs. 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 1322A A/D converter using p-clamp 8.0.1 software. The trace was processed for presentation using Origin 6.1 (Origin lab. Corporation, MA, USA) and Canvas 7.0 (Deneba, FL, USA) software.

The drugs used were tetrodotoxin (TTX), Histamine, and CsCl (Wako, Osaka, Japan), (±)-α-amino-3-hydroxy-5-methyl-isoxazole-4-propionic acid (AMPA), *N*-methyl-D-aspartic acid (NMDA), 1,2,3,4-tetrahydro-6-nitro-2,3-dioxobenzo[f]quinoxaline-7-sulfonamide (NBQX), Muscimol, Bicuculline, QX-314, Carbachol, Acetylcholine, Atropine, Serotonin, Dopamine, and Noradrenaline (Sigma, St. Louis, MO, USA), 4-ethylphenylamino-1,2-dimethyl-6-methylaminopyrimidinium, (ZD7288, Tocris, MO, USA), and D(−)-2-amino-5-phosphonopentanoic acid, (AP-5, Calbiochem, Germany). Drugs were dissolved in Hepes-buffered solution and applied by bath-application or local application through a thin polyethylene tube positioned near recording cells.

Results

Detection of orexin neurons by EGFP fluorescence

Green fluorescent cells were confined to the lateral hypothalamic area in which orexin neurons are localized [4]. We used the transgenic mice line in which approximately 80% of orexin-immunoreactive neurons expressed EGFP [24]. Ectopic expression was not observed in any regions of the brain. In 250 μm-thickness slice

preparations, EGFP fluorescence detected not only soma but also dendrite and axon of orexin neurons (Fig. 1A). Green fluorescent cells were bright and could be easily identified through the microscope or 3 CCD camera. The neurons whose coordinates and orientation matched those of fluorescent cells seen in the fluorescence image were identified on the IR-DIC video monitor screen and were subsequently subjected to whole cell patch clamp recording.

Action potentials and active membrane properties of orexin neurons

Neurons with a positive resting membrane potential at a rate of more than −45 mV or input resistance of less than 100 MΩ were not used for the experiment. In whole cell current clamp mode, orexin neurons were quiescent or spontaneously fired at a rate of 5.5 ± 3.9 Hz (range 0–16.6 Hz, $n = 34$) and had a resting membrane potential of -60.5 ± 5.9 mV (range −50.5 to −67.5, $n = 34$) and input resistance of 390 ± 110 MΩ (range 193–513 MΩ, $n = 34$). Fig. 2A shows a typical trace of an action potential which was evoked by 40 pA current injection recorded from orexin neurons. Action potentials were followed by biphasic after hyperpolarization (-59.3 ± 6.2 mV, range −47.6 to −70.6, $n = 34$). All orexin neurons examined showed the same shape of action potentials, regardless of the donor age (3–6 weeks), gender, or transgenic line. Electrical membrane properties (membrane capacitance, threshold, amplitude, duration at half amplitude, and rate of rise or fall) were corrected and the values were compared with those of randomly selected non-EGFP expressing hypothalamic neurons (Table 1). Membrane capacitance of orexin neurons tended to be larger and the falling rates of action potentials were slower than other neurons in the hypothalamus. However, none of these parameters showed statistically differences between EGFP expressing orexin neurons and non-EGFP expressing neurons. This fact suggests that orexin neurons are indistinguishable from other neurons in the hypothalamus in respect to electrophysiological characters. Fig. 2B shows the spontaneous firing pattern of orexin neurons in current clamp mode. Most orexin neurons displayed spontaneous action potentials which were preceded by an excitable postsynaptic potential (EPSP) (top). Some showed spontaneous burst firing (bottom). This type of neuron fired in clusters or episodes of repetitive burst (burst duration 1328 ± 265 ms, burst interval 1954 ± 207 ms, $n = 9$).

Both action potentials evoked spontaneously, or positive current injection were completely blocked by the sodium channel blocker TTX (1 μM), suggesting that the action potentials were based on TTX-sensitive sodium current (Fig. 2C). In voltage clamp mode under imperfect space-clamp conditions, orexin neurons displayed transient inward sodium current and sustained outward potassium current in response to 100 ms depolarizing voltage steps. This inward sodium current was also completely abolished by TTX (1 μM) treatment (data not shown). To determine the active membrane properties of orexin neurons, a positive current (duration of 300 ms, 20 pA increment from 0 to 200 pA) was injected. Positive current injection evoked firing in orexin neurons. Fig. 2D shows evoked firing frequency and injected-current relationship in orexin neurons. The number of action potentials was monotonically related to current intensity ($n = 9$, Fig. 2E). Orexin neurons can generate relatively high frequency firing by positive current injection (87.8 ± 7.0 Hz, 180 pA current injection). A current injection larger than 200 pA did not evoke a more distinct shape of action potentials in orexin neurons. Virtually firing frequency adaptation, which has been reported in pyramidal neurons [25], was not observed by depolarizing pulse injection (Fig. 2F).

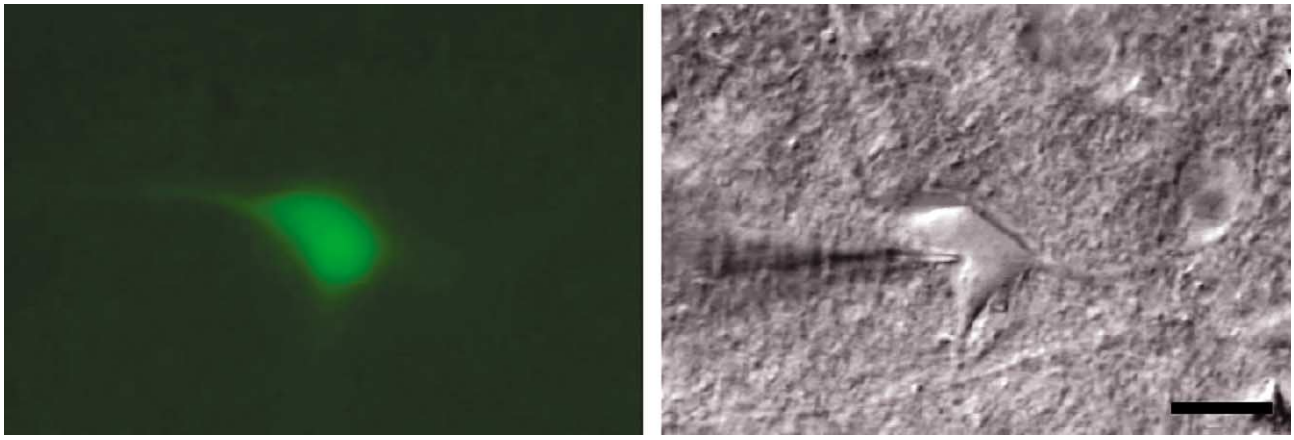

Fig. 1. Identification of orexin neurons using EGFP fluorescence in a hypothalamic slice preparation from the orexin/EGFP transgenic mouse. Fluorescence (left) and IR-DIC (right) images of an EGFP expressing orexin neuron. Scale bar: 20 μm.

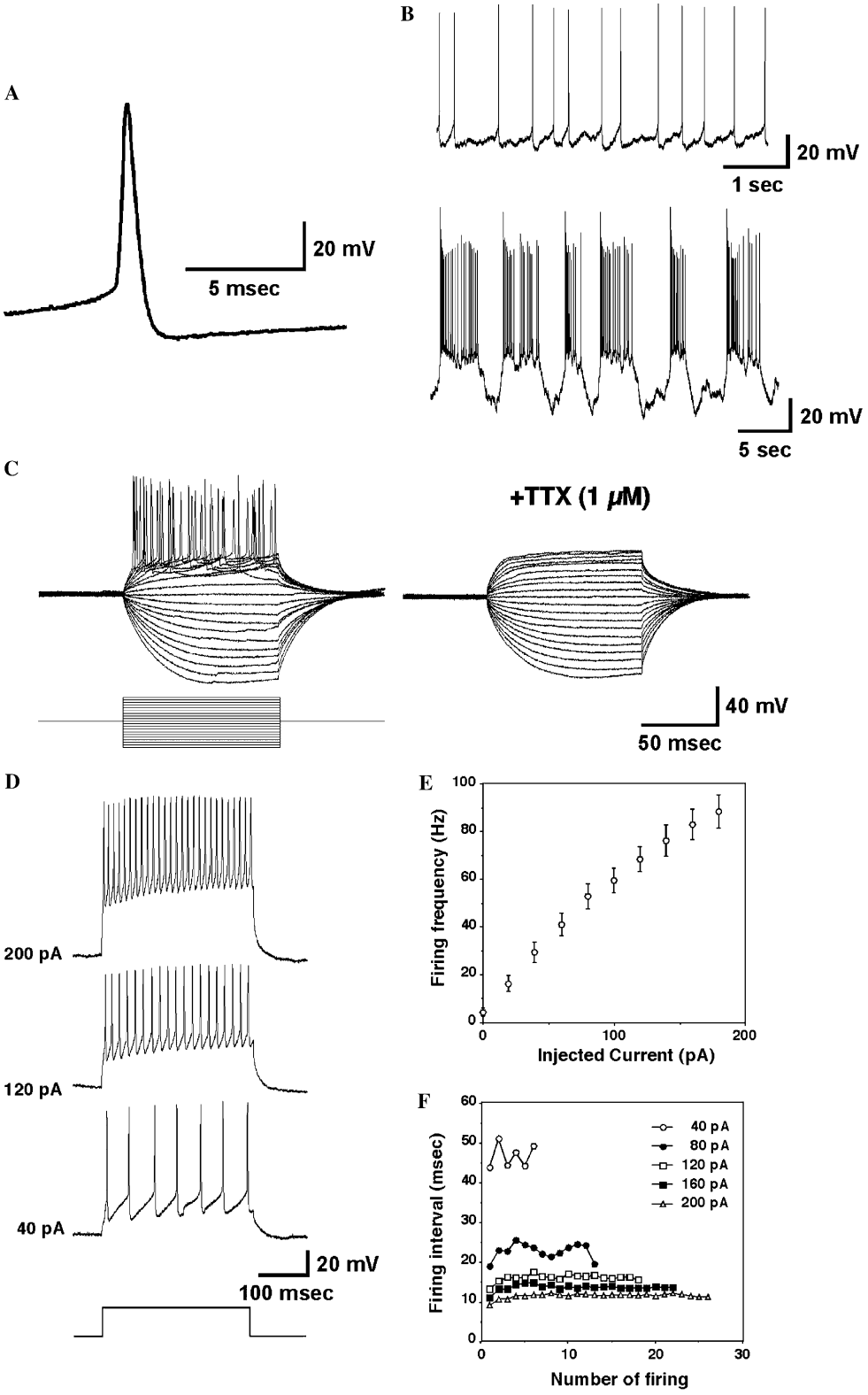

Fig. 2. (A, B) The typical action potential of orexin neurons in whole cell current clamp mode. (A) Enlarged trace of an action potential evoked by 40 pA current injection. (B) Spontaneous action potential of orexin neurons in current clamp mode. Spontaneous action potentials preceded by EPSP (top), burst firing neurons (bottom). (C–F) Active membrane properties of orexin neurons. (C) Records of membrane potential in response to a series of 100 ms current steps (in 20 pA increments) from resting potential (−60 mV) in the absence (left) or presence (right) of TTX (1 μM). Firing evoked by positive current injection was completely blocked by TTX. (D) Positive current injection (40, 120, and 200 pA) for 300 ms induced action potentials in orexin neurons. (E) Relation between average firing frequency and the injected current. Values are means ± SE ($n = 9$). (F) Inter firing interval between successive firing recorded at different current intensities (40, 80, 120, 160, and 200 pA).

Table 1
Comparison of electrical properties in orexin neurons and non-orexin neurons

	EGFP-expressing orexin neurons ($n = 34$)	Non-EGFP-expressing neurons ($n = 23$)
Resting membrane potentials (mV)	-60.5 ± 5.9	-65.3 ± 7.9
Spontaneous firing (Hz)	5.5 ± 3.9	5.8 ± 7.4
Input resistance (MΩ)	390 ± 110	310 ± 220
Membrane capacitance (pF)	30.5 ± 8.9	21.6 ± 9.1
Threshold (mV)	-36.2 ± 5.8	-39.4 ± 6.0
Peak (mV)	37.1 ± 8.6	33.6 ± 12.3
After hyperpolarization (mV)	-59.3 ± 6.2	-59.1 ± 6.7
dV/dt Rise (mV/ms)	132.1 ± 41.3	153.7 ± 50
dV/dt Fall (mV/ms)	79.9 ± 25.4	101.2 ± 37.3
Duration of half amplitude (ms)	0.76 ± 0.17	0.65 ± 0.23

The resting membrane potential was measured just after a rupture of patch membrane. 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. dV/dt Rise, slope of rising phase (threshold to peak) of the action potential; dV/dt Fall, slope of the falling phase (peak to threshold) of the action potential. Values are means ± SD.

Passive membrane properties of orexin neurons

Orexin neurons have a hyperpolarization-activated current (I_h current). Some orexin neurons showed a time- and voltage-dependent sag of membrane potential evoked by hyperpolarizing current steps and anodal break rebound depolarizations, which were often accompanied by action potential firing (arrow in Fig. 3A). This response is usually considered to be representative of I_h channels [26]. This sag of membrane potential was investigated in more detail. In Fig. 3B, current–voltage relationship was plotted separately at instantaneous phase (peak of transient hyperpolarization, open circle in Fig. 3A) and at steady phase (end of 200 ms current injection, closed circle in Fig. 3A). In the current–voltage relationship, instantaneous phase showed a linear manner, while steady phase showed an inward rectification of more than −100 mV. The membrane potentials of initiation phase and steady phase were significantly dissociated by more than −100 mV; e.g., -152.0 ± 3.8 and -139.4 ± 3.7 mV, at −200 pA current injection, respectively ($n = 12$). This depolarizing sag in membrane voltage was blocked by specific I_h channel blocker ZD7288 (100 μM, $n = 3$, Fig. 3C) or cesium (1 mM, $n = 2$, data not shown).

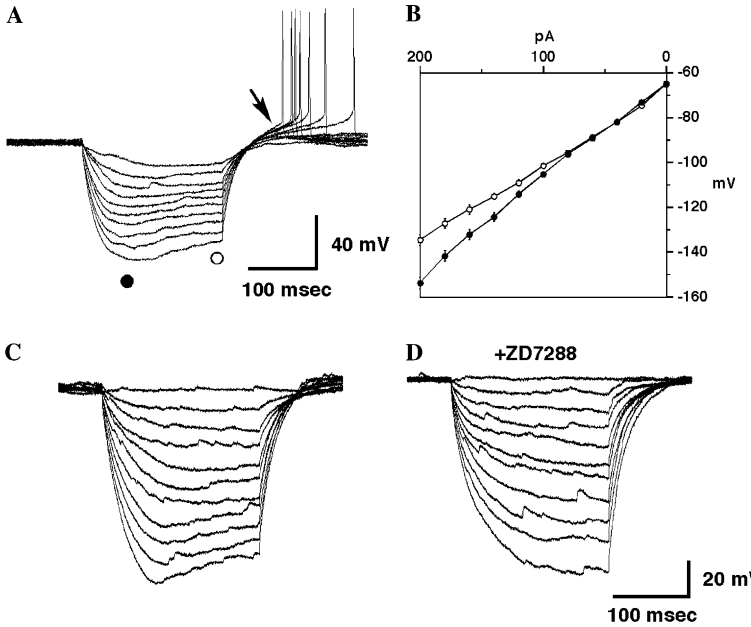

Fig. 3. Passive membrane properties of orexin neurons. (A) Records of membrane potential in response to a series of 200 ms current steps (from −200 to −20 pA in 20 pA increments) from resting potential (−60 mV). The arrow shows that anodal break rebound depolarization induced action potentials. The instantaneous potential (steady state potential circle in A) and steady state potential (instantaneous potential circle in A) are plotted in current–voltage relationship. (C) Records of membrane potential in response to a series of 200 ms current steps (from −200 to −0 pA in 20 pA increments). Bath application of ZD7288 (100 μM), a specific blocker of I_h, for 10 min abolishes the sag in membrane voltage (right).

AMPA and NMDA depolarize, while muscimol hyperpolarize, orexin neurons

To study the effect of classical neurotransmitters glutamate and GABA on orexin neurons, NMDA and AMPA, glutamate receptor agonists and muscimol, a $GABA_A$ receptor agonist was used. In whole cell current clamp, orexin neurons were depolarized by AMPA (50 μM) and NMDA (200 μM), while muscimol (200 μM) hyperpolarized orexin neurons (Fig. 4). AMPA and NMDA depolarized 27.9 ± 0.9 and 31.8 ± 7.7 mV, respectively ($n = 5$), while muscimol hyperpolarized 6.4 ± 2.5 mV ($n = 5$) in the presence of TTX (1 μM). Under voltage clamp mode in the presence of TTX, AMPA (50 μM) and NMDA (200 μM) application evoked inward current in orexin neurons, 148.7 ± 15.9 and 54.5 ± 2.2 pA, respectively ($n = 5$), with the membrane potential held at −60 mV. Muscimol (200 μM) application induced outward current in orexin neurons, 91.7 ± 30.3 pA ($n = 6$) when the membrane potential was held at −45 mV. Glycine, also known as an inhibitory neurotransmitter, had little or no effect on the membrane potential of orexin neurons in current clamp mode in the presence of TTX ($n = 3$, data not shown). To determine whether orexin neurons actually received glutametergic or GABAergic input in the brain, EPSCs and IPSCs were recorded at a holding potential of −30 mV. Under this condition, EPSC and IPSC were observed as an inward and an outward current, respectively (Fig. 4D, top). Simultaneous application of AP-5 (50 μM), NBQX (50 μM), glutamate receptor antagonists, and bicuculline (50 μM), a $GABA_A$ receptor antagonist, almost blocked EPSCs and IPSCs, suggesting that transmission via glutamate and GABA is the primary synaptic input to orexin neurons in the slice preparation (Fig. 4D, middle). EPSCs and IPSCs were observed again after washout of these antagonists (Fig. 4D, bottom).

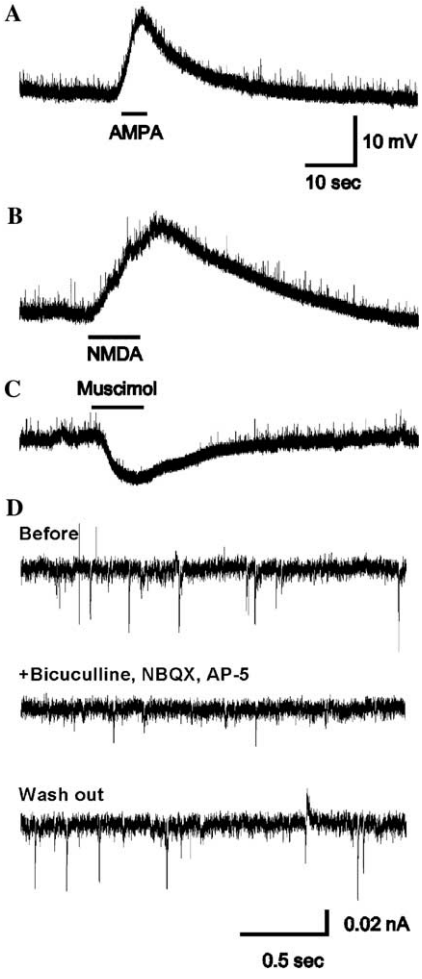

Fig. 4. Effect of AMPA (50 μM), NMDA (200 μM), and muscimol (200 μM) on orexin neurons. In current clamp mode, NMDA and AMPA depolarized, while muscimol hyperpolarized the membrane potential of orexin neurons. Membrane potential was set at −60 and −45 mV before NMDA, AMPA, and muscimol application, respectively, by the current injection. All recordings were performed in the presence of TTX (1 μM). TTX was applied by bath application; NMDA, AMPA, and muscimol were applied locally through a fine polyethylene tube during the period indicated by the bars. (D) Voltage clamp recording held at −30 mV shows EPSCs and IPSCs (top). AP-5 (50 μM) and NBQX (50 μM), glutamate receptor antagonists, and bicuculline (50 μM), a $GABA_A$ receptor antagonist were applied by bath application. EPSCs and IPSCs were blocked by simultaneous treatment of these antagonists (middle) and recovered by removal of these antagonists (bottom).

Effects of neurotransmitters on orexin neurons

The effects of several neurotransmitters implicated in sleep/wake regulation on the orexin neurons were examined. Orexin neurons densely innervate the aminergic nuclei (LC, raphe nucleus, TMN, and VTA) and orexins have been shown to activate these nuclei [3,4]. These facts suggest the possibility that orexin neurons have reciprocal connections from these nuclei for either positive or negative feedback loop. Therefore, we tried the amines, i.e. noradrenaline, serotonin, and dopamine. In whole cell current clamp mode absent of TTX, noradrenaline (100 μM) and serotonin (100 μM) hyperpolarized orexin neurons. In the presence of TTX (1 μM), noradrenaline and serotonin hyperpolarized the membrane potentials -14.6 ± 1.5 mV ($n = 6$) and -15.5 ± 4.5 mV ($n = 5$), respectively, when the membrane potentials were initially set at −45 mV. Dopamine (30 and 100 μM) also hyperpolarized orexin neurons ($n = 4$, data not shown) without TTX. However, it is not clear whether this response is mediated by dopamine receptors or not, because dopamine affects not only dopamine receptors but also adrenergic receptors. Acetylcholine is a major neurotransmitter in the brain. The cholinergic nuclei, laterodorsal tegmental nucleus (LDT) and peduncle pontine nucleus (PPN), are implicated in the regulation of REM sleep and arousal. Orexin neurons also densely innervate the LDT and PPN, and activate

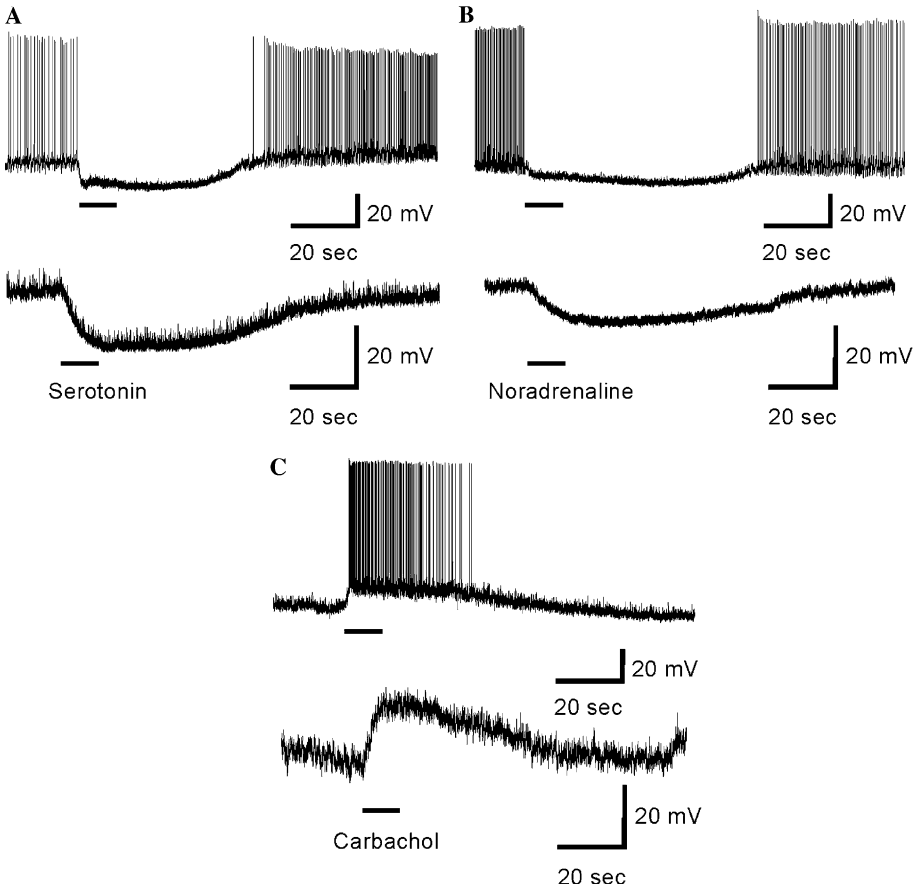

Fig. 5. In current clamp mode, noradrenaline (100 μM), serotonin (100 μM), and carbachol (100 μM) were applied on orexin neurons in the absence (upper) or presence (lower) of TTX (1 μM). Before the experiments, the membrane potential was set at −45 mV (serotonin and noradrenaline) or −60 mV (carbachol) by the current injection. TTX was applied by bath application; noradrenaline, serotonin, and carbachol were applied locally through a fine polyethylene tube during the period indicated by the bars.

the neurons in these nuclei [3,4]. To elucidate the effect of acetylcholine on the orexin neurons, the cholinergic agonist carbachol was used instead of acetylcholine. Carbachol depolarized and activated orexin neurons with or without TTX (Fig. 5C). In the presence of TTX (1 μM), carbachol (100 μM) depolarized the membrane potential 9.0 ± 1.1 mV ($n = 5$) when the membrane potentials were initially set at −60 mV. The metabolic type of acetylcholine receptor (muscarinic receptor) antagonist, atropine (10 μM), abolished this effect completely, suggesting that muscarinic receptor is involved in this response (data not shown). The effect of acetylcholine (100 μM) on the orexin neurons was the same as that of carbachol in the presence of TTX ($n = 2$).

It is well known that histamine neurons in the brain play an important role in arousal. The TMN is a histaminergic nucleus that highly expresses OX_2R and receives many orexin nerve endings [13,27]. Histamine neurons are directly activated by orexins. However, histamine (100 μM) had little or no effect on any of the orexin neurons we examined in this study ($n = 6$) in the absence of TTX.

Discussion

Recent studies revealed that orexin neurons are important for the maintenance of vigilance states, and a lack in the orexin system causes narcolepsy in animals and humans [17–22]. Orexin neurons are located specifically in the perifornical, dorsomedial, lateral, and posterior hypothalamus, but project to almost of all regions in the brain except the cerebellum. Especially dense projection was observed in the nuclei which were known to be involved in sleep/wakefulness regulation, i.e., noradrenergic LC, serotonergic raphe, acetylcholinergic LDT and PPN, histaminergic TMN, and dopaminergic VTA. Orexins directly or indirectly activate the neurons in these nuclei [8–16,28,29]. The neurons in the LC and raphe fire during wakefulness, decrease their activity during non-REM sleep, and are virtually silent during REM-sleep. Although the activity of orexin neurons during sleep and waking has not been described, in vivo recording from the perifornical lateral hypothalamus showed that most neurons in this area are active during waking and REM sleep and

inactive during sleep [30]. The location of these wake-related neurons is consistent with the region in which orexin neurons are located. Additionally, microdialysis assay showed that orexin-A release was higher during the active wake [31]. These results suggest that orexin neurons are also active during the animal's waking period. In this synchronization of activity between orexin neurons and LC, raphe nucleus in wake state might be accomplished by reciprocal connections. We showed that the activity of orexin neurons is directly inhibited by noradrenaline and serotonin in the presence of TTX. Although it is not known whether noradrenergic neurons from LC or serotonergic neurons from raphe nucleus directly synapse to orexin neurons or not, our data suggest that orexin neurons have a negative feedback loop from noradrenergic and serotonergic neurons. Dopamine (100 μM) also hyperpolarized orexin neurons, though it is not clear whether dopamine exerts via dopamine receptor or not, because this high concentration of dopamine acts not only on dopamine receptors but also on α- and β-adrenaline receptors [32]. Previous report suggests the involvement of dopaminergic VTA neurons in sleep/wake regulation [33]. Additionally it has been reported that orexins activate dopaminergic neurons in the VTA [9,16]. Further detailed experiments are needed to elucidate the participation of dopaminergic neurons. On the other hand, carbachol activated all orexin neurons we examined. If acetylcholine neurons from PPN or LDT directly synapse to orexin neurons, this forms a positive feedback circuitry. LDT and PPN neurons are activated in the REM sleep period [28,29]. A half number of wake-related neurons in the perifornical lateral hypothalamic area are also active in the REM sleep period (wake/REM-related neurons). If orexin neurons are wake/REM-related neurons, this cholinergic input has an important role to decide the activity of wake/REM-related neurons in the REM sleep period [30]. This negative or positive feedback from these nuclei to orexin neurons might be important for fine regulation or synchronization of activity of nuclei implicated in the regulation of the vigilance states. Interestingly, histamine had little effect on any of the orexin neurons we tested in this experiment, despite the fact that TMN and histamine have been thought to be important for orexin induced arousal response [13]. This result, however, does not completely rule out the effect of histamine on orexin neurons, because a few of orexin neurons might respond to histamine or indirect pathway via any other nucleus might exist for the feedback loop.

The experiments for passive membrane properties of orexin neurons revealed that time- and voltage-dependent sag of membrane potential evoked by hyperpolarizing current steps and anodal break rebound depolarizations. The specific I_h channel inhibitor ZD7288 (100 μM) or low concentration of cesium (1 mM) blocked these responses, suggesting that the involvement of I_h current. I_h has been observed in a diverse group of cell types in various species, including cardiac pacemaker cells, smooth muscle cells, and neurons [26,34,35]. We observed I_h current not only in orexin neurons but also in some non-EGFP expressing neurons in the lateral hypothalamic area, though this I_h current might be involved in physiological membrane properties of orexin neurons such as rhythmic burst firing observed in some orexin neurons and relatively high firing frequency with little adaptation evoked by positive current injection. Studies in many types of neurons have shown that one of the key conductances controlling rhythmic burst is the slow hyperpolarization-activated inward current [26,35]. I_h current was observed to be more than −100 mV hyperpolarization in orexin neurons. This hyperpolarization is unlikely to occur in a physiological state, because after hyperpolarization of orexin neurons do not reach lower than −100 mV. However, recent research reports suggested the positive modulation of I_h by an intracellular second messenger, such as cyclic adenosine monophosphate (cAMP) or calcium [36,37]. Actually, a high concentration of NMDA (200 μM) treatment often triggered membrane oscillation and started rhythmical burst firing in quiescent or normal firing orexin neurons. Thus, it is probable that I_h current shifts to a physiological range in orexin neurons in vivo. Further studies are needed to clarify the physiological importance of I_h current of orexin neurons.

In conclusion, we revealed electrophysiological properties of orexin neurons by applying a patch clamp technique to transgenic mice which specifically express EGFP in orexin neurons. Orexin neurons have no distinct electrophysiological features from other neurons in the hypothalamus, suggesting difficulty in identifying orexin neurons in vivo experiments. Serotonin and noradrenaline hyperpolarized, while acetylcholine (carbachol) depolarized orexin neurons. These data suggest that orexin neurons have serotonergic, noradrenergic, and acetylcholinergic input. Probably, orexin neurons are projected from the raphe nucleus, LC, and PPN to form a feed back circuitry. These feedback loops might be important for sleep/wake regulation by orexin neurons.

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