

Orexins Excite Neurons of the Rat Cerebellar Nucleus Interpositus Via Orexin 2 Receptors In Vitro

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Abstract Orexins are newfound hypothalamic neuropeptides implicated in the regulation of feeding behavior, sleep–wakefulness cycle, nociception, addiction, emotions, as well as narcolepsy. However, little is known about roles of orexins in motor control. Therefore, the present study was designed to investigate the effect of orexins on neuronal activity in the cerebellum, an important subcortical center for motor control. In this study, perfusing slices with orexin A (100 nM–1 μ M) or orexin B (100 nM–1 μ M) both produced neurons in the rat cerebellar interpositus nucleus (IN) a concentration-dependent excitatory response (96/143, 67.1%). Furthermore, both of the excitations induced by orexin A and B were not blocked by the low-Ca²⁺/high-Mg²⁺ medium ($n=8$), supporting a direct postsynaptic action of the peptides. Highly selective orexin 1 receptor antagonist SB-334867 did not block the excitatory response of cerebellar IN neurons to orexins ($n=22$), but [Ala¹¹, D-Leu¹⁵] orexin B, a highly selective orexin 2 receptor (OX₂R) agonist, mimicked the excitatory effect of orexins on the cerebellar neurons ($n=18$). These results demonstrate that orexins excite the cerebellar IN neurons through OX₂R and suggest that the central orexinergic nervous system may actively participate in motor control through its modulation on one of the final outputs of the spinocerebellum.

Keywords Cerebellar interpositus nucleus · Orexin A · Orexin B · Orexin 1 receptors · Orexin 2 receptors · Motor control

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Introduction

Orexin A and orexin B (also called hypocretin-1 and -2), both derived from the common precursor prepro-orexin, are synthesized exclusively within the central nervous system [1, 2]. Although the cell bodies of neurons producing orexins are narrowly concentrated in the lateral hypothalamus/perifornical region, the orexinergic fibers widely project to various areas in the brain [3, 4]. Molecular and immunohistochemical studies have revealed that orexin receptors, members of G protein-coupled receptors, are located at the projection sites of orexinergic neurons [5–7]. There are two types of orexin receptors, the orexin 1 receptor (OX₁R) and the orexin 2 receptor (OX₂R), having been identified, in which the OX₁R has one-order-of-magnitude greater affinity for orexin A than orexin B, whereas the OX₂R binds orexin A and orexin B with similar affinities [1, 8].

Originally, orexins were considered to be neurotransmitters that centrally stimulate food intake [1, 9], but it soon became evident that orexins are also involved in many basic physiological processes including energy homeostasis, sleep–awake cycle, nociception, food and drug addiction, as well as emotions [3, 4]. However, although a large number of immunohistochemical studies have revealed that various subcortical motor structures including the cerebellum receive the hypothalamic orexinergic innervations [3, 4] and both OX₁R and OX₂R are present in those centers for motor control [10–12], the effects of orexins on neuronal activity of those central motor structures are still largely unknown. In this study, the effects of orexins on the unitary firing of neurons in the cerebellar interpositus nucleus (IN), one of the final outputs of spinocerebellum coordinating body and limb movements, were investigated. The results showed that both orexin A and orexin B excited rat cerebellar IN neurons exclusively via the mediation of OX₂R.

Materials and Methods

Under ether anesthesia, 56 Sprague-Dawley rats (150–200 g) were decapitated, and their cerebella were quickly taken out and washed in ice-cold oxygenated artificial cerebrospinal fluid (ACSF, composition in mM: NaCl 124, KCl 5, MgSO₄ 1.3, KH₂PO₄ 1.2, NaHCO₃ 26, CaCl₂ 2.4, and D-glucose 10). Sagittal plane slices (400 μm thick) through the cerebellum were cut at 4°C by using a vibroslicer (VT 1000 S, Leica, Germany). The cerebellar slices containing the IN were obtained and identified according to the rat brain atlas of Paxinos and Watson [13] and then transferred to a recording chamber which was continuously perfused with ACSF equilibrated with 95% O₂/5% CO₂ (pH 7.4, 33±0.2°C, flow rate 2 ml/min). All slices were incubated for a minimum of 40 min before recording. In some experiments, a low-Ca²⁺/high-Mg²⁺ medium was used to reduce presynaptic transmitter release. In these cases, the concentration of Ca²⁺ was lowered to 0.3 mM, and Mg²⁺ was raised to 9.0 mM [14–16]. All experiments completely conformed to the US National Institutes of Health Guide for the Care and Use of Laboratory Animals (NIH Publication 80-23, revised 1996). All efforts were made to minimize the number of animals used and their suffering.

Spontaneous unitary activity of cerebellar IN neurons was recorded extracellularly from the slices using glass microelectrodes filled with 2 M NaCl (impedance 5–10 MΩ). Before bath application of orexinergic compounds at known concentrations, the discharge rate of the recorded neuron was observed for at least 15 min to assure stability. At present, commercial orexinergic compounds are still strictly limited, only orexin A, orexin B, [Ala¹¹, D-Leu¹⁵] orexin B (highly selective OX₂R agonist), and SB-334867 (highly selective OX₁R antagonist) are available for the study. These orexinergic compounds were freshly dissolved in ACSF before the experiments and equilibrated with 95% O₂/5% CO₂ before superfusing the slices. Since orexin A has the same affinity to both of the orexin receptors OX₁R and OX₂R, it was first added to the perfusing ACSF to stimulate the recorded neurons for a test period of 90 s. If the neurons responded to the stimulation of orexin A (Tocris, UK), orexin B (Tocris, UK) and highly selective OX₂R agonist [Ala¹¹, D-Leu¹⁵] orexin B (Tocris, UK) were then applied (90 s test period as well), respectively. Thereafter, perfusing medium was switched from normal ACSF to the ACSF containing selective OX₁R antagonist SB-334867 (Tocris, UK). After the slice was equilibrated with the ACSF containing SB-334867 for at least 15 min, orexin A, orexin B, or [Ala¹¹, D-Leu¹⁵] orexin B was re-applied, and the effect of antagonist on the response of the cerebellar IN neurons to orexins or the OX₂R agonist was observed. The discharges of the recorded neurons were

amplified and displayed conventionally and fed into a window discriminator simultaneously. The standard rectangle pulses (5 V, 1 ms) triggered from the spikes were sent through an interface (1404 Plus, CED, UK) to a laboratory microcomputer, which was used to analyze the discharge rate on-line by the software Spike 2 (CED, UK). Peristimulus time histograms (sampling interval=1 s) of neuronal discharges were generated by the computer to assess the effects of orexins and orexin receptor agonist on the cerebellar IN neurons. Drug-induced effects on spontaneous unitary activity of cerebellar IN neurons were considered to be substance-specific provided they were reversible and reproducible. The response magnitude of a neuron to the stimulation of orexins and orexin receptor agonist was calculated as the percentage change in the cell's peak discharge rate following stimulation with respect to its basal firing rate. Student's *t* test was employed for statistical analysis of the data, and *P* values of <0.05 were considered to be significant.

Results

Effect of Orexin A and Orexin B on Spontaneous Firing of Cerebellar IN Neurons

One hundred and forty-three IN neurons with a stable tonic discharge were recorded from 72 cerebellar slices. The spontaneous firing rate of the cells ranged from 10.1 to 57.5 spikes/s, and the mean firing rate of the cells was 33.0±12.3 spikes/s (mean ± SD), which is similar to those of our and other authors' previous reports [15–17].

Of the 143 recorded IN neurons, 96 (67.1%) were excited by both of the orexin A (100 nM–1 μM) and orexin B (100 nM–1 μM) stimulations, and the rest, 47, had no response to the orexins superfusion (bath concentration up to 1 μM). These results indicate that both orexin A and orexin B are capable of increasing the firing rates of recorded IN neurons (Fig. 1, see also Fig. 2a, c). As illustrated in Fig. 1, the recorded IN neuron exhibited a concentration-related excitatory responses to 100 nM, 300 nM, and 1 μM orexin A stimulations, respectively, with 11.4%, 22.22%, and 54.71% increase in the peak discharge rate compared with its basal firing rate (Fig. 1b; *P*<0.05 or 0.01). Additionally, the same neuron was also concentration-dependently excited by 100 nM, 300 nM, and 1 μM orexin B with a 10.9%, 24.84%, and 57.7% increase over the basal firing rate (Fig. 1d; *P*<0.05 or 0.01), respectively. There was no significant difference between the increases of firing rate induced by orexin A and orexin B (*P*>0.05) in the same concentration.

In order to exclude the possibility that the excitatory response of the cerebellar IN neurons to orexins was

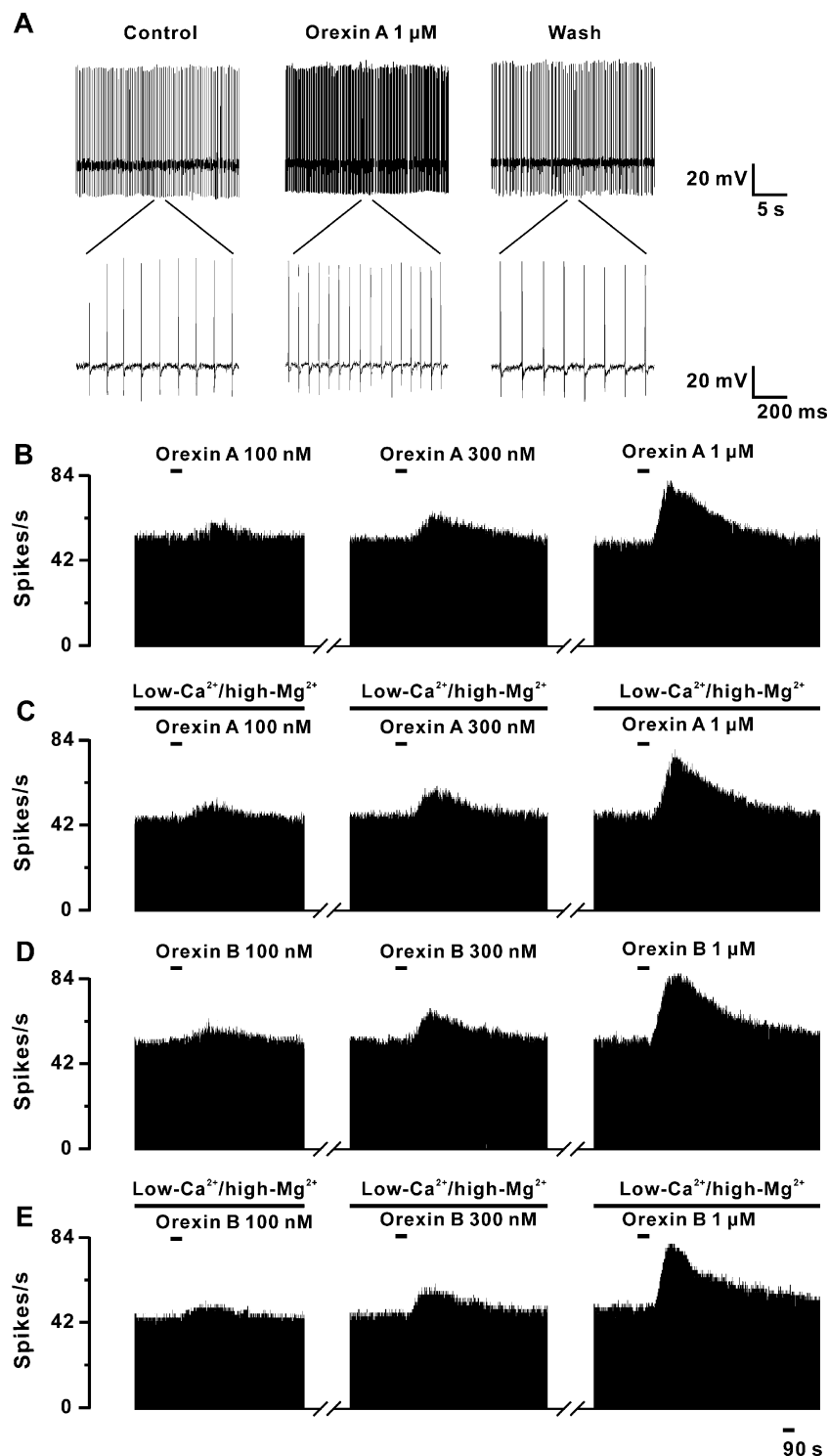


Fig. 1. Excitatory responses of a cerebellar interpositus nucleus (IN) neuron to orexin A and orexin B stimulations. **a** Oscilloscope traces show the action potentials of the recorded cerebellar IN neuron and the neuron's response to 1 μM orexin A. The mean firing rate of the neuron before, during, and after application of orexin A was 48.6 ± 0.97 , 75.2 ± 1.12 , 49.2 ± 0.83 spikes/s, respectively. **b, c** Histograms show concentration-related excitatory responses of the neuron to 100 nM, 300 nM, and 1 μM orexin A when the slice was successively equilibrated with normal artificial cerebrospinal fluid (ACSF; **b**) and

low- Ca^{2+} /high- Mg^{2+} medium (**c**). **d, e** The same cell also showed a concentration-related excitation to 100 nM, 300 nM, and 1 μM orexin B when the slice was equilibrated with both normal ACSF (**d**) and low- Ca^{2+} /high- Mg^{2+} medium (**e**). In this and the following figures, the short horizontal bars above the histograms indicate the orexin A, orexin B, or orexin receptor agonist stimulation that last for 90 s, and the concentration of orexinergic reagents applied to the slice is also given for each experiment

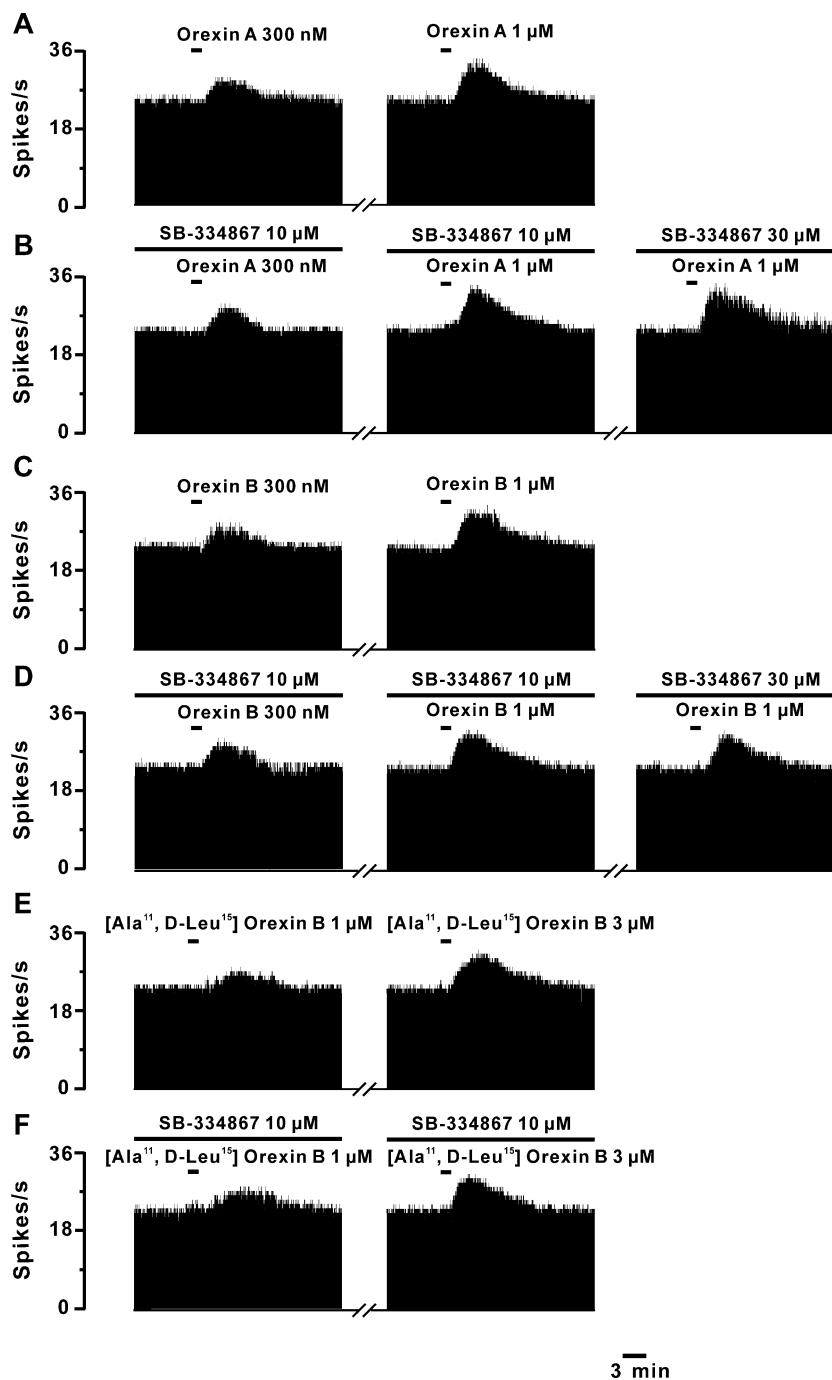


Fig. 2. Histograms showing the effect of orexins or orexin receptor agonist on a cerebellar interpositus nucleus (IN) neuron and the effects of orexin receptor antagonist on the excitations induced by orexins and orexin receptor agonist. **a** Concentration-related excitatory responses of a cerebellar IN neuron to orexin A. **b** Selective OX₁R antagonist SB-334867 (10–30 μM) did not block the neuronal excitations induced by orexin A. **c** The same neuron also showed a

concentration-dependent excitation to orexin B. **d** Selective OX₁R antagonist SB-334867 (10–30 μM) still did not block the neuronal excitations induced by orexin B. **e** Highly selective OX₂R agonist [Ala¹¹, D-Leu¹⁵] orexin B mimicked the excitatory effect of orexins on the cell. **f** Selective OX₁R antagonist SB-334867 (10–30 μM) did not block the neuronal excitations induced by selective OX₂R agonist [Ala¹¹, D-Leu¹⁵] orexin B

indirectly induced or contained components indirectly induced by the effect of orexin on presynaptic elements, we tested the effects of orexin A and orexin B on the IN cells when the normal ACSF had been replaced with a low-

Ca²⁺/high-Mg²⁺ medium ($n=8$). The results showed that the low-Ca²⁺/high-Mg²⁺ medium did not block the excitatory responses of IN cells to both orexin A and orexin B, which suggested that the responses were due to a direct

postsynaptic effect of orexins on the neurons. As it can be seen from Fig. 1c and e, after the slice was exposed to the low- Ca^{2+} /high- Mg^{2+} medium for 20 min, the recorded cell still showed an excitatory response to the orexins stimulation, which has no significant difference in comparison with the excitation induced in normal ACSF ($P > 0.05$ for each case). The slight decrease in spontaneous firing rate of the cell during the perfusion of low- Ca^{2+} /high- Mg^{2+} medium might be related to a disturbance of normal Ca^{2+} concentration in local milieu and/or to actions of Mg^{2+} on intracellular Ca^{2+} -dependent processes [15, 16, 18, 19].

Effect of Selective OX_1R Antagonist SB-334867 on Orexins-induced Excitation

Since there is only one selective antagonist for orexin receptors available, i.e., SB-334867, a highly selective OX_1R antagonist, we used it to examine what kinds of orexin receptors mediated the excitatory response of the cerebellar IN neurons to orexin A and orexin B. The results showed that SB-334867 (10–30 μM) did not block the excitatory effects of orexins on the neurons ($n=22$). As illustrated in Fig. 2, the recorded IN neuron exhibited concentration-related excitatory responses to not only 300 nM and 1 μM orexin A with 20.8% and 39.1% increase in the peak discharge rate compared with its basal firing rate (Fig. 2a, $P < 0.05$) but also 300 nM and 1 μM orexin B with an increase of firing rate by 24.1% and 39.1%, respectively (Fig. 2c, $P < 0.05$), but selective OX_1R antagonist SB-334867 did not block the excitations on cerebellar IN neurons induced by orexin A and orexin B. After the slice was equilibrated with ACSF containing SB-334867 (10 μM) for 15 min, 300 nM and 1 μM orexin A still excited the cerebellar IN neurons with 22.72% and 37.5% increase in the firing rate, which was not significantly changed in comparison with that of the control experiment (Fig. 2a, b; $P > 0.05$ for each case), so did orexin B (Fig. 2c, d; $P > 0.05$ for each case). It is also noted that even the concentration of SB-334867 was raised to 30 μM ; the excitations induced by 1- μM orexin A and orexin B on the same neuron was still not blocked (the right panel in Fig. 2b, d). These results indicated that the OX_2R instead of the OX_1R mediated the excitatory effects of orexin A and orexin B on the cerebellar IN neurons.

Effect of Selective OX_2R Agonist [Ala¹¹, D-Leu¹⁵] Orexin B on Cerebellar IN Neuronal Spontaneous Firing

The above results suggest that the excitatory response of cerebellar IN neuron to orexin A and orexin B may be mediated by the OX_2R . To confirm these results, we further

observed the effects of the highly selective OX_2R agonist [Ala¹¹, D-Leu¹⁵] orexin B, which showed about a 400-fold selectivity for the OX_2R over the OX_1R [20], on the cerebellar IN neuronal spontaneous firing. The agonist mimicked the excitatory effect of orexins on cerebellar IN cells. As illustrated in Fig. 2e and f, the recorded cerebellar IN neuron exhibited a concentration-related excitatory response to 1 and 3 μM [Ala¹¹, D-Leu¹⁵] orexin B with 20.15% and 35.64% increase in the peak discharge rate compared with its basal firing rate (Fig. 2e; $P < 0.05$ for both cases), respectively; after the slice was equilibrated with ACSF containing SB-334867, a selective OX_1R antagonist, for 15 min, the peak amplitudes of the cell's excitatory responses elicited by [Ala¹¹, D-Leu¹⁵] orexin B were not decreased (Fig. 2f; $P > 0.05$ for both cases, compared with the control experiments showed in Fig. 2e). The results indicated that orexin A and orexin B excited the cerebellar IN neurons by the mediation of OX_2R .

As summarized in Fig. 3, all of the recorded cerebellar IN cells showed a unique concentration-dependent excitatory response to stimulation of orexin A, orexin B, or selective OX_2R agonist [Ala¹¹, D-Leu¹⁵] orexin B (Fig. 3a), and all of the excitations evoked by orexin A, orexin B, and [Ala¹¹, D-Leu¹⁵] orexin B were not blocked by SB-334867, a selective OX_1R antagonist (Fig. 3b).

Discussion

Several in vivo and in vitro studies have shown that orexins have an excitatory effect on various brain regions involved in autonomic regulation, such as the lateral and medial hypothalamus [21], the arcuate nucleus [22], and tuberomammillary nucleus [23] in the hypothalamus, the nucleus accumbens [24], and the locus coeruleus [25]. The present study demonstrates that the hypothalamic peptides orexin A and orexin B also excited the IN neurons in the cerebellum, an important subcortical motor structure primarily coordinating execution of ongoing movements. The recorded cerebellar IN neurons (96/143, 67.1%) responded to both orexin A and orexin B with a unique excitatory response (Figs. 1 and 3). These concentration-dependent excitation induced by orexin A and orexin B were not blocked by superfusing the slices with the low- Ca^{2+} /high- Mg^{2+} medium (Fig. 1), suggesting a direct postsynaptic action of orexin A and orexin B on the cerebellar IN neurons. Since the magnitudes of the excitatory responses of the cerebellar IN evoked by orexin A and orexin B in the same concentration have no significant difference in this study (Figs. 1 and 3) and considering that orexin A and orexin B have the same affinities for the OX_2R whereas orexin A has higher affinity for the OX_1R than orexin B, it is likely that

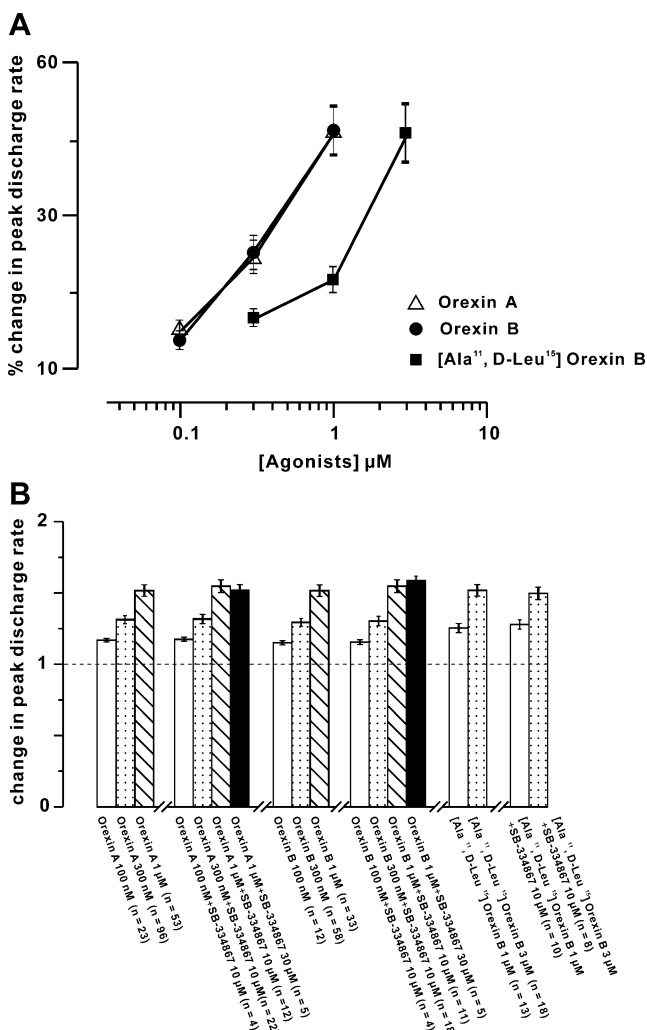


Fig. 3. Averaged dose–response curves showing the effects of orexin A ($n=23, 96$ and 53 for three used concentration, respectively), orexin B ($n=12, 58$ and 33 , respectively) and selective OX₂R agonist [Ala¹¹, D-Leu¹⁵] orexin B ($n=5, 13$ and 18 , respectively) on the discharge rate of recorded cerebellar interpositus nucleus (IN) neurons (a). Statistical histogram showed effects of orexins and orexin receptor agonist on the IN neurons, as well as the effects of orexin receptor antagonist on excitations induced by orexins and orexin receptor agonist (b). All data were normalized and expressed as mean \pm SEM. The peak firing rate of each group of cells in response to orexins or orexin receptor agonist was compared with its basal firing rate before the orexinergic reagents was applied to the slices

the excitatory effects of orexin A and orexin B on the cerebellar IN neurons are mediated by the OX₂R instead of the OX₁R. Although so far there are still only two kinds of orexinergic compounds available for judging the types of orexin receptors, the receptor mechanism underlying the excitation induced by orexins on the cerebellar IN neurons was substantially determined in this study. Selective OX₂R agonist [Ala¹¹, D-Leu¹⁵] orexin B mimicked the excitatory effect of orexins on the cerebellar IN neurons, but selective OX₁R antagonist SB-334867 did not block any excitatory responses of the cerebellar IN cells to orexin A, orexin B,

and [Ala¹¹, D-Leu¹⁵] orexin B (Figs. 2 and 3). All of these results suggest that the excitatory effect of orexins on the cerebellar IN cells is exclusively mediated by the OX₂R. Although in situ hybridization and immunohistochemical studies have revealed that both the OX₁R and OX₂R are expressed and distributed in the IN of the cerebellum in rats [10, 11], the present study demonstrates that only the OX₂R mediates the electrophysiological effect of orexins on the cerebellar IN neurons.

Central orexinergic nervous system originates from the hypothalamus and participates in many basic autonomic functions including food intake, sleep–wakefulness, emotion, and reward [1, 3, 4, 9]. Although little is known about exact roles of the central orexinergic system in motor control, several works indicated that it may participate in regulation of motor behavior. It has been reported that injection of orexin A into cerebral ventricles or specific areas in the midbrain or medulla facilitated muscle tone and maintained a higher level of locomotor activity in the animal, whereas disturbance of orexinergic system functions elicited muscular atonia or cataplexy [26–28]. However, the mechanisms underlying the regulation of central orexinergic system on motor control and the actions of orexins on those important motor structures in central nervous system remain obscure. The present study demonstrates that orexins excite the cerebellar IN neurons through activation of the OX₂R. Additionally, orexinergic neurons in the hypothalamus also exert an excitatory action on Purkinje cells in flocculus of the cerebellum [29]. Considering the neurons in the cerebellar IN and the Purkinje cells in the flocculus are ultimate outputs of the spinocerebellum and the vestibulocerebellum, respectively, we speculate that the orexinergic afferents from the hypothalamus may actively modulate the cerebellar final outputs through their innervations of the flocculus Purkinje cells as well as the cerebellar IN neurons. It has been well known that most of the orexinergic endings (varicosities) do not typically form synaptic specializations, and both of the orexin receptors are metabotropic [30]. Therefore, we suggest that orexins and the orexinergic inputs from the hypothalamus may bias neuronal activity in the cerebellum and endogenously regulate the cerebellar circuitry through which the sensorimotor integration is fulfilled. Since the spinocerebellum is the cerebellar subdivision connecting with the lateral and medial brain stem descending systems, in which the cerebellar IN precisely governs the ongoing movements of distal muscles of the limbs and digits via its modulation on the rubrospinal tract [31–33], the cerebellar orexinergic afferent inputs may actively participate in cerebellar motor coordination.

On the other hand, a growing body of evidence shows that orexin deficiency results in narcolepsy-cataplexy, which is characterized by sudden loss of muscle tone, in

humans, dogs, and rodents [3, 4, 26–28]. Interestingly, the cerebellum is one of the subcortical motor structures for regulating muscle tone through its connections with spinal gamma motor neurons [31–35]. Thus, the present result that orexins excite the cerebellar neurons strongly suggests that the excitatory orexinergic drive for the cerebellum may be helpful for maintaining a proper muscle tone in normal conditions and the loss of excitation by orexinergic innervations on the cerebellum may be closely related to cataplexy.

Series of neuroanatomical and neurophysiological studies have revealed that there are direct projections from the hypothalamus to the cerebellum. These hypothalamocerebellar projections, together with the direct cerebellohypothalamic projections, constitute the cerebellar-hypothalamic circuits, which may be potential pathways underlying the cerebellar non-somatic and autonomic modulation such as feeding, cardiovascular, respiratory, micturition, immune, learning, emotional, as well as cognitive regulation [36–38]. Therefore, orexins may not only be neuropeptides regulating basic autonomic functions but also candidates for the neurotransmitters/neuromodulators in the hypothalamocerebellar projections, which may forward hypothalamic visceral information to the cerebellum and participate in those cerebellar non-somatic functions and somatic-visceral integration, like histamine in the hypothalamocerebellar histaminergic projections [37].

In summary, the present study revealed that orexins excited the cerebellar IN neurons via the activation of the postsynaptic OX₂R and suggested that through the excitatory innervations on the cerebellar IN neurons, the orexinergic inputs from the hypothalamus to the cerebellum may actively modulate the cerebellar motor functions and help the central nervous system to generate integrated and coordinated somatic-visceral responses to the changes of internal and external environments.

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