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Low-voltage-activated A-current controls the firing dynamics of mouse hypothalamic orexin neurons

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Abstract

The activity of hypothalamic neurons that release the neuropeptides orexin-A and orexin-B is essential for normal wakefulness. Orexin neurons fire spontaneously and are hyperpolarized and inhibited by physiological neuromodulators, but the intrinsic determinants of their electrical activity are poorly understood. We show that mouse orexin neurons coexpress orexin-A and orexin-B, and possess a low-voltage-activated A-type K⁺ current (A-current) likely to be composed of Kv4.3 subunits. The A-current enhances the inhibitory influence of hyperpolarizing currents via two mechanisms: by delaying the resumption of spiking after hyperpolarization and by increasing the slope of the relation between the firing frequency and injected current. These results identify an important determinant of the firing dynamics of orexin neurons, and support the idea that the A-current can control neuronal gain.

Keywords

A-current; hypocretin; hypothalamus; orexin; wakefulness

Introduction

Lateral hypothalamic neurons that release the neuropeptides orexins (hypocretins) (de Lecea, *et al.*, 1998; Sakurai *et al.*, 1998) are essential for normal wakefulness. Orexin neurons provide excitatory inputs to multiple arousal centres, and lack of orexins, orexin receptors or orexin neurons causes narcolepsy in humans and in spontaneous or transgenic animal models (Willie *et al.*, 2001; Sutcliffe & de Lecea, 2002; Taheri *et al.*, 2002). The two orexin peptides, orexin-A (hypocretin-1) and orexin-B (hypocretin-2), are derived from the same precursor (Willie *et al.*, 2001), but whether they are expressed by overlapping or distinct groups of neurons has not been directly tested.

Orexin neurons exhibit tonic spontaneous firing but are hyperpolarized and inhibited by arousal-modulating transmitters, and by appetite-suppressing hormones and nutrients (Li *et*

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et al., 2002; Eggermann *et al.*, 2003; Yamanaka *et al.*, 2003a, b). The biophysical and molecular determinants of the firing dynamics of orexin neurons are poorly understood, although membrane potential responses of orexin cells indicate the presence of ion channels that can control neuronal firing, such as low-voltage-activated Ca^{2+} channels and I_{H} channels (Eggermann *et al.*, 2003; Yamanaka *et al.*, 2003b). Low-voltage-activated A-type K^{+} channels can also profoundly shape both spontaneous and evoked firing dynamics of neurons (Connor & Stevens, 1971; Rush & Rinzel, 1995; Liss *et al.*, 2001), but whether orexin neurons express such channels is unknown.

Here, we examine the relative localization of orexin-A and orexin-B peptides in the mouse lateral hypothalamus (LH) using double-label immunocytochemistry. We then analyse the biophysical properties, molecular identity, and functional roles of A-type currents (A-currents) in electrically and neurochemically identified LH orexin neurons.

Materials and methods

Electrophysiology

Procedures involving animals were carried out in accordance with the Animals (Scientific Procedures) Act 1986 (UK). Preparation of coronal brain slices (250–300- μm thick) containing the LH from male C57BL6 mice (13–16 days postnatal) and patch-clamp recordings were performed as previously described (Burdakov & Ashcroft, 2002). The mice were killed by cervical dislocation. Data acquisition, and switching between voltage-clamp and current-clamp modes, was performed using an EPC-9 amplifier with PULSE software (HEKA, Lambrecht, Germany). In voltage-clamp recordings, the series resistance was compensated electronically by 70%. Experiments were performed at room temperature (24–26 °C). Data are given as mean $\pm$ SEM.

Pipettes were filled with internal solution (in mM): 120 K-gluconate, 20 KCl, 10 HEPES, 0.1 EGTA, 1 NaCl, 2 MgCl_2 , 5 $\text{K}_2\text{-ATP}$, 0.2% neurobiotin, pH 7.3 with KOH. The extracellular solution (ACSF) contained (in mM): 118 NaCl, 25 NaHCO_3 , 3 KCl, 1.2 NaH_2PO_4 , 2 CaCl_2 , 2 MgCl_2 , 10 glucose, plus tetrodotoxin (TTX, Sigma) where indicated in the figure legends. ACSF was gassed with 95% O_2 and 5% CO_2 . 4-Aminopyridine (4-AP) (Sigma) was applied locally (Burdakov & Ashcroft, 2002). To avoid the effects of changes in presynaptic A-currents, the action of 4-AP on cell firing was examined using ‘low Ca^{2+} ’ solution (0.3 mM CaCl_2 and 9 mM MgCl_2). Low Ca^{2+} solution alone does not affect the firing of orexin cells (Eggermann *et al.*, 2003). A supramaximal concentration of 4-AP (10 mM) was used in all experiments, because control experiments showed that lower concentrations induced only a partial block of the A-current and altered its kinetics (as observed in other cells, Liss *et al.*, 2001), making data interpretation difficult. Ten millimolar 4-AP did not alter the leak current (Fig. 2A), action potential firing (Fig. 3A) or delayed rectifier currents ($n = 6$).

The peak A-type conductance (g_{A}) was calculated from $g_{\text{A}} = \text{peak } I_{\text{A}} / (V_{\text{test}} - V_{\text{rev}})$, where $V_{\text{rev}} = -100$ mV (based on the Nernst K^{+} equilibrium potential for our solutions). The voltage-dependence of I_{A} activation (Fig. 2C) and inactivation (Fig. 2D) were fit to:

$$(\text{activation}) g_A / g_{A \max} = m_\infty^3 \quad (1)$$

and

$$(\text{inactivation}) I_A / I_{A \max} = h_\infty \quad (2)$$

where h_∞ and m_∞ follow the sigmoidal function $f(V_m) = 1 / \{1 + \exp[(V_m + k_1)/k_2]\}$. For I_A activation, the best fit (Fig. 2C) was obtained with $k_1 = 40$ and $k_2 = -13.9$. For I_A inactivation, the best fit (Fig. 2D) was obtained with $k_1 = 78$ and $k_2 = 8$. Curve fitting was performed using Origin (Microcal).

Immunocytochemistry

All data presented are from immunocytochemically identified orexin neurons. Each neuron was maintained in the whole-cell configuration for 5–10 min to allow thorough infusion of neurobiotin. After electrophysiology, slices were fixed in 4% paraformaldehyde (in PBS, pH 7.4) for 1 h at room temperature, incubated with primary antibodies overnight, and then with fluorescent secondary antibodies for 3 h, following the protocol of Wolfart *et al.* (2001). The primary antibodies and labels were rabbit anti-orexin-A [1 : 250] (Phoenix), goat anti-orexin-B [1 : 250] (Santa Cruz Biotechnology), neurobiotin (Vector Laboratories), and rabbit anti-Kv4.3, anti-Kv4.2 or anti-Kv1.4 [1 : 500] (Alomone Laboratories). The corresponding secondary antibodies and labels were donkey anti-rabbit-Alexa-647 [1 : 250], donkey anti-goat-Alexa-488 [1 : 250], streptavidin-Cy3 [1 : 500] and donkey anti-rabbit-Alexa-647 [1 : 500] (Alexas from Molecular Probes, streptavidin from Amersham Biosciences). The specificity of the antibodies used has been established in previous studies (Liss *et al.*, 2001; Fadel *et al.*, 2002; Liu *et al.*, 2002). Labelled slices were analysed with a Bio-Rad Radiance 2000 confocal microscope. To ensure that there was no cross-talk between fluorescent signals, overlays of single-channel images were always used as controls for corresponding multichannel (simultaneous) images.

Results

Identification of orexin neurons in the mouse LH

Both orexin-A and orexin-B antibodies labelled the same population of LH neurons (Fig. 1A), confirming that orexin-A and orexin-B are coexpressed in mouse LH neurons. To analyse the biophysical properties of LH orexin neurons, we performed whole-cell recordings and infusion of neurobiotin, followed by double immunostaining for neurobiotin and orexin-A. LH orexin neurons were initially identified by their distinctive membrane potential dynamics (Eggermann *et al.*, 2003; Yamanaka *et al.*, 2003b): a lack of spike frequency adaptation during depolarizing pulses, an I_h -mediated depolarizing ‘sag’ upon hyperpolarization, and a low-threshold spike upon depolarization from hyperpolarized levels (Fig. 1B). Post-recording immunocytochemistry confirmed that 100% ($n = 34/34$) of LH neurons exhibiting this combination of properties expressed orexin-A (Fig. 1C). In all

subsequent electrophysiological experiments, LH orexin neurons were identified both by their electrical signature (Fig. 1B) and by subsequent immunocytochemical confirmation of orexin-A or orexin-B expression in neurobiotin/streptavidin-Cy3 labelled cells (Fig. 1C). The membrane capacitance of orexin neurons thus identified ($n = 34$) was 32 ± 2 pF (range 15–50 pF), the input resistance was 1.1 ± 1 G Ω (range 0.8–1.5 G Ω), and the lowest interspike potential (maximum after-spike hyperpolarization) was -43 ± 1 mV (range -38 to -54 mV). All orexin neurons studied were located in the LH area with Bregma coordinates between -1.3 mm and -1.8 mm, which agrees closely with the published distribution of orexin cells in the mouse hypothalamus (Yamanaka *et al.*, 2003a).

LH orexin neurons express a low-voltage-activated A-current and Kv4.3 subunits

In LH orexin neurons identified as described above, depolarizing voltage steps to -40 mV, from a holding potential of -90 mV, evoked transient outward currents with rapid activation and slower inactivation ($n = 34$, Fig. 2A). These properties are characteristic of A-currents (Connor & Stevens, 1971; Liss *et al.*, 2001). The identity of A-currents was confirmed using the A-channel blocker 4-AP (10 mM), which specifically eliminated the transient current ($n = 7$, Fig. 2A). The pore of the A-type channels can be formed by several α -subunits, including Kv4.3, Kv4.1 and Kv1.4 (Coetzee *et al.*, 1999). Double-label immunocytochemistry indicated that LH orexin neurons express Kv4.3 protein ($n = 50$, see Fig. 2B for a typical neuron); in contrast, we did not detect Kv4.1 or Kv1.4 protein in orexin cells ($n = 20$ for each antibody; data not shown).

To measure the voltage-dependence of A-current activation, we exploited the fact that the A-current is activated by depolarization from -90 mV, but not from -40 mV (Fig. 2D). Thus the A-current was isolated using a standard two-step voltage protocol (e.g. Yang *et al.*, 2001), which involved subtracting currents activated by depolarization from a holding potential of -40 mV from those elicited from a holding potential of -90 mV. The activation threshold of the A-current was between -60 and -50 mV, and activation was half-maximal at -20 ± 2 mV ($n = 6$, Fig. 2C). To determine the voltage-dependence of A-current inactivation, we measured peak A-currents evoked by steps to 0 mV from different holding potentials. Inactivation was half-maximal at -78 ± 2 mV and complete at potentials positive to -40 mV ($n = 6$, Fig. 2D). A two-pulse protocol with variable interpulse duration showed that the A-current recovered from inactivation with a time constant of 140 ± 20 ms ($n = 7$, Fig. 2E).

A-current shapes the responses of LH orexin neurons to current inputs

To explore the role of the A-current in shaping the firing dynamics of orexin neurons, we measured the A-current magnitude, the spontaneous firing rate, and the effect of current injection on firing frequency in the same cell. To quantify the A-current magnitude, we calculated the maximal A-current amplitude at $+30$ mV (where the current is fully activated, Fig. 2C), by isolating the A-current using the two-step/subtraction protocol described above. The maximal A-current amplitude was then converted to conductance (see Materials and methods), and divided by cell capacitance to correct for differences in cell size, giving the A-type conductance density (nS/pF). Firing rates were measured by counting the number of spikes during the last 10 s of 20 s-long current-clamp records; control measurements of

inter-spike interval duration showed that the firing rate was stable and regular during this time period.

Natural variation was observed in both the A-type conductance density (range 0.15–0.9 nS/pF, mean 0.5 ± 0.06 nS/pF, $n = 15$) and the spontaneous firing rate (range 3.6–12 Hz, mean 7 ± 0.7 Hz, $n = 19$) of orexin neurons. However, these parameters were not mutually correlated ($r = 0.002$, $P > 0.9$, slope = 0.02, $n = 10$), suggesting that the A-current does not influence the spontaneous firing rate of orexin cells under our experimental conditions. Consistent with this idea, blocking the A-current with 4-AP did not significantly alter the spontaneous firing rate of orexin cells (e.g. Fig. 3A; the firing rate in the presence of 10 mM 4-AP was $87 \pm 15\%$ of control, $P > 0.2$, $n = 5$).

We next mimicked the effect of endogenous inhibitory neuromodulators by injecting hyperpolarizing currents into orexin neurons. Upon termination of hyperpolarizing pulses, orexin neurons displayed a marked rebound hyperpolarization that caused them to resume firing with a prominent delay (Figs 1B and 3A). Application of 10 mM 4-AP reversibly eliminated this delay, reducing the latency to the first spike following a 1 s pulse to -80 mV from 533 ± 114 ms to 48 ± 9 ms ($P < 0.001$, $n = 5$, Fig. 3A).

The firing rates of orexin neurons during the hyperpolarizing current injections decreased linearly as the amplitude of the injected current was increased (Fig. 3B), but more steeply in cells with larger A-currents, than in those with smaller A-currents (Fig. 3B and C). The slope of the relationship between the injected current and the firing rate provides a quantitative measure of the sensitivity of cell firing to current injection. Plotting this gradient (sensitivity value, in Hz/pA/pF) against the A-type conductance density, for each neuron, revealed a significant positive linear correlation between the two parameters ($r = 0.91$, $P < 0.005$, slope = 11, $n = 7$, Fig. 3C). These data suggest that the A-current increases the sensitivity of orexin cell firing to current inputs. Consistent with this idea, 4-AP decreased the slope of current-firing relations in orexin cells (the slope in the presence of 10 mM 4-AP was $56 \pm 9\%$ of control, $P < 0.001$, $n = 3$, Fig. 3D).

Discussion

In this study, we confirm that orexin neurons of the mouse LH contain both orexin-A and orexin-B, and show that they express low-voltage-activated A-type currents and Kv4.3 subunits. Our results suggest that in LH orexin neurons the A-current does not have a major influence on the spontaneous firing rate, but enhances the ability of hyperpolarizing currents to inhibit cell firing by (i) delaying the recovery of cell firing from hyperpolarization, and (ii) increasing the slope of the relation between the firing frequency and injected current.

A positive linear correlation between A-current magnitude and spontaneous firing was measured experimentally in other neurons (Liss *et al.*, 2001), and is suggested by computational simulations (Rush & Rinzel, 1995). However, our measurements indicated that in LH orexin neurons there is no significant correlation between A-type conductance density and spontaneous firing rate. This is probably because A-current inactivation increases with membrane depolarization (Fig. 2D), and LH orexin neurons are intrinsically

depolarized (Eggermann *et al.*, 2003). Indeed, even in the most negative region of the interspike potential that we observed during spontaneous firing of orexin neurons (-38 to -54 mV), the A-current would be almost completely inactivated (Fig. 2D) and hence unlikely to affect the intrinsic firing rate.

In contrast to spontaneous firing, the recovery of firing after hyperpolarizing current pulses was dramatically slowed by the A-current (Fig. 3A). This suggests that the A-current may significantly affect the firing responses to pulsatile hyperpolarizing inputs, which in the case of orexin neurons could potentially originate from burst-firing noradrenergic and serotonergic cells (Tung *et al.*, 1989; Hajos & Sharp, 1996; Li *et al.*, 2002; Yamanaka *et al.*, 2003b).

Our measurements also revealed a positive correlation between the A-type conductance density and the gain (slope) of the relationship between the firing frequency and hyperpolarizing current in orexin cells (Fig. 3C). A possible explanation for this phenomenon is that hyperpolarizing inputs progressively remove A-current inactivation (Fig. 2D), allowing the A-current to decrease the firing rate. The extent of this decrease will be determined by the number of A-type channels expressed by the cell. Although mathematical modelling (Rush & Rinzel, 1995) implied that changes in A-current conductance may affect neuronal gain, to the best of our knowledge this has not been previously reported for living neurons.

The size of the A-current may therefore determine how sensitive the firing of orexin neurons is to inhibition by hyperpolarizing currents; doubling the A-type conductance density would double the gain of the firing-current response (Fig. 3C). This may have important physiological implications because several endogenous neuromodulators inhibit orexin neurons by inducing hyperpolarizing postsynaptic currents (Li *et al.*, 2002; Yamanaka *et al.*, 2003a,b). Changing the magnitude of the A-current in orexin neurons could thus control the ability of modulatory signals to influence the degree of arousal. We identified Kv4.3 subunit as a likely molecular component of the A-type channels, which could be taken advantage of in further pharmacological or genetic studies. Finally, it is noteworthy that in other neurons, A-current size can be altered both acutely, by certain neurotransmitters and neurotrophic factors (Yang *et al.*, 2001; Burdakov & Ashcroft, 2002), and chronically, by changes in transcription of genes encoding A-type channels (Liss *et al.*, 2001). Identification of an endogenous signal, possibly circadian-related or stress-related, which controls the size of the A-current in orexin neurons, thus merits further investigation.

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Abbreviations

A-current	A-type K ⁺ current
4-AP	4-aminopyridine
LH	lateral hypothalamus

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Fig. 1. Neurochemical and electrical properties of orexin neurons in the mouse LH.

(A) LH brain slice double-stained with orexin-A (orx-A, blue) and orexin-B (orx-B, green) antibodies. In simultaneous confocal imaging (orx-A/orx-B), neurons that contain both peptides appear in cyan. All neurons imaged contained both peptides. (B) Electrical properties of orexin cells: nonadapting firing response to depolarization (i), depolarizing sag in response to hyperpolarization (ii), and low-threshold spike on depolarization from a hyperpolarized potential (iii). The current-clamp protocol is shown under the trace. Action potentials are truncated at 0 mV. (C) Immunocytochemical identification of an LH neuron (arrow) filled with neurobiotin (red) and expressing orexin-A (orx-A, blue).

Fig. 2. Biophysical and molecular properties of A-currents in LH orexin neurons. Currents were recorded in 1 μ M TTX.

(A) Transient outward current elicited by a step to -40 mV from -90 mV is blocked by 10 mM 4-AP ($n = 7$). (B) Immunolabelling of an LH neuron for orexin-B (orx-B, green) and Kv4.3 (blue). Simultaneous confocal imaging (orx-B/Kv4.3) shows that these proteins are found in the same cell ($n = 50$). Scale bar, 5 μ m. (C) Left, A-currents evoked by steps to -60 mV (blue), -50 mV (green) and -40 mV (red), from -90 mV. Right, voltage-dependence of A-type conductance (g_A , see Materials and methods) expressed as a fraction of maximal conductance (g_{Amax}) ($n = 6$). The curve is the best fit of Eqn 1 (see Materials and methods)

to the data. (D) Voltage-dependence of the extent of A-current inactivation. Left, A-currents induced by a test pulse to 0 mV, after an 800 ms prepulse to -40 mV (red), -50 mV (green) and -60 mV (blue). Right, peak A-current (I_A) is expressed as a fraction of that elicited following a prepulse to -120 mV (I_{Amax}), and plotted against the prepulse potential ($n = 6$). The curve is the best fit of Eqn 2 (see Materials and methods) to the data. (E) Time-dependence of I_A recovery from inactivation. Left, the neuron was held at -40 mV for 1 s to inactivate I_A , then stepped to -90 mV for varying durations to remove inactivation, following which I_A was elicited by a test pulse to -40 mV. The protocol is illustrated above the data traces. Right, mean peak I_A ($n = 7$) during the test pulse plotted against the duration of the de-inactivating -90 mV interpulse. The curve is the best fit of a single exponential function with a time constant of 140 ± 20 ms.

Fig. 3. Effects of A-currents on the firing dynamics of LH orexin neurons.

(A) Rebound hyperpolarization (arrowed) on recovery from hyperpolarizing current pulses is reversibly abolished by 10 mM 4-AP ($n = 5$). (B) Firing responses to sustained hyperpolarizing current injections of two neurons, one with a large (blue) and one with a small (red) A-current (A-currents elicited as in Fig. 2A). The magnitudes of A-currents and the injected current were divided by cell capacitance (pF) to correct for differences in cell size. Action potentials are truncated at 0 mV. (C) Summary of the data shown in B, for seven orexin cells. There is a strong positive correlation ($r = 0.91$, $P < 0.005$, slope = 11, $n = 7$)

between A-type conductance density and the sensitivity of firing to current injection (slope of the firing-current relations). (D) Blocking the A-current with 4-AP (10 mM) decreases the slope of the firing-current relation in an orexin neuron ($n = 3$).