PRESYNAPTIC INHIBITION OF GABAERIC SYNAPTIC TRANSMISSION BY ADENOSINE IN MOUSE HYPOTHALAMIC HYPOCRETIN NEURONS

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Abstract—Hypocretin neurons in the lateral hypothalamus, a new wakefulness-promoting center, have been recently regarded as an important target involved in endogenous adenosine—regulating sleep homeostasis. The GABAergic synaptic transmissions of these neurons play an important role in the regulation of excitability of these neurons. The inhibitory effect of adenosine, a homeostatic sleep-promoting factor, on the excitatory glutamatergic synaptic transmissions in hypocretin neurons has been well documented, whether adenosine also modulates these inhibitory GABAergic synaptic transmissions in these neurons has not been investigated. In this study, the effect of adenosine on inhibitory postsynaptic currents (IPSCs) in hypocretin neurons was examined by using perforated patch-clamp recordings in the acute hypothalamic slices. The findings demonstrated that adenosine suppressed the amplitude of evoked IPSCs in a dose-dependent manner, which was completely abolished by 8-cyclopentyltheophylline (CPT), a selective antagonist of adenosine A1 receptor but not adenosine A2 receptor antagonist 3,7-dimethyl-1-(2-propynyl) xanthine. A presynaptic origin was suggested as following: adenosine increased paired-pulse ratio as well as reduced GABAergic miniature IPSC amplitude without affecting the miniature IPSC amplitude. Further findings demonstrated that when the frequency of electrical stimulation was raised to 10 Hz, but not 1 Hz, a time-dependent depression of evoked IPSC amplitude was detected in hypocretin neurons, which could be partially blocked by CPT. However, under a higher frequency at 100 Hz stimulation, CPT had no action on the depressed GABAergic synaptic transmission induced by such tetanic stimulation in these hypocretin neurons. These results suggest that endogenous adenosine generated under certain stronger activities of synaptic transmissions exerts an inhibitory effect on GABAergic synaptic transmission in hypocretin neurons by activation of presynaptic adenosine A1 receptors, which may finely regulate the excitability of these neurons as well as eventually modulate the sleep–wakefulness. © 2011 IBRO. Published by Elsevier Ltd. All rights reserved.

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It is well known that in the CNS, adenosine, a product of cell energy metabolism, plays an important role in promoting sleep by inhibition of waking-active neurons in basal forebrain (Rainnie et al., 1994; Arrigoni et al., 2006) or excitation of sleep-active neurons in ventrolateral preoptic nucleus (Gallop et al., 2005). Recently, abundant evidence has strongly suggested that hypocretin neurons in the lateral hypothalamus, a new wakefulness-promoting center (Adamantidis et al., 2007; Sakurai, 2007; Chen et al., 2008; Li et al., 2010a), may be another important target involved in the hypnogenic action of adenosine in the CNS. In vivo, local perfusion of adenosine receptor agonist in the lateral hypothalamic elicits sleep (Alam et al., 2009), whereas microinjection of adenosine receptor-specific antagonist in the same area can induce increase of wakefulness (Thakkar et al., 2008; Alam et al., 2009).

By activation of adenosine A1 and A2 receptors (Basheer et al., 2004), adenosine leads to directly postsynaptic hyperpolarization in waking-active neurons or depolarization in sleep-active neurons (Rainnie et al., 1994; Gallop et al., 2005; Arrigoni et al., 2006), but more importantly, it inhibits the presynaptic release of excitatory and inhibitory neurotransmitters including glutamate and GABA in these brain regions (Arrigoni et al., 2001; Moraity et al., 2004). In vitro, an electrophysiological study by Liu and Gao has shown that adenosine significantly attenuates the frequency of action potentials in hypocretin neurons without a change in membrane potentials, while a depression of excitatory synaptic transmission to these neurons by activating presynaptic A1 receptors is thought to be a cellular mechanism for this inhibition (Liu and Gao, 2007).

In addition to excitatory glutamatergic synaptic transmissions, inhibitory GABAergic synaptic transmissions are also abundant in the hypothalamus and are the main inhibitory afferents to hypocretin neurons (Li et al., 2002; Sakurai et al., 2005; Henny and Jones, 2006; Kokare et al., 2006). The application of GABA, selective agonist muscimol or GABA into hypothalamic slices produces a hyperpolarization and a decrease of the firing rates in hypocretin neurons.
neurons (Li et al., 2002; Eggermann et al., 2003; Xie et al., 2006). Importantly, local microdialysis of GABA<sub>A</sub> receptor antagonist into lateral hypothalamus will activate hypocretin neurons and lead to the suppression of sleep and induction of arousal (Alam et al., 2005, 2010), suggesting that the GABAergic system within lateral hypothalamus plays an important role in the regulation of sleep. It has been well documented that adenosine could regulate the GABAergic synaptic transmission in several areas in hypothalamus, such as supraoptic nucleus (Oliet and Pouthain, 1999), paraventricular nucleus (Li et al., 2010b; Han et al., 2011), and histaminergic tuberomammillary nucleus (Yum et al., 2008), an important subcortical arousal system. Whether adenosine modulates the inhibitory GABAergic transmission in hypothalamic hypocretin neurons is still unknown.

In the present study, therefore, a possible role for adenosine in regulating the inhibitory synaptic transmission in hypocretin neurons was investigated by performing perforated patch-clamp recordings in hypothalamic slices from hypocretin-enhanced green fluorescent protein (EGFP) transgenic mouse.

**EXPERIMENTAL PROCEDURES**

**Hypothalamic slice preparation**

Transgenic mice (kindly provided by Dr. T. Sakurai, Kanazawa University, Ishikawa, Japan) expressing EGFP under control of the hypocretin promoter (Li et al., 2002; Yamanaka et al., 2003) were used in this study. The animals use and methods were carried out in according with the guidelines of the Shanghai Institutes for Biological Sciences Animal Research Advisory Committee (Shanghai, China). In brief, both male and female transgenic mice (21–28 day old) were killed by decapitation after sodium pentobarbital (1%) anesthesia, and the brain was quickly removed and placed in ice-cold oxygenated (95% O<sub>2</sub> and 5% CO<sub>2</sub>) solution containing (in mM) 220 sucrose, 2.5 KCl, 6 MgCl<sub>2</sub>, 1 CaCl<sub>2</sub>, 1.25 Na<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, and 10 glucose, pH 7.3–7.4 with NaOH. Coronal slices (250 μm) of the hypothalamus containing hypocretin neurons were cut with a Microm HM 650 V vibratome (Thermo Scientific Richard-Allan, Microm, Heidelberg, Germany) and then transferred to an incubating chamber at room temperature in continuously oxygenated artificial cerebral spinal fluid (ACSF) for ~1 h before use. The composition of the ACSF was (in mM): 125 NaCl, 2.5 KCl, 1.3 Na<sub>2</sub>HPO<sub>4</sub>, 25 NaHCO<sub>3</sub>, 1.3 MgCl<sub>2</sub>, 2 CaCl<sub>2</sub>, and 10 glucose saturated with 95% O<sub>2</sub> and 5% CO<sub>2</sub>.

**Perforated patch-clamp recordings**

A hypothalamic slice was transferred to a recording chamber where it was submerged and continuously superfused with 32 °C ACSF at a flow rate of 2 ml/min. Recordings were obtained with perforated patch-clamp configuration using amphotericin B (250 μg ml<sup>−1</sup>) as the pore-forming agent (Oyamada et al., 1998) dissolved in pipette solutions before each experiment. Hypocretin neurons were visually identified using a fluorescence microscope (DM LFS, Leica, Wetzlar, Germany) fitted with infrared differential interference contrast (IR-DIC) optics. Patch-clamp recording pipettes (3–5 MΩ) were filled with a solution containing (in mM) 135 KCl, 10 4-(2-Hydroxyethyl)piperazine-1-ethanesulfonic acid (HEPES), 0.2 ethylene glycol-bis-(2-aminoethyl)-N,N′,N′,N‘-tetraacetic acid (EGTA), 0.4 Na-ATP, 0.3 Na<sub>2</sub>-GTP, and 4 Na<sub>2</sub>-phosphoethrin. Data acquisition and analysis were conducted with Axon Multi 700A amplifier and Clampex 9.0 software (Molecular Devices, Sunnyvale, CA, USA). Series resistance (<50 MΩ) was monitored online at regular intervals throughout the course of the experiments with voltage pulses (~10 mV), and cells were excluded from data analysis if a >20% change occurred during the course of the experiment.

For presynaptic stimulation, either a bipolar stainless steel or a glass-stimulating electrode was placed on the surface of the slice close to the dorsolateral border of the ipsilateral hypothalamus (Wollmann et al., 2005). An isolated constant-current source, Master-8 (A.M.P.I., Jerusalem, Israel), was used to generate square wave pulses of direct current (40–100 μA; 100–200 μs duration). The stimulation trigger was controlled by Clampex 9.0 software. For recording of inhibitory postsynaptic currents (IPSCs), hypocretin neurons were voltage clamped at −70 mV. IPSCs were induced at a frequency of 0.033 Hz and represented as averages of 10 current traces, unless otherwise stated.

**Drugs**

Drugs used were adenosine, 6,7-dinitroquinoxaline-2,3(1H, 4H)-dione (DNONX), α,β-2-amino-5-phosphonoventeic acid (APV), 8-cyclopentylthylepheline (CPT), and 3,7-dimethyl-1-(2-propynyl) xanthine (DMPX) from Sigma (St. Louis, MO, USA). Amphetamine B was purchased from Calbiochem (Novabiochem, La Jolla, CA, USA), bicuculline methiodide (bicuculline) from BIOMOL International L.P. (Plymouth Meeting, PA, USA), and CGP 55845 hydrochloride (CGP 55845) from Tocris Bioscience (Bristol, UK). Tetrodotoxin (TTX) was obtained from Hebei Fisheries Research Institute (Qinghuangdao, China).

**Statistical analysis**

Results are presented as mean±SEM. The data for amplitude of inhibitory synaptic currents were normalized relative to baseline. Significance was determined using Student’s t-test and nonparametric Kolmogorov–Smirnov test (K–S test). For all tests, P<0.05 was considered statistically significant.

**RESULTS**

Adenosine suppresses evoked inhibitory synaptic transmissions in hypocretin neurons

Green fluorescent neurons, in the hypothalamic slices from hypocretin-EGFP transgenic mice, were identified under fluorescence microscope (Fig. 1A, left panel) and subsequently subjected to perforated patch-clamp recording (Fig. 1A, right panel). The basic membrane properties of hypocretin neurons in our experiment were not different from those described in previous reports (Li et al., 2002; Yamanaka et al., 2003; Muraki et al., 2004). As seen in Fig. 1B (top panel), hyperpolarization-activated currents could be induced after currents (from ~100 pA, 800 ms, 40 pA increment) injected into a hypocretin neuron. The lower panel in Fig. 1B showed that little spike frequency adaptation was seen in response to a higher depolarizing current (200 pA) in a hypocretin neuron. Electrical stimulation of the dorsolateral border of the lateral hypothalamus induced reproducible excitatory postsynaptic currents (EPSCs) in hypocretin neurons recorded in the presence of GABA<sub>A</sub> receptor antagonist bicuculline (10 μM). Application of specific α-amino-3-hydroxy-5-methyl-4-isoxazole-propionate (AMPA) receptor antagonist DNQX (10 μM) completely blocked EPSCs in all neurons tested.
indicating that glutamatergic and GABAergic transmissions were the primary synaptic inputs to hypocretin neurons. This was consistent with what was previously reported (Li et al., 2002). In the presence of AMPA receptor antagonist DNQX (10 μM), local stimulation-induced inward IPSCs were recorded (Fig. 1C, lower panel) in hypocretin neurons for the chloride-based intracellular solution used in our experiments. And the evoked IPSCs had a mean amplitude of 372.39 ± 29.91 pA (range: -112.78 pA to -759.02 pA, n=40) and were sensitive to the GABAA receptor antagonist bicuculline (10 μM), suggesting mediation by GABAA receptor activation. As illustrated in Fig. 2A, when adenosine (100 μM) was added to the bathing solution, the amplitude of GABAergic IPSCs was reduced in a hypocretin neuron and recovered after washout with normal ACSF. On average, the normalized amplitude of IPSCs was 51.42 ± 5.59% of control at 20 min after superfusion with 100 μM adenosine in all neurons tested (n=14, P<0.05) and 92.12 ± 12.17% of control at 25 min after its withdrawal (P>0.05) (Fig. 2B). The use of varying concentrations of adenosine allowed us to examine whether the inhibitory action of adenosine on IPSCs in hypocretin neurons was in a concentration-dependent manner. As shown in Fig. 2C, in the presence of 50 μM adenosine, the normalized amplitude of IPSCs was significantly reduced to 61.58 ± 7.52% of control at 20 min after application of drugs (n=7, P<0.05). The normalized amplitude of IPSCs was 75.11 ± 5.27% of control at 20 min after application of 10 μM adenosine (n=9, P<0.05). The depression in amplitude of IPSCs was less detectable after application of 1 μM adenosine (93.02 ± 7.50% of control) (n=6, P>0.05).
Adenosine A1 receptor is responsible for the effect of adenosine on GABAergic synaptic transmission

Both adenosine A1 and A2 receptors are previously implicated in the modulation of synaptic transmission (Basheer et al., 2004). Consistent with a previous study (Liu and Gao, 2007), our recent experiment has demonstrated that adenosine suppresses the activity of glutamatergic synaptic transmission in hypocretin neurons through activation of adenosine A1 receptors (Xia et al., 2009).

To clarify the nature of the adenosine receptor subtypes in mediating adenosine-induced inhibitory action on GABA transmission, the effects of the selective A1 and A2 receptor antagonists were investigated. Hypocretin neurons were superfused with external solution containing each adenosine antagonist for 10 min before simultaneous application of 50 µM adenosine. As illustrated in Fig. 3A, in the presence of adenosine A1 receptor antagonist CPT (200 nM), no inhibition on amplitude of IPSCs was observed after application of 50 µM adenosine. On average, in the presence of 200 nM CPT, the normalized amplitude of IPSCs was 98.41 ± 5.06% of control at 20 min after administration adenosine (50 µM) (n=9, P<0.05) (Fig. 3B). It was also noted that bath application of CPT alone did not affect the amplitude of IPSCs in this condition (100.05 ± 3.95% of control at 10 min after application of CPT, P>0.05). The possible involvement of adenosine A2 receptor in adenosine-induced response was then examined with adenosine A2 antagonist DMPX. Application of DMPX (10 µM) did neither alter the amplitude of IPSCs nor affect the inhibitory action of adenosine (50 µM) on the evoked IPSCs in a hypocretin neuron (Fig. 3C). Pooled data demonstrated

Fig. 2. Effect of adenosine on GABAergic IPSCs in hypocretin neurons. (A) Sample traces show the average of 10 consecutive sweeps of evoked IPSCs before and during application of 100 µM adenosine and washout in the same hypocretin neuron. Low panel shows the time course of the IPSC amplitude during the application of adenosine (100 µM). (B) Pooled data show the changes of evoked IPSC amplitude after application of 100 µM adenosine and its washout. (C) Bar histogram shows the group data of the concentration-dependent effect of adenosine on the amplitude of evoked IPSCs in hypocretin neurons. Each point shows the response of 6–14 neurons as indicated. * P<0.05 vs. control. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
that the normalized amplitude of IPSCs was 99.35±6.10% of control at 10 min after perfusion of DMPX (10 μM) alone (n=6, P>0.05), whereas it reduced to 59.19±4.71% of control at 20 min after administration of adenosine (50 μM) (P<0.05) (Fig. 3D).

**Adenosine reduces inhibitory synaptic transmission at presynaptic terminals**

To investigate the location of adenosine receptors, facilitation of GABA release elicited was analyzed when two inhibitory synaptic responses were evoked with a 100-ms interval. After administration of 50 μM adenosine for 20 min, the average paired-pulse facilitation ratio (PPR) was increased from 1.09±0.07 to 1.76±0.12 (n=9; P<0.05) (Fig. 4A), suggesting that the inhibition of IPSCs following activation of A1 receptor with adenosine had a presynaptic origin.

The presynaptic action of adenosine on GABAergic synapses was further examined by analysis of spontaneous miniature IPSCs (mIPSCs) recorded in the presence of TTX (0.5 μM) and DNQX (10 μM). The frequency of mIPSCs ranged between 0.27 and 0.90 Hz under control conditions (n=13), whereas the mean amplitude varied between 25.64 and 174.28 pA (114–334 events for each
Fig. 4. Involvement of presynaptic adenosine A1 receptors in adenosine-induced depression on IPSCs in hypocretin neurons. (A) Traces showing paired-pulse recording of IPSCs in a hypocretin neuron before and during application of 50 μM adenosine (up panel). Pooled data show the effect of adenosine on the PPR of IPSCs in hypocretin neurons (low panel). (B) Sample traces of mIPSCs before and during application of adenosine (50 μM) and washout in a hypocretin neuron. (C) and (E) show the changes of cumulative frequency in interevent interval and amplitude of mIPSCs after application of adenosine in the same hypocretin neuron. Effects of adenosine on the interevent interval (D) and amplitude (F) of mIPSCs in hypocretin neurons recorded. ** P<0.01 vs. control. For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.
Representative traces of a typical experiment are shown in Fig. 4B. When adenosine (50 μM) was added to the bathing solution, the spontaneous mIPSC activity was reduced in all neurons recorded \((n=10)\). The interevent interval and amplitude distributions of mIPSCs were then examined. As illustrated in Fig. 4C, application of adenosine (50 μM) did affect the cumulative interevent interval distribution of mIPSCs, producing a rightward shift of the curve \((P<0.05, \text{K–S test})\). Pooled data from 10 recorded neurons were represented in Fig. 4D, which demonstrated that perfusion of 50 μM adenosine for 20 min significantly increased mean mIPSCs interevent interval, reflecting a decrease in mIPSC frequency \((n=10, P<0.01)\). In contrast, Fig. 4E showed that the cumulative distribution of mIPSC amplitude was not changed after application of 50 μM adenosine \((P>0.05, \text{K–S test})\). No significant difference of amplitude distribution in mIPSCs was found during the course of the experiments \((n=10, P>0.05)\) (Fig. 4F).

**Inhibitory effect of endogenous adenosine on GABAergic synaptic transmission is detected at 10 Hz but not 100 Hz stimulation**

Our previous results have demonstrated that endogenous release of adenosine can be induced by sustained stimulation of synaptic inputs at high-frequency stimulus in the hypothalamic slices, which exerts an inhibitory effect on the excitatory glutamatergic synaptic transmission (Xia et al., 2009). Similarly, to test the possibility that inhibitory synaptic transmission to hypocretin neurons could also be depressed by endogenous adenosine under tetanic synaptic afferents, the effects of adenosine A1 receptor antagonist CPT on the GABAergic IPSCs were measured by using different frequencies stimulation higher than 0.033 Hz. APV (50 μM) and CGP 55845 (0.5 μM) were added to the external solution to prevent the possibility of NMDA receptor-dependent plasticity and GABA_B receptor-mediated depression in these experiments.

As presented in Fig. 5A, when the stimulation was applied at 1 Hz, the amplitude of IPSCs did not show any change during the stimulation period of 240 s, and the mean amplitude measured at the end of stimulation was 99.63±6.24% \((n=9)\) of that measured at the beginning of stimulation as a control. In the presence of CPT (200 nM), the mean amplitude measured at the end of 1 Hz stimulation was 99.99±3.52% of control \((n=9)\), which was not significantly different from that measured in the same cells without CPT administration \((P>0.05)\). When the frequency of stimulation was raised to 10 Hz for 120 s, the amplitude of IPSCs in hypocretin neurons immediately increased at the start of stimulation and then reduced slowly (Fig. 5B).

**Fig. 5.** Effects of adenosine receptor antagonists on 1, 10, and 100 Hz stimulation-evoked IPSCs in hypocretin neurons. Time course of the average changes in normalized amplitude of IPSCs at 1 Hz (A) or 10 Hz (B) stimulation before and after application of CPT (200 nM) or DMPX (10 μM) (C) in the same neurons. Inset, average of consecutive IPSCs during the first 5 or 1 s (a, black) and the last 5 or 1 s (b, gray) within 240 or 120 s stimulation in control and 15–25 min after application of CPT or DMPX in the same cells, respectively. (D) Sample traces showing the changes of evoked IPSC amplitude at 100 Hz stimulation with (low) or without 200 nM CPT (top). Low panel shows the time course of the average changes in normalized IPSC amplitude at 100 Hz stimulation before and after application of CPT.
The mean amplitude measured at the end of 10 Hz stimulation was 77.38 ± 5.08% of control (n = 10). After allowing the synaptic currents to almost return to their initial values (15–25 min), CPT (200 nM) was added to the bath solution. The depressed synaptic response to 10 Hz stimulation in the same cells began to be partially blocked by CPT after 45 s of stimulation (n = 10, P < 0.05), and the mean amplitude measured at the end of stimulation was 96.38 ± 4.41% of control, which was significantly different from the amplitude of IPSCs recorded in the same neurons without CPT administration. However, there was no reliable change in the 10 Hz-induced depression of IPSC amplitude in the presence of the selective A2 receptor antagonist DMPX (10 µM) (Fig. 5C). The mean amplitude of IPSCs measured at the end of 10 Hz stimulation was 79.17 ± 2.56% of control, which was not significantly different from that measured in the same cells without DMPX administration (81.80 ± 1.81% of control, n = 11) (P > 0.05).

Next, we further tested the action of endogenous release of adenosine under higher frequency stimulation on GABAergic transmission, as endogenous adenosine released under 100 Hz stimulation plays an important role in regulating induction of LTP in excitatory glutamatergic synaptic transmission on hypocretin neurons (Xia et al., 2009). Similar to the 10 Hz stimulation-induced depression of synaptic response above, the amplitude of IPSCs in hypocretin neurons initially increased as soon as the start of 100 Hz stimulation, and then a time-dependent decrease of amplitude within the course of a 1-s train was really recorded (Fig. 5D). The mean amplitude of IPSCs measured at the end of 100 Hz stimulation was 36.59 ± 13.48% of control (n = 11). In contrast, no obvious alternation of depressed IPSC amplitude was detected in the presence of CPT (200 nM) in control slices (n = 10, P > 0.05).

**DISCUSSION**

The current results provide an additional example demonstrating that functional adenosine A1 receptors are present on GABAergic nerve terminals projecting to hypothalamic hypocretin neurons, and their activation decreases the probability of presynaptic GABA release. Furthermore, under different frequencies stimulation used in our study, the action of endogenous adenosine on these inhibitory synaptic transmissions can be observed at 10 Hz but not 100 Hz stimulation.

**Exogenous adenosine inhibits the GABAergic synaptic transmission to hypocretin neurons through activation of presynaptic adenosine A1 receptors**

A growing body of evidence has shown that adenosine, as a neuromodulator in CNS, may modulate excitatory glutamatergic synaptic transmission (Lambert and Teyler, 1991; Hasuo et al., 1992), inhibitory GABAergic synaptic transmission (Chen and van den Pol, 1997; Heinbockel and Pape, 1999; Yum et al., 2008), or both in several brain regions, such as nucleus accumbens (Uchimura and North, 1991), striatum (Kirk and Richardson, 1994), thalamus (Ulrich and Huguenard, 1995), laterodorsal tegmentum (Amigoni et al., 2001), and cerebellar granule cells (Takahashi et al., 1995; Courjaret et al., 2009). Actually, earlier studies have demonstrated that adenosine exerts an inhibitory effect on glutamatergic transmission in hypocretin neurons (Liu and Gao, 2007; Xia et al., 2009). In this study, we further find that exogenous application of adenosine produces a reversible inhibition of GABAergic synaptic transmissions in these neurons in a concentration-dependent manner. These results are in accordance with the findings observed in hypothalamic supraopticus nucleus (Oliet and Poullain, 1999) and paraventricular nucleus (Li et al., 2010b; Han et al., 2011), where adenosine inhibits both glutamatergic and GABAergic neurotransmission. Also, our previous experiment with entorhinal cortex has presented that exogenous adenosine exerts an inhibitory effect on not only glutamatergic but also GABAergic synaptic transmission (Li et al., 2011). Thus, these findings suggest that, adenosine, as a neuromodulator and ubiquitous in the mammalian CNS, may exert a substantially widespread suppression on both inhibitory and excitatory neurotransmission in most brain areas including hypothalamic nucleus.

Similar to glutamatergic transmission in hypocretin neurons, the inhibition of GABAergic transmission to hypocretin neurons by adenosine is mediated by adenosine A1 receptors because this depression is blocked by the selective A1 but not A2 receptor antagonist. Several lines of evidence support the conclusion that activation of adenosine A1 receptors has a presynaptic origin because of a reduced probability of presynaptic transmitter release rather than a decrease in postsynaptic receptor sensitivity, as evidenced by increased PPR and reduction of mIPSCs frequency without altering their amplitude distribution. This is in contrast to the results performed in other brain areas (Kirk and Richardson, 1994), such as in striatum, which shows that adenosine inhibits GABA release through activation of A2a receptors, as well as the findings observed in septal nucleus (Hasuo et al., 1992), where adenosine suppresses the GABAergic transmission via not only presynaptic but also postsynaptic A1 receptors.

**Inhibitory action of endogenous adenosine induced by low-frequency but not high-frequency stimulation is observed on GABAergic synaptic transmissions in hypocretin neurons**

Until now, there have been only a few reports describing endogenous release of adenosine-regulating GABAergic synaptic transmission in the CNS, such as in supraopticus nucleus (Oliet and Poullain, 1999), a depression at GABAergic synaptic transmission is observed at 1 Hz, which is completely antagonized by adenosine A1 receptor antagonist. In this study, our findings demonstrate that at 1 Hz stimulation, no effect of endogenous adenosine is observed in hypocretin neurons recorded from acute hypothalamic slices, as shown that evoked IPSCs are not changed after application of adenosine A1 receptor antagonist. Compelling evidence suggests that endogenous adenosine is released as a result of increased metabolic activity of cells in brain slices (Dun-
rons themselves. Furthermore, the release of ATP from astrocytes, followed by rapid degrading to extracellular adenosine (Zhang et al., 2003), will be decreased after blockade of glutamatergic transmission with glutamate receptor antagonists in our experiment. Although the origin of endogenous adenosine in the lateral hypothalamus is less unknown, the role of adenosine from hypocretin neurons or astrocytes ATP in formation of extracellular adenosine could not be ignored. Thus, compared with the release of adenosine in the existence of the large afferents of excitatory glutamatergic transmission, it seems that the amount of endogenous release of adenosine without functional glutamatergic transmission in hypocretin neurons will be much less in high frequency of 100 Hz stimulation at GABAergic transmission in our study. Also, we cannot rule out the other possibility that A2a receptor may play a role in the regulation of GABAergic transmission at high-frequency stimulation, as the study of hippocampal glutamatergic synapses has revealed a more predominant A2a receptor than A1 receptor in control of synaptic efficiencies at such high-frequency stimulation (Lopes et al., 2002; Rebola et al., 2008; Costenla et al., 2011). The precise mechanisms for these discrepancies still need further investigation.

Based on these findings, we can speculate that endogenous adenosine, accumulated as increased activity of hypocretin neurons and glutamatergic transmission in wakefulness, would exert an inhibitory effect on these neurons and promote sleep eventually, while in sleep the extracellular release of adenosine will be decreased due to the large of inhibitory afferents and lacking of the excitatory transmission, which rarely affect the inhibitory action of GABAergic transmission in hypocretin neurons. This speculation is in line with the changes of hypocretin neurons firing fast during waking and ceasing in sleep (Alam et al., 2002), as well as the net inhibitory effect and sleep-promoting action of endogenous adenosine on hypocretin neurons observed in vivo behavioral study (Thakkar et al., 2008; Alam et al., 2009). On the other hand, the limit of GABAergic activity by adenosine would prevent too strong an inhibition of hypocretin neurons and maintain a minimal level of hypocretin peptide—released activity, which would eventually promote wakefulness. Therefore, it can be proposed that, at least in part, presynaptic adenosine A1 receptors expressed on GABAergic nerve terminals might play a role in preventing hypocretin neurons from being overinhibited. But how and when the adenosine finely modulates the GABAergic transmission in hypocretin neurons in vivo is still unknown, which need more experiments to be performed.

In conclusion, functional adenosine A1 receptors expressed on GABAergic nerve terminals projecting to hypocretin neurons may play a role in the fine modulation of the excitability of the hypocretin neurons as well as the regulation of sleep-wakefulness.

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