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Interaction of the hypocretins with neurotransmitters in the nucleus accumbens

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Abstract

The hypocretins (hcrt1 and hcrt2), also known as orexins, are two neuropeptides derived from the same precursor, expressed in a few thousand cells in the lateral hypothalamus. Hypocretin-containing cells project throughout the brain, including ascending projections to the olfactory bulb and cerebral cortex, through the medial septum and the nucleus accumbens. Here, we have studied the interactions of the hypocretins with different neurotransmitters by patch clamp recording of acutely dissociated cells from the nucleus accumbens. Application of hcrt1 or hcrt2 decreased postsynaptic NMDA currents, enhanced GABA currents but did not affect glycine-activated conductances. Our results strongly suggest that the hypocretin peptides may be inhibitory peptides, probably via binding hcrt receptor 2. © 2002 Elsevier Science B.V. All rights reserved.

Keywords: Orexins; Sleep; GABA; Glycine; Patch clamp; NMDA

1. Introduction

The hypocretins (hcrts), also known as orexins, are two neuropeptides derived from the same precursor, synthesized in the lateral hypothalamic area [1,2]. Hypocretin-containing neurons project widely to numerous brain regions including cortex, thalamus, hypothalamus, brainstem, accumbens and spinal cord [3–5]. This diffuse pattern of efferent fibers suggests that the hypocretinergic system is involved in the regulation of multiple physiological functions, including energy homeostasis, neuroendocrine and autonomic function, as well as sleep and wakefulness (reviewed in Refs. [6,7]).

The hypocretins bind to two G-protein-coupled receptors, hcrtr1 and hcrt2, with different affinities. In particular, hcrtr2 binds hcrt1 and hcrt2 with similar affinities in the nanomolar range, whereas hcrtr1 exhibits 30–100 times more affinity for hcrt1 than hcrt2 [2]. Evidence from hypothalamic neurons and from cell lines transfected with the receptors suggest that the hcrtr1 is coupled to Gq and its activation increases the intracellular concentration of Ca^{2+}, whereas hcrtr2 may couple to Gi/o, and/or Gqi [2].

Recent genetic, molecular and anatomical studies have linked dysfunction of the hypocretinergic system to narcolepsy, a sleep disorder characterized by irresistible sleep attacks and cataplexy. In particular, Chemelli et al. [8] described narcolepsy-like attacks in mice deficient in prepro hypocretin, and Lin et al. [9] demonstrated that a mutation in hypocretin receptor 2 is the genetic cause of canine narcolepsy. Moreover, narcoleptic patients have reduced the number of hypocretin-expressing neurons, as shown by in situ hybridization [10], immunohistochemistry [11] and radioimmunoassay of cerebrospinal fluid [12]. Based on these data, it has been suggested that the hypocretinergic system is involved in the regulation of sleep and wakefulness and in the pathophysiology of narcolepsy. However, the mechanism of action of the hypocretins, the role of several hypocretin projection areas on sleep, and the neuronal circuits involved in cataplexy are still unknown.

The nucleus accumbens (NAcc) receives dopaminergic input from the ventral tegmental area and hypocretin-containing terminals from the lateral hypothalamus. Furthermore, NAcc expresses high levels of hcrt receptor 2 [13,14]. To investigate the role of the NAcc in the hypocretinergic system, we have analyzed the effect of the hypocretins on synaptic transmitters of the NAcc. Our results show that the

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hypocretins decrease the amplitude of NMDA currents and increase GABAergic conductances in NAcc neurons. These data suggest that the hcrts can be multifaceted peptides, with consequences for the mechanism of action of the peptides and their role in sleep regulation.

2. Materials and methods

2.1. Animals, slice preparation and experimental solutions

We used male Sprague–Dawley rats (100–160 g) to prepare NAcc slices. The rats were anesthetized with 4% halothane, decapitated and the brains rapidly transferred into a cold (4 °C) oxygenated, low-calcium HEPES-buffered salt solution (in mM): 234 sucrose, 2.5 KCl, 2 NaH_2PO_4, 11 glucose, 4 $MgSO_4$, 2 $CaCl_2$, 1.5 HEPES. We glued a tissue block containing NAcc to a teflon chuck and cut it transversally with a Vibroslicer (Campden Instrument). Then, we incubated the slices (400 μM thick) for up to 6 h at room temperature (20–22 °C) in a gassed (95% O_2 and 5% CO_2) $NaHCO_3$-buffered saline solution (mM): 116.4 NaCl, 1.8 $CaCl_2$, 0.4 $MgSO_4$, 5.36 KCl, 0.89 NaH_2PO_4, 5.5 glucose, 24 $NaHCO_3$, 100 glutathione, 1 nitro-arginine, 1 kynurenic acid. pH was 7.35 adjusted with NaOH, 300–305 mosM/l. After 1 h of incubation, we dissected out the region of the NAcc with the aid of a dissecting microscope. We incubated the tissue for 25 min in an oxygenated (100% O_2 with constant stirring) HEPES-buffered solution in the inner chamber of a Cell-Stirr flask (Wheaton, Millville, NJ) containing papain (1 mg/ml) and in (mM): 136 NaCl, 0.44 KH_2PO_4, 2.2 KCl, 0.35 NaH_2PO_4, 5.5 glucose, 10 HEPES, 100 glutathione, 1 nitro-arginine, 1 kynurenic acid and 1 pyruvic acid (pH = 7.35 with NaOH, 300–305 mosM/l). The temperature of this solution was kept constant (36 °C) by a circulating water bath in the outer chamber of the flask.

After enzymatic digestion, we transferred the tissue into a centrifuge tube and rinsed it 3–4 times with a Na^+-isethionate solution. We then filled the tube with 5 ml of Na^+-isethionate solution and after 10 min, triturated the tissue using fire-polished Pasteur pipettes with successively smaller tip diameters. We plated the supernatant onto a 35-mm Petri dish placed on the stage of the inverted microscope. The cells were allowed to attach to the dish for 10 min before replacing the Na^+-isethionate solution with normal external solution at a rate of 1.5 ml/min. This solution was composed of (in mM): 142 NaCl, 2 KCl, 1 $CaCl_2$, 23 glucose, 15 HEPES, 10 glucose (pH = 7.35 with NaOH, osmolarity of 300 mosM/l).

2.2. Whole-cell recordings

We used standard whole-cell recording methods [15]. Briefly, patch electrodes were pulled from borosilicate capillary glass (Sutter instrument, CA) on a Brown-Flaming puller (Sutter Instruments, CA) to a final resistance of 1.8–2.2 MΩ. We filled the electrodes with a solution that consisted of (in mM): 120 CsF, 10 CsCl, 11 EGTA, 10 HEPES, 0.5 $CaCl_2$ (pH = 7.35 with CsOH; osmolarity, 270–275 mosM). The capillaries were first filled through the tip and then backfilled with the recording solution. We recorded in voltage-clamp mode with an Axopatch 1D amplifier and a Digidata 1200 DAC interface from Axon Instruments. The signal was filtered at 5 kHz and digitized at 1 kHz. The series resistance was not compensated. Potentials were not corrected for the liquid junction potential, but the latter is estimated to be +4 mV. Access resistance, estimated by measuring the membrane capacitance amplitude and its decay fitted to a single exponential, was between 5 and 10 MΩ and was monitored throughout the experiments

2.3. Superfusion and drug application

Control and drug-containing solutions were applied by gravity at a rate of 1.5 ml/min, using a rapid three-barrel capillary superfusion device (Warner Instrument, CT) with the pipette tips placed about 200 μM from the recorded cell. The flow of solutions was controlled by solenoid valves. Each capillary had a tip diameter of 500 μM and the distance from center to center was 700 μM. The pipette assembly was attached to a motor, allowing fast lateral motions controlled by pClamp6 (Axon Instruments, CA), the acquisition software. A drug onset time of 20 ms for the application system was determined by measuring the changes in the tip potential of the recording pipette filled with intracellular solution as the perfusion was switched from a normal to a 1:2 dilution of the extracellular recording solution.

We evoked NMDA currents at −60 mV whereas glycine and GABA-mediated currents were recorded at 0 mV. After recording a series of stable currents, NAcc neurons were exposed to 1 μM hcrt1 or hcrt2 for 4 min. We evoked currents 2 and 4 min following drug application and 2 min after washout. Cells whose currents showed no recovery on washout were discarded.

The results were compared before and after drug treatment using a one-way analysis of variance (ANOVA).

2.4. Drug

Hcrt1, hcrt2 and control peptide were synthesized at the Microchem Core Facility at The Scripps Research Institute as previously described [16]. As a control, we used a peptide fragment that corresponds to the C-terminal of hcrt1 (GNHAAGILTL). This peptide, named GILTL, does not bind to hcrt receptors [17]. The structure and purity of the peptides was assessed by mass spectrometry and HPLC, and were confirmed to be identical to the sequence of the orexins reported by Sakurai et al. [2]. All peptides were >95% pure. Glycine, GABA, and NMDA were purchased from Sigma (St Louis, MO).

2.5. RT-PCR

Total RNA (5 μg) from rat nucleus accumbens, cortex and cerebellum, prepared using the guanidinium method [18], was reverse transcribed at 37 °C (1 h) using random primers and the MMLV virus reverse transcriptase (Amersham Pharmacia). This served as a template for PCR using the following primers:

Hcrtr1F-5′	$_{1628}$CCTATCATCTACAACTTCCTCAGTTGG;
R-5′	$_{2123}$CCAAGCTCTGATAGGGTGAAGTGAC;
Hcrtr2F-5′	$_{1110}$TGGCTTGTGTATGCCAATAGTGC;
R-5′	$_{1363}$TTGGCTGCTGGGAGTGTGCTTATG;

The primers correspond to different exons in the hcrt receptor genes, to avoid amplification of genomic DNA. After an initial denaturation step at 94 °C for 2 min, the PCR conditions were as follows: 94 °C, 20 s; 58 °C, 25 s; and 72 °C, 30 s for 35 cycles, followed by a final extension step at 72 °C for 10 min. In controls, reverse transcriptase was omitted. The PCR products were resolved in 2% agarose gel with ethidium bromide. In each case, a single PCR product was observed by gel electrophoresis.

3. Result

3.1. The nucleus accumbens expresses hcrtr1 and hcrtr2 receptors

Hypocretin-immunoreactive fibers have been detected in the basal forebrain and NAcc. To determine which receptors are expressed in this brain region, we prepared slices from NAcc, extracted RNA and performed RT PCR analysis of the hcrtr1 and hcrtr2 receptors. A band corresponding to each of the receptors was detected in the cDNA samples of NAcc and cerebral cortex, with no signals in the samples where no reverse transcriptase was added (Fig. 1).

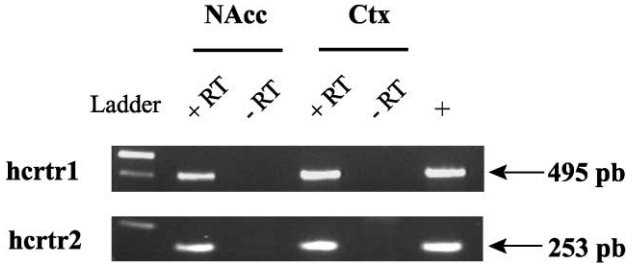

Fig. 1. Hcrtr1 and hcrtr2 receptors are expressed in the rat NAcc. RT PCR analysis of hcrt1 and hcrtr2 show strong expression of both receptors in the NAcc. RT-PCR with hcrt receptor-specific primers was performed on total RNA from NAcc and cerebral cortex (ctx). Omission of RT from the reaction (lanes 3 and 5) served as negative control. A rat whole brain cDNA library was used as a positive control template (+). Lane 1, DNA ladder.

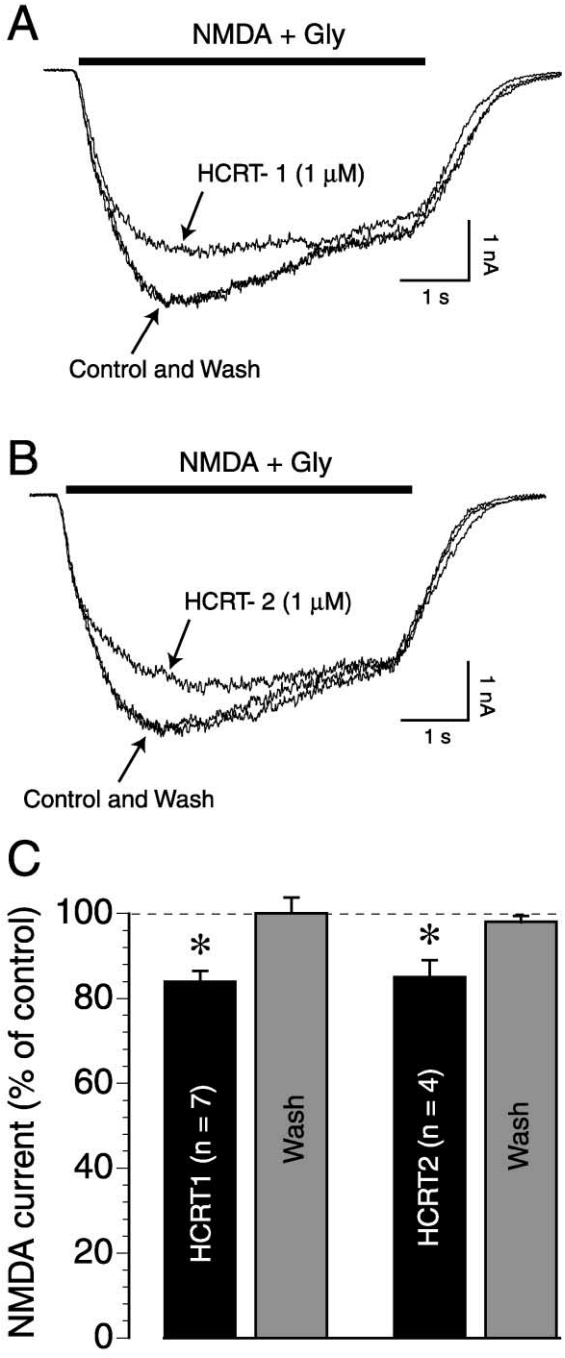

Fig. 2. Application of hcrt1 and hcrt2 (1 μM) decreases NMDA currents in NAcc medium spiny neurons. (A) Inward NMDA currents recorded at − 60 mV before (control), 4 min after hcrt1 application and 2 min after washout (Wash). (B) NMDA currents recorded before (control) during and after hcrt2 application (Wash). (C) Mean NMDA peak current amplitudes 4 min after 1 μM hcrt1 ($n=7$) and hcrt2 ($n=5$) application and 2 min after washout (Wash). Control currents were normalized to 100% (broken line). Asterisks represent statistical significance by one-way ANOVA ($p<0.05$).

3.2. Hcrt1 and hcrt2 reduce NMDA currents

To assess the effect of the hypocretin peptides on NMDA currents, we evoked NMDA currents at − 60 mV by local

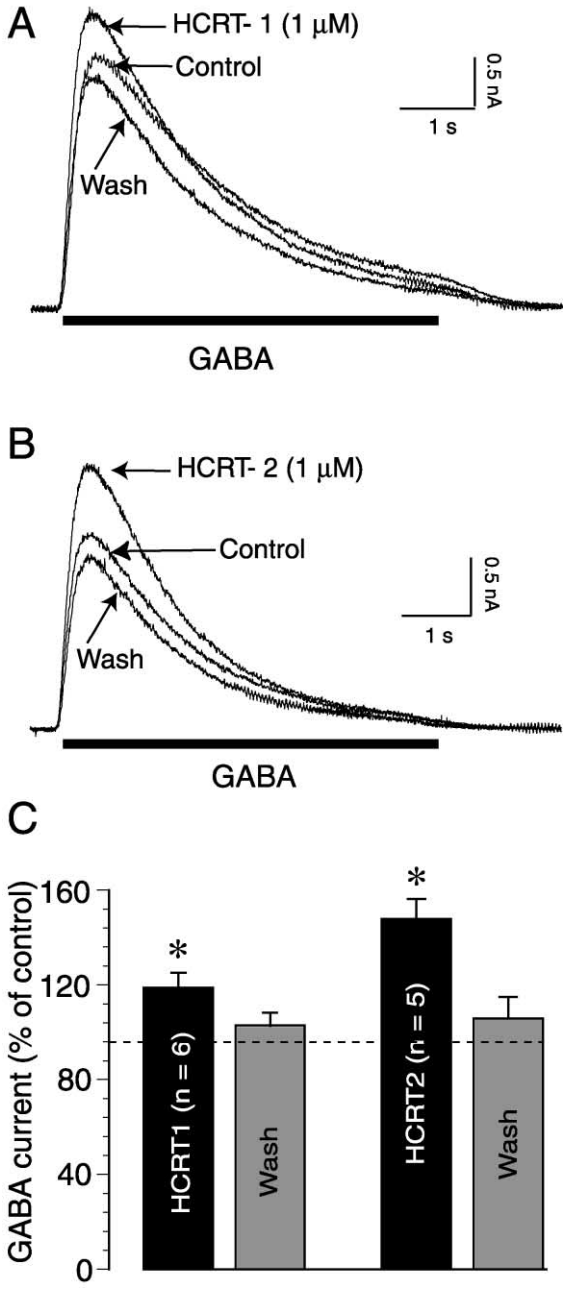

Fig. 3. Hcrt1 and hcrt2 (1 μM) increases $GABA_A$ currents in medium spiny neurons. (A) Outward currents evoked by local application of 0.5mM GABA recorded at 0 mV before (control), 4 min after hcrt1 application and 2 min after washout (Wash). (B) GABA currents recorded before (control) during and after hcrt2 application (Wash). (C) Mean GABA peak current amplitudes 4 min after 1 μM hcrt1 ($n = 6$) and hcrt2 ($n = 5$) applications and on washout (Wash). Control values were normalized to 100% (broken line). Asterisks represent statistical significance by ANOVA test.

co-application of 200 μM NMDA plus 100 μM of its co-agonist glycine. In seven out of nine cells tested, 1 μM hcrt1 clearly decreased NMDA current amplitude (Fig. 2A), an effect followed by recovery on washout. Interestingly, even if peak NMDA current was diminished, the later desensitized current was much less affected by hcrt1 (Fig. 2A and C). Averaged over seven cells, 1 μM hcrt1 significantly decreased NMDA peak current amplitudes by $16 \pm 2.5\%$, compared to the washout ($F(1, 13) = 21.58$; $p < 0.005$). Hcrt1 had no clear effect in two neurons, as it increased and decreased NMDA current to only 102% and 97% of control values. Hcrt2 (1 μM) had similar effects on NMDA current, decreasing NMDA currents with a more pronounced effect on the peak amplitude than on the later desensitized current (Fig. 2B). Averaged over five neurons, 1 μM hcrt2 significantly ($F(1, 7) = 18.68$; $p < 0.05$) decreased NMDA current by $14 \pm 3.9\%$ followed by an almost full recovery of NMDA responses 2 min after washout ($98 \pm 1.5\%$ of control). Hcrt2 failed to induce any change of NMDA currents in one neuron.

To test the specificity of the response to the hypocretins, we superfused a C-terminal fragment of hcrt1 peptide (GILTL), which does not bind to hcrt receptors. No significant changes were observed in the responses to NMDA currents upon application of this peptide (data not shown).

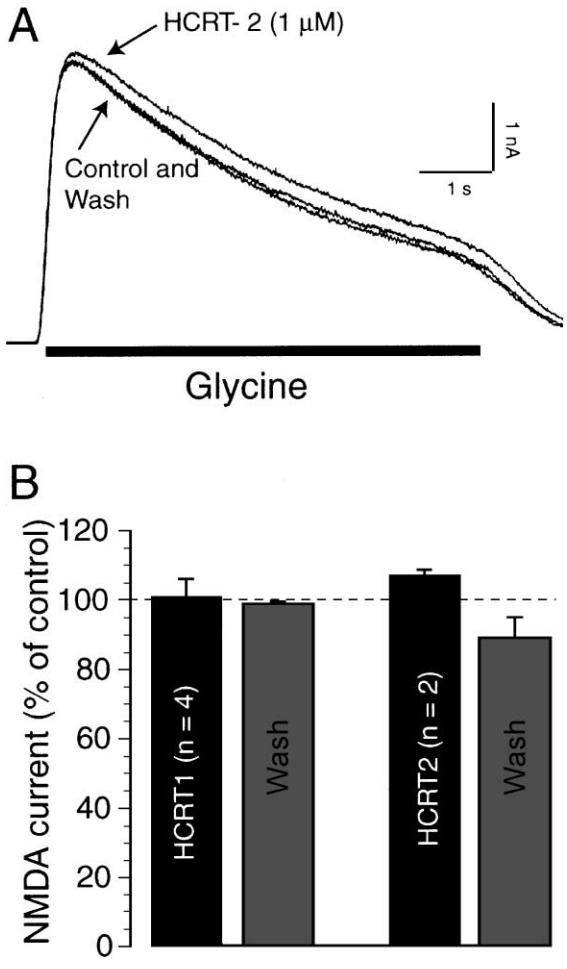

Fig. 4. Relative lack of effects of 1 μM hcrt1 or hcrt2 on currents evoked by 0.5 mM glycine at 0 mV. (A) 1 μM hcrt 2 only slightly increased glycine currents in NAcc medium spiny neurons. (B) Mean glycine peak current amplitudes 4 min after 1 μM hcrt1 ($n = 5$) and 2 ($n = 2$) application and on washout. Control values were normalized to 100% (broken line).

3.3. Hcrt1 and hcrt2 enhance $GABA_A$ currents

Due to presence of cesium in the recording pipette, outward currents evoked at 0 mV by local application of 0.5 mM GABA were purely mediated by $GABA_A$ receptors. In contrast to its effects on NMDA currents, 1 μM hcrt1 increased GABA peak amplitudes (Fig. 3A). Two minutes after wash, GABA currents fully recovered and in some cases returned below the level of control traces (Fig. 3A). On average ($n=6$), hcrt1 significantly ($F(1, 9)=15.43$; $p<0.05$) increased GABA current by $19 \pm 7.4\%$ with an almost full recovery two minutes after washout (GABA current $103 \pm 9.3\%$ of controls; Fig. 3C). Hcrt1 failed to enhance GABA currents in two neurons.

Hcrt2 also increased GABA current amplitude, although this effect was clearly more pronounced than with hcrt1 (Fig. 3B). On average ($n=5$), hcrt2 significantly ($F(1,9)=8.28$; $p<0.05$, compared to washout) increased GABA current amplitudes by $48 \pm 7.5\%$, followed by recovery ($106 \pm 7.7\%$ of control) on washout.

3.4. Hcrt1 and hcrt2 do not affect glycine currents

We have recently identified a novel inhibitory ionotropic receptor in NAcc that is permeable to chloride, activated by glycine and is expressed in about 20% of medium spiny neurons and all interneurons (GM and GRS, unpublished results). We tested the effects of hcrt1 and hcrt2 (1 μM) on 0.5 mM glycine-mediated currents evoked on medium spiny neurons. Four minutes of hcrt1 (1 μM) application did not significantly ($n=5$; $p=0.36$) alter glycine current amplitudes ($101 \pm 5.2\%$ of control) (Fig. 4B). Hcrt2 slightly increased glycine current (Fig. 4A) as the mean glycine current after hcrt2 was $107 \pm 1.7\%$ of control ($n=2$) (Fig. 4B).

4. Discussion

Here, we have shown that both hcrt peptides increase GABAergic currents in isolated neurons of the NAcc, while decreasing the amplitude of NMDA currents. The peptides had no significant effect on glycine conductances. Although we only used a saturating dose of hcrt peptides (1 μM), hcrt2 was relatively more potent than hcrt 1 in decreasing the NMDA currents, and significantly more potent at increasing the amplitude of GABA-evoked currents. A peptide fragment (GILTL) that corresponds to the C-terminal portion of hcrt1, but does not bind to hcrt receptors [17], did not show any effect on GABA or NMDA currents, confirming that the observed effects for the hypocretin peptides were specific.

The hcrt receptors exhibit differential affinities for the hcrt peptides. Hcrtr1 is selective for hcrt1 whereas hcrtr2 binds hcrt1 and hcrt2 with similar affinity [2]. Previous in situ hybridization studies [14], and the RT PCR data shown here, indicate that the NAcc expresses both hcrtr1 and hcrtr2, with a higher concentration of hcrtr2. The fact that both hcrt1 and hcrt2 peptides exhibit similar effects on the conductances analyzed strongly suggests that the hcrt effects are mediated through hcrtr2. The difference in the effects of hcrt1 and hcrt2 may be due to the presence of both hcrtr1 and hcrtr2 receptors in the NAcc as shown in Fig. 1. Since hcrtr1 binds hcrt1 with higher affinity, one may expect to observe different effects upon the application of hcrt1 or hcrt2 peptides, if both receptors are colocalized in the same neurons.

The hypocretin peptides increase neuronal activity in many brain regions, including the hypothalamus [1,19,20], raphe nucleus [21], dopaminergic neurons in the ventral tegmental area [22], the locus coeruleus [23–25] and spinal cord [5]. This previously reported activity contrasts with the lack of depolarization of NAcc neurons by hcrts (G. Martin, unpublished observations) and with postsynaptic effects of the hcrts on both NMDA and GABA currents shown here, which are indicative of an inhibitory action of the peptides in NAcc neurotransmission. The inhibitory effects observed in the NAcc are likely mediated by hcrtr2 [26]. It should be noted, however, that stimulation of hcrtr2 receptors can also lead to neuronal excitation, since local application of hcrt peptides in the histaminergic tuberomammilary nucleus, which expresses high levels of hcrtr2, causes increased cell firing [27]. Thus, the inhibitory effects of hcrts may be specific to the NAcc due to the presence of particular signal transduction components coupled to hcrtr2 in this brain region. Indeed, hcrtr2 appears to couple to different G-proteins in different cell types [28]. Also supporting this hypothesis are molecular profiling studies of the striatum and NAcc, indicating that this brain area contains a collection of mRNAs that encode distinct signal transduction proteins [29], and several signal transduction molecules have isoforms exclusively expressed in the striatum/accumbens region (e.g. adenylyl cyclase type VII [30], G_{olf} [31], RGS9 [32] and others [29,33]). These particular signal transduction pathways in the NAcc may cause different postsynaptic modifications of the GABA and NMDA receptors in the NAcc compared with other regions. Another possibility is that the other regions' receptors have excitatory effects not involving NMDA- or GABA receptors (e.g. direct effect on K+ channels [24]).

The dual excitatory/inhibitory effects of the hypocretins at the electrophysiological level have also been found at the behavioral level. Local injections of hcrt1 in the LC substantially increase wakefulness and inhibit REM sleep, probably by interacting with the noradrenergic system [23,25,34]. Recent data have also demonstrated that hypocretins increase arousal by interacting with the histaminergic system [35]. It thus appears that the hypocretinergic system is capable of modulating multiple neurotransmitter systems, including glutamatergic, GABAergic [19], noradrenergic [25], dopaminergic [22,36], histaminergic [35] and serotonergic [21] circuits. These results suggest that the hypocretins are part of the circuitry that makes decisions on the output of possibly conflicting physiological signals and determine when REM sleep is entered. In narcoleptic

patients, where hypocretin is greatly reduced, there is no control over intersecting circuits, resulting in uncontrolled onset of REM sleep.

As for the accumbens, it is unknown what behavioral function hypocretin might influence. The hypocretins are known to stimulate dopaminergic neurons in the ventral tegmental area [36], one of the main sources of afferents to the NAcc. The NAcc is known to play a role in motivated movements and drug-seeking behavior [37]. In this regard, we find it highly suggestive that the hypocretins act in NAcc like ethanol, which also reduces NMDA currents [38] and enhances GABA response [39].

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