Prefrontal Cortex–Projecting Glutamatergic Thalamic Paraventricular Nucleus-Excited by Hypocretin: A Feedforward Circuit That May Enhance Cognitive Arousal

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Prefrontal cortex–projecting glutamatergic thalamic paraventricular nucleus-excited by hypocretin: a feedforward circuit that may enhance cognitive arousal. J Neurophysiol 95: 1656–1668, 2006; doi:10.1152/jn.00927.2005. The paraventricular thalamic nucleus (PVT) receives one of the most dense innervations by hypothalamic hypocretin/orexin (Hcrt) neurons, which play important roles in sleep-wakefulness, attention, and autonomic function. The PVT projects to several loci, including the medial prefrontal cortex (mPFC), a cortical region involved in associative function and attention. To study the effect of Hcrt on excitatory PVT neurons that project to the mPFC, we used a new line of transgenic mice expressing green fluorescent protein (GFP) under the control of the vesicular glutamate-transporter-2 promoter. These neurons were retrogradely labeled with cholera toxin subunit B that had been microinjected into the mPFC. Membrane characteristics and responses to hypocretin-1 and -2 (Hcrt-1 and -2) were studied using whole cell recording (n = 300). PVT neurons showed distinct membrane properties including inward rectification, H-type potassium currents, low threshold spikes, and spike frequency adaptation. Cortically projecting neurons were depolarized and excited by Hcrt-2. Hcrt-2 actions were stronger than those of Hcrt-1, and the action persisted in TTX and in low calcium/high magnesium artificial cerebrospinal fluid, consistent with direct actions mediated by Hcrt receptor-2. Two mechanisms of Hcrt excitation were found: an increase in input resistance caused by closure of potassium channels and activation of nonselective cation channels. The robust excitation evoked by Hcrt-2 on cortically projecting glutamate PVT neurons could generate substantial excitation in multiple layers of the mPFC, adding to the more selective direct excitatory actions of Hcrt in the mPFC and potentially increasing cortical arousal and attention to limbic or visceral states.

INTRODUCTION

The paraventricular nucleus of the thalamus (PVT) is a unique midline intralaminar nucleus that has been implicated in the regulation of autonomic and visceral functions (Bhatnagar and Dallman 1999; Van der Werf et al. 2002). PVT neurons also respond to stress and to peripherally administered psycho-stimulants cocaine and amphetamine (Bubser and Deutch 1998; Deutch et al. 1998). PVT receives heavy monoamine inputs that include histamine, dopamine, noradrenaline, and serotonin fibers (Cornwall and Phillipson 1988; Otake and Ruggiero 1995). All of which have been implicated in the promotion and maintenance of wakefulness (Jones 2003; Siegel 2004). PVT also receives inhibitory fibers from the suprachiasmatic nucleus (Peng and Bentivoglio 2004) and fibers containing NO (Otake and Ruggiero 1995), which may modulate sleep and arousal (Aston-Jones et al. 2001; Cespuglio et al. 1998; Williams et al. 1997). In addition, PVT receives inputs from homeostatic control regions of the brain including the nucleus of the solitary tract (Otake and Ruggiero 1995), parabrachial nucleus (Krout and Loewy 2000), and from many regions of hypothalamus (Chen and Su 1990; Cornwall and Phillipson 1988; Otake and Ruggiero 1995).

PVT efferents are unique among the midline thalamic nuclei and project to the ventral aspects of medial prefrontal cortex (mPFC), particularly the infralimbic and prelimbic cortices, nucleus accumbens, and amygdala (Berendse and Groenewegen 1991; Bubser and Deutch 1998; Conde et al. 1990; Moga et al. 1995; Su and Bentivoglio 1990), all of which are associated with limbic function including motivation and attention (Cardinal et al. 2002; Christakou et al. 2004). Many of these projections are excitatory (Christie et al. 1987; Frassoni et al. 1997). Because the ventral parts of the mPFC play key roles in executive aspects of attention and a broad spectrum of limbic and associative functions, that PVT neurons that project here are of particular interest. Thus the axonal projections are one characteristic that set PVT apart from other midline thalamic nuclei.

Hypocretin/orexin (Hcrt) neurons, found exclusively in the lateral hypothalamus and perifornical area (de Lecea et al. 1998; Sakurai et al. 1998), have been implicated in the regulation of sleep-wakefulness, feeding, neuroendocrine, and autonomic functions (Ferguson and Samson 2003; Sakurai 2002). Hcrt increases arousal (Hagan et al. 1999), whereas disruption of the Hcrt system leads to narcolepsy (Chemelli et al. 1999; Lin et al. 1999; Nishino et al. 2000). Two Hcrt peptides (Hcrt-1 and Hcrt-2) are synthesized by the same neurons and act on two receptors (Hcrt-R1 and Hcrt-R2) (Sakurai et al. 1998). Hcrt fibers and receptors show a widespread distribution throughout the brain and spinal cord, with high levels in some selected brain regions, particularly the PVT (Marcus et al. 2001; Peyron et al. 1998; van den Pol 1999).

High levels of Hcrt immunoreactive axons and receptors suggest the PVT is an important CNS site where Hcrt acts to influence arousal, autonomic functions, and limbic activities. This study uses whole cell recording and anatomical tracing in transgenic mice that express green fluorescent protein (GFP) under control of a glutamate vesicular transporter-2 (vGluT2)
promoter to examine Hcrt modulation of the glutamatergic PVT cells that project to the mPFC.

METHODS
Preparation of thalamic slices

Thalamic slices were prepared from nontransgenic control or vGluT2-GFP transgenic mice that express enhanced GFP selectively in presumptive glutamatergic neurons, as described previously for hypothalamic slices (Li et al. 2002).

Briefly, 14- to 21-d-old mice maintained in a 12/12-h light/dark cycle were given an overdose of pentobarbital sodium (100 mg/kg) during the light part of the cycle (11:00 am to 4:00 pm), and the brains were removed rapidly and placed in ice-cold oxygenated (95% O2-5% CO2) 1 MgCl2, 10 HEPES, 1.1 EGTA (or 10 BAPTA, as indicated), pH 7.4 (when equilibrated with a mixture of 95% O2-5% CO2). A thalamic block was prepared, and coronal slices (220–300 μm thick) were cut on a vibratome. After a 1- to 2-h recovery period, slices were moved to a recording chamber mounted on a BX51WI upright microscope (Olympus, Tokyo, Japan) equipped with video-enhanced infrared-differential interference contrast (DIC) and fluorescence. Slices were perfused with a continuous flow of gassed artificial cerebrospinal fluid (ACSF; 95% O2-5% CO2) that contained (in mM) 124 NaCl, 2.5 KCl, 1 CaCl2, 1.23 NaH2PO4, 26 NaHCO3, and 10 glucose, pH 7.4. Experiments in which equilibrium potentials for potassium (E钾) was changed were done with the following ACSF (in mM): 111.5 NaCl, 15 KCl, 2 MgCl2, 2 CaCl2, 1.23 NaH2PO4, 26 NaHCO3, and 10 glucose. According to the Nernst equation, E钾 was −108.3 mV in control ACSF ([K+]o = 2.5 mM) and −60.5 mV in modified ACSF ([K+]o = 15 mM). Tissues were continuously perfused with ACSF solution. The ACSF temperature in the chamber was maintained at 35 ± 1°C using a dual-channel heat controller (Warner Instruments, Hamden, CT). Neurons were visualized with an Olympus ×40 water-immersion lens. The Yale University Committee on Animal Care and Use approved all procedures used in this study.

Patch-clamp recording

Whole cell current- and voltage-clamp recordings were performed using pipettes with 4- to 6-MΩ resistance after being filled with pipette solution. The pipettes were made of borosilicate glass (World Precision Instruments, Sarasota, FL) using a PP-83 vertical puller (Narishige, Tokyo, Japan). For most recordings, the composition of the pipette solution was as follows (in mM): 145 KMeSO4 (or KCl for Hcrt-2) and 10 glucose, pH 7.4. When equilibrated with a mixture of 95% O2-5% CO2, K was maintained at 35°C using a dual-channel heat controller (Warner Instruments, Hamden, CT). Neurons were visualized with an Olympus ×40 water-immersion lens. The Yale University Committee on Animal Care and Use approved all procedures used in this study.

Vesicular glutamate transporter 2 drives GFP expression in transgenic mouse

Transgenic mice in which the vGluT2 promoter 1.8 kb upstream from the vGluT2 sequence were used to drive GFP expression. Two short oligomers, one forward primer with sequences 5′-ATCTC-GAGACGCACTCCCCTGGTGTATTAG-3′ and one reverse primer containing 5′-CCGCGGTAACCTCTTGAAAGACTGTGT-CCAGCTTACCAGATTTA-3′ corresponding to the amino terminal region immediately upstream of the mouse vGluT2 coding region were used to synthesize a 1.8-kb PCR fragment from mouse whole brain genomic DNA template. This 1.8-kb PCR fragment was cloned in the TA-TOPO II cloning system, and the sequence was verified. The 1.8-kb promoter fragment was cut out from the TOPO II plasmid by digestion with restriction enzymes XhoI and KpnI, purified and subsequently cloned at XhoI and KpnI sites into a pEGFP-F1 lacking a promoter. The constructed vGluT2-GFP plasmid was digested with BspH1 and AfeI to isolate the 3,369-bp fragment, which contains the mouse vGluT2 promoter followed by GFP coding sequences and SV40 poly(A) signal sequences. This 3.3-kb DNA fragment was purified and prepared for microinjection to generate vGluT2 transgenic mice, using methods previously described (van den Pol and Ghosh 1998; van den Pol et al. 2002, 2004). In these mice, GFP-expressing neurons were found only in selected regions of the brain that previously have been shown to express vGluT2, including the PVT (Fremaux et al. 2001).

Identification of PVT neurons that project to the prefrontal cortex

Mice were anesthetized, and the retrograde tracer cholera toxin subunit B (CTB, Alexa Fluor 594 conjugate, 0.5%, 400 nl, dissolved in sterile PBS) was injected into the mPFC area through a glass pipette (tip diameter, 20–40 μm) attached to a Hamilton syringe. After a 5- to 7-day survival period, the animals were anesthetized and perfused with 4% paraformaldehyde in PBS. Coronal sections of thalamus were cut at 20–30 μm on a freezing microtome, and sections were mounted on gelatinized slides, dried, coverslipped, and examined using fluorescent microscopy. A Texas red filter was used to detect CTB. Other brains were used to prepare slices as described above and were used for whole cell recording.

Immunocytochemistry

Immunostaining was performed as described previously (van den Pol 1999; van den Pol et al. 1998). Briefly, mice were anesthetized and perfused transcardially with a fixative containing 4% paraformaldehyde in 0.1 M phosphate buffer. Sections were cut on a cryostat at 20–30 μm (or 16 μm for counting immunoreactive Hcrt axons and boutons). After washing in normal buffer containing 0.1% lysine, 1% bovine serum albumin, 1% normal goat serum, and 0.3% Triton X-100, sections were incubated overnight in primary guinea pig antiserum against vGluT2 (Chemicon International, Temecula, CA; diluted 1:2,500), rabbit antiserum against the neuroactive peptide Hcrt-2, or rabbit antiserum against GFP. Specificity of Hcrt-2 antiserum is described elsewhere (van den Pol 1999; van den Pol et al. 1998). A secondary antiserum of donkey anti-guinea pig or goat anti-rabbit immunoglobulin conjugated to Texas red (1:200; Molec-
ular Probes) was used. In some experiments, sections were incubated in biotinylated goat anti-rabbit Ig, washed, and treated with avidin-biotin-peroxidase complex (Vector Laboratories, Burlingame, CA). Sections were reacted with diaminobenzidine and hydrogen peroxide to reveal the horseradish peroxidase complex.

Quantification of immunoreactive Hcrt axons and boutons

Immunoreactive Hcrt axons and boutons were quantified with a ×40 objective, combined with an additional ×1.5 magnification. Three different sections were used for each brain, and the number of axons and boutons were counted in three different areas for each section. The density of axons and boutons were studied in fields of 7,500 μm². Both single terminal boutons and boutons en passant along the fibers were counted. The relative density of immunoreactive axons was determined by counting axon intersections with a superimposed test grid placed over the histological image of the axons. The test grid consists of three parallel lines and three lines perpendicular to those. Hcrt immunoreactive PVT and locus coeruleus sections cut at the same thickness from the same mice were compared.

Drugs and drug application

Dl-2-amino-5-phosphonopentanoic acid (AP5), bicuculline methiodide (BIC), 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), (±)-amino-3-hydroxy-5-methylisoxazole-4-propionic acid hydrobromide (AMPA), N-methyl-D-aspartate (NMDA), 5-aminoethyl-3-hydroxyisoxazole (muscimol), and L-glutamate were purchased from Sigma (St. Louis, MO). Hcrt-1/orexin A (mouse) was obtained from Phoenix Pharmaceutical. Hcrt-2 was synthesized by the Stanford University Peptide Facility. TTX was obtained from Alomone Labs (Jerusalem, Israel). CTB was obtained from Molecular Probes (Eugene, OR). All drugs were given by flow pipette application, directed at the recorded cell, unless otherwise noted. Drug solutions were prepared by diluting the appropriate stock solution with ACSF, unless otherwise noted.

RESULTS

Strong Hcrt axon innervation of PVT

The PVT receives axonal input from hypothalamic Hcrt neurons (Peyron et al. 1998). To get a general perspective of the level of innervation, we compared the density of Hcrt immunoreactive boutons and axons in the PVT with the locus coeruleus, a brain region considered to have one of the highest immunoreactive boutons and axons in the PVT with the locus coeruleus, a brain region considered to have one of the highest densities of Hcrt immunoreactive boutons and axons in the PVT, respectively (n = 4, P > 0.1). The mean density of immunoreactive Hcrt boutons in PVT and LC was 81 ± 9 and 93 ± 11/field, respectively (n = 4, P > 0.1).

vGluT2-GFP transgenic mouse

Previous work has suggested that a subpopulation (~60%) of PVT neurons uses glutamate as a neurotransmitter, whereas other PVT neurons use unidentified transmitters (Christie et al. 1987; Frassoni et al. 1997). Three vesicular glutamate transporters have been cloned (Fremeau et al. 2004); and in situ hybridization studies have shown that glutamatergic cells in the PVT express vGluT2 (Fremeau et al. 2001; Herzog et al. 2001; Hur and Zaborszky 2005). We used a transgenic mouse that showed strong GFP expression in populations of presumptive neurons in the thalamus and hypothalamus. Substantially less GFP expression was found in cortical areas where vGluT1 is the primary vesicular glutamate transporter (Fremeau et al. 2001; Herzog et al. 2001). Figure 1C shows GFP-immunoreactive neurons and terminals in PVT. Figure 1, D–F, shows the distribution of vGluT2-GFP–positive neurons in the anterior, central, and posterior parts of the PVT. The expression of GFP driven by the vGluT2 promoter was consistent with the pattern of in situ hybridization showing vGluT2 mRNA in the PVT (Fremeau et al. 2001; Herzog et al. 2001; Hur and Zaborszky 2005). Single cell RT-PCR was used to confirm that green cells expressed vGluT2. All six GFP-containing neurons in the PVT aspired into recording pipettes showed positive mRNA expression.

After immunocytochemical staining for the vGluT2 antigen, the distribution of vGluT2 immunoreactive axons in the PVT and also in the mPFC was examined. Figure 1G shows the vGluT2-immunostaining in the PVT. In the mPFC, vGluT2 immunoreactive axonal boutons are densely distributed in layers I, V, and VI, and appear slightly less dense in deeper layer VI b than VI a (data not shown). Figure 1H shows the vGluT2-immunostaining in layer V of the mPFC.

Membrane properties of vGluT2-GFP–positive neurons

The active and passive membrane properties of PVT neurons have not been substantively characterized. Our first goal was to examine the membrane properties of vGluT2-GFP–positive PVT neurons. In vGluT2-GFP–positive PVT neurons, cells were either quiescent or fired regular spontaneous action potentials (active cells) with a mean firing rate for active cells of 1.52 ± 0.21 Hz (n = 33; Table 1). The RMP was 57.9 ± 0.4 mV (n = 103), and the mean input resistance was 465 ± 18 mΩ (n = 25). As reported previously (Ishibashi et al. 2005), the spontaneous action potentials were followed by an afterhyperpolarization (AHP; Fig. 2B) and were TTX-sensitive (data not shown), suggesting that they are sodium-mediated action potentials. The mean input resistance was calculated from the slope of the current-voltage relation. In all neurons studied, the current-voltage relation was linear between 0 and −50 mV and showed a time-independent (Fig. 2, A1 and A2) inward rectification between −50 and −80 mV (Fig. 2A3), indicating the existence of inwardly rectifying K⁺ conductance. As shown in Fig. 2A2 and Table 1, most of the neurons displayed a membrane rectification characterized by a sag that has been identified as a time- and voltage-dependent rectification resulting from the presence of an H-type potassium current (H-current) in other neurons. When the neurons were depolarized from a hyperpolarized level, all the neurons recorded showed a low-threshold spike (LTS; Fig. 2C1) (Ishibashi et al. 2005; Richter et al. 2005). The LTS was calcium-dependent, because it always persisted in the presence of TTX but was eliminated by nickel at 200 μM (n = 6; Fig. 2, C2–C4), indicating the presence of inwardly rectifying K⁺ conductance. As shown in Fig. 2A2 and Table 1, most of the neurons displayed clear spike frequency adaptation (SFA; the frequency of spikes is reduced over time) or spike failure at higher currents. A small number (5 of 19) of neurons showed only modest SFA (Fig. 2, E1 and E2; Table 1).
FIG. 1. Glutamate vesicular transporter-2 (vGluT2)-green fluorescent protein (GFP)-positive neurons in the paraventricular thalamic nucleus (PVT). A and B: hypocretin/orexin (Hcrt) immunoreactive axons and boutons in PVT (A) and locus coeruleus (LC) (B). C: peroxidase-labeled GFP-immunoreactive neurons and terminals in PVT. D–F: vGluT2-GFP–positive neurons in anterior (D), central (E), and posterior (F) parts of PVT. 3V, third ventricle; IMD, intermediodorsal nucleus. G and H: immunoreactive vGluT2 terminals in PVT (G) and layer V of medial prefrontal cortex (mPFC) (H). Scale bar: A–C, 15 μm; D–F, 50 μm; G and H, 5 μm.
TABLE 1. Properties of neurons in PVT

<table>
<thead>
<tr>
<th></th>
<th>Nontransgenic Mice</th>
<th></th>
<th>vGluT2-GFP+ Neurons</th>
<th></th>
<th>vGluT2-GFP− Neurons</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Resting potential, mV</td>
<td>−58.1 ± 0.44</td>
<td>101</td>
<td>−57.9 ± 0.41</td>
<td>103</td>
<td>−59.4 ± 0.45</td>
<td>97</td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>430 ± 14</td>
<td>12</td>
<td>465 ± 18</td>
<td>25</td>
<td>437 ± 17</td>
<td>31</td>
</tr>
<tr>
<td>Spike frequency, Hz</td>
<td>1.41 ± 0.24</td>
<td>48</td>
<td>1.52 ± 0.21</td>
<td>33</td>
<td>1.46 ± 0.38</td>
<td>30</td>
</tr>
<tr>
<td>Percent of cells firing</td>
<td>57% (48/84)</td>
<td></td>
<td>66% (33/50)</td>
<td></td>
<td>64% (30/47)</td>
<td></td>
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<tr>
<td>Percent of cells with clear Ih</td>
<td>71% (12/17)</td>
<td></td>
<td>69% (20/29)</td>
<td></td>
<td>54% (13/24)</td>
<td></td>
</tr>
<tr>
<td>Percent of cells with clear SFA</td>
<td>No data collected</td>
<td></td>
<td>74% (14/19)</td>
<td></td>
<td>54% (13/24)</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Active cells</td>
<td></td>
<td>Silent cells</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Resting potential, mV</td>
<td>−58.4 ± 0.4</td>
<td>106</td>
<td>−59.1 ± 0.6</td>
<td>68</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Input resistance, MΩ</td>
<td>451 ± 11</td>
<td>38</td>
<td>435 ± 19</td>
<td>17</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent of cells with clear Ih</td>
<td>81% (26/32)</td>
<td></td>
<td>42% (5/12)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Percent of cells with clear SFA</td>
<td>58% (14/24)</td>
<td></td>
<td>80% (8/10)</td>
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</table>

Values are means ± SD. Numbers in parentheses refer to the number of cells with that property—the total number of cells tested. $I_h$, H current; SFA, spike frequency adaptation.

To determine if the membrane properties of vGluT2-GFP–positive cells differ from vGluT2-GFP–negative cells, we recorded from neurons that showed no GFP in the PVT of vGluT2 transgenic mice. As a second control, to ensure that transgenic mice were not different from other mice and that GFP did not alter membrane properties, we also recorded from age-matched PVT neurons in nontransgenic mice. We did not find any substantive differences in RMP, spike frequency, or input resistance between the three groups of neurons (Table 1).

All three groups of neurons had both active and silent cells, in similar proportions. We grouped all the cells into active ($n = 111$) or silent ($n = 70$) cells. Active cells showed a more substantial H-current and somewhat less SFA (Table 1).

Hcrt excites vGluT2-GFP–positive neurons

vGluT2-GFP–positive PVT neurons were recorded in current clamp using the whole cell mode and compared with vGluT2-GFP–negative cells in the same PVT slice. As shown in Fig. 3, A and B, and Table 2, −71% (54/76) of all the neurons recorded (whether silent or active) were potently depolarized and excited by flow pipette application of Hcrt-2. Because no difference was found between vGluT2-GFP–positive and vGluT2-GFP–negative cells relative to increases in spike frequency, amplitude of depolarization, or percent of responding cells (Table 2), data are grouped together. Similar results were also found in neurons from nontransgenic mice (Table 2). As shown in Fig. 3C, 50 nM Hcrt-2 significantly increased the average action potential frequency by over threefold, from 0.95 ± 0.19 to 3.15 ± 0.36 Hz ($n = 27, P < 0.01$, ANOVA), and recovered to 1.15 ± 0.28 Hz after washout for 5–30 min. The depolarizing responses of PVT to Hcrt-2 application were found to be concentration dependent at concentrations ranging from 10 to 1,000 nM; the EC$_{50}$ value was 25 nM (Fig. 3D; neurons that did not respond to Hcrt were not included). Active and silent cells were also compared in their response to Hcrt; no differences were found (Table 2). Additionally, there was no obvious difference in location within the PVT of neurons showing different responses to Hcrt (data not shown). To exclude the possibility that the absence of a response in the remaining PVT neurons to 50 nM Hcrt-2 was caused by poor sensitivity to low peptide concentrations, bath application of 50 nM Hcrt-2 or flow pipette application of 1 μM Hcrt-2 was used. Similar results were obtained. Bath application of 50 nM Hcrt-2 and flow pipette application of 1 μM Hcrt-2 both depolarized and excited five in seven neurons and six in eight neurons, respectively (data not shown).

To explore the receptors involved in the depolarizing action of Hcrt, the effect of Hcrt-1 and Hcrt-2 was compared in the same neurons. Both Hcrt-1 and Hcrt-2 at 50 nM depolarized and excited PVT neurons. Hcrt-2 produced an effect that was as strong as or stronger than Hcrt-1 (Fig. 3E). The mean depolarization by 50 nM Hcrt-1 and Hcrt-2 was 4.1 ± 0.37 and 7.5 ± 0.54 mV, respectively ($n = 5, P < 0.01$, paired Student’s t-test; Fig. 3F).

The mechanism of Hcrt depolarization was further characterized. The depolarizing effect of Hcrt-2 (50 nM) persisted in the presence of either TTX (1 μM; Fig. 3G) or in modified ACSF (0.1 mM Ca$^{2+}$/10 mM Mg$^{2+}$; data not shown) that blocks synaptic transmission. The effect of Hcrt-2 also persisted in ACSF containing GABA$_A$ and glutamate receptor antagonists (BIC, AP5, and CNQX; $n = 5$, data not shown). There is no significant difference in the depolarization induced by 50 nM Hcrt among neurons in the normal ACSF condition, TTX condition, or low calcium/high magnesium conditions, and the mean depolarization was 7.76 ± 0.47 (n = 18), 7.5 ± 0.60 (n = 8), and 8.07 ± 0.67 mV (n = 10), respectively ($P > 0.5$, ANOVA). These results suggest that Hcrt action was a direct postsynaptic effect. Figures 3G and 6G show a thickening of the trace after Hcrt-2 application even though synaptic potentials were blocked, suggesting an opening of some channels after Hcrt application.

**Hcrt depolarization is attributable to two mechanisms: closure of a potassium channel and activation of nonselective cationic channels**

The following experiments were designed to elucidate the mechanism of the depolarization caused by Hcrt-2. First, we studied whether the input membrane resistance was changed after Hcrt application. In 8 of 24 neurons depolarized and excited by 50 nM Hcrt-2, the input resistance was increased after application of Hcrt-2 (Fig. 4A; mean increase, 12.2 ± 2.7%; $P < 0.01$, paired Student’s t-test). An increase in input resistance could be caused by the closure of potassium channels. To test this hypothesis, we compared the effect of Hcrt-2 on neurons held at a membrane potential of −60 mV in two different conditions of external potassium concentration ([K]$_o$).
To further analyze the contribution of K⁺ channels to the depolarization, we undertook an additional experiment. Voltage clamp was used to study the effect of Hcrt on membrane current. Current-voltage plots (I-V curves) were obtained in the presence of TTX, CNQX, AP5, and BIC. In normal ACSF with [K]ₒ at 2.5 mM, a comparison of voltage-clamp ramps in control and in the presence of Hcrt (Fig. 4, C and E) indicated a reversal at approximately −110 mV of the Hcrt-induced current in 7 of 15 neurons which had clear inward currents after application of 50 nM Hcrt-2. In the seven cells (Fig. 4E, inset), the mean reversal was −110.0 ± 3.4 mV, very close to the estimated E_K. Performing the same protocol in an ACSF with [K]ₒ at 15 mM in other PVT neurons indicated a reversal of approximately −60 mV of Hcrt-induced currents in five of eight neurons (Fig. 4, D and E) which had clear inward currents after Hcrt-2 application. In these five cells (Fig. 4E, inset), the mean reversal was −63.0 ± 2.8 mV, very close to the theoretical E_K. Together, these results indicate that the depolarizing effect of Hcrt in these PVT neurons is mediated by the reduction of a potassium conductance.

resulting in two different E_Ks. As shown in Fig. 4B1, in the first condition ([K]_ₒ = 2.5 mM; estimated E_K = −108.3 mV), Hcrt-2 powerfully depolarized PVT neurons. In contrast, when tested again in the second condition with high K⁺ (Fig. 4B2; [K]_ₒ = 15 mM; estimated E_K = −60.5 mV), application of Hcrt-2 had no clear effect. The depolarization by 50 nM Hcrt-2 in these two conditions was 9.8 ± 0.55 and 1.4 ± 0.8 mV, respectively (n = 5, P < 0.01, unpaired Student’s t-test; Fig. 4B3). The effect of Hcrt on membrane depolarization showed little desensitization. In normal conditions, the depolarization by Hcrt-2 (50 nM) was 7.8 ± 0.3 and 7.6 ± 0.4 mV (n = 3) for the first and second application of Hcrt with a 15-min interstimulus interval, respectively.

FIG. 2. Membrane properties of vGluT2-GFP–positive PVT neurons. A1 and A2: voltage responses to current injection in 2 different neurons. One neuron has a clear sag (A2, dot), suggesting the presence of H-current. The other neuron does not have a clear sag (A1, dot). Note presence of time-independent (A1 and A2) inward rectification, activated in response to large amplitude current pulses (Δ). A3: mean current-voltage relationship in 55 neurons. Values were taken at the end of pulses (dots in A3). B: note afterhyperpolarization (AHP). C1: response to current steps from a hyperpolarized level, showing strong low threshold spike (LTS). C2–C4: traces show that LTS persists in the presence of TTX (C2), is eliminated by nickel (C3), and can recover after washout of nickel (C4). D1 and D2: voltage responses to depolarizing 40- and 120-pA currents, showing clear spike frequency adaptation (SFA) (D1) and spike failure (D2). E1 and E2: another cell shows only modest SFA and no spike failure.

FIG. 3. Hypocretins excite GFP-positive PVT neurons. A and B: Hcrt-2 depolarizes and excites a silent neuron (A, resting membrane potential (RMP), −59.2 mV) or an active (B, RMP, −58.8 mV) neuron. C: mean effect of Hcrt-2 (50 nM) on spike frequency of PVT (**P < 0.01, n = 27). D: dose–response curve for the effect of Hcrt-2 on depolarization in PVT neurons (n = 5 at 5 nM, 4 at 10 nM, 4 at 20 nM, 15 at 50 nM, 4 at 100 nM, and 7 at 200 nM). E: response to Hcrt-1 and Hcrt-2 (both at 50 nM) of a single neuron (RMP, −59.3 mV). F: comparison of the depolarization induced by Hcrt-1 and Hcrt-2 (applied at 50 nM; **P < 0.01, n = 5). G: in the presence of TTX (1 μM), Hcrt-2 depolarized the membrane potential and caused a thickening of the trace, probably because of channel openings (RMP, −59.2 mV).
In addition to an increase in input resistance, of the 24 neurons depolarized and excited by 50 nM Hcrt-2 described above, 9 other neurons showed a decrease (Fig. 5A; mean decrease, 11.9 ± 2.4%; P < 0.01, paired Student’s t-test), and the remaining 7 neurons showed no clear change (Fig. 5B; mean increase, 1.0 ± 1.3%; P > 0.5, paired Student’s t-test) in input resistance. Furthermore, in 8 of the 15 neurons described in the paragraph above that displayed a clear inward current after application of 50 nM Hcrt-2 in voltage clamp, the reversal of the Hcrt-induced current was −38.0 ± 3.1 mV in one-half (4) of them (Fig. 5, C1 and C2), and in the remaining one-half (4) of the neurons, the I-V lines remained either parallel or did not converge over the entire voltage range tested (Fig. 5, D1 and D2).

The above result that Hcrt-induced currents reversed at −38 mV is consistent with nonselective cationic channels as the basis for the currents and suggest that activation of nonselective cationic channels contribute to the depolarizing effect of Hcrt. However, another mechanism that might contribute to the inward current would be the electrogenic Na\(^{+}\)/Ca\(^{2+}\) exchanger. In 16 neurons that had clear inward currents after application of 50 nM Hcrt-2 in nominal zero calcium ACSF, we still found that in 6 neurons the Hcrt-induced currents reversed at −35.8 ± 2.38 mV; in 7 neurons the currents reversed at −109.2 ± 3.93 mV; and in the remaining 3 neurons, the I-V lines remained parallel or did not converge (data not shown). These results are consistent with the previous findings and suggest that the Hcrt-induced currents that reversed around −38 mV are likely caused by the activation of nonselective cationic channels and not by the Na\(^{+}\)/Ca\(^{2+}\) exchanger.

Different PVT neurons showed different mechanisms of response. We found no obvious anatomical differences in different groups of responding neurons or any anatomical correlation with the existence of H-current or spontaneous activity (data not shown).

Cortically projecting vGlut2-GFP–positive PVT neurons are excited by Hcrt

Retrograde tracer CTB injections into mPFC typically involved both the prelimbic and infralimbic areas of the PFC (Fig. 6A). This cortical injection of tracer labeled cells throughout the anteroposterior extent of the PVT. Figure 6B is an example showing the labeling of neurons in the PVT. Labeling was mainly on one side, ipsilateral to the side of tracer injection (Fig. 6B). Within the thalamus, retrogradely labeled neurons were found in the PVT and also in the mediodorsal and reuniens nuclei of thalamus. In contrast, few neurons of the central medial thalamus nucleus showed labeling. Figure 6, C and D, shows the labeling of the vGlut2-GFP–positive neurons and red tracer labeled neurons in the same micrograph. Figure 6, C–E, shows that some vGlut2-GFP–positive neurons were retrogradely labeled (indicated by the arrows), indicating that these PVT neurons projecting to the mPFC are probably glutamatergic. Because our CTB injection sites in the mPFC were small, the percent of retrogradely labeled PVT neurons is probably an underestimate of the total number of cells that project from the PVT to the mPFC.

To investigate whether the GFP-expressing neurons projecting to mPFC in PVT were Hcrt responders or not, we recorded from red PVT neurons retrogradely labeled with CTB that had been injected into the mPFC in vGlut2-GFP mice.

The retrogradely labeled neurons were either quiescent or fired regular spontaneous action potentials. The RMP was −60.0 ± 0.6 mV (n = 60). The input membrane resistance was 358 ± 10 MΩ (n = 37). Forty-seven percent (18 of 38) of the neurons had clear H-currents. Most neurons (8 of 10) showed clear SFA or spike failure with greater current injections. All the neurons studied showed inward rectification and LTS. Application of Hcrt-2 (50 nM) depolarized and excited ~76% (25 of 33) of the retrogradely labeled neurons (Fig. 6F). After Hcrt application, the mean frequency of spontaneous action potentials increased by about threefold, from 0.93 ± 0.24 to 2.94 ± 0.55 Hz (n = 20, P < 0.01, ANOVA). In 11 CTB retrogradely labeled GFP\(^{+}\) neurons, 9 of them were depolarized and excited by Hcrt-2 (from 0.96 ± 0.26 to 2.73 ± 0.49 Hz, P < 0.01, ANOVA). In the presence of 1 μM TTX, Hcrt-2 (50 nM) still depolarized neurons that had been retrogradely labeled by CTB (Fig. 6G), suggesting again the involvement of a postsynaptic action. The mean depolarization by Hcrt-2 was 8.7 ± 1.2 mV (n = 5). The changes of input membrane resistance were also studied in these retrogradely labeled neurons. In 16 neurons that were depolarized and excited by Hcrt-2 (50 nM), the input resistance was increased in 5 neurons (mean increase, 16.4 ± 3.9%; P < 0.01, paired Student’s t-test); decreased in 4 neurons (mean decrease, 15.2 ± 4.7%; P < 0.01, paired Student’s t-test); and not significantly changed in 7 neurons (mean decrease, 1.0 ± 1.8%; P > 0.5, paired Student’s t-test), implying that multiple mechanisms were also involved in these neurons. Together, these results indicate that Hcrt has direct excitatory actions on the cortically projecting PVT neurons.
Hcrt has little effect on glutamate or GABA synaptic currents in PVT

Prior to studies of synaptic input, to ensure that PVT expressed ionotropic glutamate and GABA receptors, glutamate and its agonists and GABA agonist were applied through a flow pipe. In all the neurons tested, glutamate (50 μM, n = 5), AMPA (30 μM, n = 6), and NMDA (50 μM, n = 7) depolarized the neurons and evoked groups of spikes in current clamp (data not shown). In voltage clamp (−60 mV holding potential), inward currents were generated, and the inward currents evoked by glutamate, AMPA, and NMDA were 13.4 ± 1.9, 137.5 ± 27.3, and 44.4 ± 4.7 pA, respectively. Muscimol (20 μM), a GABA<sub>A</sub> receptor agonist, attenuated spike frequency and hyperpolarized the membrane potential by 28.1 ± 1.0 mV (n = 6). Thus glutamate and GABA receptors are expressed in PVT neurons.

Both excitatory and inhibitory synaptic activity was found in PVT. Using a recording pipette solution with KMeSO<sub>4</sub> with the GABA<sub>A</sub> antagonist BIC (30 μM) in the bath, spontaneous excitatory postsynaptic currents (EPSCs) were identified (−60 mV holding potential). These EPSCs were completely blocked by glutamate ionotropic antagonists AP5 (50 μM) and CNQX (10 μM) and recovered after antagonist washout (n = 6; Fig. 7A). In the presence of AP5 and CNQX in the bath, spontaneous inhibitory postsynaptic currents (IPSCs) were recorded using a recording pipette solution containing KCl (−60 mV holding potential). These currents were blocked by BIC and recovered after BIC washout (Fig. 7B). PVT received a robust

![Figure 4](image_url)

**FIG. 4.** Hypocretin excites PVT by blocking a K<sup>+</sup> current. A: comparison of hyperpolarizing pulses before (1) and during (2) effect of Hcrt-2. RMP, −59.3 mV. Bottom inset: enlargement of pulses showing increase in input membrane resistance in the presence of Hcrt-2. B1 and B2: depolarizing effect of Hcrt-2 is suppressed (B2) after changing [K]<sub>i</sub>, from 2.5 to 15 mM when neurons are held at −60 mV. B3: comparison of depolarization induced by Hcrt-2 in external solution with [K]<sub>i</sub>, of 2.5 or 15 mM (**P < 0.01, n = 5). C: voltage-clamp ramps in the absence (b) or presence (a) of Hcrt-2 with [K]<sub>i</sub>, of 2.5 mM. D: voltage-clamp ramps in the absence (d) or presence (c) of Hcrt-2 with [K]<sub>i</sub>, of 15 mM. E: subtraction of curves from C (a and b) and D (c and d). Inset: reversal potentials for both conditions with respect to Nernst relationship.

Hcrt has little effect on glutamate or GABA synaptic currents in PVT

Prior to studies of synaptic input, to ensure that PVT expressed ionotropic glutamate and GABA receptors, glutamate

![Figure 5](image_url)

**FIG. 5.** Hypocretin excites PVT through activation of nonselective cationic currents. A and B: comparison of hyperpolarizing pulses before (1) and during (2) effect of Hcrt-2 in 2 neurons. RMP, −58.9 mV in A; −59.2 mV in B. Bottom inset: enlargement of pulses showing decrease (A) or lack of change (B) in input membrane resistance in the presence of Hcrt-2. C1 and D1: voltage-clamp ramps in the presence (a) and absence (b) of Hcrt in 2 neurons. C2 and D2: subtraction of curves (a and b) from C1 and D1, respectively. C1 and C2: Hcrt-2–induced current reversed at −38 mV. D1 and D2: Hcrt-2–induced current remains parallel.
FIG. 6. Direct effect of hypocretin on vGluT2-GFP–positive neurons in PVT that project to prefrontal cortex. A: representative injection site. Cholera toxin subunit B (CTB) was injected in the mPFC. IL, infralimbic area; PL, prelimbic area. Arrows show midline of brain. B: PVT was selectively labeled, ipsilateral to injection side. Mediodorsal (MD), and reuniens nucleus (RE) were also labeled, whereas few central medial (CM) nucleus were labeled. C and D: GFP-positive neurons (GFP$^+$, C) and retrogradely labeled neurons (CTB$^+$, D) in a same layer of PVT. E: both GFP$^+$ and CTB$^+$ neurons are in PVT (boxed area in C and D). Double-labeled neurons show a yellow color. Arrows in C–E show 3 neurons that are double-labeled. Arrowheads in B–D show middle of ventricle. F: retrogradely labeled vGluT2-GFP–positive neuron is depolarized and excited by Hcrt-2 (50 nM). RMP, −58.9 mV. G: Hcrt-2 (50 nM) depolarized a retrogradely labeled vGluT2-GFP–positive neuron in the presence of TTX (1 μM). RMP, −59.7 mV. Scale bars: A, 200 μm; B, 100 μm; C and D, 50 μm; E, 125 μm.
GABA-mediated input, shown by high levels of GABA-mediated synaptic currents.

In other regions of the brain, Hcrt has been reported to increase the release of amino acid transmitters by presynaptic mechanisms (Burlet et al. 2002; Liu et al. 2002; van den Pol et al. 1998). To determine whether Hcrt modulates the synaptic inputs to PVT, whole cell recordings were performed, and synaptic currents were recorded under voltage clamp at a holding potential of −60 mV. In the first set of experiments, EPSCs were recorded with KMeSO₄ pipette in the presence of BIC. In the neurons that showed clear inward currents after Hcrt-2 application, Hcrt-2 at 50 nM (Fig. 7, C and D) showed no clear effect on the frequency or amplitude of EPSCs. The mean frequency and amplitude of EPSCs in the presence of 50 nM Hcrt was 99.3 ± 7.72% (from 0.98 ± 0.32 to 0.94 ± 0.27 Hz) and 98.8 ± 1.45% of control, respectively (n = 7, P > 0.5, ANOVA). Then IPSCs were recorded with KCl pipette in the presence of AP5 and CNQX. Application of Hcrt-2 at 50 nM (Fig. 7, E and F) showed no distinct effect on the frequency or amplitude of IPSCs. The mean frequency and amplitude of IPSCs in the presence of 50 nM Hcrt was 97.7 ± 5.68% (from 1.31 ± 0.27 to 1.22 ± 0.22 Hz) and 98.8 ± 3.16% of control, respectively (n = 8, P > 0.5, ANOVA).

Discussion

In this study, we found that GFP-positive neurons that project from the PVT to the prefrontal cortex show strong excitatory responses to low concentrations of Hcrt-2. These actions were mediated by two different mechanisms: a decrease in potassium currents and an activation of nonselective cationic currents. Hcrt axons project directly to selected regions of the prefrontal cortex; the more robust and widespread innervation of different layers of selected regions of the mPFC by excitatory PVT neurons may allow excitatory actions of Hcrt on PVT to excite additional layers of the cortex. The robust excitation of Hcrt on cortically projecting excitatory PVT neurons may serve as a state-dependent amplifier for the actions of the Hcrt arousal system to enhance cortical arousal and attention, particularly with regard to limbic or visceral states.

**Hcrt acts through postsynaptic Hcrt-2 receptors**

A number of studies have shown that Hcrt produces an excitatory effect on some but not all CNS neurons (for review, see Siegel 2004). Direct and/or indirect actions have been shown to mediate Hcrt excitatory effects (Siegel 2004). In this study, the effect of Hcrt on depolarization persists in the presence of TTX or after blockade of synaptic input, indicating that the action of Hcrt is direct and postsynaptic. In the PVT, Hcrt-1 has an excitatory effect that is stronger than Hcrt-2, suggesting that the action is mediated by Hcrt-R2. The interpretation that Hcrt-R2 is responsible for the excitatory actions of Hcrt is further supported by in situ hybridization data showing that Hcrt-R2 is the primary Hcrt receptor mRNA in the PVT (Marcus et al. 2001; Trivedi et al. 1998). Hcrt-R1 shows a much greater response to Hcrt-1 than -2; in contrast, the responses of the Hcrt-R2 to Hcrt-1 and -2 have been suggested to be similar pharmacologically. However, those data were acquired in nonneuronal cells transfected with the receptor (Sakurai et al. 1998). Our data showing greater responses to Hcrt-2 than to Hcrt-1 in the same neuron is consistent with other work on populations of midline thalamic nuclei, including the centromedial and rhomboid, which showed greater responses to Hcrt-2 (Bayer et al. 2002; Ishibashi et al. 2005). Perhaps some difference exists between these G protein–coupled receptors when expressed naturally in neurons as opposed to when transfected into other cell types that might account for the greater response to Hcrt-2.
Two mechanisms underlie actions of Hcrt: closure of potassium channels and activation of nonselective cationic channels

For neurons that display an increased input membrane resistance after application of Hcrt, the depolarizing action of Hcrt likely results from the closure of a potassium conductance. This is suggested by the finding that these neurons held at \( E_K \) show no response to Hcrt, and the Hcrt-induced current reversed at \(-110 \) mV, close to the estimated \( E_K \). A similar mechanism has been found in thalamic nuclei (Bayer et al. 2002; Ishibashi et al. 2005), cortical neurons (Bayer et al. 2004), locus coeruleus neurons (Ivanov and Aston-Jones 2000), and dorsal motor nucleus of the vagus (Grabauskas and Moises 2003). Another mechanism underlying the effect of Hcrt is likely attributable to the activation of nonselective cationic channels. In these neurons, Hcrt induced a decreased input resistance and Hcrt-induced currents reversed at \(-38 \) mV, consistent with nonselective cationic currents. Although inward current induced by the \( \text{Na}^+ / \text{Ca}^{2+} \) exchanger may have a similar reversal potential (Burdakov et al. 2003; Ehara et al. 1989), the result that Hcrt-induced currents still reverse at \(-35.8 \) mV, similar to the reversal potential for \( \text{Ca}^{2+} \), suggests that the \( \text{Na}^+ / \text{Ca}^{2+} \) exchanger does not contribute to the Hcrt-induced current in these neurons. In addition, the increased thickening of the trace line after Hcrt application suggests an opening of ion channels. Alternatively, an opening of both sodium and potassium channels could also generate a reversal that might lie between the two individual reversal potentials; this seems less likely because the TTX used during these experiments would block most of the voltage-sensitive sodium channels. Activation of nonselective cationic channels was not found in studies of Hcrt responses in other midline thalamic nuclei, including the centromedial and rhomboid nucleus (Bayer et al. 2002), but has been found in the hypothalamic paraventricular nucleus (Follwell and Ferguson 2002), dorsal raphe (Liu et al. 2002), nucleus tractus solitarius (Yang and Ferguson 2003), and area postrema (Yang and Ferguson 2002). In a study of rat PVT, Hcrt caused an increase in input resistance and closure of potassium channels (Ishibashi et al. 2005). In our work on the mouse PVT, we found some cells that showed a similar response to Hcrt as described in the rat PVT, but we also found at least one other mechanism based on a reduction in input resistance and activation of nonselective cation channels. The difference between the two studies may be due to the species used.

Multiple mechanisms have been shown to mediate the effect of Hcrt on depolarization in the same neurons, including nucleus tractus solitarius neurons (Yang and Ferguson 2003), dorsal motor nucleus of vagus (Hwang et al. 2001) (activation of a nonselective cationic current and inhibition of \( K^+ \) current), and septohippocampal cholinergic neurons (Wu et al. 2004) (suppression of a \( K^+ \) current and activation of a \( \text{Na}^+ / \text{Ca}^{2+} \) exchanger). However, in this study, it seems that the two mechanisms involved may be in different PVT cells, because the reversal potential of Hcrt-induced current was close to either \(-110 \) or \(-38 \) mV, similar to the reversal potential for \( K^+ \) or the reversal potential for a nonselective cationic current, respectively. There is also a possibility that a minor block of a \( K^+ \) current in some neurons might be obscured by activation of the mixed cationic conductance. Additional mechanisms of Hcrt excitation may also exist here, for instance, as suggested in neurons with no change in input resistance or in neurons in which the \( I-V \) curves remained parallel after Hcrt application. In an attempt to identify cells showing different response characteristics, we used retrograde labeling from the mPFC, GFP expression driven by the vGlut2 T promoter, a combination of GFP expression and retrograde labeling, and nontransgenic control mice. All groups of cells showed similar distributions of response profiles. This raises the question of false negatives; that is, not all cells projecting to the cortex were necessarily labeled with the retrogradely transported dye, and although the expression of GFP in the PVT is consistent with in vGlut2 mRNA situ hybridization, in some cells the GFP was weak, raising the possibility that not all glutamatergic neurons expressed sufficient GFP to be detected in a thick slice.

Functional implications

The states of awareness and sleep involve interactions between thalamus and cortex (McCormick and Bal 1997). The midline intralaminar nuclei are activated during attention-demanding tasks (Kinomura et al. 1996). The midline intralaminar thalamic nuclei have important roles in the regulation of awareness (Van der Werf et al. 2002). Unlike other midline and intralaminar nuclei that project to other nearby regions of the cortex, PVT has a unique and very strong reciprocal connection with the mPFC (Berendse and Groenewegen 1991; Hurley et al. 1991; Katz and Robertson 1981), particularly limbic and infralimbic regions, and also sends a strong output to the medial nucleus accumbens and amygdala, which also projects to the mPFC (Moga et al. 1995; Su and Bentivoglio 1990). Because the mPFC, nucleus accumbens, and amygdala are associated with the modulation of visceral/limbic function (Cardinal et al. 2002; Christakou et al. 2004; Price 1999), Hcrt may enhance viscerosensory awareness or motivational arousal through activation of PVT neurons. Hcrt is important in sleep and arousal (Sakurai 2002; Sutcliffe and de Lecea 2002). Of interest is the question of the role of dual regulation of the mPFC by hypothalamic Hcrt neurons, with a direct innervation by Hcrt axons (Fadel and Deutch 2002; Peyron et al. 1998), coupled with an indirect regulation acting through an excitatory thalamic relay that receives a very strong projection from hypothalamic Hcrt axons. Hcrt is reported to act very selectively on layer VI b neurons in the cortex, with neurons in other cortical layers showing little direct response to Hcrt (Bayer et al. 2004), consistent with selective expression of Hcrt receptors in the same restricted region of the deep cortex (Marcus et al. 2001). In contrast, glutamate released by PVT afferents would activate a much broader group of cells, as suggested by the nonspecific innervation pattern of excitatory thalamic efferents to the mPFC that would stimulate more cortical layers and that were previously reported to terminate most strongly in layers I, V, and VI (Berendse and Groenewegen 1991). The strong vGlut2 immunolabeling of multiple layers of the mPFC, coupled with the retrograde transport of dye from mPFC back to vGlut2-positive PVT neurons in this study, is consistent with this. Hcrt could directly activate layer VI b neurons and could also potentially antidromically fire thalamic neurons by actions on thalamic axon input to layer V cells (Lambe and Aghajanian 2003). Antidromic activation of thalamic neurons could facil-
ity of thalamic bursting activity (Gutnick and Prince 1972; McCormick and Contreras 2001; Pinault 1995).

The excitatory actions of Hcrt on cortically projecting excitatory cells of the PVT could act as a feedforward circuit to enhance cognitive arousal, with the substantial facilitative and depressive inputs from other brain regions also modulating PVT activity. Although we found a strong postsynaptic action, we found no demonstrable presynaptic action of Hcrt on GABA or glutamate axons in the PVT; it is, however, possible that Hcrt may presynaptically enhance release of other neuromodulators from axons terminating in the PVT, further altering the activity of PVT neurons.

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