

# Presynaptic and Postsynaptic Actions and Modulation of Neuroendocrine Neurons by a New Hypothalamic Peptide, Hypocretin/Orexin

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A new orexigenic peptide called hypocretin (orexin) has recently been described in neurons of the lateral hypothalamus and perifornical area. The medial and lateral hypothalamus have been loosely called satiety and feeding centers of the brain, respectively. Approximately one-third of all medial and lateral hypothalamic neurons tested, but not hippocampal neurons, show a striking nanomolar sensitivity to hypocretin. As studied with calcium digital imaging with fura-2, hypocretin raises cytoplasmic calcium via a mechanism based on G-protein enhancement of calcium influx through plasma membrane channels. The peptide has a potent effect at both presynaptic and postsynaptic receptors. Most synaptic activity in hypothalamic circuits is attributable to axonal release of GABA or glutamate. With whole-cell patch-clamp recording, we show that hypocretin, acting directly at axon terminals, can increase the release of

each of these amino acid transmitters. Two hypocretin peptides, hypocretin-1 and hypocretin-2, are coded by a single gene; neurons that respond to one peptide also respond to the other. In addition to its effect on feeding, we find that this peptide also regulates the synaptic activity of physiologically identified neuroendocrine neurons studied in hypothalamic slices containing the arcuate nucleus, suggesting a second function of hypocretin in hormone regulation. The widespread distribution of hypocretin axons, coupled with the strong response to the peptide at both presynaptic and postsynaptic sites, suggests that the peptide probably modulates a variety of hypothalamic regulatory systems and could regulate the axonal input to these regions presynaptically.

**Key words:** hypothalamus; presynaptic; neuroendocrine; neuromodulation; neuropeptide; glutamate; GABA; feeding

The hypothalamus has been called the control center of the brain for homeostasis, and neurons here regulate a substantial part of the internal milieu. Although the medial hypothalamus has been a fertile ground for the discovery of many neuroactive peptides, the lateral hypothalamus (LH) has been relatively barren. We recently described a new peptide, hypocretin, which shows robust expression in a restricted subset of lateral hypothalamic and perifornical neurons, with little apparent expression in neurons outside the hypothalamus (de Lecea et al., 1998). Axons from these cells show a strong innervation of the hypothalamus and ramify widely throughout the brain, including in areas that also have wide axonal distributions, such as the septal nuclei, preoptic area, central gray, and locus ceruleus. These data suggest that the cells that synthesize the peptide may exert a strong modulatory action on many different brain functions.

Subsequent to our description of hypocretin, a different group independently described the same peptide, which they called orexin, and found that it increased feeding in rats and that the peptide mRNA was upregulated in hungry animals (Sakurai et al., 1998). The hypocretin gene maps to a presumptive locus at chromosome 17q21 (de Lecea et al., 1998; Sakurai et al., 1998), a

site that has been implicated in severe human neurodegenerative disorders (Wilhelmsen et al., 1994; Wijkers et al., 1996), raising the possibility that a hypocretin deficit may contribute to this problem. Despite the excitement this peptide is generating on many fronts, there previously has been no characterization of its physiological actions on neurons and no assessment of neuroendocrine involvement. These are the focus of the present paper.

## MATERIALS AND METHODS

**Whole-cell recording in culture.** Whole-cell patch-clamp experiments were performed in synaptically coupled medial hypothalamic neurons cultured for 10–21 d *in vitro* as described previously (Gao et al., 1998). The bath solution contained (in mM): 150 NaCl, 2.5 KCl, 2 CaCl<sub>2</sub>, 10 HEPES, and 10 glucose, pH 7.3 with NaOH. Whole-cell voltage clamp was used to observe spontaneous and miniature postsynaptic currents at –60 mV with a List (HEKA Elektronik) EPC-7 amplifier. Patch pipettes had a tip resistance of 4–6 MΩ when filled with pipette solution, which contained (in mM): 145 KCl, 1 MgCl<sub>2</sub>, 10 HEPES, 1.1 EGTA, 4 Mg-ATP, and 0.5 Na<sub>2</sub>-GTP, pH 7.3 with KOH. Only cells with an input resistance >0.8 GΩ were used. The series resistance was <30 MΩ and was partially compensated by the amplifier. All data were sampled at 3–10 kHz and filtered at 1–3 kHz with an Apple Macintosh computer using AxoData software (Axon Instruments). Data were analyzed with Axograph 3.5 (Axon Instruments), plotted with Igor Pro (WaveMetrics, Lake Oswego, OR), and reported as the mean ± SEM. Student's *t* test was used to compare two groups of data.

Two active peptides are cleaved from preprohypocretin, hypocretin-1 and hypocretin-2 (also called orexin A and orexin B, respectively). All whole-cell recordings and most of the digital imaging studies were done with the 27 amino acid peptide hypocretin-2, PGPPGLQGRLLQANGNHAAGILTM-NH<sub>2</sub>, made in-house. Hypocretin-1, using the structure described for orexin A (Phoenix Pharmaceuticals) (Sakurai et al., 1998), was used in some digital imaging experiments.

**Slice recording.** Arcuate nucleus (ARC) frontal slices, 350–400 μm

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thick, were obtained from 3-week-old Sprague Dawley rats. Slices were incubated in an interface chamber with constant flow (4 ml/min) of the oxygenated artificial CSF (ACSF) containing (in mM): 124 NaCl, 3.0 KCl, 2.0 CaCl<sub>2</sub>, 2.0 MgCl<sub>2</sub>, 1.23 NaH<sub>2</sub>PO<sub>4</sub>, 26 NaHCO<sub>3</sub>, 10 glucose, 0.1 D,L-2-amino-5-phosphonovalerate (AP-5), and 0.01 6-cyano-7-nitroquinoxaline-2,3-dione (CNQX), pH 7.4, at room temperature. Whole-cell patch-clamp recordings were made with an Axoclamp-2B amplifier (Axon Instruments). Glass pipettes were filled with: 145 mM potassium methylsulfate, 2 mM MgCl<sub>2</sub>, 0.1 mM CaCl<sub>2</sub>, 1.1 mM EGTA, 10 mM HEPES, 2 mM Na-ATP, 0.3 mM Na-GTP, pH 7.2, 290 mOsm, and 5–7 MΩ resistance. Hypocretin (30 μM) was dissolved in ACSF and applied to ARC slices using the microdrop technique (Christian and Dudek, 1988; Belousov and van den Pol, 1997). A small drop was pressure ejected from the hypocretin-containing pipette (7–10 μm tip diameter) onto the area of the recorded cell. To stimulate axons that innervated the recorded ARC neuron, a bipolar electrode was placed in the dorsal or ventrolateral part of the ARC. Electrical impulses (0.2 msec, 0.5 Hz, 40–250 μA) were delivered through an isolation unit from a Grass 44 stimulator. In some slices, a bipolar electrode was also placed on the median eminence to generate antidromic responses in ARC neurons, identifying them as neuroendocrine cells (stimulating current, 50–70 μA). In all slice experiments, data were monitored and stored on a Macintosh Quadra 800 computer using AxoData 1.2 with the subsequent off-line analysis by AxoGraph 3.0 (Axon Instruments) or Igor Pro (WaveMetrics). Recordings were made at 2000 Hz frequency acquisition.

**Immunocytochemistry.** Hypocretin-2 was conjugated with glutaraldehyde to keyhole limpet hemocyanin, dialyzed in phosphate buffer, mixed with complete Freund's adjuvant, and injected subcutaneously and intradermally in three rabbits. Subsequent boosts were made from the hypocretin conjugate mixed with incomplete Freund's adjuvant. Use of rabbits for this purpose was approved by the Yale University Committee on Animal Use. Each of the rabbits made antiserum against the peptide and stained the same cells in the LH. Rats were deeply anesthetized and perfused transcardially with 4% paraformaldehyde. Brains were removed, cut into 20–50-μm-thick sections, and stained with immunoperoxidase as described previously (van den Pol, 1985). Adsorption of the antigen with the antibody or elimination of the primary antiserum blocks immunostaining.

**Fura-2 imaging.** Neurons were loaded with fura-2 AM (5 μM; Molecular Probes, Eugene, OR) and studied on a Nikon inverted microscope fitted with a 40× Olympus UV objective with high 340 nm light transmittance (van den Pol et al., 1996a,b). Fluor software (Universal Imaging Corporation, West Chester, PA) was used to control a Lambda 10 Sutter filter wheel with 340 and 380 nm filters and a 150 W xenon light source. Calcium standards from Molecular Probes were used to calibrate the system according to the equation of Grynkiewicz et al. (1985). Cells were imaged while in a linear-flow chamber, allowing a complete change of solution in 5 sec (Forscher et al., 1987).

## RESULTS

### Hypocretin modulates glutamatergic and GABAergic signaling in cultured hypothalamic neurons

In our previous studies of hypothalamic neuromodulators, which included neuropeptide Y, adenosine, metabotropic glutamate receptor, and GABA<sub>B</sub> receptor agonists, we found primarily inhibitory actions (Obrietan et al., 1995; Chen and van den Pol, 1996, 1998; van den Pol et al., 1996b; Obrietan and van den Pol, 1997). In striking contrast, hypocretin increases synaptic activity in hypothalamic cultures. Almost all fast synaptic activity in the hypothalamus is generated by release of either the excitatory transmitter glutamate (van den Pol et al., 1990) or the inhibitory transmitter GABA (Randle and Renaud, 1987; Decavel and van den Pol, 1990; Renaud and Bourque, 1991). In some parts of the brain, peptide modulation is restricted to one or another of the amino acid transmitters, as in the hippocampus, in which neuropeptide Y decreases glutamate release but has little effect on GABA (Bleakman et al., 1992). For this reason, we studied GABA or glutamate activity selectively in neurons cultured from the medial hypothalamus, a brain region that has been suggested as a satiety center. To determine whether hypocretin responses would be found in

GABAergic neurons, we blocked glutamate actions with AP-5 (100 μM) and CNQX (10 μM) and studied spontaneous IPSCs (sIPSCs) in voltage-clamped neurons. Hypocretin caused an increase in the frequency of IPSCs in three of five cells (mean increase, 56%; range, 36–79%; paired *t* test, *p* < 0.05; *n* = 5) (Fig. 1*A,D*), measured 1 min after application of the peptide. Bicuculline (20 μM) blocked the IPSCs (data not shown). In parallel, we tested the effect on glutamatergic neurons in the presence of the GABA<sub>A</sub> antagonist bicuculline (20 μM) and found that hypocretin increased the frequency of spontaneous EPSCs (sEPSCs) in four of six neurons by 83% (range, 35–130%; *p* < 0.05; *n* = 5) (Fig. 1*B,C*). Glutamate receptor antagonists AP-5 (100 μM) and CNQX (10 μM) blocked the EPSCs (data not shown). These data indicate that both glutamate- and GABA-releasing neurons, which account for almost all fast synaptic activity, express hypocretin receptors.

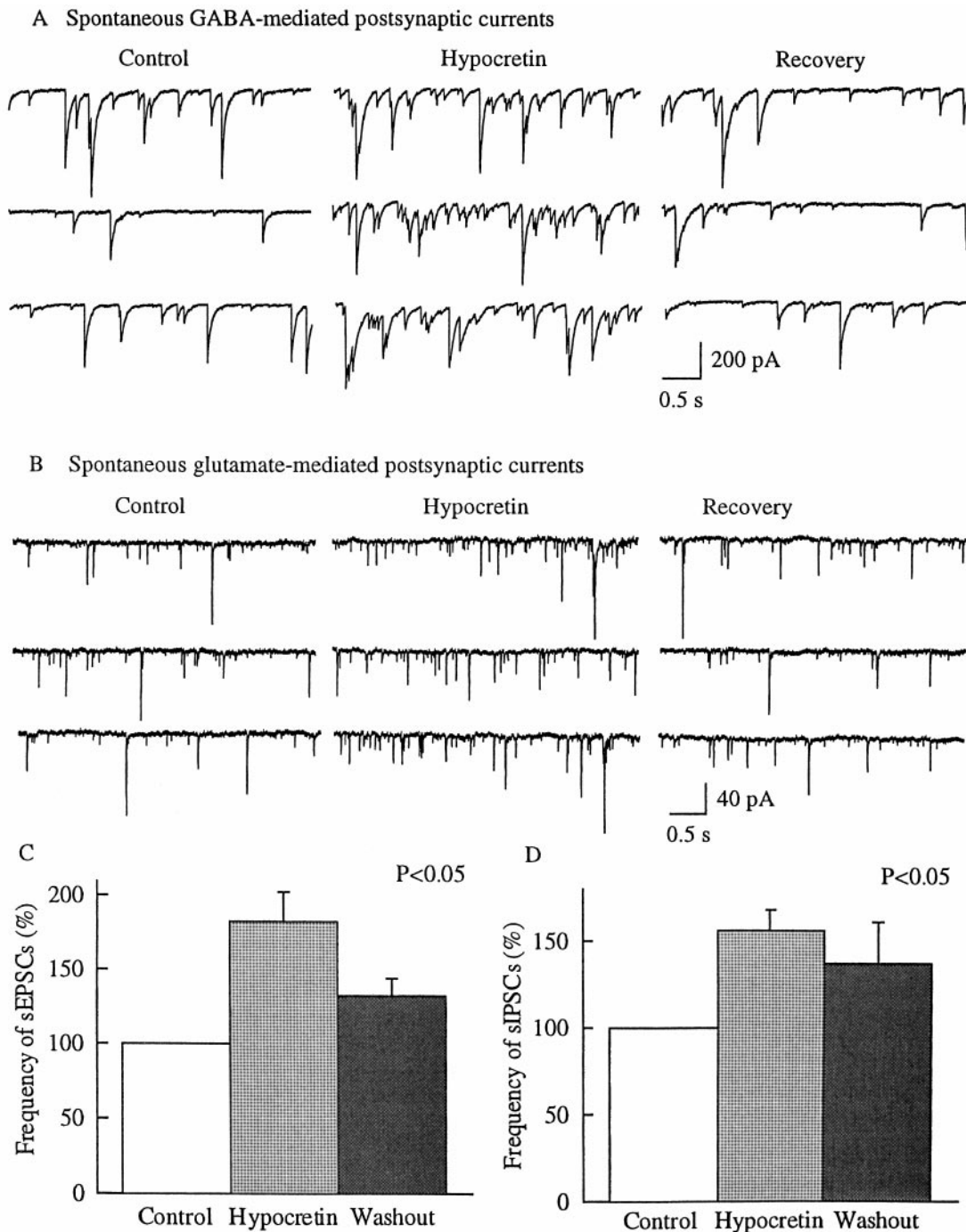
### Hypocretin enhances evoked IPSP in ARC slices

A high density of immunoreactive axons was found in the lateral and medial hypothalamus (Fig. 2*A–D*); many of the fibers had large endings suggestive of presynaptic boutons. Many terminals were found in the ARC (Fig. 2*C,D*) but not in the median eminence (Fig. 2*E*). We therefore tested the hypothesis that hypocretin regulates the activity of neuroendocrine neurons in hypothalamic slices containing the ARC. A bipolar electrode was used to stimulate the lateral edge of the ARC (Fig. 3*A*). Hypocretin was applied by microdrop as described in detail elsewhere (Christian and Dudek, 1988; Belousov and van den Pol, 1997). Six of seven current-clamped neurons showed an increase in the amplitude of the evoked IPSP in response to microdrop application of hypocretin, with a mean increase of 48% from 7.1 ± 0.9 mV to 10.6 ± 1.5 mV (range, 40–61%) based on the responding cells (Fig. 3*B*). This was a significant increase in the evoked IPSP (paired *t* test, *p* < 0.003). No cells showed a decrease in the IPSP.

Neuroendocrine neurons that secrete factors that control the anterior pituitary gland send efferent axons to the median eminence in which the factors are released from axon terminals. Antidromic stimulation of axons in the median eminence was used to detect neurosecretory neurons of the ARC. Neurons (*n* = 3) that were antidromically identified showed an increase in the amplitude of the IPSP in the presence of hypocretin. To ensure that the response to stimulation of the median eminence was attributable to antidromic activation of ARC neurons, at the end of the experiments, control bath application of Co<sup>2+</sup> was used to block orthodromic activation of recurrent collaterals (Fig. 3*C*). These data indicate that hypocretin modulates the afferent input that controls putative neuroendocrine neurons.

### Calcium response to hypocretin

Electrical stimulation of the LH leads to increased feeding (Miller, 1960); hypocretin, made in LH cell bodies, also increases feeding (Sakurai et al., 1998). Lesions of the LH cause a long-lasting reduction in body weight and food intake in both male and female rodents (Powley and Keesey, 1970; van den Pol, 1982). In addition to the cell bodies (Gautvik et al., 1996; de Lecea et al., 1998; Sakurai et al., 1998) (Fig. 2*A,B*), large numbers of hypocretin immunoreactive axons and their terminals are found in the LH (Fig. 2*A,B*), suggesting that hypocretin could have a direct effect on neurons in the LH. In our past work on hypothalamic cultures, calcium digital imaging with fura-2 proved to be sensitive in detecting responses to both transmitters and a number of peptide and nonpeptide neuromodulators (Obrietan et al., 1995; van den Pol et al., 1996a,b). Because hypocretin immunoreactive

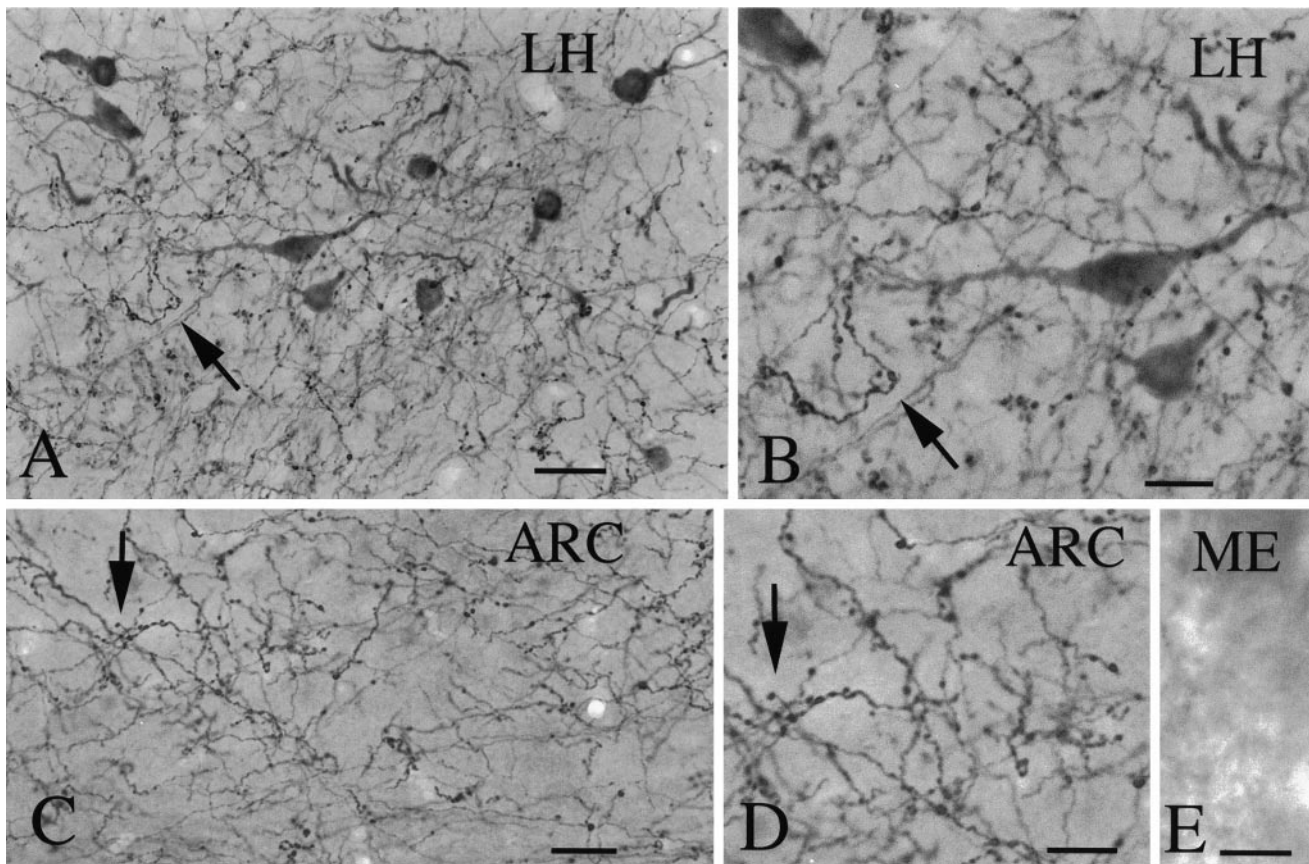


**Figure 1.** Both excitatory and inhibitory neurons express hypocretin receptors. To determine the transmitter identity of neurons that respond to hypocretin, different transmitter antagonists were used, and responses to hypocretin were studied with whole-cell recording in voltage-clamped cells held at  $-60$  mV. *A*, In the presence of glutamate receptor antagonists AP-5 ( $100 \mu\text{M}$ ) and CNQX ( $10 \mu\text{M}$ ), only sIPSPs were found, and these were completely blocked with the GABA<sub>A</sub> antagonist bicuculline ( $20 \mu\text{M}$ ) (data not shown). When hypocretin ( $1 \mu\text{M}$ ) was bath-applied, the frequency of sIPSCs increased dramatically, and after peptide washout, the frequency of sIPSCs decreased to its normal baseline. *B*, In the presence of the GABA<sub>A</sub> antagonist bicuculline ( $20 \mu\text{M}$ ), sEPSCs were found, and these were completely blocked by AP-5 ( $100 \mu\text{M}$ ) and CNQX ( $10 \mu\text{M}$ ) (data not shown). Hypocretin ( $1 \mu\text{M}$ ) increased the frequency of sEPSCs, and these returned to normal after peptide washout. Both GABA and glutamate evoked inward currents (downward deflection) attributable to the composition of the pipette solution. *C*, *D*, Mean increase in sEPSC and sIPSCs evoked by hypocretin and the return toward control levels after peptide washout.

axons terminate in both lateral (Fig. 2*B*) and medial (Fig. 2*D*) hypothalamus, we tested the hypothesis that each of these areas would express hypocretin receptors that alter cytoplasmic calcium. Both lateral (23% of 180 neurons) (Fig. 4*A*) and medial (Fig. 4*B*) hypothalamic neurons showed substantial  $\text{Ca}^{2+}$  rises in

response to hypocretin ( $1 \mu\text{M}$ ) stimulation. In 1260 medial hypothalamic neurons from 21 experiments, a mean of 29.7% of the neurons showed a response to  $1 \mu\text{M}$  hypocretin, using a criterion  $\text{Ca}^{2+}$  rise of at least 20 nM. The mean ( $\pm$  SE)  $\text{Ca}^{2+}$  rise was  $106 \pm 7$  nM, with a range of 20–930 nM.





**Figure 2.** Hypocretin immunoreactivity. *A*, Immunocytochemical staining revealed high densities of axons in both the lateral hypothalamus (*A*, *B*, *LH*) and arcuate nucleus (*C*, *D*, *ARC*) of the hypothalamus. Around immunoreactive cells in the *LH*, a high density of immunoreactive axons was found. *E*, Immunoreactive axons were not found in the external zone of the median eminence (*ME*), in which neurosecretory axons release pituitary tropins that are carried to the anterior pituitary by the portal blood system. Scale bars: *A*, 25  $\mu\text{m}$ ; *B*, 12  $\mu\text{m}$ ; *C*, 25  $\mu\text{m}$ ; *D*, *E*, 12  $\mu\text{m}$ .

As a control, a 17 amino acid peptide based on amino acids 1–17 of the C terminal of preprohypocretin was also tested. No response to the 1–17 amino acid sequence was found in 194 cells, and no response was found in 29 neurons that responded to hypocretin (Fig. 4*B*). Preprohypocretin is cleaved to form two peptides, hypocretin-1 and hypocretin-2. The actions of hypocretin-1 have not been examined previously in single neurons. In three experiments, all 28 neurons that showed a response to one peptide also responded to the other with similar amplitude  $\text{Ca}^{2+}$  rises (Fig. 4*C*). Thus, most hypothalamic neurons express receptors that are sensitive to both hypocretin-1 and hypocretin-2. Neurons responded in a dose-dependent manner (Fig. 4*D*) to  $\geq 5$  nM hypocretin.

Hypocretin could generate a  $\text{Ca}^{2+}$  rise by increasing the release of an excitatory transmitter or by increasing the frequency of action potentials. To test these hypotheses, we examined the effect of hypocretin in the presence of tetrodotoxin (TTX) (1  $\mu\text{M}$ ). Even in the presence of TTX, hypocretin evoked a  $\text{Ca}^{2+}$  rise of at least 30 nM in 27% of 248 neurons (Fig. 5*A*). In the responding cells, hypocretin evoked a mean rise of  $120 \pm 16$  nM  $\text{Ca}^{2+}$ . These results support the concept that functional hypocretin receptors are found on or near the cell body of approximately one-third of hypothalamic neurons and that the  $\text{Ca}^{2+}$  rise is not dependent on synaptic modulation or action potentials.

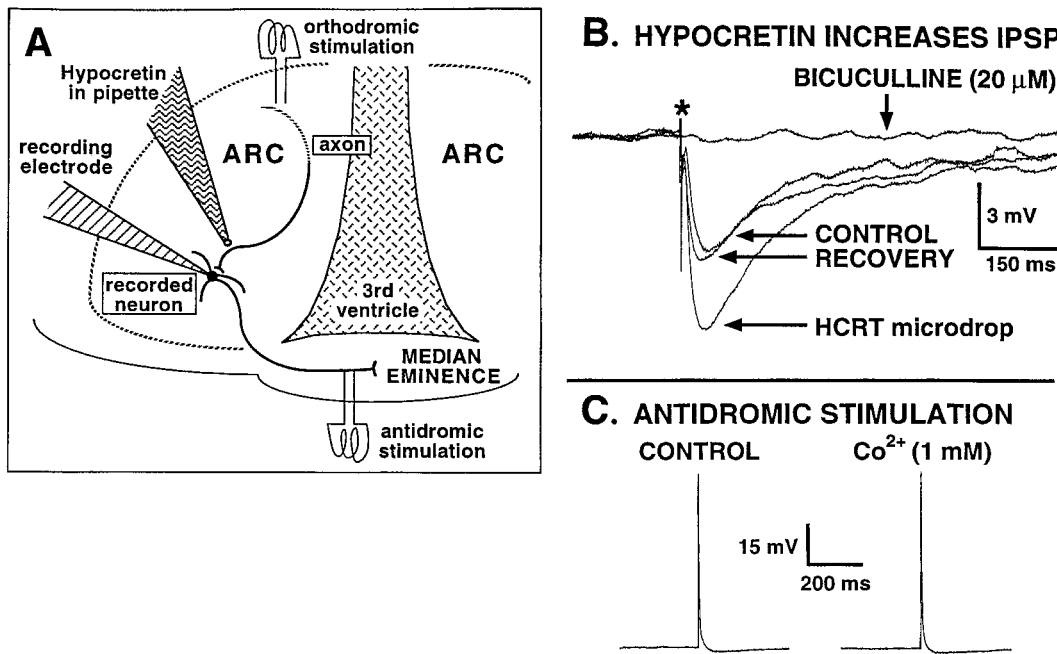
A hypocretin-evoked increase in cytoplasmic  $\text{Ca}^{2+}$  could result from release from intracellular stores or could come from outside the cell via  $\text{Ca}^{2+}$  channels in the plasma membrane. To test

whether the hypocretin-mediated increase in  $\text{Ca}^{2+}$  is dependent on intracellular stores, we depleted the intracellular stores with thapsigargin (2  $\mu\text{M}$ ). In 24 neurons that responded to the peptide, hypocretin induced a  $113 \pm 15$  nM  $\text{Ca}^{2+}$  rise; 45 min after thapsigargin treatment, hypocretin evoked virtually the same amplitude  $\text{Ca}^{2+}$  rise in these cells ( $110 \pm 12$  nM). This absence of a thapsigargin effect (Fig. 5*B*) suggests that hypocretin does not act primarily by a mechanism dependent on release of  $\text{Ca}^{2+}$  from intracellular stores.

The thapsigargin experiments suggest that hypocretin acts by increasing  $\text{Ca}^{2+}$  influx from outside the cell. To test this hypothesis further, we used a zero  $\text{Ca}^{2+}$  buffer with 1 mM EGTA. In normal  $\text{Ca}^{2+}$ -containing HEPES buffer, hypocretin evoked a  $130 \pm 12$  nM  $\text{Ca}^{2+}$  rise in 38 neurons. In contrast, in the  $\text{Ca}^{2+}$ -free buffer, hypocretin evoked a negligible  $\text{Ca}^{2+}$  rise of  $7 \pm 3$  nM in the same neurons (Fig. 5*C*). In parallel, adding  $\text{Cd}^{2+}$  to the buffer blocked any hypocretin-evoked  $\text{Ca}^{2+}$  rise (Fig. 5*D*). These data support the view that hypocretin raises intracellular  $\text{Ca}^{2+}$  by opening plasma membrane  $\text{Ca}^{2+}$  channels that allow  $\text{Ca}^{2+}$  entry from extracellular space.

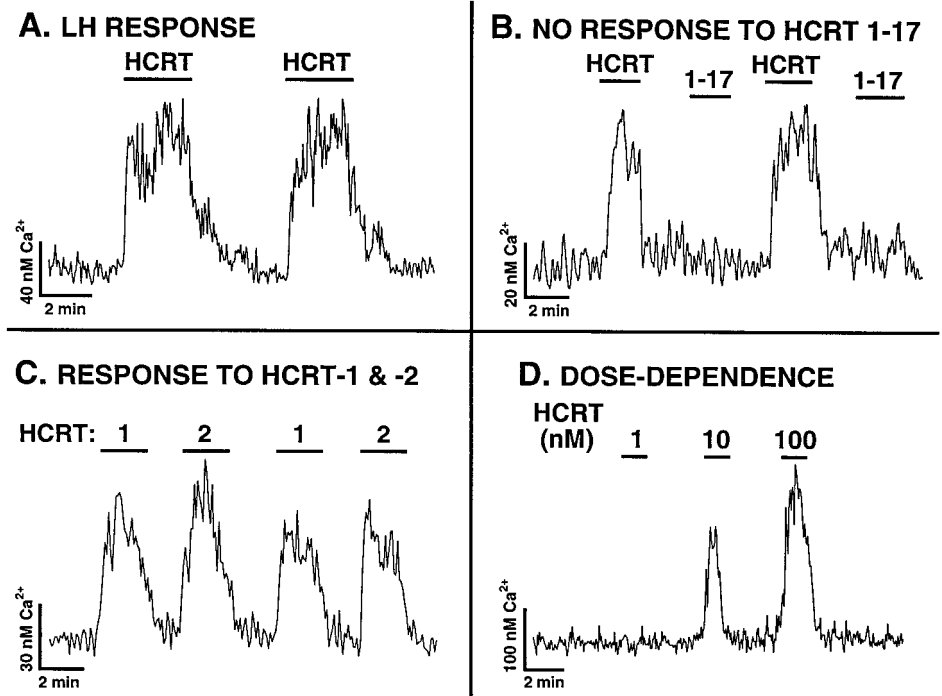
### Intracellular signaling

Next, we assessed possible intracellular signaling pathways that mediate the excitatory effects of hypocretin. cAMP levels in [ $^3\text{H}$ ]adenine-labeled cells were measured by determining the ratio of [ $^3\text{H}$ ]ATP, [ $^3\text{H}$ ]ADP and [ $^3\text{H}$ ]AMP to [ $^3\text{H}$ ]cAMP on cultures done in triplicate (Wong et al., 1991). Hypocretin (10 nM to 10  $\mu\text{M}$ )



**Figure 3.** Hypothalamic slice electrophysiology. *A*, Experimental paradigm for recording hypothalamic slices that included the arcuate nucleus (ARC). *B*, In the presence of AP-5 (100  $\mu$ M) and CNQX (10  $\mu$ M), IPSPs were evoked by orthodromic electrical stimulation (asterisk), as shown in *A*. The IPSP was blocked in the presence of bicuculline (20  $\mu$ M), indicating that it was attributable to GABA release. Hypocretin (HCRt) applied by microdrop (30  $\mu$ M) caused a substantial increase in the amplitude of the IPSP. This recovered to baseline levels after washout of the peptide. Each trace is the mean of eight sweeps. *C*, After testing hypocretin in *B*, the axon of the same cell was antidromically stimulated from the median eminence. That median eminence stimulation evoked an action potential even in the presence of control application of  $\text{Co}^{2+}$  (1 mM) indicated that the action potential response was not attributable to recurrent collaterals releasing transmitters on the recorded cell. A fixed latency of 3–5 mS between stimulus artifact and antidromic spike was routinely found. These data indicate that hypocretin enhances the action of transmitters released onto identified neuroendocrine neurons.

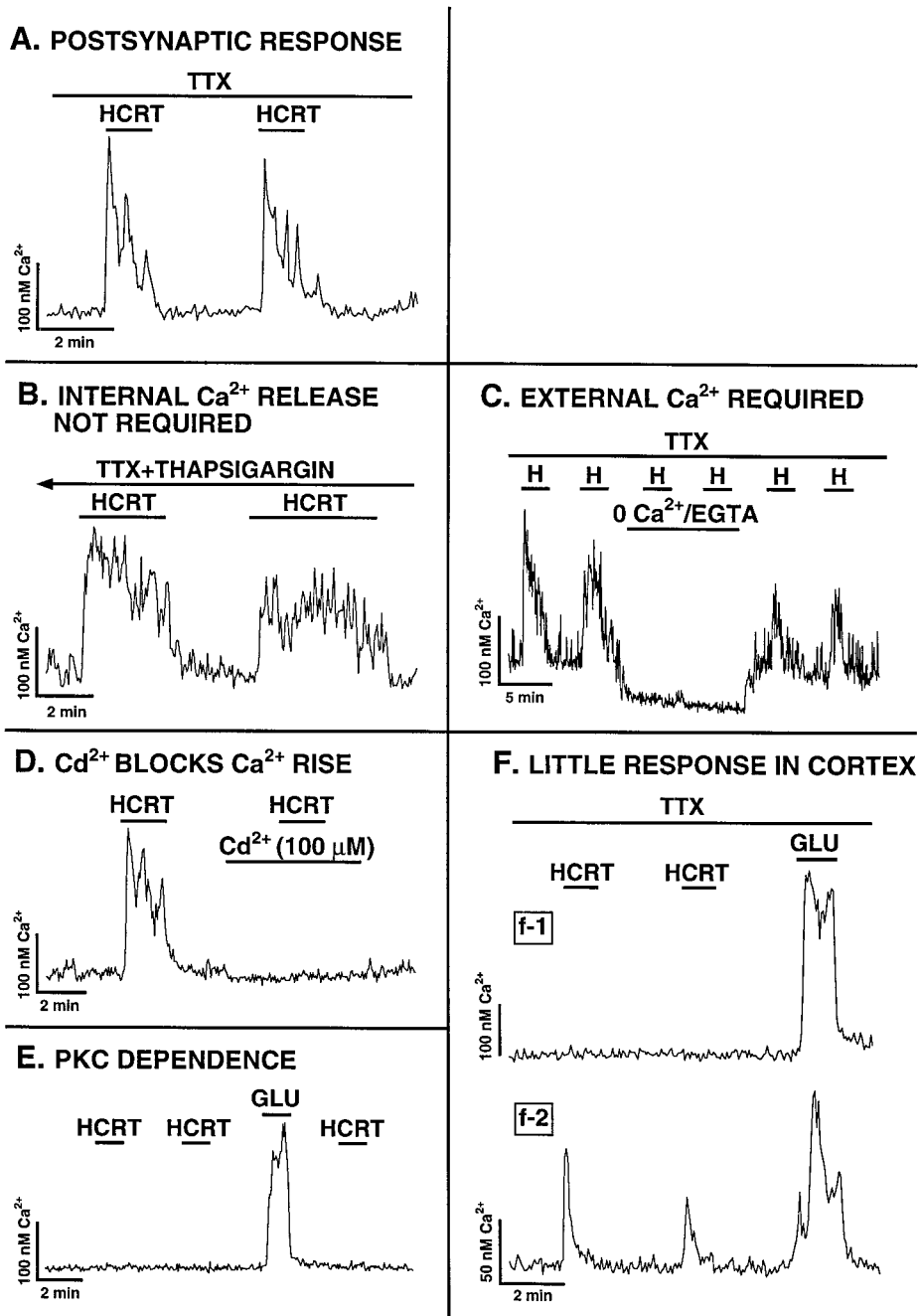
**Figure 4.** Calcium responses to hypocretin. *A*, Based on fura-2 calcium recordings, neurons from the lateral hypothalamus (LH), the site of the hypocretin immunoreactive cell bodies, showed strong responses to hypocretin (HCRt) (1  $\mu$ M) and clear recovery after two bath applications of the peptide. Horizontal lines above  $\text{Ca}^{2+}$  trace indicate time of drug application. Except for Figure 3C, all other experiments used hypocretin-2. *B*, Medial hypothalamus cells also showed strong  $\text{Ca}^{2+}$  elevations in response to hypocretin but no response to the C-terminal 1–17 (1–17) peptide of preprohypocretin (1  $\mu$ M). *C*, Hypocretin-1 (HCRt: 1), using the 33 amino acid structure with the double disulfide bonds found for orexin A, and hypocretin-2 (HCRt: 2) were compared. Each evoked a  $\text{Ca}^{2+}$  rise in this typical medial hypothalamic neuron. *D*, Hypothalamic neuron that showed an increasing peak response to increasing concentrations of the peptide, with no response to 1 nM hypocretin, a response to 10 nM hypocretin, and a bigger response to 100 nM hypocretin.



did not increase cAMP levels in primary cultures of hypothalamic neurons. In sister cultures, a  $12 \pm 0.82$  (mean  $\pm$  SE) and 25.4  $\pm$  3.6-fold increase was triggered by vasoactive intestinal peptide (VIP) (1  $\mu$ M) and forskolin (1  $\mu$ M), respectively. The VIP-evoked cAMP rise was not affected by the coadministration of hypocretin

(data not shown). These results suggest that the hypocretin receptor does not act via heteromeric  $G_i$  or  $G_s$  proteins.

Another possibility is that the hypocretin receptor couples to a  $G_q$  protein, as suggested in transfected non-neuronal cells (Sakurai et al., 1998). Activation of  $G_q$  triggers the stimulation of



**Figure 5.** Mechanisms of hypocretin elevation of calcium. *A*, To demonstrate that hypocretin can evoke Ca<sup>2+</sup> elevations directly at the cell body rather than by altering synaptic interactions, hypocretin (1 μM) was added in the presence of tetrodotoxin (TTX) (1 μM), used to block action potential-mediated release of transmitters. *B*, Depleting intracellular stores of Ca<sup>2+</sup> with thapsigargin (2 μM) pretreatment did not affect the action of hypocretin in elevating Ca<sup>2+</sup> in the presence of 1 μM TTX. *C*, Eliminating extracellular Ca<sup>2+</sup> and adding the Ca<sup>2+</sup> chelator EGTA (1 mM) completely blocked the Ca<sup>2+</sup> rise evoked by 1 μM hypocretin (HCRT). When bath Ca<sup>2+</sup> levels were returned to normal, the action of hypocretin returned. *D*, Cd<sup>2+</sup> (100 μM) blocked the Ca<sup>2+</sup> rise, indicating an extracellular origin of Ca<sup>2+</sup>. *E*, Pretreatment of hypothalamic neurons with bisindolylmaleide (1 μM for 12 hr), a PKC inhibitor, completely blocked the actions of hypocretin (1 μM). Glutamate (GLU) (10 μM), applied as a control, evoked a large Ca<sup>2+</sup> rise. *F*, Most cortical cells showed no response to hypocretin, as shown by the typical cell in *f-1*. A small percent of cortical cells did show a small Ca<sup>2+</sup> rise in response to hypocretin, as shown in *f-2*. A control application of glutamate (GLU) (10 μM) evoked a Ca<sup>2+</sup> rise.

phospholipase C, which, via the hydrolysis of phosphatidylinositol and the generation of inositol 1,4,5-trisphosphate and diacylglycerol, can activate protein kinase C (PKC) (Exton, 1994). To test whether hypocretin triggers PKC activation, hypothalamic neurons were pretreated with the PKC-specific inhibitor bisindolylmaleide (1 μM). Bisindolylmaleide completely blocked hypocretin responses (Fig. 5*E*), and no hypocretin-mediated Ca<sup>2+</sup> rise was found in 128 neurons. These results suggest that hypocretin may work via G<sub>q</sub>-mediated PKC, resulting in phosphorylation of Ca<sup>2+</sup> channels that has been reported to increase Ca<sup>2+</sup> conductance (Yang and Tsien, 1993; Stea et al., 1995).

#### Small cortical response

Hypocretin immunoreactive axons are found in the cortex but in small numbers, suggesting that a minor response to the peptide

might be found in cortical neurons. Consistent with this hypothesis, only 5% of 122 cultured cortical neurons showed a hypocretin-evoked (1 μM) Ca<sup>2+</sup> rise >30 nM (Fig. 5*F*). In the six responding cells, the mean Ca<sup>2+</sup> rise was 65 ± 6 nM Ca<sup>2+</sup>, approximately half the amplitude of that found in hypothalamic neurons. No Ca<sup>2+</sup> responses were found in 128 hippocampal neurons, and in parallel, no effect of hypocretin (1 μM) on synaptic activity was found in synaptically coupled hippocampal neurons (*n* = 7).

#### Presynaptic actions of hypocretin

Although hypocretin evoked a substantial increase in synaptic activity, little direct effect on membrane potential was found in 28 neurons tested, even in cells in which increases in synaptic activity were detected. To test the hypothesis that hypocretin exerted an



effect directly on presynaptic axons to alter release of neurotransmitter, we examined the effect of the peptide on miniature postsynaptic currents (mPSCs) in the presence of TTX *in vitro*. To study modulation of GABA-mediated mIPSCs, glutamate receptors were blocked with AP-5 (100  $\mu\text{M}$ ) and CNQX (10  $\mu\text{M}$ ). The miniature events that were found in this buffer were caused by the release of GABA and were blocked by bicuculline (20  $\mu\text{M}$ ) (data not shown). In three of five neurons, hypocretin generated a substantial increase in the frequency of mIPSCs, with a mean increase of  $54.7 \pm 12.3\%$  (range, 30.2–67.4%; paired *t* test,  $p < 0.05$ ;  $n = 5$ ) (Fig. 6A,D). To determine whether hypocretin also might increase the release of glutamate-secreting cells, we did parallel experiments in TTX (1  $\mu\text{M}$ ) and a GABA receptor antagonist. Hypocretin caused an increase in the frequency of glutamate-mediated mEPSCs by  $48.2 \pm 9.7\%$  (range, 40.6–65.1%) in three of five synaptically coupled neurons ( $p < 0.05$ ;  $n = 5$ ) (Fig. 6B,C); AP-5 and CNQX blocked the mEPSCs (data not shown). We found no change in the amplitude of the mEPSC in cells that showed an increase in frequency (Fig. 6E), suggesting that the increase in frequency of mPSCs was mediated at a presynaptic site of hypocretin action.

## DISCUSSION

In the present set of experiments, we demonstrate that hypocretin increases synaptic activity of both glutamatergic and GABAergic neurons, and in part, this is attributable to actions at presynaptic receptors on GABA- and glutamate-secreting axons. In addition, we find that the afferent input to physiologically identified neuroendocrine neurons is modulated by hypocretin. Mechanistically, hypocretin elevates cytosolic calcium levels via plasmalemmal calcium channels. These actions are consistent with activation of a  $G_q$  protein acting via PKC to phosphorylate voltage-activated calcium channels.

### Neuroendocrine modulation

Most, if not all, fast synaptic activity in the hypothalamus appears to be mediated by GABA and glutamate release (van den Pol et al., 1990; Randle and Renaud, 1987; Renaud and Bourque, 1991; van den Pol and Trombley, 1993), and this is also true of the ARC (Belousov and van den Pol, 1997), an area of the hypothalamus that regulates the endocrine system. Immunocytochemical analysis of hypocretin axons indicates a strong innervation of the ARC. Our finding that hypocretin modulates the afferent input to physiologically identified neuroendocrine neurons in the hypothalamus suggests that hypocretin could modulate the final common neuronal pathway regulating the hormone system. The antidromically identified neuroendocrine neurons probably maintain axon terminals in the external zone of the median eminence but may also send axons to the neurohypophysis. Pituitary tropins are released into the pituitary portal system in the median eminence with a dependence on the electrical activity of the neuroendocrine neurons. That hypocretin-containing axons innervate the ARC and hypocretin enhances signaling to these neurons suggests that by influencing the electrical activity of ARC neurons, hypocretin could play a pivotal role in hormone regulation. A number of different pituitary tropins are contained in neurons of the ARC, and the identity of the specific neuroendocrine neurons that respond to hypocretin remains to be determined. Although many different transmitters and modulators are found in axon terminals in the external zone of the median eminence, the striking lack of hypocretin fibers here suggests

that hypocretin-containing cells probably do not directly control pituitary secretions but may indirectly participate in neuroendocrine regulation by modulating the activity of neurons that do directly control the pituitary.

Hypocretin immunoreactive axons are found not only in the ARC but also in other medial regions of the hypothalamus that may play a role in endocrine regulation and in other hypothalamic functions. Innervation of the LH is also found. In parallel, that both lateral and medial hypothalamic neurons respond to hypocretin suggests that hypocretin may play a role in the general modulation of a number of hypothalamic functions. This is consistent with the effects of hypocretin on hypothalamic neurons that contain either GABA or glutamate.

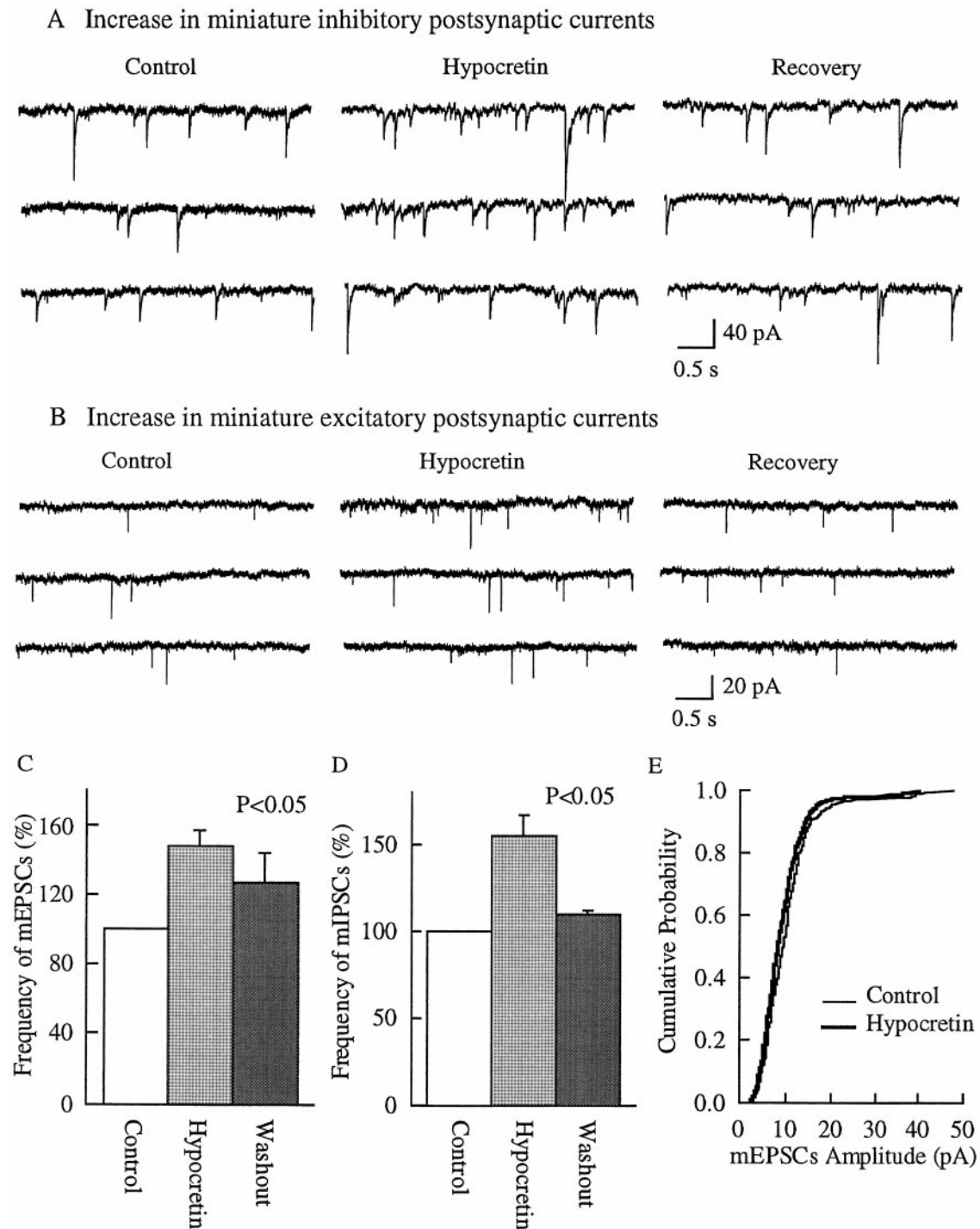
### Presynaptic actions and nonhypothalamic responses

That functional hypocretin receptors appear to exist on cell bodies and on presynaptic axons suggests that hypocretin could have a direct effect on cytosolic calcium at the cell body, and in addition, could modulate incoming axonal input from a wide variety of sources. It remains to be determined if extrahypothalamic projections to the hypothalamus express presynaptic hypocretin receptors on their axons. Because hypocretin can increase cytosolic calcium levels in neurons independent of action potentials, this suggests that hypocretin may enhance presynaptic transmitter release by a parallel mechanism of increasing calcium levels of the presynaptic bouton, which would enhance calcium-dependent transmitter release. Calcium increases in response to hypocretin have been described in non-neuronal cells transfected with hypocretin receptor cDNA (Sakurai et al., 1998). The lack of a direct effect of hypocretin on membrane potential is consistent with a neuro-modulator function of the peptide.

Although the focus in the present study is on hypothalamic actions of hypocretin, there are a small number of fibers that innervate the cerebral cortex, and consistent with that, we find that a small percentage of cortical cells tested show small responses to hypocretin. Because hypocretin axons are found in many brain regions, these data suggest that hypocretin receptors may also have a widespread distribution and that the receptor density may parallel the level of innervation. This remains to be substantiated in other brain regions and with other approaches, including *in situ* hybridization and immunostaining with hypocretin receptor antisera. That cells in some areas of the rat brain, for instance the hippocampus, appear unresponsive to hypocretin, suggests that functional hypocretin receptors are expressed in a limited population of CNS neurons.

### Hypocretin and energy homeostasis

In light of our data, the role of hypocretin in physiological homeostasis is not restricted to the enhancement of feeding (Sakurai et al., 1998) but is also involved in the regulation of the neuroendocrine system in the ARC, perhaps related to endocrine regulation of energy balances. ARC neurons not only synthesize and release neuropeptide Y, involved in feeding (Leibowitz, 1991; Stanley, 1993) and endocrine (McDonald and Koenig, 1993) regulation, but also express receptors for leptin (Hakansson et al., 1998), the signal that adipose tissue releases into the vascular system that reduces food intake and plays a key role in some forms of obesity (Zhang et al., 1994); falling leptin levels may initiate mechanisms for energy conservation (Flier and Maratos-Flier, 1998). Our finding that hypocretin regulates the



**Figure 6.** Presynaptic mechanism for hypocretin to enhance GABA or glutamate secretion. In the presence of TTX ( $1 \mu\text{M}$ ), mPSCs were studied with whole-cell voltage-clamp recording. *A*, In the presence of the glutamate receptor antagonists AP-5 ( $100 \mu\text{M}$ ) and CNQX ( $10 \mu\text{M}$ ), hypocretin ( $1 \mu\text{M}$ ) increased the frequency of mIPSCs. After washout of the peptide, the frequency of mIPSCs returned to baseline levels. *B*, An example of hypocretin ( $1 \mu\text{M}$ ) increasing the frequency of mEPSCs in the presence of bicuculline ( $20 \mu\text{M}$ ). *C, D*, Mean increase in mEPSCs and mIPSCs and the return toward baseline levels after peptide washout. *E*, Cumulative probability for mEPSC amplitude in the presence of hypocretin ( $1 \mu\text{M}$ ) or in its absence (control). Superimposition of the two lines indicates that although hypocretin increased the frequency of mEPSCs, it did not change the amplitude, suggesting that the peptide acted at a presynaptic site to enhance transmitter release.

synaptic input to these arcuate cells and that hypocretin-containing axons strongly innervate this area suggests the hypocretin is in a key position to influence neurons that are the hub for vascular signals from the periphery and afferent and efferent axonal signals involved in feeding. Feeding studies have demon-

strated that both GABA and glutamate can regulate hypothalamic control of food intake (Maldonado-Irizarry et al., 1995; Stanley et al., 1996), suggesting that one mode of hypocretin action based on the present paper is to modulate presynaptically GABA and glutamate circuits involved in energy regulation. The



fact that hypocretin can modulate neurons containing either the primary excitatory or primary inhibitory transmitter in the hypothalamus suggests that the local microcircuitry, particularly the proximity of hypocretin axons to other axons, may be critical in hypocretin function. This raises the general question of how to best interpret animal responses to mass injections of hypocretin into the hypothalamus or ventricular system that may activate circuits other than those that would be activated by discrete axonal release of the peptide.

That approximately one-third of all hypothalamic neurons express hypocretin receptors and that hypocretin axon terminals are found throughout the hypothalamus suggests that hypocretin could influence the general level of activity in many hypothalamic systems. Included are other recently identified medial and lateral hypothalamic systems involved in regulating energy balances, including those related to melanin-concentrating hormone, melanocyte-stimulating hormone, Agouti-related protein, (Mountjoy et al., 1994; Qu et al., 1996; Huszar et al., 1997; Ollmann et al., 1997), and neurons that respond to glucose (Oomura, 1983). These are found in the same hypothalamic regions as high densities of hypocretin axons, suggesting that many opportunities exist for hypocretin cells to not only directly affect postsynaptic neurons, but also, based on data presented here, to modulate the synaptic input to these areas at the axonal terminal. Thus, hypocretin may be an important link in the chain of regulatory cells that orchestrate behavioral, metabolic, and endocrine systems to maintain energy homeostasis.

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