Input organization and plasticity of hypocretin neurons: Possible clues to obesity's association with insomnia

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Summary

The lateral hypothalamic hypocretin (also called orexin) neurons have emerged as instrumental in triggering arousal and regulating energy metabolism. The lack of hypocretin signaling is the cause of narcolepsy while elevated hypocretin levels induce arousal, elevated food intake, and adiposity. Here, we report an unorthodox synaptic organization on the hypocretin neurons in which excitatory synaptic currents and asymmetric synapses exert control on the cell bodies of these long-projective neurons with minimal inhibitory input. Overnight food deprivation promotes the formation of more excitatory synapses and synaptic currents onto hypocretin cells; this is reversed by re-feeding and blocked by leptin administration. This unique wiring and acute stress-induced plasticity of the hypocretin neurons correlates well with their being involved in the control of arousal and alertness that are so vital to survival, but this circuitry may also be an underlying cause of insomnia and associated metabolic disturbances, including obesity.

Introduction

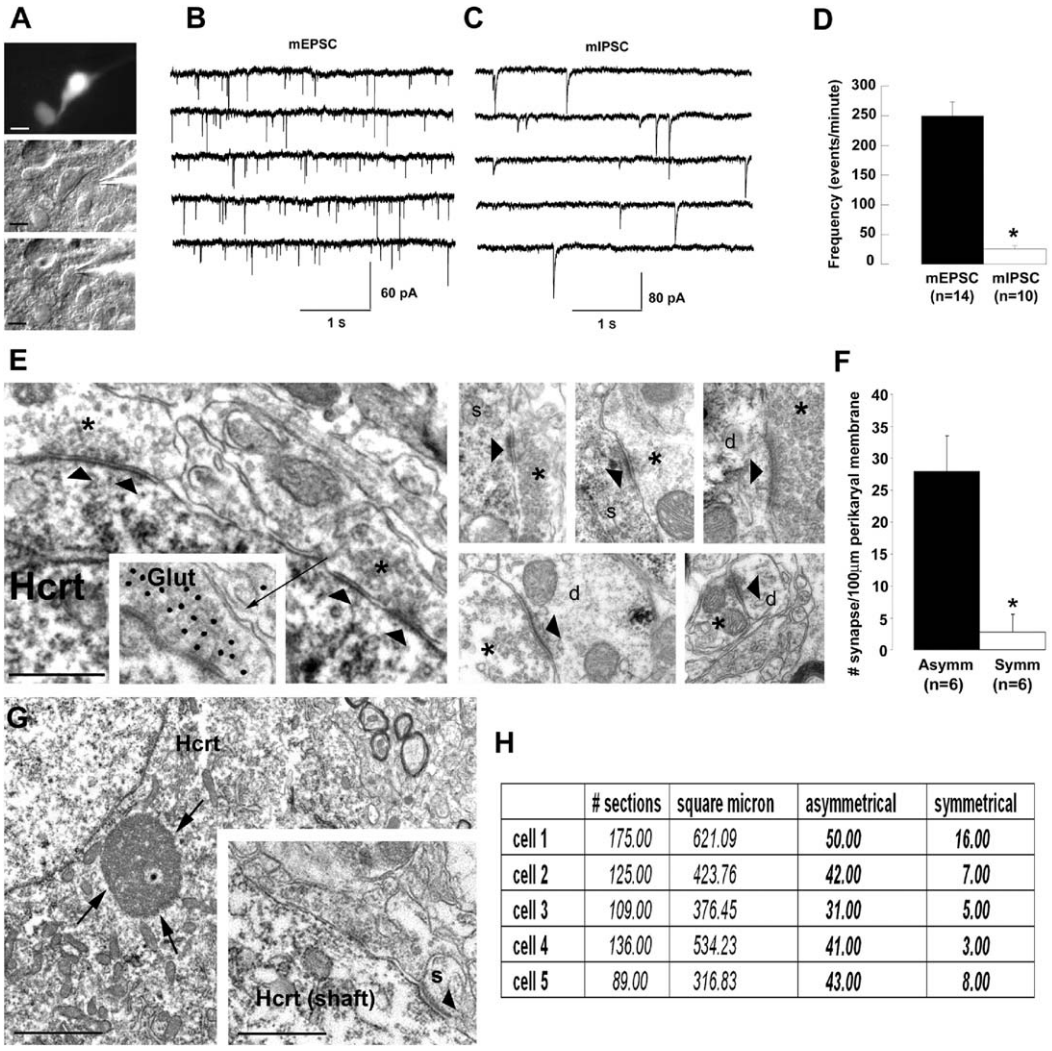
The appropriate functioning of hypothalamic circuits in support of adequate energy balance relies on specific, yet redundant connectivity between intra- and extrahypothalamic regions. This redundancy is further impacted by ongoing rewiring of some of the connections, for example the inputs of the arcuate nucleus melanocortin system (Pinto et al., 2004). These observations raised the possibility that plasticity is an inherent and mandatory characteristic of hypothalamic circuits for adequate regulation of energy balance (Horvath and Diano, 2004). An intriguing question is whether rapid synaptic reorganization is specific to the neuropeptide Y (NPY) and pro-opiomelanocortin (POMC) neurons of the arcuate nucleus, a region that is partially outside of the blood-brain barrier, or whether is it a more general phenomenon involving other hypothalamic peptidergic systems. To address this question, in this study we have analyzed the lateral hypothalamic hypocretin-producing neuronal system, a circuitry that is situated outside of the blood-brain barrier and plays important roles in a variety of brain functions, most notably energy metabolism (Sakurai et al., 1998) and arousal (Chemelli et al., 1999).

Hypocretin (also called orexin) is produced in a distinct subset of lateral hypothalamic neurons (de Lecea et al., 1998; Sakurai et al., 1998). Hypocretin first emerged as a critical regulator of feeding (Sakurai et al., 1998) and, subsequently, of arousal (Chemelli et al., 1999). The lack of hypocretin (Chemelli et al., 1999; Hara et al., 2001) or its receptors (Lin et al., 1999) are the underlying causes of narcolepsy. Hypocretin neurons project to all regions of the brain, including the hypothalamus (de Lecea et al., 1998; Peyron et al., 1998; Horvath et al., 1999a), cerebral cortex, brain stem and spinal cord (de Lecea et al., 1998; Peyron et al., 1998; van den Pol, 1999), and locus coeruleus (Horvath et al., 1999b). Thus, the hypocretin circuitry may serve as a critical integrator of diverse inputs for the appropriate synchronization of various autonomic, endocrine, and metabolic processes. In this regard, it is intriguing to note that obesity has been associated with increased wakefulness at the expense of sleep (Taheri et al., 2004; Spiegel et al., 2004), albeit to date no neurobiological explanation for this phenomenon exists (Flier and Elmquist, 2004). Metabolic disturbances caused by the lack of sleep could very well relate to the activity of the hypocretin/orexin system since these neurons promote arousal and feeding (Chemelli et al., 1999; Sakurai et al., 1998). Characterizing the input onto the lateral hypothalamic hypocretin neurons is likely to provide important new insights to the understanding of arousal and associated metabolic disturbances. Therefore, using quantitative and qualitative synaptology and targeted patch-clamp electrophysiology, we have determined the synaptic input organization and stability of hypocretin (Hcrt) cell bodies (perikarya).

Results

Mini postsynaptic currents of Hcrt perikarya

We analyzed the afferent inputs to hypocretin neurons using patch-clamp recordings in lateral hypothalamic slice preparations from transgenic animals in which hypocretin neurons are visualized by green fluorescence protein (Li et al., 2002). Hypocretin cells were held at –60 mV in the whole-cell voltage-clamp configuration and the number of miniature excitatory and inhibitory postsynaptic currents (mEPSC/mIPSC) was determined by using tetrodotoxin (TTX) to block all action potential-driven PSCs. Mini postsynaptic currents arise from spontaneous vesicle fusion and thus, most likely reflect the number of transmitter release sites and hence presynaptic terminals (Regehr and Stevens, 2001). Strikingly, hypocretin perikarya had approximately 10-fold higher levels of mEPSCs than mIPSCs (Figures 1A–1D). The frequency of mEPSCs was



	# sections	square micron	asymmetrical	symmetrical
cell 1	175.00	621.09	50.00	16.00
cell 2	125.00	423.76	42.00	7.00
cell 3	109.00	376.45	31.00	5.00
cell 4	136.00	534.23	41.00	3.00
cell 5	89.00	316.83	43.00	8.00

Figure 1. Stimulatory and Inhibitory Input organization on Hcrt perikarya

A) a GFP-hypocretin-containing neuron was identified and whole-cell patch clamp recording was made on this neuron. Scale bar: 10 μm.

B and C) raw traces of recorded mEPSCs and mIPSCs are presented. mEPSCs were recorded in the presence of TTX (1 μM) and bicuculline (30 μM), while mIPSCs were recorded in the presence of TTX (1 μM), CNQX (10 μM), and AP-5 (50 μM).

D) pooled data representing mEPSC and mIPSC frequencies (events recorded per minute) from hypocretin-containing neurons are presented.

E) Electron micrographs showing asymmetric synaptic contacts between unidentified axon terminals (asterisk) and Hcrt somata (s) and dendrites (d). Arrowheads point to synaptic membrane specializations. Arrow on left panel originates on a bouton establishing an asymmetric synapse on an Hcrt perikaryon and points to the insert showing the same bouton in a subsequent section after postembedding immunostaining for glutamate (5 nm immunogold particles). Bar scale on left panel indicates 1 μm for all panels of (**E**).

F) Statistical analysis revealed that asymmetric, putative excitatory axon terminals dominate inhibitory contacts on Hcrt perikarya.

G) Cytoplasmic organelles consisting of aggregating transport vesicles (black arrows) were frequently found in Hcrt-immunopositive cells. Bar scale represents 2 μm. Insert on (**G**) shows a dendritic shaft of a hypocretrin neuron to be pre-synaptic to unidentified dendritic spine (s) in an asymmetric synaptic configuration (arrowhead). Bar scales represent 3 μm (main panel of [**G**]) and 1 μm (insert of [**G**]).

H) Table showing results of 3D reconstruction of five randomly selected hypocretin-immunopositive neurons from intact, ad libitum fed animals (n = 5). Ultrathin sections were 70 nm thick. The surface area of each neuron was determined by multiplying total perikaryal membrane length (from each plane) by 70 nm.

249 ± 23 per minute (n = 14), while the frequency of mIPSCs was 26 ± 6 per minute (n = 10).

Excitatory/inhibitory synapse ratio on Hcrt perikarya

In wild-type mice (n = 5), hypocretin perikarya were dominated by asymmetric synapses, in which the putative excitatory synapses robustly outnumbered symmetric, putatively inhibitory contacts (27.87 ± 5.62 versus 4.86 ± 2.79/100 μm Hcrt perikaryal membrane; $p < 0.01$; Figures 1E and 1F).

To complement the conclusions of the unbiased analyses, selected boutons of six hypocretin neurons were followed through 600 consecutive serial ultrathin sections. In this assessment, 10 boutons adjacent to each hypocretin perikaryon (a total of 60 boutons) were followed. Every selected element of the analysis was chosen at random: the 10 boutons at high power magnification; the hypocretin-immunoreative perikarya; and the plane of each transected hypocretin cell. Fifty-three of these contacts were found to establish asymmetric synapses,

four synapses were symmetric, and three did not appear to develop either type of contact and did not establish parallel membranes with hypocretin perikaryal membranes.

We did three-dimensional (3D) reconstruction of five hypocretin-immunopositive perikarya of the lateral hypothalamus from five animals and assessed their entire perikaryal synaptology. Figure 1H shows that on each of these five cells, asymmetric synapses outnumbered symmetric connections by several magnitudes. During the 3D reconstruction process, we noted that some hypocretin cells contained a cyoplasmic organelle (Figure 1G) that has previously been referred to as either a nematosome (Leranth et al., 1985; Naftolin et al., 1988) or butrysome (Kind et al., 1997). These cytoplasmic organelles are aggregations of transport vesicles and have been associated with both developmental and adult synaptic plasticity (Naftolin et al., 1988; Kind et al., 1997).

Excitatory/inhibitory synapse ratio on Hcrt dendrites

While perikaryal (cell body) synaptology is a critical measure of the overall excitability of a neuron, the majority of the synaptic input is received by the dendrites. Therefore, we quantified synaptic input based on ultrastructural characteristics on hypocretin-immunopositive dendritic profiles. The analysis revealed that asymmetric contacts dominate over symmetric synapses on dendrites of hypocretin neurons (10.984 ± 1.174 versus 4.466 ± 1.112; $p < 0.05$). An intriguing finding from this analysis was that dendro-dendritic interactions between hypocretin-immunopositive shafts and unidentified dendritic spines were not uncommon (Figure 1G).

Qualitative assessment of putative inputs on hypocretin perikarya

To delineate putative stimulatory and inhibitory connections, we analyzed glutamatergic and GABAergic inputs on hypocretin neurons, using immunolabeling for vesicular glutamate transporter 2 (vGlut2; to identify excitatory glutamatergic fibers) and for glutamic acid decarboxylase (GAD; the rate-limiting enzyme for the synthesis of the inhibitory neurotransmitter, γ-amino butyric acid, GABA). These multiple labeling studies confirmed the presence of an extensive (Li et al., 2002) interaction between hypocretin perikarya and vGlut2-expressing axon terminals (Figures 2A–2C). Strikingly, in contrast to the vGlut2-Hcrt interaction, there were very few instances in which GAD-immunopositive, putative GABAergic boutons were found in close proximity to Hcrt perikarya (Figures 2D–2F). This is in sharp contrast to perikaryal arrangements of GABA boutons in other areas, such as the arcuate nucleus, where GAD-immunoreactive boutons dominate neuronal perikarya (Figure 2G).

To assess the relative abundance of vGlut2- versus GAD-immunolabeled boutons in close proximity to Hcrt perikarya, either vGlut2 or GAD axon terminals that appeared to be in direct apposition to 20 Hcrt-immunopositive perikarya were counted. This calculation revealed that for every 10 putative vGluT2 inputs, there were 2 ± 0.32 GAD-containing axons encountered.

Using correlated light and electron microscopy, we now show that vGlut2-containing axon terminals do indeed establish synaptic membrane specializations on Hcrt perikarya (Figures 2H–2L).

While occasional proximity was noted between NPY, POMC, serotonin, or catecholamines axon terminals and Hcrt neurons, the frequency of these interactions was minimal, limited to one or two putative contacts on 10%–20% of Hcrt perikarya (Figures 2M–2P). Attention was focused on these putative inputs of Hcrt neurons because previous electrophysiological and anatomical studies suggested direct regulatory roles for these peptides/neurotransmitters on Hcrt neuronal activity (Broberger et al., 1998; Elias et al., 1998; Horvath et al., 1999a; Li et al., 2002; Yamanaka et al., 2003b; Muraki et al., 2004).

Instability of postsynaptic currents and synapses on Hcrt perikarya

To test whether the synaptology of Hcrt neuronal perikarya is hard wired, an analysis and comparison of the synaptic organization and PSCs of hypocretin neurons were performed between fed, fasted (24 hr), fasted plus leptin-replaced, and fasted re-fed (24 hr) mice. Patch-clamp electrophysiological analyses of hypocretin-GFP neurons in slice preparation revealed that mIPSCs did not differ between fed and fasted mice (Figure 3B): the frequency of mIPSCs recorded from hypocretin neurons was 26 ± 6 per minute (n = 10) in control mice and 29 ± 5 per minute (n = 11) in fasted mice. This difference is not significant ($p > 0.1$). In contrast, the overnight fast underlies significant elevations in mEPSCs compared to controls (Figure 3A): the frequency of mEPSCs was 250 ± 23 per minute (n = 14) in control mice and 349 ± 35 per minute (n = 14) in fasted mice, which represents a significant difference ($p < 0.05$). In addition, the inter-event intervals between detected mEPSC events from control and fasted mice were examined and the cumulative probabilities of the inter-event interval for both groups were plotted (data not shown). The left shift of the cumulative probability curve obtained from fasted mice was significant ($p < 0.01$). Thus, our data showed that fasting significantly increased the frequency of mEPSCs but not mIPSCs in hypocretin/orexin neurons (Figure 2C). Unbiased ultrastructural analyses confirmed that this elevation in mEPSCs is correlated with an elevated number of asymmetric, putatively excitatory synapses on hypocretin perikarya of fasted animals (n = 6) compared to fed controls (n = 6) (39.16 ± 3.44 versus 24.85 ± 4.08, respectively; $p < 0.01$; Figure 2D).

Next, we analyzed whether diminishing leptin levels during fasting may be a key signal to trigger the observed changes in excitatory inputs of hypocretin cells. Hypocretin neurons were previously found to express leptin receptors (Horvath et al., 1999a; Håkansson et al., 1999). Hypocretin-GFP mice were food deprived for 24 hr but they received a single injection of recombinant leptin (50 μg per animal, i.p.) at the beginning of the fast. Twenty-four hours later, leptin-treated animals as well as a set of intact, ad lib fed animals were killed and their hypothalami prepared for slice electrophysiology. In this set of experiments, the frequency of mEPSCs from control animals was 269 ± 90 per minute (n = 7), while that of the fasted and leptin-treated group was 294 ± 61 events/minute (n = 7). The modest increase (109.4% ± 22.6% of control, Figure 3E) was not statistically significant ($p > 0.05$). In parallel studies, we analyzed synaptology of hypocretin neurons from intact, ad lib fed (n = 6) and fasted plus leptin-treated (50 μg per animal, i.p.; n = 6) animals. Quantitative synaptology revealed no differences between intact and fasted plus leptin-replaced animals regarding their asymmetric synapses on hypocretin perikarya (35.16 ± 4.12 versus 31.43 ± 6.53; $p > 0.05$, Figure 3F). Together, these data suggest that the fasting-induced increase in mEPSC fre-

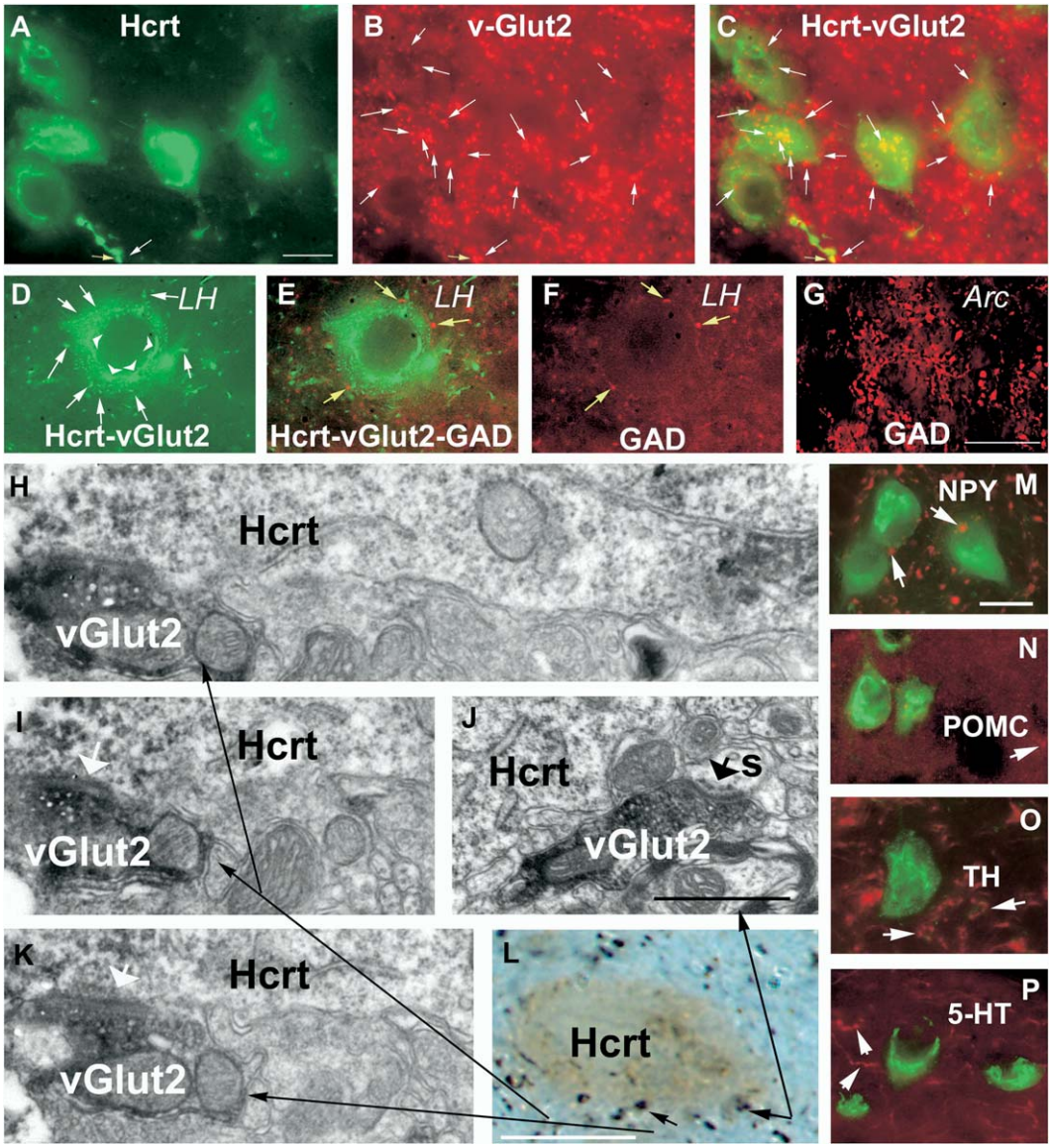


Figure 2. Various inputs of Hcrt perikarya

A–C) Double immunostaining for Hcrt (**A**) and vGlut2 (**B**) reveals a robust interaction between red, vGlut2-immunopositive, putatively excitatory axon terminals (white arrows) and green, Hcrt-containing cells (**C**). Bar scale on (**A**) represents 10 μm for (**A**)–(**C**).

D–G) Multiple labeling for vGlut2, Hcrt (both labeled with green fluorescence; **D** and **E**) and GAD (red fluorescence; **F** and **G**). vGlut2 terminals (white arrows on [**D**]) outnumber GAD-containing putative boutons (**E** and **F**) on this Hcrt-producing neuron (white arrowheads on [**D**] point to cytoplasmic membranous structures, a characteristic feature of Hcrt immunoreactivity). Most neuronal cell bodies in the hypothalamus are dominated by inhibitory GABA input as seen on an unlabeled arcuate nucleus neuron on (**G**) after GAD labeling. Bar scale on (**G**) represents 10 μm for panels (**D**)–(**G**).

H–L) vGlut2-immunoreactive axon terminal establishes asymmetric synaptic contacts with the perikarya of Hcrt neurons. **H–K)** Consecutive electron micrographs showing the establishment of an asymmetric synapse by a vGlut2-immunoreactive axon terminal indicated on the light micrograph (black arrow on [**L**]). The white arrows on (**I**) and (**K**) point to the postsynaptic density. **J**) Asymmetric contact (red arrow) between another v-Glut2-immunoreactive axon terminal (indicated on panel [**L**]) and a dendrite adjacent to a hypocretin perikaryon. Bar scale on (**J**) represents 1 μm for (**H**)–(**K**). Bar scale on (**L**) represents 10 μm.

M–P) Double-labeling fluorescence microscopy showed a limited interaction between NPY- (white arrows indicate putative contacts on [**M**]), POMC- (**N**), TH- (**O**), and serotonin- (5-HT, **P**) immunoreactive boutons (red) and Hcrt perikarya (green). Bar scale on (**M**) represents 10 μm for (**M**)–(**P**).

quency is triggered, at least in part, by declining levels of circulating leptin.

Subsequently, we tested whether the increase in mEPSC frequency induced by 24 hr of food deprivation is reversed by 24 hr of re-feeding. Hypocretin-GFP mice were food deprived for 24 hr after which food was made available ad libitum. Animals were sacrificed 24 hr after food replacement. The control group included animals that were not fasted. The mean frequency of mEPSCs in fasted/re-fed animals, while elevated, was not significantly different compared to control values (401 ± 54 per minute [n = 12] versus 308 ± 67 per minute [n = 9]; $p > 0.05$, Figure 3E). Asymmetric synapses on hypocretin perikarya of fasted re-fed animals (n = 6) did not significantly differ from that of intact controls (n = 60 [42.65 ± 7.24 versus 35.16 ±

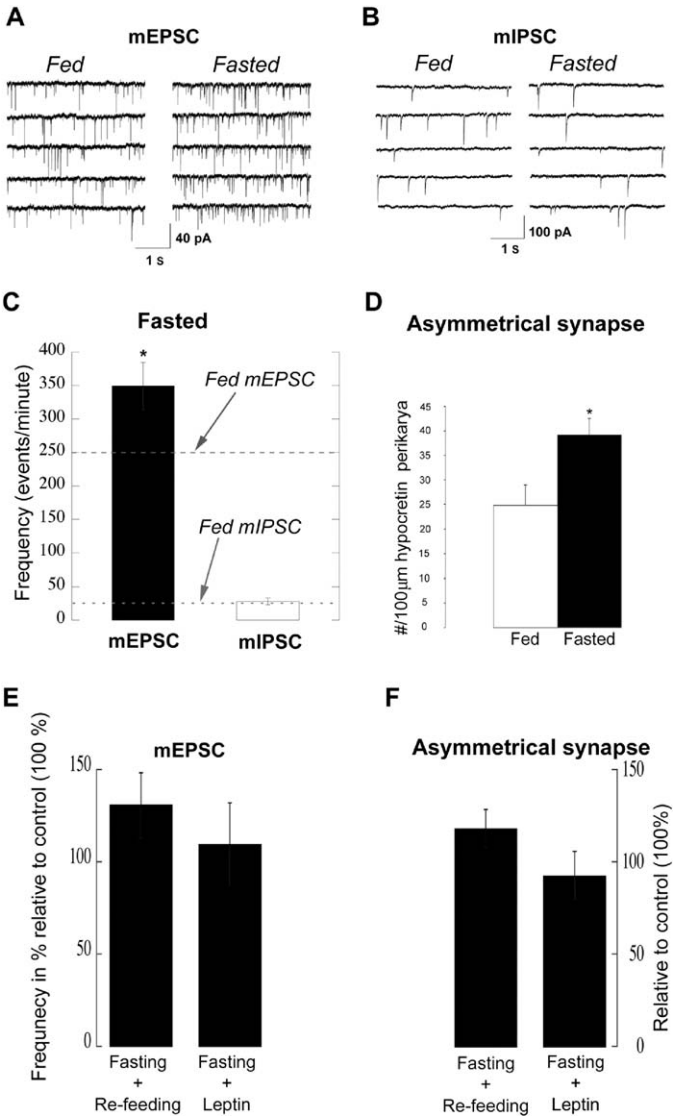


Figure 3. Fasting potentiates excitatory but not inhibitory inputs onto hypocretin neurons

A) The frequency of mEPSCs recorded from hypocretin neurons was enhanced after a 24 hr fast.

B) Raw traces of mIPSCs recorded from hypocretin neurons from control and fasted animals; the frequency of mIPSCs recorded from hypocretin neurons did not change after a 24 hr fast.

C) Pooled data from all experiments are presented here. Averaged mEPSC frequency and mIPSC frequency (events/min) recorded in hypocretin neurons from fasted animals. The dotted lines represent control levels of mEPSC and mIPSC frequencies.

D) Fasting (filled column) induced a significant (* = $p < 0.05$) elevation in the number of asymmetric, putative excitatory synapses on Hcrt perikarya compared to the values of fed controls (empty column).

E) Re-feeding after fasting or leptin injections in parallel with fasting abolished significant differences in mEPSC frequencies of control (100%) and fasted animals.

F) Re-feeding after fasting or leptin injections in parallel with fasting abolished significant differences in perikaryal asymmetric synapse numbers of control (100%) and fasted animals.

4.12; $p > 0.05$], Figure 3F). Thus, re-feeding triggers a rearrangement of the perikaryal synaptic input organization of hypocretin neurons.

Discussion

The present results revealed an unorthodox synaptic organization on the hypocretin neurons in which excitatory synaptic currents and asymmetric synapses exert control on the perikarya of these long-projective neurons with minimal inhibitory input. Overnight food deprivation promoted the formation of more excitatory synapses and synaptic currents onto hypocretin perikarya; this formation was reversed by re-feeding and blocked by leptin administration. The wiring and fasting-induced synaptic remodeling of the hypocretin neurons correlates well with their being involved in the control of arousal and alertness that are so vital to survival; but, this circuitry may also be an underlying cause of insomnia and associated metabolic disturbances, including obesity.

Anatomical considerations

While glutamatergic axons accounted for the majority of asymmetric inputs on Hcrt perikarya, the source of these excitatory synapses remains to be confirmed. They likely originate, at least in part, from local interneurons (Li et al., 2002), but they may also arise from other hypothalamic and extrahypothalamic sites as well. Since melanin concentrating hormone (MCH)-containing axon terminals also establish asymmetric contacts (Bittencourt et al., 1992), it is likely that the MCH input contributes to the asymmetric input ratio on Hcrt neurons as well (Guan et al., 2002). Recently, we also demonstrated that at least some of the incoming asymmetric, stimulatory inputs on Hcrt perikarya originate from the corticotrophin releasing hormone (CRH)-producing hypothalamic neurons, suggesting a possible pathway for stress-induced activation of the Hcrt system (Winsky-Sommerer et al., 2004). It is possible that the limited number of GABA boutons seen on Hcrt cells are those that also contain NPY (Horvath et al., 1997) and originate in the arcuate nucleus (Broberger et al., 1998; Elias et al., 1998; Horvath et al., 1999a).

Neuronal cell bodies in the central nervous system are either dominated by inhibitory inputs (long-projective neurons), or have an approximate ratio of excitatory to inhibitory inputs of 1:1 (for example, on the perikarya of interneurons; Figure 4; Shepherd, 2004; Douglas et al., 2004; Pinto et al., 2004). The lateral hypothalamic hypocretin neurons are long-projective cells (Peyron et al., 1998; Horvath et al., 1999b). Our work described here provides the first evidence that a set of long-projective excitatory neurons, the lateral hypothalamic hypocretin cells, are controlled by excitatory inputs onto their perikarya which are further increased by a stressor such as fasting (Figures 4A–4D).

Plasticity of synapses on Hcrt perikarya

When animals were fasted, additional stimulatory synapses appeared to be formed on Hcrt perikarya as revealed by both ultrastructural and electrophysiological analyses. Our observations clearly indicate that the input organization of Hcrt neurons is not hard wired but, rather, changes dynamically in the face of a fluctuating environment, similar to the recent revelations of the arcuate nucleus melanocortin system (Pinto et al., 2004).

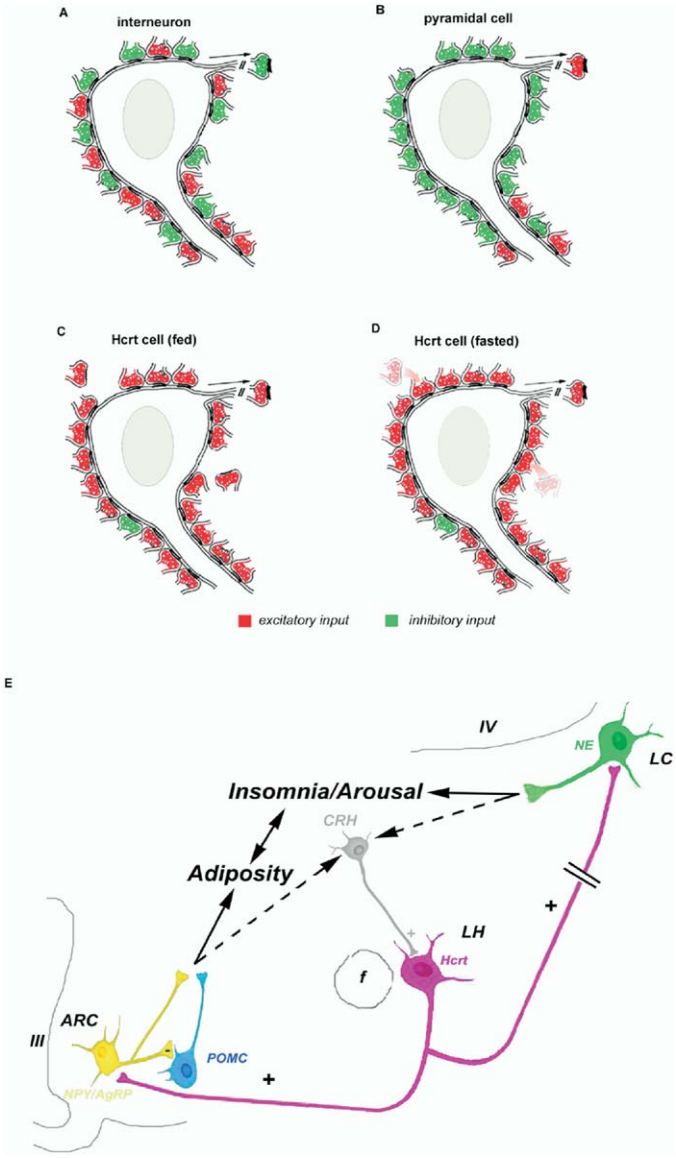


Figure 4. Input organization of hypocretin neurons and its impact on energy homeostasis and insomnia/arousal

A–D) Schematic illustration of the inhibitory (green) and excitatory (red) input organization of various cell types in the brain: interneurons (**A**), long-projective neurons, such as pyramidal cells (**B**). Hypocretin cells in the fed state (**C**) are controlled by excitatory synaptic inputs, which outnumber inhibitory connections 10:1. Food restriction underlies further recruitment (curved, light red arrows) of excitatory inputs onto Hcrt perikarya (**D**).

E) The connectivity and synaptic input organization of lateral hypothalamic hypocretin neurons provide a simple and straightforward explanation for the relationship between insomnia and adiposity: because of the easy excitability of hypocretin neurons, any signal that triggers their activity, regardless of the homeostatic needs, will elevate the orexigenic tone of the arcuate nucleus melanocortin system while also promoting wakefulness through activation of medullary locus coeruleus (LC) noradrenergic (NE) neurons. Elevated orexigenic output of the melanocortin system as well as sustained arousal at the expense of sleep promote the activity of corticotrophin releasing hormone (CRH)-producing neurons in the hypothalamus, which, in turn, can further trigger hypocretin neuronal firing. This vicious cycle, in which hypocretin neurons play the role of both trigger and accelerator, can simultaneously promote and worsen adiposity and insomnia.

The regulatory signals for these synaptic changes are not yet well defined. Re-feeding after fasting partially reversed fasting-induced changes on Hcrt synaptology, and leptin administration concomitant to food withdrawal prevented fasting-induced plasticity as well. Regarding leptin's role, leptin receptors have been identified in Hcrt cells of different species (Horvath et al., 1999a; Håkansson et al., 1999) and leptin has been shown to affect the synaptic organization of other hypothalamic peptidergic systems (Pinto et al., 2004). On the other hand, fasting entails various peripheral signals that are associated with both metabolism as well as the stress axis. Thus, it is not unlikely that the alterations we encountered on Hcrt perikarya are the consequence of multiple humoral and neuronal signals. For example, ghrelin binding was also detected in lateral hypothalamic slices (Cowley et al., 2003) and ghrelin administration to the lateral hypothalamus was shown to induce c-Fos activation in Hcrt perikarya (Olszewski et al., 2003), an event that is also triggered by fasting (Diano et al., 2003). It remains to be tested whether circulating ghrelin can reach lateral hypothalamic ghrelin neurons and affect synaptic input organization. Further studies are needed to determine the molecular underpinnings of the dynamic synaptic fluctuation on Hcrt perikarya.

Functional considerations

The Hcrt system has emerged as a critical regulator of arousal and energy metabolism (Sakurai et al., 1998; Chemelli et al., 1999; Lin et al., 1999; Date et al., 1999; Peyron et al., 2000; Yamanaka et al. 2003a). Arousal is a vital behavior in all species. It is essential to an animal's survival that it possesses the ability to adequately respond to threats such as a predator or other life-threatening circumstance (i.e., food deprivation). We suggest that the unique input organization and plasticity of Hcrt neurons, in which excitatory contacts dominate inhibitory ones, is a necessary element for the maintenance of a low threshold for arousal and alertness. The synaptic organization onto Hcrt neurons makes it likely that, for the Hcrt system, noise becomes signal, thus allowing for this system to be easily activated leading to arousal. Conversely, since arousal inherently disturbs sleep, it is also reasonable to infer that this unorthodox synaptology and synaptic plasticity of the lateral hypothalamic arousal center may be responsible for the emergence of insomnia under certain circumstances. Since the hypocretin/orexin system is also a potent regulator of metabolism (Sakurai et al., 1998; Sakurai, 2002), our observations may also help us to better understand the uncovered correlation between insomnia and obesity in humans (Taheri et al., 2004; Spiegel et al., 2004; Figure 4E): easy excitability of hypocretin neurons can support orexigenic output of the melanocortin system in parallel with arousal at the expense of sleep (insomnia). Elevated output of the melanocortin system (Dhillo et al., 2002) as well as sustained arousal at the expense of sleep (Buckley and Schatzberg, 2005) can promote the activity of corticotrophin releasing hormone (CRH)-producing neurons in the hypothalamus (activation of the stress axis), which, in turn, can further trigger hypocretin neuronal firing (Winsky-Sommerer et al., 2004). This vicious cycle, in which hypocretin neurons play a role as both the trigger and accelerator, can simultaneously promote and worsen adiposity and insomnia. Future studies are needed to test whether selective interference of the activation of the hypocretin neuronal circuitry may offer an approach to diminish disfunctions of energy homeostasis, including obe-

sity, associated with sustained arousal or insomnia (Taheri et al., 2004; Spiegel et al., 2004).

Experimental procedures

Animals

Transgenic and wild-type male mice were used in the present studies. The transgenic mice used for the targeted electrophysiological recordings from Hcrt neurons were generated by Dr. T Sakurai and described in detail elsewhere (Li et al. 2002). For ultrastructural studies, C57Bl6 animals were used. Animals were kept under standard laboratory conditions in a 12/12 hr light/dark cycle and food and water available ad libitum. Groups of animals (6–8 weeks of age) were either normally fed (n = 6), fasted for 24 hr (n = 6), fasted for 24 hr and treated with a single intraperitoneal injection of recombinant leptin (50 μg in 100 μl saline; Peprotec, Inc., Rocky Hill, New Jersey) at the time of food withdrawal (n = 6), or fasted for 24 hr followed by ad libitum re-feeding for 24 hr (n = 6). Thirteen pairs of mice were used for electrophysiological experiments. All procedures described were approved by the Institutional Animal Care and Use Committee of Yale University.

Electrophysiology

Whole-cell recordings were made from hypocretin/orexin neurons identified by GFP expression under the control of the hypocretin promoter (from Dr. T. Sakurai; Li et al., 2002). Lateral hypothalamic slices were maintained at 33°C and perfused with ACSF. The bath solution (ACSF) consisted of (in mM): NaCl, 124; KCl, 3; $CaCl_2$, 2; $MgCl_2$, 2; NaH_2PO_4, 1.23; $NaHCO_3$, 26; glucose, 10 (pH 7.4) with NaOH and was continuously bubbled with 5% CO_2 and 95% O_2. The patch pipettes were made of borosilicate glass (World Precision Instruments) with a Sutter micropipette puller (PP-97). The tip resistance of the recording pipettes was 4–6 MΩ after filling with a pipette solution containing (mM): $KMeSO_4$ (or KCl), 145; $MgCl_2$, 1; Hepes, 10; EGTA, 1.1, Mg-ATP, 2; Na2-GTP, 0.5 (pH 7.3) with KOH. After a gigohm seal and whole-cell access were achieved, the series resistance was between 20 and 40 MΩ and partially compensated by the amplifier. Both input resistance and series resistance were monitored throughout the experiments. mEPSCs and mIPSCs were recorded under voltage clamp in the presence of TTX and bicuculline (for mEPSCs) or TTX and CNQX plus AP-5 (for mIPSCs) with a multiclamp 700A amplifier (Axon Instruments, Inc). Hypocretin/orexin neurons (expressing GFP exclusively) were identified under an upright Olympus BX51WI microscope with IR-DIC optics and a GFP filter cube and were held at –60 mV. Detection of mEPSC and mIPSC events were performed offline with the software Axograph 4.9 (Axon Instruments, Inc.), as described previously (Gao and van den Pol, 2001). Frequency and Amplitude of mEPSCs (or mIPSCs) were generated after detection of mEPSC events, as described previously (Gao and van den Pol, 2001). Frequency of mEPSCs was presented as mean ± SEM. Student's t test and Komogorov-Smirnov test were performed to examine the significance of difference (Gao and van den Pol, 2001).

Microscopy

To visualize various inputs of Hcrt neurons, fluorescence immunostaining for NPY, POMC, serotonin, tyrosine hydroxylase, vGlut2, and GAD was performed on vibratome sections of GFP-Hcrt animals using previously published protocols and heterologous antisera for the various tissue antigens (Horvath et al., 1999a; Horvath et al., 1999b; Horvath et al., 1999c) and Alexa fluor 594 for red fluorescence and Alexa fluor 488 for green fluorescence (Molecular Probes, Eugene, Oregon).

Ultrastructural analysis of Hcrt perikarya

The lateral hypothalamus-perifornical region was dissected from each brain. Processing of the tissue for Hcrt immunocytochemsitry and electron microscopy were done as described elsewhere (Horvath et al. 1999a). Hcrt-immunolabeled cells with equal immunoperoxidase density were studied from all groups. Few embedded ultrathin sections were immunostained for glutamate as described in our recent publication (Pinto et al., 2004).

The analysis of synapses was performed in a blind fashion as described in our recent paper (Pinto et al., 2004). The synaptic counts were expressed as numbers of synapses on a membrane length unit of 100 μm. For statistics, the number of animals analyzed reflects the number of n for each group. For each animal, the mean number of synapses was calculated from nine Hcrt cells. F-test analyses have revealed significant homogeneity of variance within groups and significant nonhomogeneity of variances between groups (e.g., fed versus fasted). Thus, for the intergroup multiple statistical comparisons, the Kruskal-Wallis one-way nonparametric analysis of variance test was selected. The Mann-Whitney U-test was used to determine significance of differences between groups. A level of confidence of $p < 0.05$ was employed for statistical significance.

Three-dimensional reconstruction of perikaryal synapses of hypocretin neurons

We randomly selected five hypocretin-immunopositive perikarya of intact, ad lib fed wild-type animals (n = 5) in flat embedded blocks. We completely reconstructed them at the EM level using serial sections throughout the cell. In each plane, the labeled perikarya were photographed (between 5 and 7 photographs per plane at magnification 16,800) and reconstructed into a collage. Depending on the cell's depth in the block, a cell spanned eighty-nine (89) to one-hundred seventy-five (175) 70 nm thick electron microscopic planes. Thus, a cell was reconstituted using 600–1200 electron micrographs. The planes were then put into sequence and each and every bouton was followed and its type of membrane specialization (asymmetric or symmetric) was determined.

Correlated light and electron microscopic analyses of vGlu2 input on Hcrt neurons

Correlated light and electron microscopic analysis of vGlut2 innervation of Hcrt-immunopositive neurons was done using the same approach as described in our previous work (Horvath et al., 1999a).

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