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The Activity Profile of Hypocretin Neurons in the Freely Moving Rat

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I. Introduction

Since discovery in 1998 of the hypothalamic neuropeptide hypocretin (Hcrt), also known as orexin, the study of Hcrt behavioral function has mainly been carried out by using of c-Fos immunostaining. It has been found increased c-Fos expression during locomotion, active wakefulness and decreased number of c-Fos positive Hcrt neurons during quite wakefulness and even more so during NREM sleep. Results have been controversial for rapid eye movement (REM) sleep. This method has a number of important limitations, in particular insufficient temporal resolution and dissociation of c-Fos expression andneuronal firing (1,2). A number of major questions that cannot be addressed using c-Fos also still need to be addressed. First, what is the baseline activity of Hcrt neurons in the brain during wakefulness? Second, why does such small portion of entire Hcrt population show c-Fos expression in response on variety of behavioral challenges? Third, why does the medial part of Hcrt cell field express c-Fos more readily than the lateral portion? Finally, what is the activity of these cells during REM sleep, whether tonic or phasic.

Only the extracellular recording of Hcrt cells during these behaviors in freely moving animals can answer these questions. Two recent attempts were made to describe discharge patterns of perifornical-lateral hypothalamic (PFH-LH) neurons across S-W cycle (3,4). Unfortunately, the neurotransmitter phenotype of the recorded cells was not determined in either of these studies. Combining juxtacellular cell labeling, micropipette and microwire recordings with spike waveform analysis, we have developed electrophysiological criteria for the identification of Hcrt neurons and showed some behavioral correlates of their activity in freely moving rats (5).

II. Electrophysiological Identification of Hcrt Neurons

We used micropipette unit recording in the PFH-LH region followed by juxtacellular Neurobiotin labeling and immunostaining of Hcrt to determine the specific spike waveform profile of Hcrt neurons in anesthetized rats. Hcrt neurons were also identified

antidromically during electrical stimulation of the Ventral Tegmental Area (VTA). This latter technique allowed the identification of the same subset of Hcrt neurons during subsequent experiments in freely moving rats. The VTA receives dense hypothalamic projections and about 20% of PFH-LH cells that project to the VTA contain Hcrt. Therefore, VTA stimulation would be likely to antidromicaly activate a large percentage of Hcrt neurons.

We found that antidromically and immunohistochemically identified Hcrt neurons have broad spikes with long-lasing Later Positive Deflection (LPD) that distinguished them from adjacent antidromically identified PFH-LH cells. The spike LPD of Hcrt neurons ranged from 0.82 ms to 1.2 ms with a mean of 0.93 ± 0.01 ms (n = 26) and was significantly broader than the spike LPD of nonHcrt neurons ($t = 12.3$, $p < 0.0001$). Labeling and appropriate immunostaining of PFH-LH cells with LPD in this range allowed the practical identification of Hcrt neurons that responded antidromically to electrical stimulation of the VTA. To apply the LPD criterion for unit recording with microwires, we recorded Hcrt neurons with composite electrodes that consisted of a glass micropipette to which tungsten microwire (12.5 μm) was attached and allowed simultaneous recording through both electrodes. We have determined that the spike LPD for Hcrt neurons exceeds 0.56 ms during microwire recordings.

In freely moving rats, we found 9 PFH-LH neurons with spikes that met our criteria for Hcrt cells in anesthetized animals. All had (1) a spike LPD between 0.56 ms and 0.77 ms (Fig. la), (2) responded antidromically to VTA stimulation (Fig. lb,c), and (3) were located in the PFH-LH. Discharge patterns of these Hcrt neurons were analyzed during different behavioral states.

III. Hcrt Neuronal Activity During Sleep

The finding that most patients suffering from narcolepsy with cataplexy have lost most Hcrt neurons suggests that this hypothalamic neuropeptide plays a crucial role in maintaining wakefulness (6,7). Indeed, intracerebroventricular injections of Hcrt-1 produce a dose-dependent increase in the duration of wakefulness and substantially reduce REM sleep and slow wave (SW) sleep in rats (8). Investigation of the relation between Hcrt cell activity and sleep in rats showed that c-Fos expression in Hcrt neurons is circadianly modulated and negatively correlates with SW and REM sleep amounts. In contrast, the number of Hcrt+/c-Fos+ neurons significantly increases during sleep deprivation. After sleep recovery or REM sleep rebound Hcrt neurons show reduced c-Fos expression (9-11).

Our electrophysiological studies of the activity of identified Hcrt neurons across the sleep-wakefulness (S-W) cycle in freely moving rats showed that these neurons discharged with maximal frequency during active waking (AW), strongly decrease their firing rates during quiet wakefulness (QW) and practically cease activity in SW as well as in tonic REM sleep (Fig. 1d and Fig. 2a-d). During the phasic periods of REM sleep, Hcrt neuronal discharge became more frequent and sometimes correlated with spontaneous muscle twitches. A similar discharge pattern of identified Hcrt cells across S-W cycle was described by Lee et al. (12) who recorded and labeled Hcrt neurons in head restrained rats. The firing rate of Hcrt neurons increased during EEG desynchronization and negatively correlated with EEG spectral power in delta,

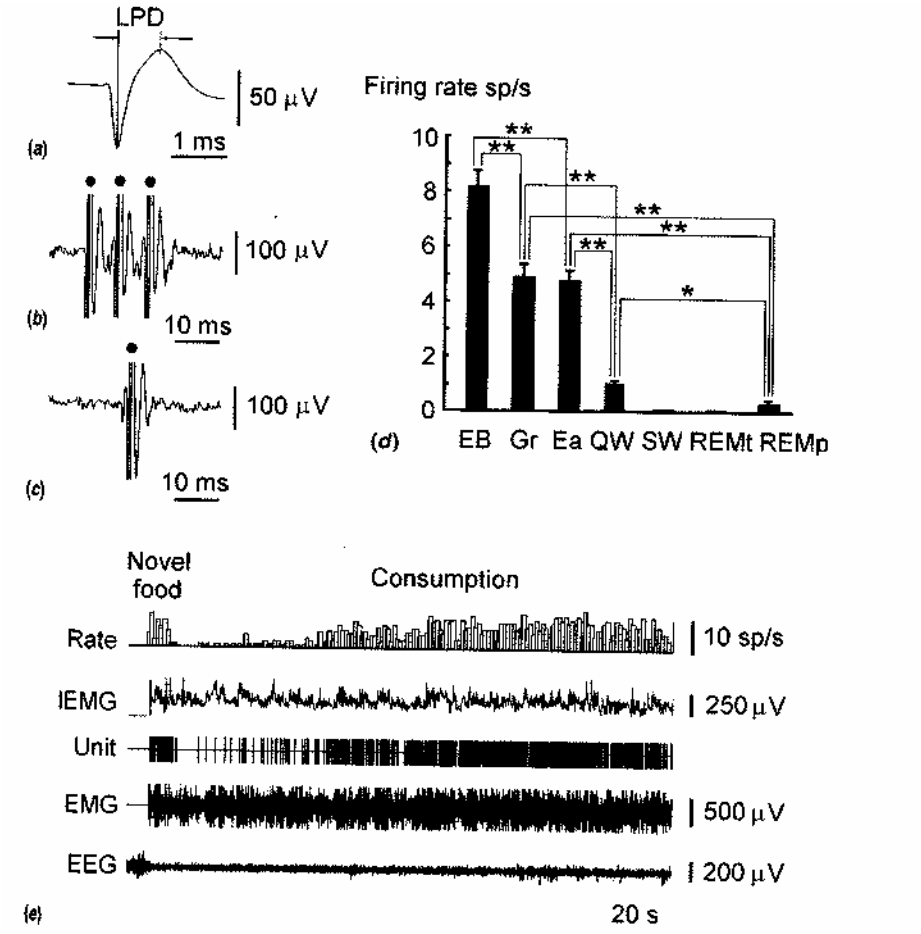

Figure 1 Example of electrophysiological identification of an Hcrt neuron in a freely moving rat and discharge pattern of Hcrt cells during different behavioral states. (a) An averaged spike waveform of the Hcrt neuron recorded with microwires. *(b)* Antidromic spikes of the Hcrt neuron to VTA train electrical stimulation. *(c)* Collision of orthodromic and antidromic spikes in axon of a Hcrt neuron. *(d)* Firing rate of Hcrt neurons in waking and sleep behaviors. *(e)* Transient decrease of Hcrt cell activity in response to the presentation of a novel food (chicken). During the observed decrease in firing rate, the rat sniffed, tasted and backed away from food. Hcrt cell firing accelerated in conjunction with the onset of food consumption. *Abbreviations:* AW, active waking; Ea, eating; EB, exploratory behavior; EEG, electroencephalogram; EMG, neck muscle electromyogram; Gr, grooming; QW, quiet waking; REMt and REMp, tonic and phasic REM sleep; Error bars indicate SEM; SW, slow wave sleep.

theta, alpha, and beta frequency bands (Fig. 2e-h). On the other hand, increased Hcrt cell activity was accompanied by an elevation of EEG spectral power in the gamma frequency band (Fig. 2i). It is worth noting that cortical desynchronization was always seen if Hcrt cells increased their firing rate to 1-2 Hz. This suggests that a low level

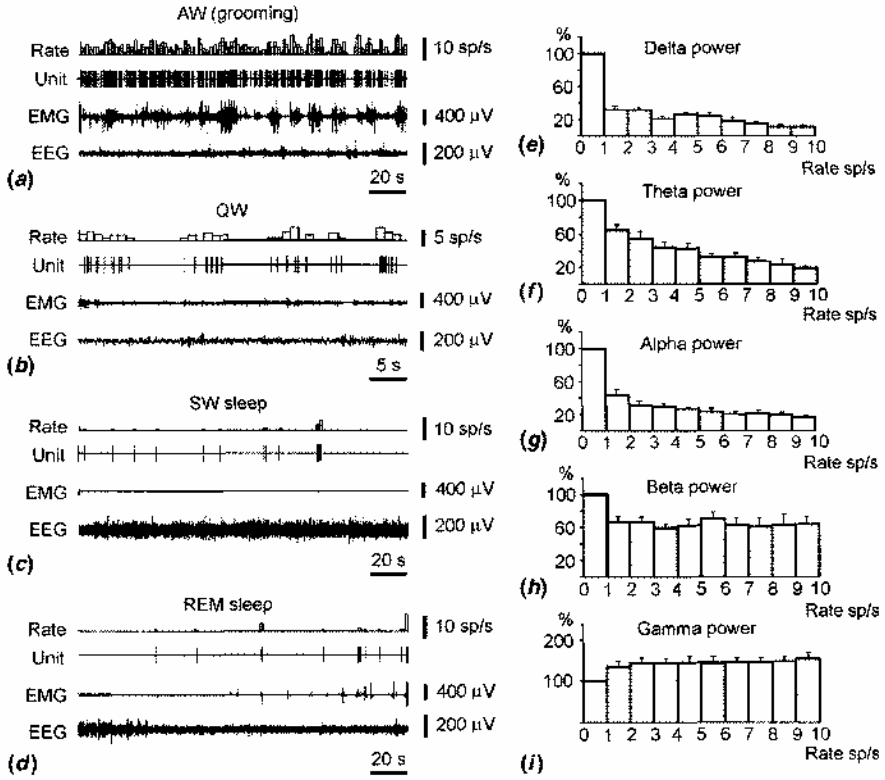

Figure 2 The discharge pattern of a representative Hcrt neuron across the sleep-wakefulness cycle in the freely moving rat and the alteration of EEG spectral power during periods of increased firing of Hcrt neurons. (a) High firing rates of an Hcrt neuron as seen during AW (grooming). *(b)* Reduced firing rate or cessation of Hcrt cell activity as seen in QW. (c) A further decrease or cessation of firing is seen during SW sleep. *(d)* Minimal firing rate is seen during the tonic phase of REM sleep. Brief Hcrt cell discharge bursts are correlated with muscle twitches during the phasic events of REM sleep. *(e-h)* The decrease of EEG powers in delta, theta, alpha, and beta frequency bands. *(i)* The increase of EEG power in gamma frequency band. Error bars indicate SEM. See abbreviations in Figure 1.

of the Hcrt neuronal activity is sufficient to induce and maintain ascending EEG activation. Hcrt neurons may contribute to cortical arousal through the excitation of ascending monoaminergic, cholinergic, reticular, and thalamocortical systems as well as through direct Hcrt projections to the cortex.

The behavioral correlates of Hcrt neuronal activity across the sleep cycle share similarities with those of the norepinephrine (13), serotonin (14), and histamine (15) cells to which Hcrt cells are reciprocally connected. However, unlike monoaminergic cells that predominantly show tonic "clock-like" activity during QW, Hcrt neurons have an irregular pattern of discharge sometimes alternating with periods of silence. During REM sleep, Hcrt neurons show sporadic discharges that sometimes correlate with muscle twitches, whereas most monoaminergic neurons cease firing completely.

The mechanisms underlying the suppression of Hcrt cell activity during sleep suggest that these neurons may be under GABAergic control from sleep-promoting regions of the preoptic area. Perfusion of the preoptic area with the GABA agonist muscimol or blockage of GABAergic function in the PFH-LH with bicuculline significantly increases the number of Hcrt+/c-Fos+ neurons (16,17). Thus, there is strong evidence for an active inhibition of Hcrt neurons during sleep.

IV. Hcrt Neuronal Activity and Feeding Behavior

Intracerebroventricular administration of Hcrt-1 and Hcrt-2 increases food consumption, suggesting an important role of these peptides in the regulation of feeding (18,19). c-Fos expression in Hcrt neurons is significantly increased when glucose level are decreased by the administration of insulin (20,21). Similarly, intracerebroventricular injections of ghrelin-releasing peptide that stimulates food intake as well as intraperito-neal administrations of intralipids producing robust elevation in the levels of triglycer-ides increase substantially the number of Hcrt+/c-Fos+ neurons (22,23). Both in rodent and primates, c-Fos expression in Hcrt neurons increases in responses to fasting and restricted feeding (24,25). Activation of Hcrt cells during these conditions suggests that they have a role in the maintenance of energy homeostasis through behavioral mechanisms, in particular triggering food-seeking behavior and regulating appetitive phase of feeding. This hypothesis is supported by the finding that the activity of Hcrt neurons markedly increases when food is anticipated under a condition of restricted feeding in wild-type mice. Additionally, Hcrt neuron-ablated mice display abnormal low food-anticipatory activity (26,27). Finally, c-Fos expression in Hcrt neurons after the administration of the GABA agonist muscimol into the nucleus accumbens shell (28) suggests that Hcrt systems may be also relevant to the regulation of the hedonic aspects of feeding. In our electrophysiological studies, identified Hcrt neurons showed moderate activity when rat consumed a familiar food (Fig. 1d). Interestingly, however, they strongly decreased their firing rate during the period of the initial food aversion induced by the presentation of a novel food (Fig. 1e). This suppression of Hcrt cell discharges continued for 20-50 s despite strong EEG desynchronization and the presence of substantial motor activity that is usually associated with Hcrt cell activation. After tasting the novel (highly palatable) food, subsequent consumption was accompanied by a gradual elevation of Hcrt discharge frequency without any obvious EEG or motor alterations. Presented together, these results suggest that Hcrt neurons provide an important link between metabolic requirements and behaviors, modulating cortical arousal, motor activity, and regulating emotional/motivational aspects of feeding.

V. Hcrt Neuronal Activity and Motor Activity

The level of Hcrt-1 in the CSF of freely moving animals increases with motor activity (29,30). A greater number of Hcrt+/c-Fos+ neurons in cats is however observed during exploratory behavior when compared to stereotype locomotion on a treadmill (31). Similarly, we found that Hcrt cells discharge with a significantly higher frequency

during exploratory behavior when compared to grooming and eating (Fig. 1d). This is consistent with the idea that Hcrt neurons are involved in the integration of arousal and motor activity. Indeed, amphetamine and caffeine administration, which evoke a general behavioral activation, significantly increase the number of Hcrt+/c-Fos+ neurons in the medial part of the PFH-LH (32,33). We found that although the firing rate of Hcrt cells was correlated with the presence of motor activity (Fig. 3a,b), in some cases, this correlation was very weak or absent. Sound for example produced EEG desynchronization, elicited burst Hcrt cell discharge but did not substantially alter motor output (Fig. 3c). Similarly, behaviors with equal levels of motor activity could be accompanied by very different discharge frequencies of Hcrt neurons. As mentioned above, Hcrt cell activity decreased during food aversion despite the presence of substantially increased motor activity that manifested as repeated approaches and withdrawals. These results show that the discharge frequency of Hcrt neurons is modulated by sensory stimuli as well as emotional and/or motivational states independently of the activity of the motor system.

VI. Hcrt Neuronal Activity and Cataplexy

The data presented above suggest that Hcrt cell activation correlates with behaviors that may be associated with the triggering of cataplexy in human and animals. In particular, positive emotional events such as laughter, feelings of excitation or elation are the most frequently reported trigger for cataplexy in patients with narcolepsy (34,35). In contrast, sadness and pain very rarely induce cataplectic attacks. In narcoleptic dogs, cataplexy is triggered by the consumption of highly palatable food and by excited play, but not by regular food or a noxious stimuli (36,37). In rodents, triggering of cataplexy is most frequently linked to exploration, burrowing, seeking, and grooming (38,39), behaviors all associated with increased Hcrt cell activity (5). The excitation of the Hcrt system in the normal brain may thus appear to counter the activation of mechanisms that are responsible for a decrease in muscle tone and an inhibition of motor activity in response to positive emotional stimuli. The specific circuitry that underlie the emotional triggering of cataplexy is however unknown but may involve the interaction of mesocorticolimbic dopaminergic and Hcrt systems.

VII. Hcrt Neuronal Activity and Stress

Stress an unpleasant stimuli, has been suggested to activate Hcrt neurons in some conditions. Selected stress-like states (novelty-stress, immobilization, foot-shock) elicit c-Fos expression in Hcrt neurons (40-42). Analysis of the animal's behavior in these paradigms shows both a high level of arousal and of motor activity. Moreover, Hcrt neurons are activated by noxious stimuli and descending projections involved in descending regulation of analgesia (43). Thus, stress-inducing conditions related to motor activation and analgesia are accompanied by an excitation of the Hcrt system. In contrast, fear conditioning that is characterized by decreased motor activity and enhanced pain sensitivity does not elicit c-Fos expression in Hcrt neurons (41). Because c-Fos is not expressed in cells when the background activity is stable and

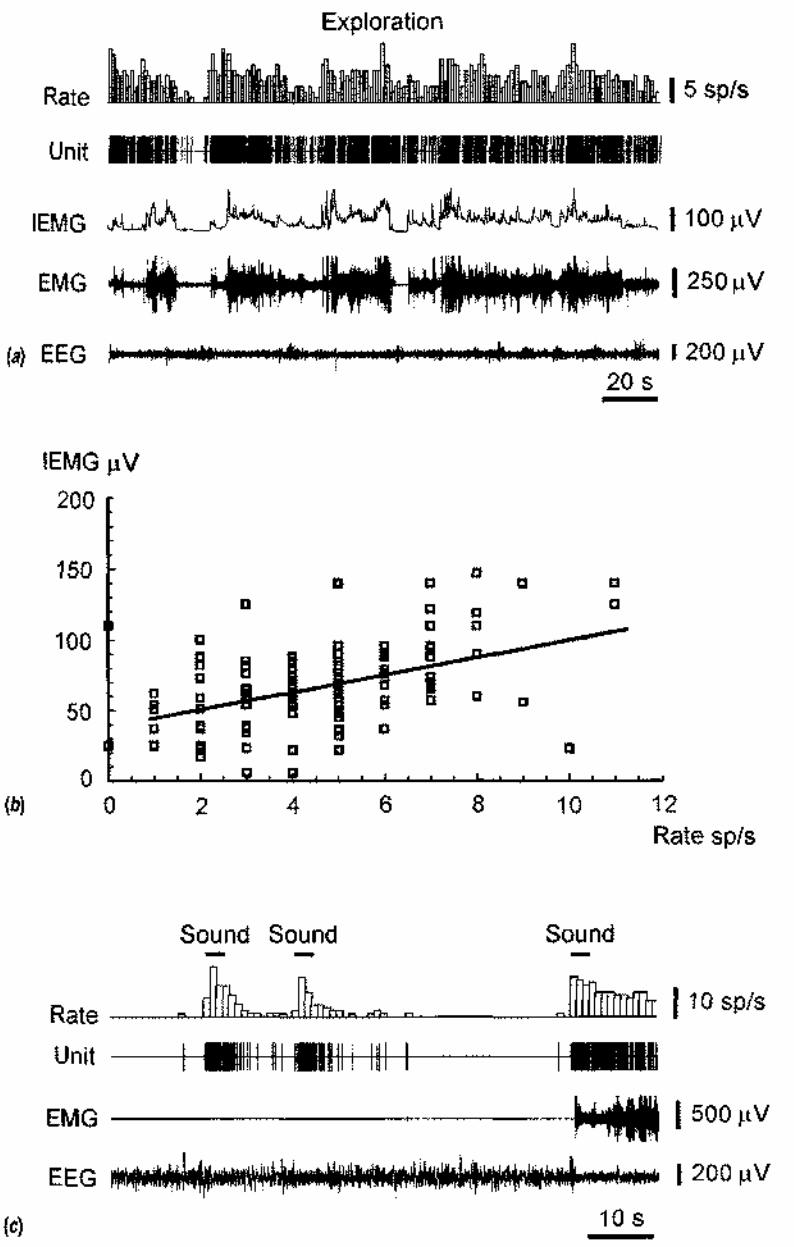

Figure 3 Example of the correlation between the firing rate of a Hcrt neuron and the amplitude of the neck EMG during exploratory behavior and short-lasting Hcrt cell excitation in response to sound stimuli. (a) The alteration of Hcrt cell firing rate, neck EMG, integrated neck EMG (IEMG), and EEG during exploratory behavior of the freely moving rat. *(b)* The correlation between Hcrt cell firing rate and the amplitude of the neck IEMG during exploratory behavior. *(c)* Sound stimuli induce Hcrt discharges independently of marked neck muscle activation. See abbreviations in Figure 1.

under inhibitory influences, it remains to be determined whether Hcrt cells keep their low baseline firing rate or if they are inhibited by fear. In this context, our data demonstrating strong reduction of Hcrt cell discharges during the presentation of novel food (neophobia) suggests an inhibition of Hcrt neurons by fear.

VIII. Hcrt Neuronal Activity and Psychotropic Drugs

Psychotropic drugs modulating dopaminergic tone have been shown to influence c-Fos activation in Hcrt cells. In rats, administration of methamphetamine and amphetamine increases the percent of Hcrt neurons expressing c-Fos in rats (9,32). The increased c-Fos expression correlates significantly with prolonged wakefulness and increased motor activity. Antipsychotic drugs clozapine, olanzapine, and risperidone also significantly increase the number of Hcrt+/c-Fos+ neurons after intraperitoneal administration. Interestingly, however, clozapine predominantly induces c-Fos in Hcrt neurons located in the lateral part of the PFH-LH, whereas other antipsychotics increase the number c-Fos positive Hcrt neurons in the medial hypothalamic region. Ziprasi-done, haloperidol, and fluphenazine are ineffective in inducing c-Fos expression in Hcrt cells (32). Low doses of the wake-promoting drug modafinil significantly increase c-Fos immunoreactivity in Hcrt and tuberomammillary nucleus neurons suggesting that modafinil may promote wakefulness through both Hcrt and histamine systems (44). However, later studies showed that the wake-promoting effects of modafinil and methamphetamine are predominantly related to activation of dopaminergic system, because dopamine transporter knockout mice are unresponsive to administration of these drugs (45). Moreover, recent studies have shown that modafinil more effectively increases wakefulness in hypocretin knockout mice when compared to wild-type mice (46). Administration of mixed and selective dopamine receptor agonists increases the number of c-Fos positive Hcrt neurons. This effect must however involve transsynaptic mechanisms because Hcrt neurons rarely express dopamine receptors (47). Moreover, dopamine directly hyperpolarizes Hcrt cell membrane as shown in rodent slices (48). Georgescu et al. (49) reported that 50% of Hcrt neurons express μ-opioid receptors and about 30% express c-Fos after chronic morphine administration or during acute withdrawal. These authors also found that Hcrt knockout mice develop attenuated morphine dependence. The number of Hcrt+/c-Fos+ neurons also increases in conditioned rats during exposure to environments previously paired with morphine, cocaine or food. Furthermore, there is a significant correlation between the amount of place preference and the number of Hcrt+/c-Fos+ neurons (50). These results suggest that Hcrt neurons may be involved in the action of some psychotropic drugs modulating arousal, emotional states and motor activity. The interaction of Hcrt neurons with structures of the limbic system, dopaminergic nuclei, and brainstem regions related to regulation muscle tone and motor output may underlie these effects.

IX. Conclusion

On the basic of juxtacellular labeling with Neurobiotin and immunohistochemical staining for Hcrt, we found that Hcrt neurons could be identified by antidromic

activation from the VTA and the presence of a broad LPD in their spike waveform. We found that Hcrt neurons discharge with maximal frequency during exploratory behavior and are relatively inactive in QW. During grooming and eating, Hcrt neurons have moderate and approximately equal levels of activity. Hcrt neurons are silent in SW sleep and tonic periods of REM sleep with occasional burst discharges that sometimes correlate with muscle twitches in phasic REM sleep. Responding to metabolic cues, Hcrt neurons may maintain energy homeostasis by triggering food-seeking behaviors. Some stress-like states accompanied by strong motor activity and analgesia increase the number of excited Hcrt neurons. Sensory stimuli and emotional cues may modulate Hcrt cell firing rate independently from cortical arousal and motor output. The presented data suggest that Hcrt neurons are involved in the regulation of emotional states, modulating arousal and motor activity, and are excited during conditions similar to those that trigger cataplexy in narcoleptic animals. Hcrt activity may thus be needed to counteract a state of physiological atonia trigerred by emotions.

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