

The Hypocretin Story

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Abstract The main topic of this book stems from the discovery of two neuropeptides derived from the same precursor, conducted in parallel by two different groups (de Lecea et al. in *Proc Natl Acad Sci USA* 95:322–327, 1998; Sakurai et al. in *Cell* 92:573–585, 1998). Here is the account of the discovery of the hypocretin peptides, which was the result of a larger effort to characterize patterns of gene expression in the brain. The original hypothesis leading to the discovery of the peptides was that new mechanisms underlying the many functions of the hypothalamus (e.g. feeding, thermoregulation, circadian rhythmicity, sexual behavior and arousal) could be unraveled by identifying the expression patterns of hundreds of “cell-type” specific transcripts. This hypothesis ended up converging with a reverse pharmacology approach followed by Yanagisawa and colleagues, which is described in detail in other chapters. With the hypocretin story in the background, I also describe here the first implementation of optogenetic methods in a freely moving animal, which has led to a revolution in systems neuroscience.

1 Clones of Hypothalamus-Enriched mRNAs

The hypothalamus has long been recognized as a site for central regulation of homeostasis (Bloom 1987). In contrast to laminar cortical structures such as the cerebellum and hippocampus whose final functions rely on input from the thalamus and brain stem, the hypothalamus is organized as a collection of distinct, autonomously active nuclei with discrete functions (Swanson 1999). Ablation and electrical stimulation studies have implicated several of these nuclei as central regulatory centers for major autonomic and endocrine homeostatic systems mediating processes such as reproduction, lactation, fluid balance, blood pressure, thermoregulation,

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metabolism, and aspects of behaviors, such as circadian rhythmicity, basic emotions, the sleep/wake cycle, feeding and drinking, mating activities, and responses to stress, as well as normal development of the immune system. Distinct hormones and releasing factors have been associated with some of these nuclei.

To illuminate additional molecules that contribute to the specialized functions of hypothalamic nuclei, Bloom and Sutcliffe embarked on a systematic analysis of the mRNAs whose expression is restricted to or enriched in the hypothalamus (Gautvik et al. 1996; Milner et al. 1987). The plan involved the following steps: Directional tag PCR subtractive hybridization (Usui et al. 1994) was used to enrich a cDNA library for clones of mRNA species selectively expressed in the hypothalamus. Candidate clones identified by their hybridization to a subtracted hypothalamus probe were validated in three stages. First, a high throughput cDNA library Southern blot was used to demonstrate that the candidate corresponded to a species enriched in the subtracted library. Second, candidate clones positive in the first assay were used as probes for Northern blots with RNA from several brain regions and peripheral tissues. Finally, candidate clones that were still positive were subjected to in situ hybridization analysis to detect the hypothalamic regions that express the corresponding mRNAs (Gautvik et al. 1996).

I joined the Sutcliffe lab as a postdoc in 1992. Hiroshi Usui, Mark Erlander and Ana Dopazo perfected the subtraction hybridization technique, and we used an in situ hybridization screening method I developed in Barcelona to generate a list of mRNAs enriched in the striatum (Usui et al. 1994). Using the subtraction and in situ screening methods, we reported the discovery of Cortistatin, a novel somatostatin-like gene expressed in the neocortex and hippocampus with neuronal depressant and sleep-inducing properties (de Lecea et al. 1996).

Kaare and Vigdis Gautvik of the University of Oslo visited Greg Sutcliffe's lab for a sabbatical year to learn the subtraction method. They were given the hypothalamus project and applied the directional tag PCR subtractive hybridization method to construct a rat hypothalamic cDNA library from which, in two consecutive steps, cerebellar and hippocampal sequences had been depleted, enriching 20-to-30 fold for sequences expressed selectively in the hypothalamus. They made a radioactive probe from the library inserts and used it to screen 648 clones from the subtracted library that they had spotted into grid arrays. Approximately 70 % of the colonies gave significant signals with the subtracted target probe compared to 50 % with an unsubtracted target probe. Only 10 % of the colonies gave signals with a mixed-driver probe. These data indicated that the enrichment by subtraction was substantial. Among the clones showing the highest degree of hypothalamus enrichment were cDNAs for oxytocin, vasopressin, CART, melanin concentrating hormone, POMC, VAT-1, and a novel species called clone 35.

As part of the screening effort, we conducted in situ hybridization on coronal sections of brain from adult male rats, using the inserts from clones representing many of the RNAs, including clone 35 (Fig. 1). The clone 35 mRNA displayed a striking pattern of bilaterally symmetric expression restricted to a few cells in the dorsolateral hypothalamic area, with no signals outside the hypothalamus (de Lecea et al. 1998). Other novel mRNAs showed substantial enrichment in basal

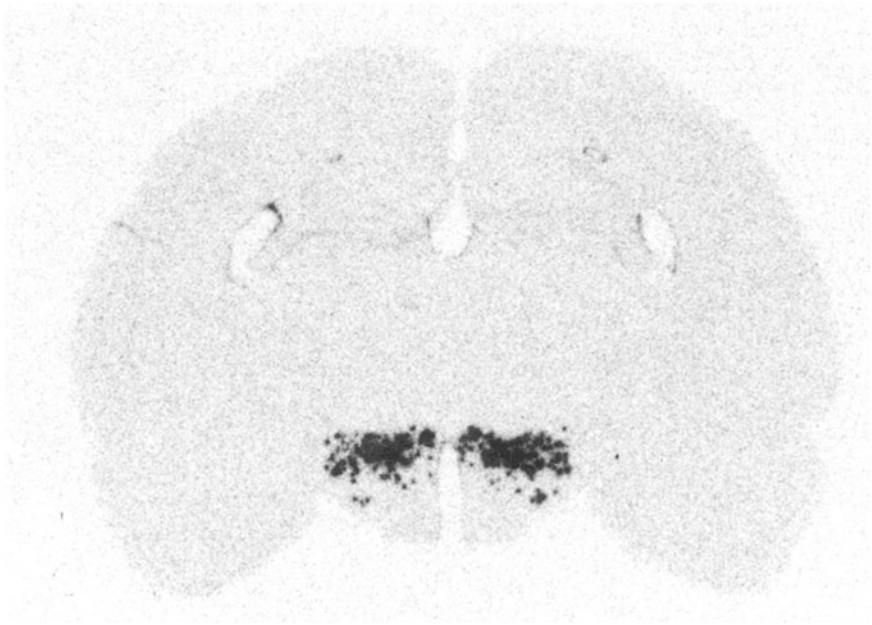


Fig. 1 The first glimpse of the hypocretin system. In situ hybridization of rat coronal section with cDNA isolated in subtractive hybridization study detecting a few thousand neurons in the dorsal-lateral hypothalamus [from Bloom (1987)]

diencephalic structures, particularly the hypothalamus, without restriction to single hypothalamic nuclei.

Among the novel species we identified, only clone 35 met our starting criterion in its strictest sense: the mRNA appeared to be restricted to a single nucleus in the dorsolateral area of the hypothalamus (the in situ hybridization image in reference 2 represents the first published description of the hypocretins).

2 Clone H35

We used the original rat cDNA clone 35 to isolate full-length cDNAs for both rat and mouse. The 569-nucleotide rat sequence (de Lecea et al. 1998) suggested that the corresponding mRNA encoded a 130-residue putative secretory protein with four pairs of tandem basic residues for potential proteolytic processing. We then isolated a cDNA clone from mouse libraries and its sequence revealed only that two of the four possible rat maturation products were conserved. Both of these terminated with glycine residues, which in proteolytically processed secretory peptides typically are substrates for peptidylglycine alpha-amidating monooxygenase, leaving a C-terminal amide in the mature peptide. These features suggested that the

product of the clone 35 hypothalamic mRNA served as a preprohormone for two C-terminally amidated, secreted peptides. One of these, which was later to be named hypocretin 2 (hcrt2), was, on the basis of the putative preprohormone amino-acid sequence, predicted to contain precisely 28 residues.

The C-terminal 19 residues of these two putative peptides shared 13 amino acid identities. This region of one of the peptides contained a 7/7 match with secretin, suggesting that the preprohormone gave rise to two peptide products that were structurally related closely to each other and distantly to secretin. We initially commented on the secretin similarity. Sequence similarities with various members of the incretin family, especially secretin, suggested that the gene was formed from the secretin gene by three genetic rearrangements: first, a duplication of the secretin gene; second, deletions of the N-terminal portion of the 5' duplicate and the C-terminal portion of the 3' duplicate to yield a secretin with its N- and C- termini leap-frogged (circularly permuted); and third, a further duplication of the permuted gene, followed by modifications, to form a secretin derivative that encoded two related hypocretin peptides.

3 Nomenclature

As we began to write the paper describing our discovery of the peptides via cDNA cloning, their immunohistochemical detection, their presence in dense core vesicles at synapses, and their neuroexcitatory properties on hypothalamic neurons, we realized that we needed a name other than clone 35. There were several possible functions for the peptides, but direct evidence for none. Based on our previous discovery of Cortistatin (which we named after its predominantly *cortical* expression and its similarity with *somatostatin*), (de Lecea et al. 1996) we came up with several non-pejorative possibilities, most of which were variations on syllables abstracted from *hypothalamic* member of the *incretin* family. The most straightforward of these possibilities was “hypocretin”. At the 1997 Society for Neuroscience Meeting, Greg Sutcliffe presented a poster describing the sequence of the new protein, the expression of its mRNA exclusively in a small number of neurons in the dorsolateral hypothalamus, the electron microscopic detection of immunoreactive vesicles in presynaptic boutons, and the neuroexcitatory properties of the amidated hcrt2 peptide. At the adjacent poster, Cristelle Peyron and Tom Kilduff presented the data detecting hypocretin immunoreactivity in the dorsolateral hypothalamic neurons and immunoreactive fibers through the CNS (Peyron et al. 1998).

At the poster, a ballot listing several of the names under consideration was listed and asked poster attendees to express their preference. The plurality of the votes were cast for hypocretin. Thus, this became the first democratically named neurotransmitter.

We sent a reformatted text using the revised name hypocretin to the *PNAS*, where it was accepted and published on January 6, 1998 (de Lecea et al. 1998). In the paper we noted that the existence of two hcrt peptides that differ in their amino acid sequences might indicate two Hcrt receptor subtypes (de Lecea et al. 1998).

4 Independent Discovery

The February 20, 1998 issue of *Cell* carried an article describing the identification of two endogenous peptides that stimulated calcium flux in cells transfected with an orphan G protein-coupled receptor (Sakurai et al. 1998). Sakurai and colleagues demonstrated that intracerebroventricular administration of either hcr1 or hcr2 increased food consumption in rats. Furthermore, rats fasted for 48 h increased the concentration of hypocretin mRNA and peptides. Based on these observations, they proposed the alternative name, orexins, for the hypocretin peptides (Sakurai et al. 1998).

This study demonstrated the actual presence of the two proteolytically processed hypocretin peptides within the brain, and elucidated by mass spectroscopy the exact structures of these endogenous peptides, which could not be deduced from nucleic acid sequences alone. The structure of orexin B was the same as that predicted from the hcr2 cDNA sequence. The N-terminus of orexin A (hcr1) was defined and found to correspond to a genetically encoded glutamine derivatized as pyroglutamate. The two intrachain disulfide bonds within hcr1 were also defined.

A commentary about new hypothalamic factors accompanied the *Cell* report. It mentioned both the hypocretins and the orexins, without realizing that they were the same peptides. The omission was inadvertent but, unfortunately, this was the beginning of a dual nomenclature. The Yanagisawa group published an addendum in the March 6 *Cell* indicating that the orexins are the same as the hypocretins (Sakurai et al. 1998).

5 Hypocretin Functions

The papers describing the independent discovery and characterization of the hypocretins/orexins have catalyzed a great body of work. There are now already over 12,000 papers concerning aspects of these neurotransmitters, including their prominent role in arousal state transitions, brain reward, stress, panic and other neuropsychiatric conditions. The perifornical region of the hypothalamus has been associated with nutritional homeostasis, blood pressure and thermal regulation, neural control of endocrine secretion, brain reward and arousal. Thus, these activities ranked among those affected by the peptides (Sakurai 2014; Li et al. 2014).

The association of Hcr1 deficiency with narcolepsy conducted in parallel in two different laboratories using, again, two independent approaches, clearly demonstrated that Hcr1 system is a non-redundant peptidergic system whose critical function is to stabilize arousal states (de Lecea and Huerta 2014).

How do Hcr1 neurons maintain wakefulness? In 2005, the groups of Siegel and Jones independently reported the recording of identified Hcr1 neurons from awake animals (Mileykovskiy et al. 2005; Lee et al. 2005). These recordings revealed that Hcr1 neurons are phasically active during active waking, and practically silent

during quiet waking and sleep. Interestingly, the highest activity was observed during transitions between rapid eye movement sleep and wakefulness. This discovery also showed that the pharmacological approach to studying the role of Hcrt in sleep and wakefulness was not appropriate, as drugs take several minutes to diffuse into brain structures and therefore span several sleep/wake cycles in rodents. In order to determine precisely whether Hcrt release was instructive or permissive to wakefulness, other methodologies needed to be applied.

6 Optogenetics

To further explore the mechanism by which phasic Hcrt activity maintains wakefulness, we used a newly developed optogenetic approach to manipulate the activity of Hcrt neurons *in vivo* (Adamantidis et al. 2007). Optogenetics uses actuator opsin molecules (e.g., channelrhodopsin-2 (ChR2) or halorhodopsin—NpHR) to selectively activate or silence genetically-targeted cells, respectively, with flashes of light at specific wavelength (Boyden et al. 2005). Further information about optogenetic technology can be found in many other excellent reviews (Fenno et al. 2011).

In an effort to better understand the temporal dynamics of neural circuits of wakefulness, we applied optogenetics to reversibly and selectively manipulate the activity of hcr neurons in freely-moving animals. To deliver light to the hcr or LC field, we designed optical-neural interfaces in which optical fibers were chronically implanted on the mouse skull (Fig. 2). Using this strategy, we were able to control

Fig. 2 Light delivery into the brain. Picture of a mouse transduced with a virus expressing Channelrhodopsin in Hcrt neurons and cannulated with an optical fiber to stimulate Hcrt neurons with millisecond precision



hcrt neural activity both *in vitro* and *in vivo* with millisecond-precise optical stimulation (Adamantidis et al. 2007). The high temporal and spatial precision of stimulation allowed us to mimic the physiological range of hypocretin neuron discharge rates (1–30 Hz) (Lee et al. 2005; Hassani et al. 2009).

Photostimulations of mice transduced with a Hcrt::ChR2 lentivirus at frequencies 5 Hz and higher dramatically decreased the latency to wakefulness compared with mice transduced with a control Hcrt::mCherry lentivirus. An important control included stimulations of Hcrt neurons in Hcrt knockout animals, and demonstrated that the reduction on the latency to wakefulness was strictly dependent on Hcrt release. Interestingly, chronic stimulation studies revealed that persistent stimulation of Hcrt neurons cannot maintain wakefulness (Adamantidis et al. 2007).

Importantly, these results demonstrate a causal link between hcrtn neuron activation and sleep-to-wake transitions, consistent with previous correlative studies. This was further supported by the fact that optical silencing of hcrtn neurons promote SWS (Tsunematsu et al. 2011).

In a followup study (Carter et al. 2010) we genetically targeted noradrenergic neurons in the locus coeruleus, a major target of Hcrt cells, by stereotaxic injection of a Cre recombinase- dependent adeno-associated virus (rAAV) into knock-in mice selectively expressing Cre in tyrosine hydroxylase (TH) neurons. We found that both NpHR and ChR2 were functional and could inhibit and activate, respectively, LC-NE neurons both *in vitro* and *in vivo*. Optogenetic stimulation of LC-NE neurons at low frequencies (1–10 Hz) caused immediate (less than 5 s) sleep-to-wake transitions from both SWS and REM sleep. Approximately 20 pulses of light delivered over 5 s in the LC (presumably each leading to an action potential) were sufficient to induce an awakening deterministically (i.e. with a probability of 1). Stimulation of LC neurons during wakefulness increased locomotor activity and the total time spent awake, confirming the strong arousal effect. In contrast, NpHR-mediated silencing of LC-NE neurons decreased the duration of wake episodes but did not block sleep-to-wake transitions when animals were asleep. Taken together, this study showed that activation of LC-NE neurons is necessary for maintaining normal durations of wakefulness (NpHR experiment), and sufficient to induce immediate sleep-to-wake transitions, sustained wakefulness, and increased locomotor arousal. Thus, we proposed that the LC-NE neurons act as a fast tuning system to promote sleep-to-wake transitions and general arousal.

The question remained as to whether Hcrt and LC neurons, which had already been shown to be anatomically connected, were also functionally connected. We therefore used combinatorial optogenetics to determine whether LC neurons were necessary for Hcrt-induced awakenings. Simultaneous photostimulation of Hcrt neurons at 10 Hz and photoinhibition of LC neurons did not result in reduced latencies to wakefulness, strongly suggesting that noradrenergic transmission is necessary for at least one component of the awakenings elicited by Hcrt (Carter et al. 2012).

Since the first set of manuscripts using optogenetics to study behavioral state transitions, more than 5000 publications have used similar approaches *in vivo*. Optogenetics was selected as “Method of the Year” in 2010 and one of the

“Breakthroughs of the Decade” by the journal *Nature*. Clearly, the ability to manipulate genetically defined cell populations with millisecond precision is advancing our understanding of the mesoscale organization of the brain (Carter and de Lecea 2011; Deisseroth 2014). With regard to the Hcrt neurons, optogenetics has allowed us to functionally map connections with other neuronal structures associated with arousal and brain reward, two of the main functions of the hypocretinergic system (Carter et al. 2013).

Thus, the discovery of the hypocretins/orexins has led to many important advances in the neurosciences: providing a mechanism for narcolepsy and the regulation of sleep and wakefulness (Partinen et al. 2014); the first-in-class molecule for the treatment of primary insomnia (Osborne 2013), and is also leading the way of circuit functional mapping of neuromodulators (Giardino and de Lecea 2014). This is a lot to celebrate in only 15 years of existence.

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