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Peptides 23 (2002) 2203–2211

PEPTIDES

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Measurement of hypocretin/orexin content in the mouse brain using an enzyme immunoassay: the effect of circadian time, age and genetic background

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Received 17 April 2002; accepted 8 July 2002

Abstract

The hypocretins (1 and 2) have emerged as key regulators of sleep and wakefulness. We developed a high-throughput enzyme immunoassay (EIA) to measure total brain hypocretin levels from large numbers of mice. Hypocretin levels were not altered by circadian time or age. However, significant differences in one or both hypocretin peptides were observed between different mouse strains. We studied hypocretin levels in knockout and transgenic mouse models with obesity, circadian gene mutations or monoaminergic defects. Compared to controls, only histamine receptor knockouts had lower hypocretin levels. This was most pronounced in H1 receptor knockouts suggesting the existence of a positive feedback loop between hypocretin and histaminergic neurons.

Keywords: Hypocretin; Orexin; Narcolepsy; Enzyme immunoassay; Histamine receptor; Knockout

1. Introduction

Hypocretin-1 and -2 (also called orexin A and B) are neuropeptides synthesized by neurons in the lateral hypothalamus [6,35]. These neurons project throughout the brain [26,35], but particularly to regions involved in the control of feeding, sleep-wake cycle, hormone release, body temperature, and autonomic control [23,35]. Hypocretins have mainly excitatory effects on neuronal activity [23]. Notably, they stimulate monoaminergic neurons of the locus coeruleus, raphe magnus, ventral tegmental area and tuberomamillary nucleus neurons [1,2,9,14,50].

One of the most important functions of these peptides may be the regulation of sleep [17,20,23,45]. Hypocretin deficiency causes the sleep disorder narcolepsy in various models and in human patients. Narcolepsy is a sleep disorder characterized by excessive daytime sleepiness, cataplexy and a tendency to enter rapid eye movement (REM) sleep with an abnormally short latency [31,32]. In a canine model, the disease is caused by mutations in the hypocretin receptor-2 gene [16,22]. Disruption of the preprohypocretin gene in mice produces a syndrome similar to human and canine narcolepsy [5]. In humans, however, mutations of hypocretin genes (ligand and receptor) are not commonly observed. Only one unusual case of narcolepsy has been associated with a mutation in the preprohypocretin gene [34]. Despite the absence of mutations in the vast majority of cases, narcoleptic patients have a remarkable reduction in the number of hypocretin neurons [34,42], and hypocretin peptides are undetectable in CSF from most cases [29,30,36].

A role for hypocretins in the regulation of energy metabolism and food intake was initially suggested by their intrahypothalamic localization [6,37]. The finding that hypocretin deficiency causes narcolepsy suggested a role for sleep regulation in coordination with metabolic status. Intracerebroventricular injection of hypocretins stimulates appetite, increases temperature and blood pressure, activates sympathetic outflow and releases cortisol [17]. Animals are awake and active with increased energy consumption. Studies of the diurnal variation of hypocretin peptide content in CSF throughout the day and food deprivation in rats suggest

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0196-9781/02/$ – see front matter
PII: S0196-9781(02)00251-6

a link between circadian and homeostatic control of sleep and energy homeostasis via the hypocretin system [11]. Taheri et al. [39] demonstrated a significant diurnal fluctuation in hypocretin-1 levels in the pons, and preoptic/anterior hypothalamic region of the rat brain. Studies of immediate early gene activation in rats indicate that hypocretin activity is high when the animal is awake and active and low during sleep [10].

Our goal was to establish a high-throughput enzyme immunoassay (EIA) to measure hypocretin-1 and -2 levels in whole mouse brains, making it possible to investigate sources of variation in peptide content. Hypocretin levels were measured in mice at different ages, in different strains, and with various genetic mutations. As most investigators have used rats to study hypocretin regulation, little is known about hypocretin levels in mice, which are more commonly used in genetic manipulation studies. Further studies using mice models will be required to identify related genes and gene products that impinge on hypocretin neurotransmission, providing greater understanding of the role of hypocretin in regulating sleep, circadian rhythms, and other homeostatic mechanisms.

2. Materials and methods

2.1. Animals and procedures

Experiments were carried out under locally approved protocols. Eight-week-old male C57BL/6J mice (Jackson laboratory, Cold Spring Harbor) were maintained under constant temperature (25 °C) and 12 h:12 h light:dark cycles (lights on 06:30 h) for 1 week prior to the circadian time experiment. A total of 168 mice were divided into three groups: 32 h constant darkness (DD, $n = 63$), 32 h 12 h:12 h light:dark (LD, $n = 63$), and 32 h constant light (LL, $n = 42$). Seven mice were taken out from each group at 0, 4, 8, 12, 16, 20, 24, 28, and 32 h after the onset of the experiment and the mouse brains were removed immediately after euthanasia.

Twenty-five C57BL/6J mice brains were collected for the aging study. Their ages ranged from 8 to 122 weeks. To study different mouse strains, C57BL/6J ($n = 8$), CAST/Ei ($n = 10$), SJL/J ($n = 8$), DBA/2J ($n = 8$) were used. These animals were sacrificed in the early light phase of a 12 h:12 h LD cycle.

Clock mutant mice (Clock: +/+, $n = 6$; +/−, $n = 5$; −/−, $n = 5$) are coisogenic C57BL/6J and maintained in the Association for Assessment and Accreditation of Laboratory Animal Care accredited Center for Experimental Animal Resources at Northwestern University. Dopamine transporter (DAT: +/+, $n = 10$; +/−, $n = 8$; −/−, $n = 8$) and vesicular monoamine transporter mice (VMAT: +/+, $n = 10$; +/−, $n = 10$) are C57BL/6J background and were provided by the Molecular Neurobiology Branch, National Institute on Drug Abuse. Histamine H1 receptor deficient mice (H1KO: +/+, $n = 11$; −/−, $n = 17$) and histamine receptor H2 deficient mice (H2KO: +/+, $n = 8$; −/−, $n = 13$) are C57BL/6J background and were donated by the Department of Pharmacology, Tohoku University. Histamine H3 receptor deficient mice (H3KO: +/+, $n = 11$; −/−, $n = 12$) are C57BL/6J background and were provided by Center for Sleep and Circadian Biology, Northwestern University. Leptin deficient *ob*/*ob* (+/+, $n = 8$; −/−, $n = 8$) and leptin receptor deficient *db*/*db* (+/+, $n = 8$; −/−, $n = 8$) mice are C57BL/6J background and were purchased from the Jackson laboratory.

Mice were anaesthetized individually by carbon dioxide inhalation followed by decapitation. The whole mouse brain was removed immediately and kept in a 24-well box (Whatman, 10 ml, polypropylene, Clifton, NJ), frozen on dry ice and stored at −80 °C prior to assay. In cases in which direct comparisons were made, all animal samples were extracted and assayed in the same experiment.

2.2. Peptide extraction

Frozen brains were weighed and boiled in 2 ml of Milli-Q water in 24-well plates for 10 min. After cooling on ice, the plate was spun briefly and acetic acid and HCl were added to each well making a final concentration of 1 M and 20 mM, respectively. Each brain was homogenized at 40,000 rpm for 30 s. Two milliliters of peptide extraction buffer (1 M acetic acid and 20 mM HCl) were added to each well followed by centrifugation at 5500 rpm for 20 min. The supernatants were aliquoted into 96-well plates and kept at −80 °C.

2.3. Enzyme immunoassay (EIA)

The principle of the assay is based on a competitive EIA protocol. A 96-well plate is pre-coated with secondary antibody and the non-specific binding sites are blocked. The secondary antibody can bind to the Fc fragment of the peptide specific antibody while the Fab fragment of the peptide-specific antibody will be competitively bound to both biotinylated peptide and targeted peptide in samples. The biotinylated peptide interacts with the enzyme which catalyzes the substrate to produce a colored solution. The intensity of color is inversely proportional to the amount of the peptide in the sample. Hypocretin levels were determined using a standard curve (range 0.1–40 ng/ml) prepared from synthetic peptide (Phoenix Pharmaceuticals, Belmont, CA) running with every single plate.

EIA were performed on polystyrene 96-well EIA/RIA plates (Costar, Fisher Scientific, Santa Clara., CA). The plates were coated with 1 ug/ml goat anti-rabbit antibody (Zymed, San Francisco, CA) in coating buffer, 250 ul per well for 2 h at 37 °C. The plate was washed four times with TNT buffer (0.05 M Tris, pH 7.5; 0.15 M NaCl; 0.05% Tween-20), and then blocked with TNTB buffer (TNT plus 1% BSA) at 37 °C for 1 h. After pouring off the blocking buffer, the blocked plates were air dried, sealed by adhesive

sealing film (Rainin, Emeryville, CA) and stored at 4 °C until use.

The peptide solution was dried in siliconized (Sigmacote Sigma, St. Louis, MD) 96-well plates (Nalge Nunc, Roskilde, Denmark) under a fume cupboard hood overnight. Peptide was resuspended in TNTB buffer, added to pre-coated plate and incubated with hypocretin-1 or -2 antibodies (Phoenix Pharmaceuticals, Belmont, CA) at room temperature for 2 h. Biotinylated hypocretin-1 or -2 (provided by Neurocrine, San Diego, CA) were added to each well followed by incubation at room temperature for another 2 h. The plate was washed five times with TNT buffer, streptavidin–horseradish peroxidase conjugate (Biosource, Camarillo, CA) was added to each well and incubated for 1 h at room temperature. Wells were washed six times with TNT buffer, then incubated for 1 h in TMB liquid (3,3′,5,5′-tetramethyl-benxidine substrate, Sigma, Steinheim, Germany). To stop the enzyme reaction, 2N HCl was added to each well. Absorbance at 450 nM was measured with a microplate reader (Molecular Devices, Sunnyvale, CA).

The sensitivity of the EIA is 0.1–40 ng/ml. Intra-assay coefficients of variation (CV) are 6.6 and 4.9% for hypocretin-1 and -2, respectively. To adjust for inter-assay variation, we included a reference sample in every assay and adjusted final values accordingly. All comparative measurements were done in a single assay and every sample was assayed in duplicate or triplicate. We have done multiple comparisons of the results generated from the EIA with parallel assays from the RIA. We have also, furthermore, done multiple comparisons of the same sample using the EIA technique to show reproducibility.

2.4. Radioimmunoassay (RIA)

Hypocretin-1 and -2 were measured using a commercially available ^{125}I RIA kits (Phoenix Pharmaceuticals, Belmont, CA). Briefly, dried peptide was resuspended in RIA buffer and duplicate samples were assayed. Levels were determined against a known standard. The intra-assay CVs were 2.9 and 4.6% for hypocretin-1 and -2, respectively.

2.5. Statistical analysis

All results are presented as mean ± S.E.M. with statistical analysis by the unpaired *t*-test with Bonferroni adjustment.

3. Results

3.1. Correlation between EIA and RIA measurements

The RIA method has high sensitivity; its detection limit is 4 pg per tube for both hypocretin-1 and -2. The intra-assay variation is less than 5%. This method is suitable for detecting hypocretin at very low levels such as in cerebrospinal fluid [29,36]. With the EIA method, the detection limit is 0.6 ng/ml. The intra-assay variation is less than 7%. There is good correlation between hypocretin levels measured by EIA and RIA: the correlation coefficients are 0.75 ($n = 34$, $P < 0.0001$) for hypocretin-1 and 0.84 for hypocretin-2 ($n = 85$, $P < 0.0001$) (Fig. 1). The samples involved in the correlation study included samples from the different strains studied, and H1 and H2 knockout mice including their wild-type

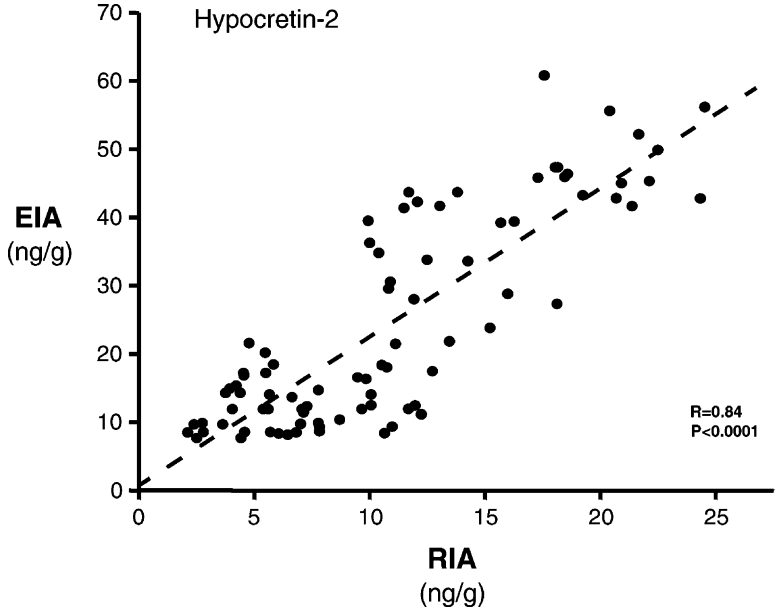

Fig. 1. Correlation between the RIA and EIA methods for hypocretin-2. The samples involved in the correlation study included samples from the different strains studied, and H1 and H2 knockout mice including their wild-type controls. These samples were selected because they provided the greatest contrast in hypocretin levels. There was good correlation between levels measured using the two techniques ($r = 0.84$; $P < 0.0001$). The hypocretin-1 EIA (not shown) also showed good correlation with the RIA ($r = 0.75$; $P < 0.0001$).

controls. These samples were selected because they provided the greatest contrast in hypocretin levels.

3.2. Effect of circadian time and age on hypocretin-1 and -2 levels

Circadian time did not significantly influence hypocretin-1 or -2 levels in whole mouse brain (Fig. 2). This data also was confirmed by RIA method. There was a non-significant tendency towards higher hypocretin-1 levels with aging (Table 1).

3.3. Hypocretin contents in selected mice strains

Four mouse strains were used in this study: C57BL/6J (most commonly used strain), CAST/Ei (usually used for genetic studies), SJL/J (widely used for cancer, cardiovascular, diabetes, obesity, immunology, and inflammation studies), and DBA/2J (used for cardiovascular and neurobiology research). The contents of hypocretin-1 and -2 showed a significant difference between mouse strains (Fig. 3). CAST/Ei mice had the highest hypocretin-1 and -2 levels while SJL/J mice had high hypocretin-1 but the

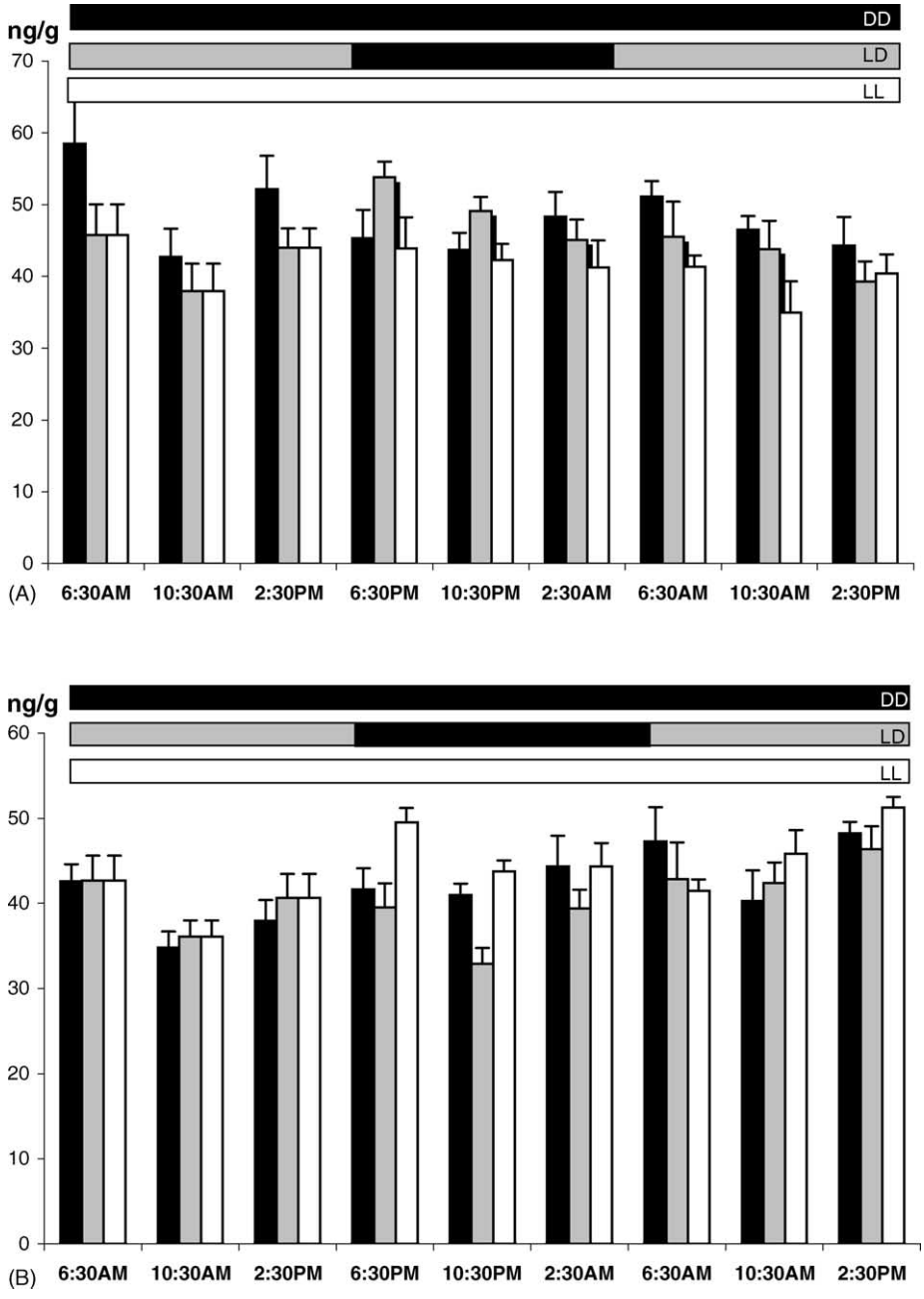

Fig. 2. Circadian alteration in hypocretin-1 (A) and -2 (B) levels. DD: 32 h darkness, LD: 12 h:12 h:12 h light:dark:light, and LL: 32 h lightness. No significant difference was observed for the various time points and alterations in light exposure.

Table 1
Hypocretin content in aging and genetic background study

	n	Hypocretin-1 (ng/g; mean ± S.E.M.)	Hypocretin-2 (ng/g; mean ± S.E.M.)
Age			
8–10 weeks	8	40.91 ± 1.70	43.16 ± 2.15
13 weeks	3	37.62 ± 6.37	34.94 ± 1.39
18–20 weeks	10	53.59 ± 4.59	48.32 ± 2.87
>30 weeks	4	55.15 ± 14.23	42.82 ± 2.33
Genotype			
C57BL/6J	8	55.79 ± 3.13	35.58 ± 2.10
ob/*ob*	8	59.51 ± 3.01	39.98 ± 1.26
C57BL/6J	8	31.08 ± 6.53	35.03 ± 1.04
db/*db*	8	35.29 ± 4.05	40.11 ± 2.18
VMAT+/+	10	36.08 ± 2.44	30.66 ± 1.15
VMAT+/−	10	33.82 ± 3.17	30.69 ± 1.39
DAT+/+	10	37.052 ± 5.04	30.78 ± 0.97
DAT+/−	8	48.68 ± 7.26	32.57 ± 1.93
DAT−/−	8	49.155 ± 4.43	35.06 ± 1.82
Clock+/+	6	36.62 ± 2.15	10.31 ± 0.71
Clock+/−	5	36.03 ± 1.25	10.90 ± 0.82
Clock−/−	5	33.76 ± 1.06	9.59 ± 0.62

n indicates the total number of mouse studied.

lowest hypocretin-2 levels. DBA/2J had the second highest level of hypocretin-2 contents. C57BL/6J mice had relatively low levels for both peptides compared to other strains. Interestingly, the hypocretin-1/hypocretin-2 ratio was not constant among strains.

3.4. Hypocretin contents in various mutant mice

Hypocretin contents were measured in mice harboring null mutations of sleep regulator and metabolism-related genes (Table 1). We found significantly lower hypocretin-1 and -2 levels in histamine H1 knockout mice compared to wild-type (Fig. 4). Histamine H2 knockout mice also showed a much lower hypocretin-2 level, but not hypocretin-1, than wild-type mice. Histamine H3 knockout mice, however, did not show any difference in either of the hypocretins compared to wild-type. No significant difference in hypocretin contents was observed in clock, *db*/*db*, *ob*/*ob*, DAT, and VMAT knockout mice (Table 1).

4. Discussion

In this report, we validated the establishment of an EIA protocol to measure hypocretin peptide content in a large number of samples. The EIA technique provides simple, cheap, fast, and accurate measurement of brain hypocretin-1 and -2 levels. Results correlated tightly with those obtained with a previously validated RIA assay. We now have measured over 1000 samples with high reliability. This approach can be applied to screen large number of samples (results available in 1 day) without the limitations posed by using ^{125}I. This technique can be used to determine major alterations in hypocretin levels resulting from genetic or environmental influences.

To further validate this assay, we first explored whether total hypocretin content varied with age and zeitgeber time. Surprisingly, total hypocretin-1 and -2 brain contents did not vary significantly in different lighting conditions across the entire circadian cycle (this was verified using both EIA and RIA assays, data not shown). This result contrasted with the report by Taheri et al. [40] which indicated diurnal variation in hypocretin-1 content in selected brain areas in rat; in this study, a 50% hypocretin-1 reduction occurred in the preoptic/anterior hypothalamus from 09:00 to 21:00 h, while

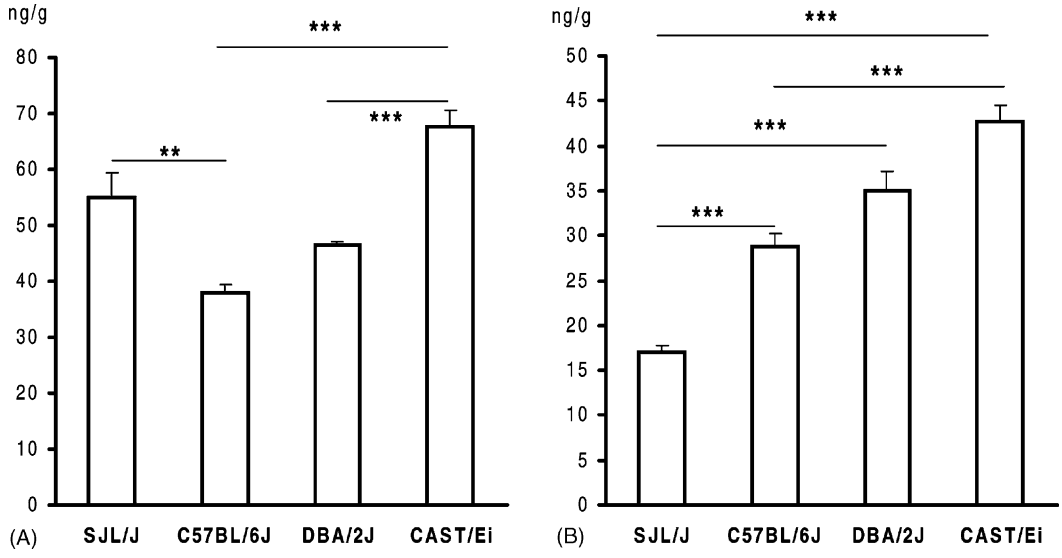

Fig. 3. Hypocretin immunoreactive contents (mean ± S.E.M.) from four different mouse strains. (A) Hypocretin-1. (B) Hypocretin-2. (**) indicates $P < 0.001$. (***) indicates $P < 0.0001$.

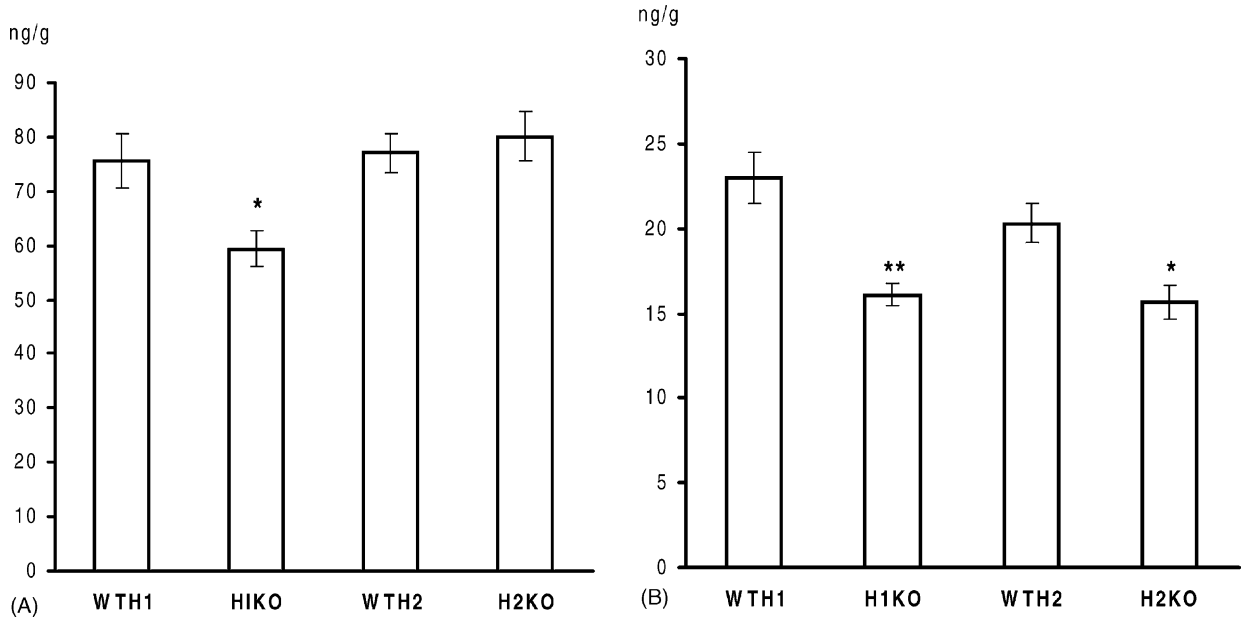

Fig. 4. Hypocretin immunoreactive contents (mean ± S.E.M.) in histamine receptor knockout and control mice. (A) Hypocretin-1 content. H1KO has significantly lower hypocretin-1 levels compared to WTH1 ($^{*}P < 0.01$). (B) Hypocretin-2 immunoreactive content: both H1KO and H2KO have significantly lower levels compared to WTH1 ($^{**}P < 0.001$) and WTH2 ($^{*}P < 0.01$), respectively.

hypocretin-1 levels increased during the same period in the pons. Similarly, recent results indicate significant fluctuations in hypocretin-1 levels across the 24 h cycle in both the CSF and in vivo dialysis extracellular fluid. Hypocretin release is highest at the end of the dark period when animals are most active [11,52]. These studies demonstrate the existence of a diurnal rhythm for hypocretin-1 in rat hypothalamus, CSF, and specific brain areas. The finding that total brain levels do not fluctuate is thus most likely the sum of opposite regional effects. It is also possible that hypocretin-1 levels in terminal regions may change in phase with hypocretin release while levels are building up in the hypothalamic cell bodies.

Hypocretin levels were also not significantly influenced by age past sexual maturity. A statistically non-significant tendency for older mice to have higher hypocretin-1 levels compared to younger mice was observed. This result, together with CSF data in normal humans of indicating no decrease in hypocretin-1 with age or Alzheimer's Disease [29,36] rather suggest that this system is not involved in mediating sleep disturbances associated with aging.

Another interesting result in this study was the observation that hypocretin contents differ substantially in different inbred mouse strains. These differences may be useful in the context of further quantitative trait loci (QTL) analysis. High hypocretin-1 and -2 levels in CAST/Ei mouse may be particularly interesting as these animals have unusually high behavior/locomotor activity during the light period when compared to other strains [21]. Other investigators have indicated lower amounts of sleep and limited sleep rebound after sleep deprivation in this strain [46], a result that could reflect higher hypocretin tone than other strains. It would be particularly interesting to study the behavior/locomotor activity of SJL/J mice since this strain showed very low hypocretin-2 level, and moderate hypocretin-1 level.

There are several controversial reports regarding hypocretin levels in rodents with deficient leptin action. Some researchers report that hypothalamic peptide and prepro-orexin mRNA concentrations in *ob*/*ob* and *db*/*db* mice and Zucker fatty rats are significantly lower than controls [4,24,48,49] while others have reported no significant difference or even higher levels in obese models [13,24,39,43]. In this study, our result showed similar hypocretin-1 and -2 levels between *ob*/*ob*, *db*/*db* and control mice. These results demonstrate that leptin deficiency does not profoundly affect the overall brain content of hypocretin peptides. As pointed before, however, overall brain hypocretin content is likely to have a very different regulation than hypocretin release or hypocretin stores in selected projection areas. It is thus possible that changes can be detected in discrete hypothalamic nuclei.

We also explored hypocretin levels in various knockout models with impaired monoaminergic transmission. Vesicular monoamine transporters (VMAT) concentrate monoamines (catecholamine, serotonin, histamine) from the cytoplasm into vesicles. VMAT2 immunoreactivity is found in the histaminergic tuberomammillary neuronal cell bodies of the rat posterior hypothalamus [8,33]. VMAT2 mRNA has also been observed in the human hypothalamus [12]. VMAT+/− animals are viable but have abnormal catecholamine levels and are supersensitive to psychostimulant compounds [41,44]. To understand whether impaired

vesicular function alters the level of hypocretins, we measured both peptides in VMAT+/− and VMAT+/+ mice. No changes in hypocretin contents were found, indicating that heterozygous knockout VMAT2 mice with half of wild-type levels of expression of the vesicular monoamine transporter [19] do not directly affect the overall levels of hypocretin peptide in whole mouse brains. We also explored hypocretin levels in DAT knock out animals. DATs play an important role in sleep regulation. In mice, deletion of the DAT gene results in normal circadian patterns but reduced non-REM sleep time and increased wakefulness [47]. These mice are unresponsive to the wake promoting effects of amphetamine-like stimulants and modafinil, drugs used in the treatment of narcolepsy [47]. Our result showed that there is no difference in the levels of hypocretin peptides between DAT knockout and control mice. These results do not support the hypothesis that the dopaminergic system strongly regulates the hypocretin system.

Clock mutant mice have significantly lower non-REM sleep times and longer circadian cycle awake time than wild-type mice [27]. These findings parallel data obtained in DAT knockout mice [47]. In spite of these changes, mutant mice brains did not show any difference in hypocretin levels compared to wild-type suggesting that circadian deregulation may not affect hypocretin content. As can be seen in Table 1, both the wild-type and litter-mate clock knockout mice had a much lower concentration of hypocretin-2 than is observed in mice of other genetic backgrounds. Given this unusual finding, we replicated the EIA multiple times and independently confirmed the results with RIA. In all assays, the clock knockout mice and their controls had low hypocretin-2 levels. It is possible that in the creation of coisogenic clock knockout mice, the inbreeding caused a fortuitous change in a genetic element responsible for regulation of hypocretin-2 transcription, translation, or post-translational modification. This possibility, however, remains to be explored.

Hypocretin-1 and -2 were significantly lower in histamine H1 receptor knockout mice while levels of hypocretin-2 only were lower in H2 knockout mice when compared to wild-type mice. Histamine has been shown to play a major role in the regulation of sleep and appetite. The activation of the histaminergic system promotes wakefulness through the activation of the histamine H1 receptor, a generally excitatory receptor [3,25]. Recently, Huang et al. [15] reported that the arousal effect of hypocretin-1 depends on activation of the histaminergic system. In their study, hypocretin-1 perfusion in the CSF increased wakefulness in rats and wild-type mice, but not in H1 receptor knockout animals, a result partially substantiated by pharmacological experiment with H1 antagonist [50]. This suggested that the hypocretin effects on wakefulness may be primarily mediated by a downstream activation of the tuberomamillary system. In this direction, histaminergic cells primarily contain the hypocretin receptor-2, the main receptor mediating the narcolepsy phenotype. We have also shown decreased histamine content in hypocretin receptor-2 mutated narcoleptic dogs [28].

Our finding that H1 knockout animals have lower hypocretin levels suggests reciprocal interactions between hypocretin and histaminergic systems. Histamine H1 receptors have high density in the hypothalamus and other limbic regions [3]. The highest density of histaminergic fibers is in the hypothalamus, with all nuclei receiving high to moderate innervations [3]. Hypocretin neurons have been recently shown to be heavily innervated by histaminergic fibers [9]. This colocolization suggests that hypocretin neurons are not only excitatory to tuberomammillary neurons but may also be an important target for the histaminergic system themselves. As H1 receptors are generally excitatory, it is possible that hypocretin levels are lower in H1KO mice due to the loss of excitatory inputs from histaminergic neurons. These inputs may form part of a not previously studied positive feedback loop between hypocretin and histaminergic neurons.

Although H1KO mice show a significantly decreased locomotor activity and reduced exploratory behavior in a novel environment [18,51], their sleep behavior and circadian rhythms are comparable to those of wild-type mice [15]. This indicates that decreased hypocretin -1 and -2 levels may not influence baseline sleep behavior but have greater effects on locomotor activity and exploratory behavior. Similarly, overall locomotor activity of H2KO mice is lower than in wild-type mice. However, exploratory behavior during daytime in H2KO is higher [38]. H2KO mice showed a significantly low level of hypocretin-2 but not hypocretin-1 suggesting that the H2 receptor may alter hypocretin peptide processing/turnover, and/or that the two hypocretin peptides have different functions. The H3 receptor is predominantly a presynaptic receptor. H3KO mice have been reported to exhibit a significant reduction in the amount of REM sleep during daytime and a decrease in locomotor activity in the dark phase [7]. Surprisingly, no differences in whole brain hypocretin levels were observed between H3 knockout and wild-type control mice. However, a study of more discrete brain regions at different time points (light versus dark phase) may be more informative in this model.

The role of hypocretins in normal sleep physiology remains to be determined. The EIA technique provides an excellent technique with high sensitivity and reproducibility for study of brain hypocretin levels with various physiological manipulations and in mice with alterations in the expression of various genes. Our results support the existence of reciprocal neuronal connections between hypocretin and histaminergic neurons. Furthermore, reduced hypocretin levels may contribute to the locomotor phenotype of histamine receptor knockout mice.

Acknowledgments

Research supported by NIH Grant NS23724.

References

[1] Bayer L, Eggermann E, Serafin M, Saint-Mleux B, Machard D, Jones B, et al. Orexins (hypocretins) directly excite tuberomammillary neurons. Eur J Neurosci 2001;14:1571–5.

[2] Brown RE, Sergeeva O, Eriksson KS, Haas HL. Orexin A excites serotonergic neurons in the dorsal raphe nucleus of the rat. Neuropharmacology 2001;40:457–9.

[3] Brown RE, Stevens DR, Haas HL. The physiology of brain histamine. Prog Neurobiol 2001;63:637–72.

[4] Cai XJ, Lister CA, Buckingham RE, Pickavance L, Wilding J, Arch JR, et al. Down-regulation of orexin gene expression by severe obesity in the rats: studies in Zucker fatty and Zucker diabetic fatty rats and effects of rosiglitazone. Brain Res Mol Brain Res 2000;77:131–7.

[5] Chemelli RM, Willie JT, Sinton CM, Elmquist JK, Scammell T, Lee C, et al. Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. Cell 1999;98:437–51.

[6] de Lecea L, Kilduff TS, Peyron C, Gao X, Foye PE, Danielson PE, et al. The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. Proc Natl Acad Sci USA 1998;95:322–7.

[7] Toyota H, Dugovic C, Koehl M, Laposky AD, Weber C, Ngo K, et al. Behavioral characterization of mice lacking histamine H(3) receptors. Mol Pharmacol 2002;62:389–97.

[8] Erickson JD, Eiden LE, Schafer MK, Weihe E. Reserpine- and tetrabenazine-sensitive transport of (3)H-histamine by the neuronal isoform of the vesicular monoamine transporter. J Mol Neurosci 1995;6:277–87.

[9] Eriksson KS, Sergeeva O, Brown RE, Haas HL. Orexin/hypocretin excites the histaminergic neurons of the tuberomammillary nucleus. J Neurosci 2001;21:9273–9.

[10] Estabrooke IV, McCarthy MT, Ko E, Chou TC, Chemelli RM, Yanagisawa M, et al. Fos expression in orexin neurons varies with behavioral state. J Neurosci 2001;21:1656–62.

[11] Fujiki N, Yoshida Y, Ripley B, Honda K, Mignot E, Nishino S. Changes in CSF hypocretin-1 (orexin A) levels in rats across 24 h and in response to food deprivation. NeuroReport 2001;12:993–7.

[12] Gonzalez AM, Walther D, Pazos A, Uhl GR. Synaptic vesicular monoamine transporter expression: distribution and pharmacologic profile. Brain Res Mol Brain Res 1994;22:219–26.

[13] Hakansson M, de Lecea L, Sutcliffe JG, Yanagisawa M, Meister B. Leptin receptor- and STAT3-immunoreactivities in hypocretin/orexin neurones of the lateral hypothalamus. J Neuroendocrinol 1999;11:653–63.

[14] Horvath TL, Peyron C, Diano S, Ivanov A, Aston-Jones G, Kilduff TS, et al. Hypocretin (orexin) activation and synaptic innervation of the locus coeruleus noradrenergic system. J Comp Neurol 1999;13:145–59.

[15] Huang ZL, Qu WM, Li WD, Mochizuki T, Eguchi N, Watanabe T, et al. Arousal effect of orexin A depends on activation of the histaminergic system. Proc Natl Acad Sci USA 2001;98:9965–70.

[16] Hungs M, Fan J, Lin L, Lin X, Maki RA, Mignot E. Identification and functional analysis of mutations in the hypocretin (orexin) genes of narcoleptic canines. Genome Res 2001;11:531–9.

[17] Hungs M, Mignot E. Hypocretin/orexin, sleep and narcolepsy. BioEssays 2001;23:397–408.

[18] Inoue I, Yanai K, Kitamura D, Taniuchi I, Kobayashi T, Niimura K, et al. Impaired locomotor activity and exploratory behavior in mice lacking histamine H1 receptors. Proc Natl Acad Sci USA 1996;93:13316–20.

[19] Itokawa K, Sora I, Schindler CW, Itokawa M, Takahashi N, Uhl GR. Heterozygous VMAT2 knockout mice display prolonged QT intervals: possible contributions to sudden death. Brain Res Mol Brain Res 1999;25:354–7.

[20] Kilduff TS, Peyron C. The hypocretin/orexin ligand-receptor system: implications for sleep and sleep disorders. Trends Neurosci 2000;23:359–65.

[21] Koide T, Moriwaki K, Ikeda K, Niki H, Shiroishi T. Multi-phenotype behavioral characterization of inbred strains derived from wild stocks of Mus musculus. Mamm Genome 2000;11:664–70.

[22] Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, et al. The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. Cell 1999;98:365–76.

[23] Mignot E. A commentary on the neurobiology of the hypocretin/orexin system. Neuropsychopharmacology 2001;25:S5–13.

[24] Mondal MS, Nakazato M, Matsukura S. Characterization of orexins (hypocretins) and melanin-concentrating hormone in genetically obese mice. Regul Pept 2002;15:21–5.

[25] Monti JM, Jantos H, Leschke C, Elz S, Schunack W. The selective histamine H1-receptor agonist 2-(3-trifluoromethylphenyl)histamine increases waking in the rat. Eur Neuropsychopharmacol 1994;4:459–62.

[26] Nambu T, Sakurai T, Mizukami K, Hosoya Y, Yanagisawa M, Goto K. Distribution of orexin neurons in the adult rat brain. Brain Res 1999;827:243–60.

[27] Naylor E, Bergmann BM, Krauski K, Zee PC, Takahashi JS, Vitaterna MH, et al. The circadian clock mutation alters sleep homeostasis in the mouse. J Neurosci 2000;20:8138–43.

[28] Nishino S, Fujiki N, Ripley B, Sakurai E, Kato M, Watanabe T, et al. Decreased brain histamine content in hypocretin/orexin receptor-2 mutated narcoleptic dogs. Neurosci Lett 2001;9:125–8.

[29] Nishino S, Ripley B, Overeem S, Lammers GJ, Mignot E. Hypocretin (orexin) deficiency in human narcolepsy. Lancet 2000;1:39–40.

[30] Nishino S, Ripley B, Overeem S, Nevsimalova S, Lammers GJ, Vankova J, et al. Low cerebrospinal fluid hypocretin (orexzin) and altered energy homeostasis in human narcolepsy. Ann Neurol 2001;50:381–8.

[31] Okun ML, Lin L, Pelin Z, Hong S, Mignot E. Clinical aspects of narcolepsy-cataplexy across ethnic groups. Sleep 2002;1:27–35.

[32] Overeem S, Mignot E, Gert van Dijk J, Lammers GJ. Narcolepsy: clinical features, new pathophysiologic insights, and future perspectives. J Clin Neurophysiol 2001;18:78–105.

[33] Peter D, Liu Y, Sternini C, de Giorgio R, Brecha N, Edwards RH. Differential expression of two vesicular monoamine transporters. J Neurosci 1995;15:6179–88.

[34] Peyron C, Faraco J, Rogers W, Ripley B, Overeem S, Charnay Y, et al. A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. Nat Med 2000;6:991–7.

[35] Peyron C, Tighe DK, van den Pol AN, de Lecea L, Heller HC, Sutcliffe JG, et al. Neurons containing hypocretin (orexin) project to multiple neuronal systems. J Neurosci 1998;18:9996–10015.

[36] Ripley B, Overeem S, Fujiki N, Nevsimalova S, Uchino M, Yesavage J, et al. CSF hypocretin/orexin levels in narcolepsy and other neurological conditions. Neurology 2001;26:2253–8.

[37] Sakurai T, Amemiya A, Ishii M, Matsuzaki I, Chemelli RM, Tanaka H, et al. Orexins and orexin receptors: a family of hypothalamic neuropeptides and G protein-coupled receptors that regulate feeding behavior. Cell 1998;92:573–85.

[38] Shiba T, Kobayashi T, Mobarakeh JI, Sakurai E, Nakagawa Y, Tamai M, et al. Behavioral characterization in mice lacking histamine H2 receptors. Neuroscience 2001 [abstract 2001, 745.15].

[39] Taheri S, Mahmoodi M, Opacka-Juffry J, Ghatei MA, Bloom SR. Distribution and quantification of immunoreactive orexin A in rat tissues. FEBS Lett 1999;457:157–61.

[40] Taheri S, Sunter D, Dakin C, Moyes S, Seal L, Gardiner J, et al. Diurnal variation in orexin A immunoreactivity and prepro-orexin mRNA in the rat central nervous system. Neurosci Lett 2000;279:109–12.

[41] Takahashi N, Miner LL, Sora I, Ujike H, Revay RS, Kostic V, et al. VMAT2 knockout mice: heterozygotes display reduced amphetamine-conditioned reward, enhanced amphetamine locomotion, and enhanced MPTP toxicity. Proc Natl Acad Sci USA 1997;2:9938–43.

[42] Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, et al. Reduced number of hypocretin neurons in human narcolepsy. Neuron 2000;27:469–74.
[43] Tritos NA, Mastaitis JW, Kokkotou E, Maratos-Flier E. Characterization of melanin concentrating hormone and preproorexin expression in the murine hypothalamus. Brain Res 2001;895:160–6.
[44] Wang YM, Gainetdinov RR, Fumagalli F, Xu F, Jones SR, Bock CB, et al. Knockout of the vesicular monoamine transporter 2 gene results in neonatal death and supersensitivity to cocaine and amphetamine. Neuron 1997;19:1285–96.
[45] Willie JT, Chemelli RM, Sinton CM, Yanagisawa M. To eat or to sleep? Orexin in the regulation of feeding and wakefulness. Annu Rev Neurosci 2001;24:429–58.
[46] Wisor J, Edgar D. Genetic and temporal segregation of sleep homeostatic processes in mice. In: Word Federation Meeting, Sleep Research On Line, vol. 2; 1999. p. 562.
[47] Wisor JP, Nishino S, Sora I, Uhl GH, Mignot E, Edgar DM. Dopaminergic role in stimulant-induced wakefulness. J Neurosci 2001;21:1787–94.
[48] Yamamoto Y, Ueta Y, Date Y, Nakazato M, Hara Y, Serino R, et al. Down regulation of the *prepro-orexin* gene expression in genetically obese mice. Mol Brain Res 1999;65:14–22.
[49] Yamamoto Y, Ueta Y, Serino R, Monura M, Shibuya I, Yamashita H. Effects of food restriction on the hypothalamic prepro-orexin gene expression in genetically obese mice. Brain Res 2000;51: 515–21.
[50] Yamanaka A, Tsujino N, Funahashi H, Honda K, Guan JJ, Wang QP, et al. Orexins activate histaminergic neurons via the orexin 2 receptor. Biochem Biophys Res Commun 2002;1:1237–45.
[51] Yanai K, Son LZ, Endou M, Sakurai E, Nakagawasai O, Tadano T, et al. Behavioural characterization and amounts of brain monoamines and their metabolites in mice lacking histamine H1 receptors. Neuroscience 1998;87:479–87.
[52] Yoshida Y, Fujiki N, Nakajima T, Ripley B, Matsumura H, Yoneda H, et al. Fluctuation of extracellular hypocretin-1 (orexin A) levels in the rat in relation to the light–dark cycle and sleep–wake activities. Eur J Neurosci 2001;14:1075–81.