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Effects of hypocretin–saporin injections into the medial septum on sleep and hippocampal theta ☆

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Abstract

Neurons containing the peptide hypocretin, also known as orexin, were recently implicated in the human sleep disorder narcolepsy. Hypocretin neurons are located only in the lateral hypothalamus from where they innervate virtually the entire brain and spinal cord. This peptide is believed to be involved in regulating feeding and wakefulness. However, to fully understand what other behaviors are regulated by this peptide it is necessary to investigate each hypocretin target site. In the present study, we focus on one hypocretin target site, the medial septum, where there is a dense collection of hypocretin-2 receptor-containing cells, and degenerating axons are present here in canines with narcolepsy [J. Neurosci. 19 (1999) 248]. We utilize a saporin toxin conjugated to the hypocretin receptor binding ligand, hypocretin-2, and find that when this toxin is injected into the medial septum, it lesions the parvalbumin and cholinergic neurons. We contrast the effects of the hypocretin–saporin with another saporin conjugated toxin, 192 IgG–saporin, that lesions only the cholinergic neurons in the basal forebrain. 192 IgG–saporin reduced theta activity, a finding consistent with previous reports [J. Neurophysiol. 79 (1998) 1633; Neurodegeneration 4 (1995) 61; Neuroscience 62 (1994) 1033]. However, hypocretin–saporin completely eliminated hippocampal theta activity by day 12, indicating that parvalbumin-containing cells in the medial septum generate theta. The daily amount of sleep and wakefulness were not different between hypocretin–saporin, 192 IgG–saporin, or saline-treated rats. The homeostatic response to 12 h prolonged wakefulness was also not affected in hypocretin–saporin lesioned rats. These findings suggest that hypocretin neurons could facilitate theta generation during episodes of purposeful behavior by activating GABAergic neurons in the MS/VDB. In this way, hypocretin, which is implicated in feeding, energy metabolism and wakefulness, serves to influence cognitive processes critical for the animal's survival.

Theme: Neural basis of behavior

Topic: Biological rhythms and sleep

Keywords: Theta rhythm; Medial septum; Hypocretin; Sleep; Immunohistochemistry

1. Introduction

Recently, the neuropeptide hypocretin, also known as orexin, was implicated in maintaining wakefulness [20,34]. Loss of hypocretin neurons is associated with sleepiness and increased rapid eye movement (REM) sleep propensity in the sleep disorder narcolepsy [33,40]. Consistent with such a neuronal loss, levels of hypocretin-1 are undetectable in the cerebrospinal fluid of narcoleptic patients [30]. Narcoleptic-like behavior is evident in hypocretin gene knockout mice [7] and in mice where the hypocretin neurons degenerate in adulthood [16]. In adult rats lesions of hypocretin neurons produce hypersomnolence and increased REM sleep within a few days after the lesion [13], further indicating a common function of these neurons across species. The distribution of hypocretin-containing neurons has been described in the mouse [37], rat [10,34], cat [43,48] and humans [4]. Neurons containing this peptide are located only in the lateral hypothalamus, from where they project to virtually the entire brain and spinal cord [4,10,29,34,37]. Hypocretin neurons were originally implicated in energy metabolism and feeding since they

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are located in the lateral hypothalamus. However, it is very likely that this peptide has a multifunctional role because of the widespread projection of hypocretin neurons. To understand what behaviors are regulated by hypocretin, it is necessary to investigate each of the projection sites separately.

The medial septal area [the medial septal nucleus (MS) and vertical limb of the diagonal band of Broca (VDB)] of the basal forebrain is one of the targets of the hypocretin neurons [8,29], and the hypocretin-2 receptor is present here [41]. In canine narcolepsy this receptor is mutated [24] and a higher number of degenerating axons are found in the medial septum, and in other limbic regions such as the amygdala [38]. Septal neurons are believed to generate hippocampal theta rhythm, a nearly sinusoidal rhythm of 4–12 Hz which is present in the hippocampus during exploratory movements in waking and during REM sleep. Electrical or chemical stimulation of the medial septal area drives theta rhythm [6,46]. The theta rhythm is present in all species in which REM sleep occurs and this rhythm is thought to be involved in learning and memory. The theta rhythm facilitates the induction of long-term potentiation in hippocampal circuits and also facilitates and controls the flow of information to the hippocampus or through the hippocampus to the targets [6,23]. Spatial memory is impaired following disruption of the theta rhythm by chemical inactivation of the MS/VDB or following excitotoxic septal lesions [23,28].

To determine whether the hypocretin innervation of the medial septum influences sleep–wake regulation including theta activity, in the present study we targeted the hypocretin-receptor bearing neurons in the MS/VDB for destruction using the ligand hypocretin-2 conjugated to the ribosomal inactivating enzyme, saporin. The effects of the hypocretin2–saporin were compared with another saporin toxin, 192 immunoglobulin (Ig) G–saporin, that binds to the p75 nerve growth factor receptor. Since this receptor is found exclusively on cholinergic neurons in the MS/VDB, the 192 IgG–saporin selectively lesions the cholinergic medial septal neurons [2,3,22]. We reasoned that by employing two different saporin conjugates, one that targets the cholinergic neurons (192 IgG–saporin) and the other (hypocretin2–saporin) that destroys hypocretin-receptor bearing neurons, the relative contribution of hypocretin on septal cholinergic versus non-cholinergic neurons in sleep–wake homeostasis and theta activity could be better understood.

2. Materials and methods

2.1. Subjects and surgery

Fourteen male Sprague-Dawley rats (400–450 g) were anesthetized [IM injection of cocktail of acepromazine (0.75 mg/kg), xylazine (2.5 mg/kg) and ketamine (22 mg/kg)] and using a stereotaxic apparatus injected with the test substances (see next paragraph) and then implanted with electrodes to record the electroencephalogram (EEG) and electromyogram (EMG). Four stainless steel screw electrodes were positioned in the skull to sit on the surface of the cortex and were used to record the EEG. Two miniature screws were inserted 2 mm on either side of the midline and 3 mm anterior to bregma (frontal cortex). The other two screws were located 2 mm on either side of the midline and 6 mm behind bregma (occipital cortex). The cortical EEG was recorded from two contralateral screws (frontal-occipital). To record hippocampal theta activity a bipolar wire electrode, insulated except at the tip, was placed in the dorsal hippocampus (AP −3.3, L +2.0, V −3.4 mm). To record muscle activity (EMG) two flexible multistranded wires were inserted in the nuchal muscles. The electrodes were placed in a plastic plug and secured onto the skull using dental cement. The animals were housed singly in Plexiglas cages with wood-shavings maintained at 21°C with lights on (100 lux) at 7 a.m. and off at 7 p.m. Rats had food and water available ad libitum.

2.2. Microinjections of saporin conjugates

The sample sizes for the three groups were as follows: saline=5; 192 IgG–saporin=4; hypocretin–saporin=5. The hypocretin–saporin conjugate (Advanced Targeting Systems, San Diego, CA, USA) was delivered via a glass micropipette using a Picospritzer. Pipettes were pulled from thick-walled borosilicate capillaries (1.0 mm OD, 0.58 mm ID, with filament, BF100-58-15, Sutter Instruments, Novato, CA, USA) in a standard electrode puller to a tip diameter of approximately 20 μm. Bilateral injections of the hypocretin–saporin (100 ng/0.5 μl) in the medial septum were made at the following coordinates relative to bregma: A +1.0; L ±0.3 mm; V −5.9 mm. After microinjection, the pipette was left in place for 5 min and then withdrawn slowly. Control rats were injected bilaterally in the medial septum with 0.5 μl of saline using the same procedure and coordinates as those used for the hypocretin–saporin injections. 192 IgG–saporin (1 μg/μl) was injected into the lateral ventricle (coordinates: 1.2 mm anterior to bregma, 1.1 mm lateral to midline, 4.8 mm below the cortical surface) in a volume of 4 μl at the rate of 0.5 μl/min. EEG and EMG recordings started 1 day after the surgery and continued for at least 1 month.

2.3. Sleep deprivation

Sleep deprivation was performed 2 weeks following hypocretin–saporin treatment. Rats were kept awake for 12 h by lightly tapping the cage, placing pieces of paper in the cage or stroking the animal's fur whenever they appeared to go to sleep. The prolonged period of wakefulness was started at 7 a.m. (light onset) and ended at 7 p.m. (lights off). The EEG and EMG were continuously monitored

during the entire period of sleep deprivation. After the 12-h period of prolonged wakefulness the animals were allowed to sleep undisturbed and the EEG data were collected for 24 h.

2.4. Analysis of sleep–wake states and EEG spectra

EEG and EMG signals were recorded on a Grass polygraph and also recorded onto a Jaz disk using an analog–digital board (National Instruments). The EEG data were filtered at 70 Hz (low pass filter) and 0.3 Hz (high pass filter) using a Grass electroencephalograph and continuously sampled at 128 Hz. The 24-h EEG and EMG recordings obtained on the 2nd, 5th and 12th day post-injection were scored manually on a computer (Icelus software, Mark Opp, Ann Arbor, MI, USA) in 12-s epochs for awake, slow-wave sleep (SWS) and REM sleep by staff blind to the type of drug administered to the rats. Wakefulness was identified by the presence of desynchronized EEG and high EMG activity. SWS consisted of high amplitude slow waves together with a low EMG tone relative to waking. REM sleep was identified by the presence of desynchronized EEG and/or theta activity coupled with absence of EMG activity. The amount of time spent in wakefulness, SWS and REM sleep was determined for each hour. After the EEG data were scored, the code was broken to reveal the identity of each rat. ANOVA and *t*-tests with Bonferroni correction (where appropriate) were used to compare changes in sleep parameters. Statistical significance was evaluated at the $P<0.05$ level. A fast-Fourier analysis of EEG recording was used to calculate EEG theta and delta power. The fast-Fourier analysis of the EEG was performed on 12-s segments of the EEG in 0.5-Hz bands of the 0.5- to 20-Hz frequency range. The EEG power density values were summed in theta (4–12 Hz) and delta (0.5–4 Hz) frequency bands for each 12-s epoch. Epochs containing artifacts were excluded from the spectral analysis of the EEG during manual scoring. The spectral data were paired with the vigilance states. Theta power was calculated during REM sleep or wakefulness bouts for the 24-h period. Delta power was calculated during SWS bouts and averaged for each hour.

2.5. Immunohistochemistry

Coronal brain sections (30-μm thick) cut on a sliding microtome were incubated overnight at room temperature in the primary antibody [monoclonal anti-parvalbumin (PARV) antibody (1:2000), Sigma; rabbit anti-choline acetyltransferase (ChAT) polyclonal antibody (1:2000), Chemicon International]. After washing, the sections were placed in the secondary antibody (1 h; 1:500) (Chemicon) and then incubated in the avidin–biotin complex (1 h; ABC) (Vector Labs). The DAB method was used to visualize the reaction product. The tissue sections were counter-stained with a Nissl stain (Neutral Red), dehydrated in graded alcohols and coverslipped.

2.6. Cell counts

One person blind to the type of drug and site of injection counted the number of ChAT-immunoreactive (ChAT-ir) and PARV-immunoreactive (PARV-ir) cells in the medial septum. Cell counts were obtained from at least 12 sections (one in five series) that included the medial septum. All somata that were ChAT- or PARV-ir were counted and the total numbers of cells were determined for each animal. The neuronal loss was calculated as a percent of immunoreactive cells in rats treated with hypocretin–saporin from the cell number in rats treated with saline.

3. Results

3.1. Loss of ChAT- and PARV-immunoreactive cells in the MS/VDB following treatment with saporin conjugates

The extent of the lesion induced by intraseptal injection of hypocretin–saporin was verified on tissue sections stained with Neutral Red. Fig. 1 shows a drawing of a typical lesion in a single representative rat (rat #300). The lesion included the whole medial septum, vertical limb of the diagonal band of Broca (VDB), and part of the horizontal limb of the diagonal band of Broca (HDB) (Fig. 1). All the rats treated with hypocretin–saporin had a significant loss of both ChAT-positive (−87%) and PARV-positive (−91%) neurons (Figs. 2 and 3). However, such an extensive loss of PARV- and ChAT-containing neurons was not accompanied by loss of fibers, and many PARV-ir fibers were seen in the lesioned area (Fig. 2). ChAT and PARV neurons elsewhere in the basal forebrain were intact.

Similar to other reports [2,3,22], we observed a complete loss of ChAT-ir neurons in the medial septal nucleus and the diagonal bands (VDB and HDB) in rats treated with 192 IgG–saporin (Figs. 2 and 3). ChAT neurons in the other basal forebrain areas were also eliminated. The distribution of PARV-ir neurons in the MS/VDB was similar between saline-treated and 192 IgG–saporin-treated rats, a finding consistent with previous reports [22] that the 192 IgG–saporin does not lesion non-cholinergic neurons in the MS/VDB. Thus, the hypocretin–saporin destroyed both PARV-ir and ChAT-ir neurons in the MS/VDB, whereas the 192 IgG–saporin destroyed only the ChAT-ir neurons in the basal forebrain.

3.2. Sleep–wakefulness in rats treated with hypocretin–saporin

Administration of hypocretin–saporin into the MS/VDB did not produce any changes in sleep even though the toxin eliminated virtually all of the PARV-ir and ChAT-ir

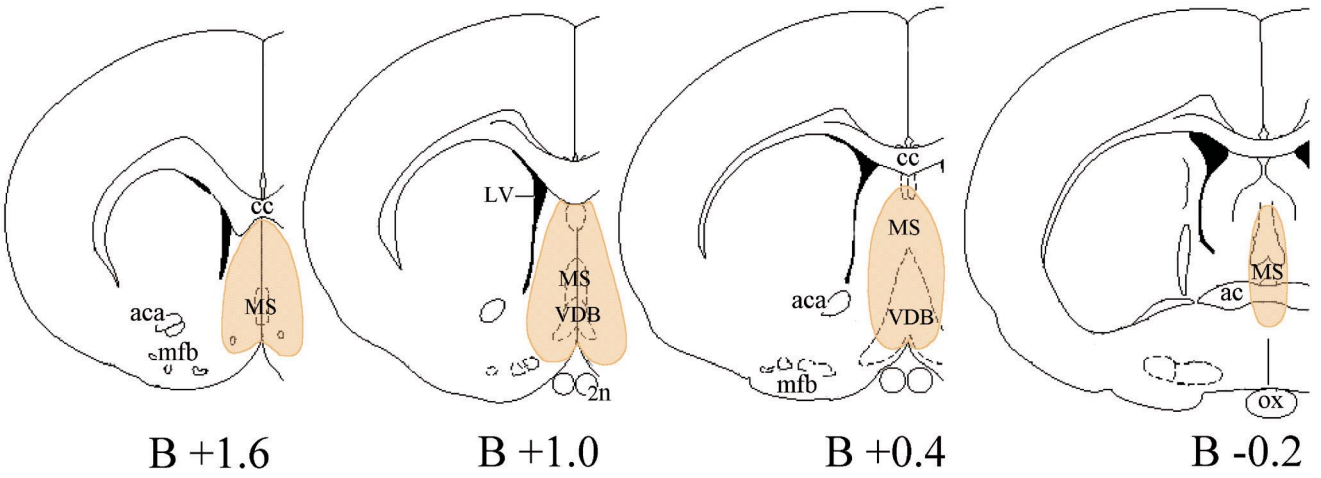

Fig. 1. Schematic drawings of the lesion area in a representative rat treated with hypocretin–saporin. Neutral Red staining identified loss of neurons. The number below each coronal section indicates the approximate distance of that section from bregma. Abbreviations: 2n, optic nerve; ac, anterior commissure; aca, anterior commissure, anterior part; cc, corpus callosum; LV, lateral ventricle; mfb, medial forebrain bundle; MS, medial septal nucleus; ox, optic chiasm; VDB, nucleus of the vertical limb of the diagonal band. Adapted from the atlas of the *Rat Brain in Stereotaxic Coordinates* by George Paxinos and Charles Watson, 1998.

neurons in the MS/VDB (Fig. 4). There were no changes in sleep (SWS and REM sleep) between the saline-, hypocretin–saporin-, and 192 IgG–saporin-treated rats on day 12 post-injection (Fig. 4). Previously, other investigators had shown that loss of the ChAT-ir neurons in the basal forebrain (including the MS and VDB) produced no change in the amounts of SWS and REM sleep in rats 7 days following 192 IgG–saporin treatment [19]. Our data now demonstrate that loss of both the cholinergic and PARV-ir neurons in the MS/VDB also produces no change in sleep. Other behavioral abnormalities, such as cataplexy, or sleep onset REM sleep periods that are present in narcolepsy, were not evident in hypocretin–saporin-treated rats.

3.3. Effect of sleep deprivation on sleep and EEG delta power in hypocretin–saporin-treated rats

To determine whether the homeostatic response to sleep loss was altered, the rats were kept awake for 12 h and then allowed to fall asleep. The 12-h period of wakefulness induced a significant compensatory increase in REM sleep and SWS (Fig. 5) in both saline- and hypocretin–saporin-treated rats, but there were no significant differences between the two groups. To further evaluate the homeostatic response to the loss of sleep, EEG delta power during SWS was measured. There was a significant increase in EEG delta power during the recovery sleep period, but there were no differences between saline- and hypocretin–saporin-treated rats (Fig. 6).

3.4. Loss of cortical and hippocampal EEG theta following treatment with hypocretin–saporin

Fig. 7 summarizes cortical theta power during REM sleep and wakefulness in rats given saline, hypocretin–saporin, or 192 IgG–saporin 12 days after injection. On day 2, theta power in the hypocretin–saporin-treated rats was not significantly different compared to saline-injected rats on day 2. However, in the hypocretin–saporin-treated rats the power in the theta band was significantly decreased on day 5 (during REM sleep by 97.2%, $P<0.005$; during wakefulness by 98.8%, $P<0.01$) and theta was abolished by day 12 (100% loss of theta power during both REM sleep and wakefulness; $P<0.01$) (Fig. 7). Theta activity did not reappear within a month of the post-lesion period as well as during the period of recovery from sleep deprivation when the REM sleep drive is very high. EEG recorded from electrodes placed in the hippocampus confirmed the loss of theta activity (Fig. 8). In contrast, rats administered 192 IgG–saporin had lower theta activity compared to saline rats ($P<0.05$), but higher theta activity compared to hypocretin–saporin rats on day 12 ($P<0.05$, Fig. 7). The attenuation of theta following cholinergic neuronal loss is consistent with other reports [3,22]. The progressive loss of theta power in rats administered hypocretin–saporin is consistent with the time-course of cell loss produced by other saporin conjugates [26,44]. The first signs of neuronal death occur about 2 or 3 days after toxin administration, and then neurons progressively continue to die [44].

4. Discussion

Our findings indicate that (1) the hypocretin–saporin toxin kills both the PARV-containing neurons and ChAT-containing neurons in the MS/VDB; (2) neuronal loss in the MS/VDB is accompanied by loss of theta activity; and (3) loss of PARV- and ChAT-containing neurons in the

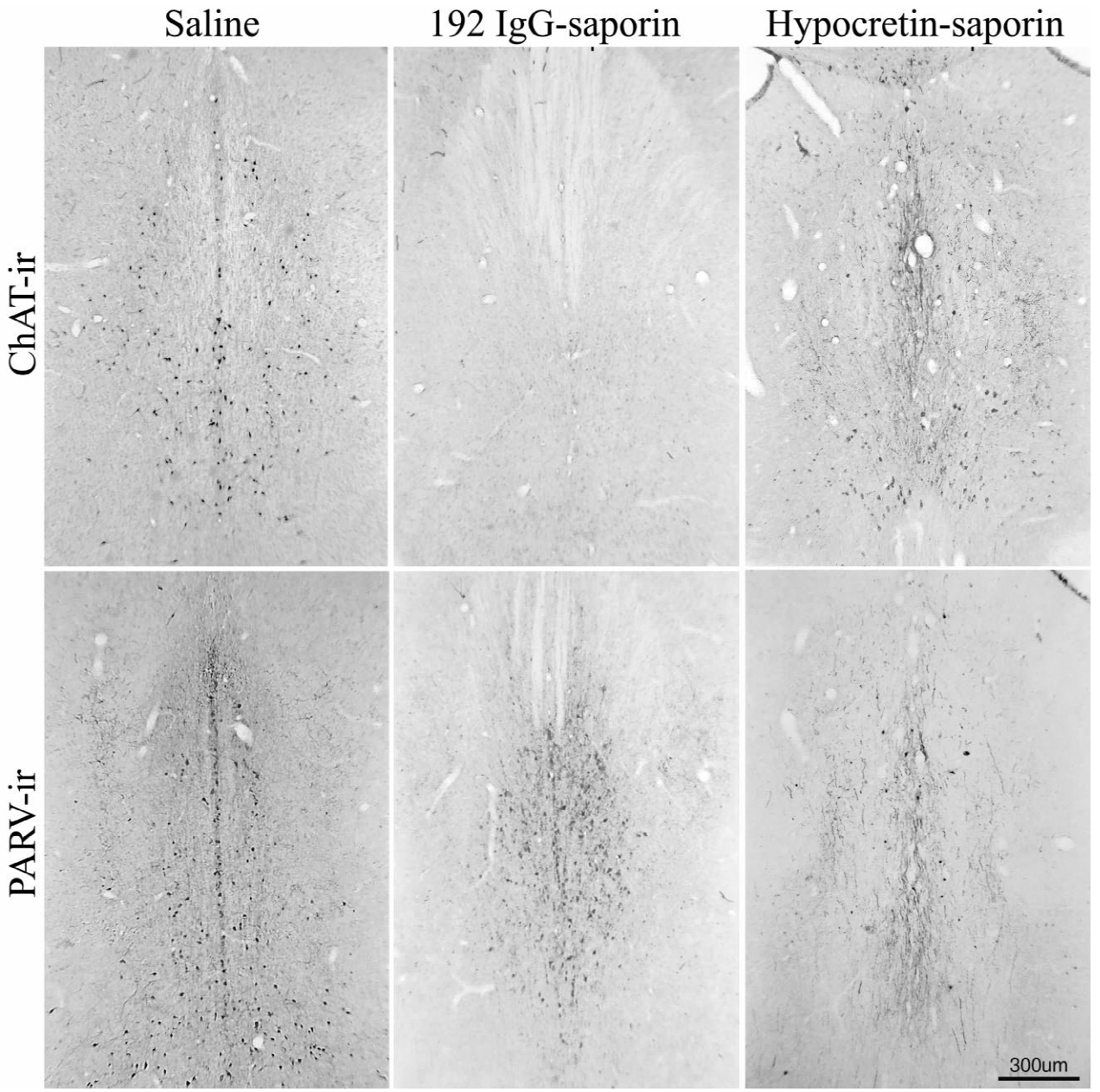

Fig. 2. Distribution of parvalbumine-immunoreactive (PARV-ir) and choline acetyltransferase-immunoreactive (ChAT-ir) cells in the medial septum/diagonal band of Broca in rats treated with saline, 192 immunoglobulin (Ig) G–saporin, or hypocretin–saporin.

MS/VDB does not produce a change in overall levels of sleep–wakefulness or diminish the drive to sleep following a 12-h period of prolonged wakefulness.

In the present study, we examined hypocretin function in the medial septal region of the basal forebrain. To accomplish this, the ligand hypocretin-2 was conjugated to saporin to lesion hypocretin-receptor bearing neurons. In vitro cell culture experiments have evaluated the specificity of the hypocretin–saporin. Using FACS analysis we have found that the conjugate binds to hypocretin receptor bearing CHO cells but not to substance-P receptor bearing cells [13]. In vivo experiments have demonstrated that the toxin lesions neurons in the tuberomammillary nucleus and the hypocretin neurons in the lateral hypothalamus which contain the hypocretin receptor [13]. On the other hand, neurons in the suprachiasmatic nucleus contain few, if any, hypocretin receptors [41], and hypocretin–saporin does not lesion these neurons (our unpublished observations). At the behavioral level, administration of hypocretin–saporin to the lateral hypothalamus, where the hypocretin neurons are located, produces narcoleptic symptoms correlated with the loss of the hypocretin neurons [13].

The hypocretin–saporin was applied directly to the MS/VDB (100 ng in a volume of 0.5 μl) whereas the 192 IgG–saporin was applied ICV (4 μg in a volume of 4 μl). The hypocretin–saporin could not be applied ICV because

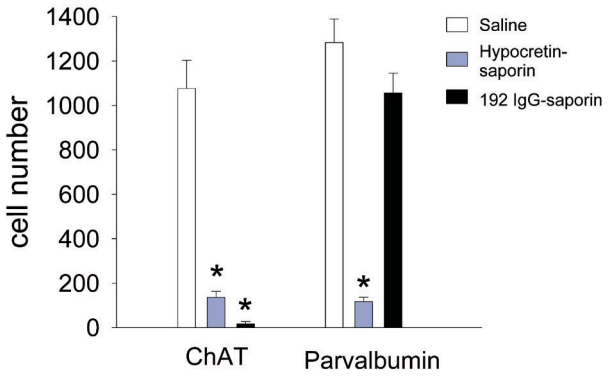

Fig. 3. Loss of parvalbumin-immunoreactive (PARV-ir) and choline acetyltransferase-immunoreactive (ChAT-ir) cells in the MS/VDB in rats treated with saline, 192 immunoglobulin G–saporin, or hypocretin–saporin. The total number of PARV-ir and ChAT-ir cells was counted in 30-μm thick sections at 12 representative levels from −0.2 mm to 1.6 mm relative to the bregma. Data are presented as mean±S.E.M. in each group of rats. *$P<0.05$ vs. saline group.

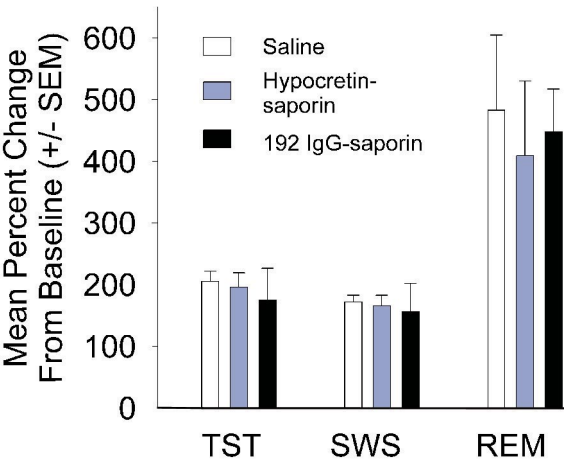

Fig. 5. Total sleep time (TST), slow wave sleep (SWS), and rapid eye movement (REM) sleep during the first 12-h period (night cycle) of recovery sleep. Rats were kept awake for 12 h (07:00–19:00) and then allowed to sleep. Bars represent mean percent difference (±S.E.M.) in TST, SWS, or REM from baseline (night cycle). There were no significant differences between control, hypocretin–saporin-treated, and 192 immunoglobulin G–saporin-treated rats.

the hypocretin receptors are present throughout the brain and spinal cord [15,41] and the toxin would have produced a non-specific lesion of all hypocretin receptor bearing neurons. On the other hand, ICV application of the 192 IgG–saporin lesions the basal forebrain cholinergic neurons, a much more limited lesion. By comparing ICV versus local injection of hypocretin–saporin we demonstrate that the cholinergic neurons are not critical for theta generation, since when all of these neurons are lost (via ICV injection of 192 IgG–saporin), theta still persists. Instead, theta is due to the non-cholinergic neurons in the medial septal nucleus because when these neurons are lost (local injection of hypocretin–saporin) theta is eliminated. Hypocretin–saporin injection into the medial septum also destroyed the cholinergic neurons but logically these

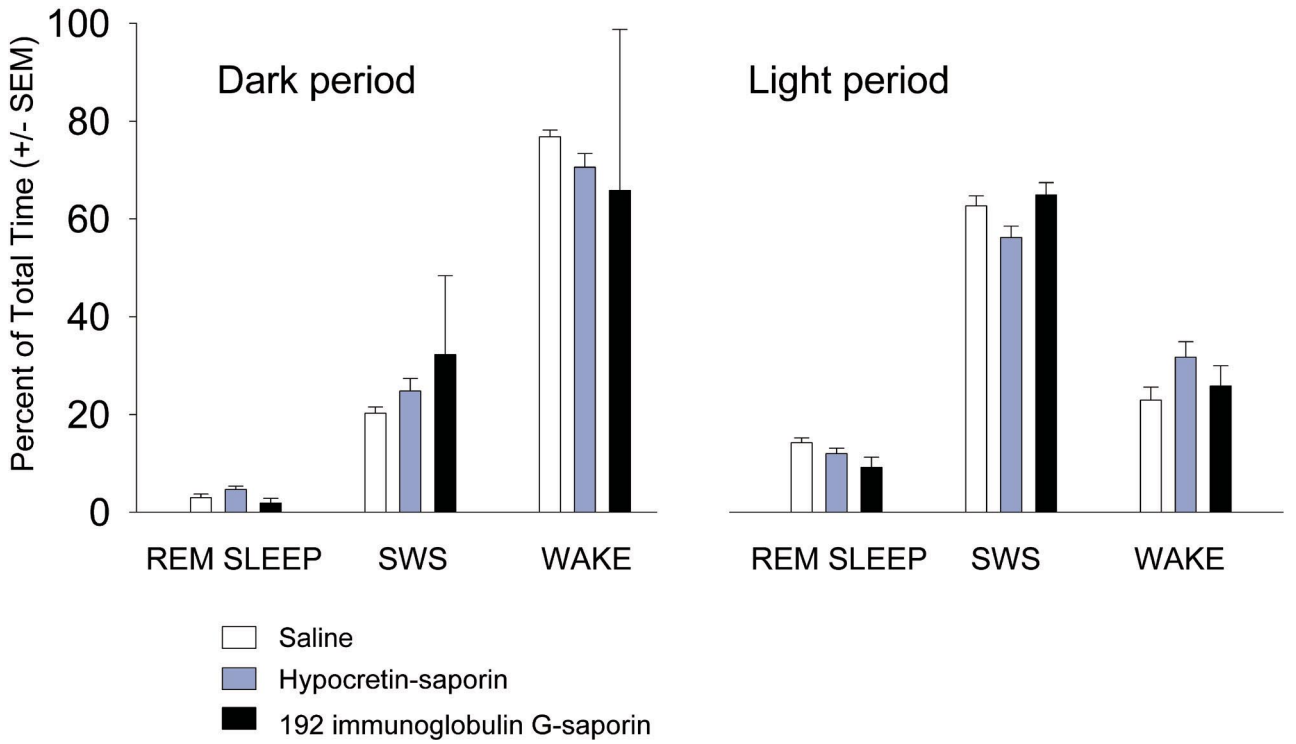

Fig. 4. Percent (mean±S.E.M.) of rapid eye movement (REM) sleep, slow wave sleep (SWS), and wakefulness in rats injected with hypocretin–saporin ($n = 4$), 192 immunoglobulin G–saporin ($n = 4$), and saline ($n = 5$) on the 12th day following the injection. There were no significant differences between groups.

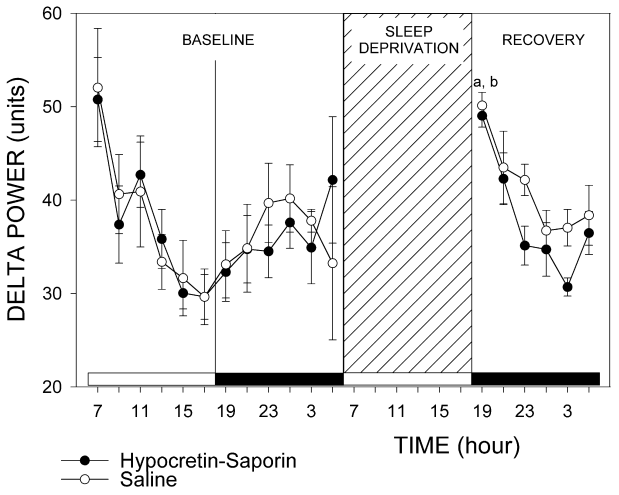

Fig. 6. Changes in slow wave sleep (SWS) delta power during baseline and recovery after sleep deprivation. Each data point represents the 4-h mean value±S.E.M. ($n = 4$). [a]Significant difference between recovery and baseline in saline-treated rats ($P<0.05$). [b]Significant difference between recovery and baseline in hypocretin–saporin-treated rats ($P<0.05$).

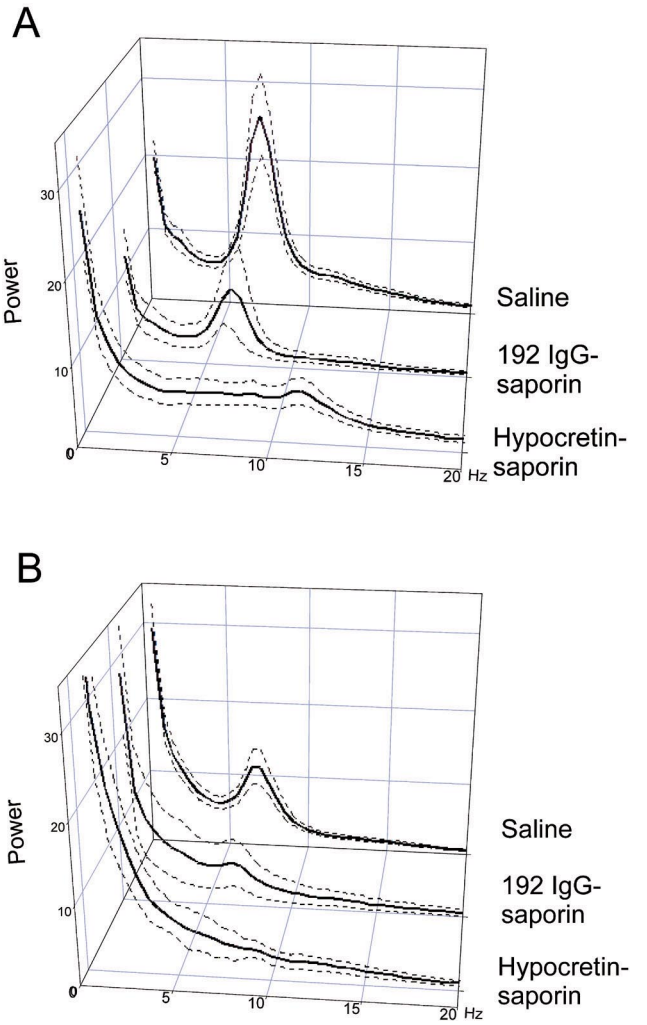

Fig. 7. Cortex EEG spectra 12 days following surgery in saline-, hypocretin–saporin-, and 192 immunoglobulin G–saporin-treated rats. (A) EEG spectra during REM sleep. (B) EEG spectra during wakefulness ($n = 4$ in each group). Areas between dashed lines represent range of S.E.M.

neurons cannot control theta because when they are lost with ICV injections as in this study or following local injection [22], theta is still present.

The 192 IgG–saporin and hypocretin–saporin were applied at a moderate dose in our experiments. High doses of saporin conjugates may cause non-specific damage of neurons and myelinated axons [17,47]. We used a moderate dose of saporin conjugate (bilateral MS injections of 0.1 μg hypocretin–saporin) and observed 91% loss of PARV-ir neurons and 87% loss of cholinergic neurons in the MS/VDB (Fig. 3). Nevertheless, it is possible that there might be some non-specific loss of non-hypocretin-receptor bearing neurons. At the dose used, the hypocretin–saporin did not destroy fibers of passage since we found many PARV-ir fibers at the core of injection (Fig. 2).

Hypocretin–saporin in the MS/VDB destroyed nearly all of the PARV-ir neurons in the MS/VDB. PARV is present in septohippocampal neurons and all PARV-containing septohippocampal cells are GABAergic [12]. Recent reports indicate that septohippocampal GABAergic mechanisms, rather than cholinergic neurons, produce theta activity [1]. The present findings support this since theta activity was still evident after complete loss of the MS/VDB cholinergic neurons, a finding consistent with other reports [3,22]. On the other hand, theta activity during both REM sleep and waking was lost when the PARV-ir neurons were also lesioned.

The high sensitivity of the ChAT-ir and PARV-ir cells to hypocretin–saporin in our experiment may be explained by a high concentration of hypocretin receptor present in the MS/VDB [41]. However, activation by hypocretin of MS/VDB neurons does not regulate overall sleep amounts, since, in the present study, loss of the MS/VDB neurons did not produce a change in sleep. Nevertheless, hypocretin does influence specific aspects of the EEG since, in the present study, the hippocampal theta rhythm was abolished following hypocretin–saporin-induced lesion of MS/VDB neurons.

We suggest that hypocretin neurons could facilitate theta generation during episodes of purposeful behavior by activating GABAergic neurons in the MS/VDB. The purpose of such activation during wakefulness might be to establish a memory trace that would be later reactivated during REM sleep. Recent findings imply that episodic memory traces are reactivated during REM sleep [25]. Activation of P-wave-generating cells in the brainstem structures during REM sleep has been shown to be important in reactivation of the forebrain and cortical memory-processing structures to reprocess recently stored

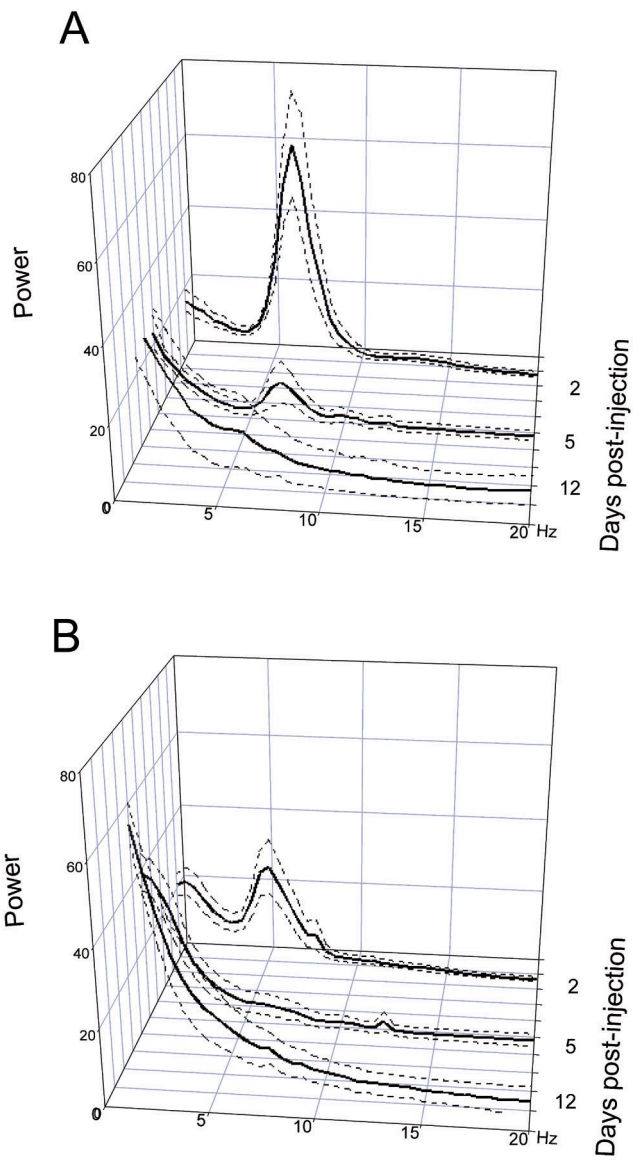

Fig. 8. Hippocampal EEG spectra 2, 5 and 12 days after hypocretin–saporin injection into the medial septum. (A) EEG spectra during REM sleep. (B) EEG spectra during wakefulness ($n = 4$ in each group). Areas between dashed lines represent range of S.E.M.

information [9]. We suggest that during wakefulness when the hypocretin neurons are active, hypocretin's action on MS/VDB neurons could facilitate learning, perhaps enabling the animal to remember a food source. Such a memory could then be reactivated during REM sleep and the memory is then transferred to long-term storage. In this way, hypocretin, which is implicated in feeding, energy metabolism and wakefulness, serves to influence cognitive processes critical for the animal's survival.

Anatomical data suggest that hypocretin neurons may directly or indirectly facilitate activity of MS/VDB neurons. Hypocretin-immunoreactive fibers are found in the MS/VDB as well as in the caudal diencephalon, namely the supramammillary nucleus and the posterior hypothalamic nucleus [8,29]. The discharge pattern of cells in these nuclei shows a distinct relationship to hippocampal field activity [5]. Electrical stimulation of the posterior hypothalamic nucleus not only elicits hippocampal theta rhythm, but also induces locomotor activity [31,45]. These effects result from activation of the ascending septo-hippocampal pathway, because injection of procaine, a local anesthetic, into the MS/VDB completely blocks stimulation-induced locomotor activation and theta rhythm [31]. Several other brain areas also control hippocampal theta by acting on the septal neurons [21,42]. Therefore, elimination of one of the sources of the MS/VDB input would not necessarily lead to a noticeable change in theta. For example, lesion of supramammillary nucleus does not alter hippocampal theta [27]. Loss of hypocretin neurons does not lead to a significant change in theta power ([13], our unpublished observation). Hypocretin knockout mice also do not show loss of theta activity [39].

The basal forebrain has been implicated in the regulation of sleep/wakefulness. Extracellular concentrations of adenosine increase in the substantia innominata/nucleus basalis regions of the basal forebrain during spontaneous wakefulness and decrease during SWS [36]. Following sleep deprivation, adenosine levels increase significantly in the cholinergic region of the basal forebrain, and, to a lesser degree, in cortex, but adenosine levels do not change in several other brain areas implicated in the regulation of sleep/wakefulness [35]. It is not known whether adenosine regulates wakefulness by decreasing the discharge of basal forebrain cholinergic neurons or it also acts on non-cholinergic arousal-related neurons in the basal forebrain, such as GABAergic neurons [18]. Adenosine A1 receptor mRNA remains prominent in the basal forebrain following 192 IgG–saporin-induced lesion of the cholinergic neurons [14], suggesting that non-cholinergic neurons in the basal forebrain could mediate the effects of adenosine.

In our experiment, most of the cholinergic as well as non-cholinergic PARV-containing neurons in the medial septal nucleus/VDB were destroyed. However, amounts of REM sleep, SWS and wakefulness in lesioned rats were similar to those of control rats. We also did not find quantitative differences in sleep after 12-h prolonged wakefulness between lesioned rats and rats treated with saline. Following 12-h sleep deprivation, SWS amounts increased almost two-fold, and REM sleep increased more than four-fold in hypocretin–saporin-lesioned rats (Fig. 5), which is similar to the values reported by other authors [11]. Slow wave activity (delta power, 0.25–4.0 Hz) in SWS, a marker of the homeostatic process S in the two-process model of sleep regulation [32], was also similar between lesioned and control rats (Fig. 6). Thus, hypocretin innervation of the MS/VDB parvalbumin (GABAergic) and cholinergic neurons may drive these neurons during wakefulness, and influence cognitive processing, but such an innervation is not critically involved in

producing wakefulness or building sleep drive following prolonged periods of wakefulness.

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