american physiological society

JNP JOURNAL OF NEUROPHYSIOLOGY

J Neurophysiol 127: 1221–1229, 2022.
First published March 30, 2022; doi:10.1152/jn.00041.2022

RESEARCH ARTICLE

Control of Homeostasis

Orexin enhances neuronal synchronization in adult rat hypothalamic culture: a model to study hypothalamic function

Vijayakumar Mavanji,[1] Apostolos P. Georgopoulos,[1,2,3,4,5,6] and Catherine M. Kotz[1,7,8,9]

[1]Research Service, Veterans Affairs Health Care System, Minneapolis, Minnesota; [2]Brain Sciences Center, Veterans Affairs Health Care System, Minneapolis, Minnesota; [3]Department of Neuroscience, University of Minnesota Medical School, Minneapolis, Minnesota; [4]Department of Psychiatry, University of Minnesota Medical School, Minneapolis, Minnesota; [5]Center for Cognitive Sciences, University of Minnesota, Minneapolis, Minnesota; [6]Department of Neurology, University of Minnesota Medical School, Minneapolis, Minnesota; [7]Minnesota Nutrition and Obesity Research Center, St. Paul, Minnesota; [8]Geriatric Research Education Clinical Center, Veterans Affairs Health Care System, Minneapolis, Minnesota; and [9]Department of Integrative Biology and Physiology, University of Minnesota, Minneapolis, Minnesota

Abstract

The regulation of sleep/wake behavior and energy homeostasis is maintained in part by the hypothalamic neuropeptide orexin A (OXA, hypocretin). Reduction in orexin signaling is associated with sleep disorders and obesity, whereas higher lateral hypothalamic (LH) orexin signaling and sensitivity promotes obesity resistance. Similarly, dysregulation of hypothalamic neural networks is associated with onset of age-related diseases, including obesity and several neurological diseases. Despite the association of obesity and aging, and that adult populations are the target for the majority of pharmaceutical and obesity studies, conventional models for neuronal networks utilize embryonic neural cultures rather than adult neurons. Synchronous activity describes correlated changes in neuronal activity between neurons and is a feature of normal brain function, and is a measure of functional connectivity and final output from a given neural structure. Earlier studies show alterations in hypothalamic synchronicity following behavioral perturbations in embryonic neurons obtained from obesity-resistant rats and following application of orexin onto embryonic hypothalamic cultures. Synchronous network dynamics in adult hypothalamic neurons remain largely undescribed. To address this, we established an adult rat hypothalamic culture in multi-electrode-array (MEA) dishes and recorded the field potentials. Then we studied the effect of exogenous orexin on network synchronization of these adult hypothalamic cultures. In addition, we studied the wake promoting effects of orexin in vivo when directly injected into the lateral hypothalamus (LH). Our results showed that the adult hypothalamic cultures are viable for nearly 3 mo in vitro, good quality MEA recordings can be obtained from these cultures in vitro, and finally, that cultured adult hypothalamus is responsive to orexin. These results support that adult rat hypothalamic cultures could be used as a model to study the neural mechanisms underlying obesity. In addition, LH administration of OXA enhanced wakefulness in rats, indicating that OXA enhances wakefulness partly by promoting neural synchrony in the hypothalamus.

NEW & NOTEWORTHY This study, for the first time, demonstrates that adult hypothalamic cultures are viable in vitro for a prolonged duration and are electrophysiologically active. In addition, the study shows that orexin enhances neural synchronization in adult hypothalamic cultures.

adult neuron; hypothalamus; orexin; sleep; synchronization

INTRODUCTION

The hypothalamus is critically involved in the central control of food intake, sleep/wake regulation, neuroendocrine integration, and spontaneous physical activity, all of which play critical roles in maintaining normal body mass index (1, 2). When this control is perturbed, the result is imbalance in energy utilization and ultimately in body weight. Obesity is

Correspondence: V. Mavanji (mavan005@umn.edu).
Submitted 7 February 2022 / Accepted 28 March 2022

0022-3077/22 Published by the American Physiological Society.

associated with hypothalamic dysfunction resulting in a chronic positive energy balance, due in part to excessive energy intake, altered sleep/wake behavior, hormonal imbalance, reduced physical activity, and decreased energy expenditure (1, 3–5). The hypothalamus is a network of neural structures that are important in the regulation of hunger and wakefulness (6, 7). These structures contain neurons that are specialized to sense changes in peripheral signals, such as plasma glucose levels (8), and also contain populations of neurons critical in regulating sleep/wake and energy balance. For example, orexin neurons in the lateral hypothalamus (LH) play a pivotal role as a key regulator or modulator of sleep/wake behavior, feeding, and energy expenditure, among other functions (1, 2, 9). Consistent with these observations, using a selectively bred rodent model of obesity (10), we have shown that obesity resistant (OR) rats have increased LH orexin gene expression, greater orexin sensitivity, increased physical activity, and enhanced sleep quality relative to obesity prone (OP) rats (11–13). In addition, we showed that sleep disturbances alter feeding and energy homeostasis (12, 14), which could be attributed to altered orexin function. Moreover, in a recent study, we demonstrated higher synchronization in obesity-resistant embryonic hypothalamus and in embryonic hypothalamic cultures following application of orexin and topiramate, which exhibit anti-obesity properties (15).

The LH orexin-producing neurons are extensively interconnected with other hypothalamic areas important for sleep and energy balance, such as the arcuate nucleus, supraoptic nucleus, preoptic area, dorsomedial nucleus, tuberomammillary nucleus, posterior hypothalamus, and hypothalamic paraventricular nucleus (5, 16–18). In addition, LH orexin neurons project to multiple additional brain regions, such as cortical/limbic areas and brainstem autonomic and motor sites, which are involved in the regulation of sleep-wake behavior, energy homeostasis, and cognitive, reward-related, and emotion-related functions (18, 19). The widespread projection pattern thus supports a critical role for orexin in modulation of intrahypothalamic signaling and hypothalamic neural output to extrahypothalamic structures to regulate energy homeostasis.

Importantly, recent evidence suggests that orexin may also play a role in neuroprotection (19, 20). Obesity in humans and animal models cause profound changes (including inflammation) to the energy balance centers of the hypothalamus, which results in the loss of central leptin and insulin sensitivity and neuronal connectivity and synaptic plasticity (5). We and others have shown that orexin treatment can protect hypothalamic neurons from damage when exposed to toxic conditions (19–21), suggesting that in addition to promoting healthy energy balance through increased physical activity, orexin signaling may also play an important role in preventing diet- or environment-induced hypothalamic perturbations that could predispose individuals to obesity (1, 2, 22).

Orexins alter neural excitation and synaptic plasticity (23, 24). In addition, application of orexin into locus coeruleus (LC) slices increases the temporal synchrony of action potentials and synchrony of LC output (25). Thus, a logical next step in understanding the relationship between orexin-mediated regulation of metabolism and sleep is investigation of the temporal dynamics/synchronicity of neural network activity in the hypothalamic neurons and how orexin modulates this activity. Complex interactions take place within the hypothalamic neural networks, which can ultimately integrate the incoming signals and determine efferent signals to maintain whole body energy homeostasis. However, little information is available on how hypothalamic neurons work together in small or large ensembles to form dynamic neural networks.

The multielectrode-array (MEA) recording technique allows real time in vitro measurement of neuronal interactions, with spatial and temporal precision (15, 26), which informs how neurons work together in small or large ensembles to form dynamic neural networks. To date, neural network studies identifying the dynamics of complex networks of brain regions involved in energy balance in adult hypothalamus are sparse. We have reported on the value of the MEA technique in understanding neural synchrony in developing neural networks (cortical and hypothalamic tissue obtained from rat embryos) (15, 26). To understand the dynamics of neural interactions within the adult hypothalamus, and to determine how they form a global network, we measured network synchronization within in vitro hypothalamic neural networks obtained from adult Sprague–Dawley rats. In addition, we studied the effect of intralateral hypothalamus administration of orexin A (OXA) on time spent in sleep/wake states to understand the effect of OXA in vivo (following administration into the LH). We hypothesized that adult neurons are viable and electrically active, and hence could be used to understand the hypothalamic dynamics in normal and disease states. Here, we report the feasibility of growing and maintaining adult hypothalamic neural culture, and the effect of orexin manipulation on the network activity of these neurons in vitro. Finally, we demonstrate that intra-LH orexin enhances wakefulness in rats, a functional outcome for orexin network stimulation.

METHODS

Use of Sprague–Dawley rats and rat tissue was approved by the local Institutional Animal Care and Use Committee at the Minneapolis VA Health Care System. All experiments were performed in accordance with the guidelines and regulations described in The US National Institutes of Health Policy on Humane Care and Use of Laboratory Animals. In addition, the study is reported in accordance with ARRIVE (Animal Research: Reporting of In Vivo Experiments) guidelines.

Cell Isolation

Hypothalamic tissue was obtained from two adult rats and was isolated and cultured according to the methodology developed by Brewer and Torricelli (27). Details regarding the preparation and volume of media and tube sizes are determined by the amount of tissue isolated and are described step by step in the study by Brewer and Torricelli (27). In brief, two adult (5-mo-old) male Sprague–Dawley rats (Charles River, Kingston, NY) were decapitated by guillotine without the use of CO_2. Brains were rapidly removed and hypothalami were dissected from the brain while bathed in sterile ice cold Hibernate-A (Invitrogen) supplemented with B27 media (referred to as HABG). Tissue was then transferred into sterile tubes containing fresh prewarmed (30°C) HABG

(without calcium) containing papain (Sigma-Aldrich, 10 U/mg protein) and then placed in a 30°C water bath. Tissue was then shaken for 8 min and fragments were transferred with a wide bore pipet to a new tube and incubated again for 30 min in a 30°C water bath. A series of homogenization titrations (10 times within 45 s) at room temperature was carried out using a siliconized 9-in. Pasteur pipet. Tissue fragments were allowed to settle within 1 min and the supernatant was transferred to another tube. This was repeated for a total of three times, and all supernatants were combined. The resulting cell suspension was then separated using an Optiprep (Sigma-Aldrich) density gradient (35%, 25%, 20%, and 15% Optiprep in HABG, vol:vol). This method facilitates the separation of neurons and glia from the growth inhibiting debris. Gradients were then centrifuged for 15 min at room temperature at a speed of 1,900 rpm. Fractions containing cells were collected with the trituration pipet (25% and 20% density gradients) and diluted with HABG to remove the Optiprep. The diluted cell suspension was then centrifuged for 2 min with 1,100 rpm at 22°C. Supernatant was discarded and pellet was re-suspended in HABG, and centrifuged again for 2 min with 1,100 rpm at 22°C. The supernatant was aspirated and the cell pellets were re-suspended in fresh NeurobasalA media (Invitrogen) with the addition of 0.5 mM glutamine and B27 supplement (referred to as NABG media).

Cell Plating

Before plating, MEAs were cleaned with 70% ethanol, autoclaved, and sterilized under UV light for 2 h. Sterilized MEAs were then coated overnight with poly-D-lysine (Sigma; 50 mg/mL in water). Poly-D-lysine was then aspirated, and MEAs were rinsed once with sterile water and allowed to dry under the hood. Cells obtained as described earlier were counted using a hemacytometer by incubating them with Trypan blue (0.4%, 1:1 cell to dye volume). Live cells were identified by dye-exclusion. For each of eight MEAs prepared, cell suspensions were pipetted directly onto the electrode area at a density of 450,000 cells per plate in 250 μL of NABG supplemented with human fibroblast growth factor (HFGF; Sigma-Aldrich, 5 mg/mL). The MEAs were then covered by a Teflon cover and placed in a 37°C cell culture incubator with 5% CO_2 and 95% humidity for 30 min to allow the cells to adhere to the MEAs. Unattached cells and debris were removed next by rinsing MEAs three times with HABG. Cultures were then replenished with 1 mL of fresh NABG with HFGF. For the duration of the experiment, fresh media was added twice a week by replacing one-half of the media (0.5 mL) in each MEA.

Immunocytology

A separate set of neural cells obtained as described earlier were allowed to grow in three chambered slides, and then stained for neuronal and glia markers. Antibodies against the following markers were used to determine the purity of the neuronal cultures: doublecortin (DCX; an immature neuron marker), glial fibrillary acidic protein (GFAP; a marker for astrocytes), and neuron-specific nuclear protein (NeuN; marker for mature neurons). Cells were fixed with Histochoice (Sigma) overnight at 4°C. For immunostaining, cells were washed three times in 0.1 M phosphate buffered saline (PBS), permeabilized for 15 min with Triton X-100 (0.15% in PBS), and then blocked with for 1 h in 5% normal donkey serum (Jackson ImmunoResearch) at room temperature. Cells were then incubated overnight at 4°C with the following antibody cocktail: mouse anti-NeuN (Millipore, 1:500), chicken anti-GFAP (Millipore, 1:1,000), and goat anti-DCX (Abcam, 1:300) prepared in blocking buffer. Cells were next washed three times in PBS and incubated with appropriate fluorescent secondary antibodies (Jackson ImmunoResearch) for 60 min: donkey anti-mouse (AlexaFluor-488, 1:800), donkey anti-chicken (AlexaFluor-647, 1:600), and donkey anti-goat (Cy3, 1:800), in blocking buffer. After rinsing four times in PBS, the slides were cover-slipped using HardSet mounting medium (Vector Labs). All slides were stored in dark, cool location until photographed.

Data Acquisition

Each MEA (MultiChannel-Systems, Reutlingen, Germany; model MEA120-2-System) is embedded with 60 electrode sites on a flat surface surrounded by a circular wall that creates a well around the electrodes. The electrodes are titanium nitride disks (30-μm diameter) arranged in a square grid with 200 μm spacing between disks. Recording of neuronal activity was performed twice weekly, beginning on the 9th day in vitro (DIV) and ending on the 83rd DIV, for each of the eight MEAs plated with cells, as described earlier (15, 26). Briefly, to record the neuronal activity, MEAs with plated cells were removed from the incubator and placed in a temperature-controlled (37°C) amplifier assembly located within a Faraday shielded metal box. Recording sessions began between 1,100 and 1,200 h. Electrical activity from each electrode was pre-amplified (lowpass filter 15 kHz), amplified (1.0–3.0 kHz bandpass filter), and sampled at 20 kHz using the amplification stages and data recording modules built into the MEA hardware. Signals from all 60 electrodes in a MEA were recorded for 1 min using the MultiChannelSystems software MC_Rack (v. 3.9.1). In the later part of the recording sessions [from 41 DIV onward (in last 12 of the recording sessions)], we also tested the effect of orexin on hypothalamic synchronous activity. To do this, orexin A (American Peptides) was suspended in artificial cerebrospinal fluid (CSF) to constitute a 300 μM solution. Prior to recording, half of the MEA cultures (n = 4) were treated with orexin (1 μL of 300 μM orexin, to obtain a final concentration of 300 nm in 1 mL), whereas the other half (n = 4) were treated with CSF vehicle (1 μL). Recording sessions began immediately following the application of either orexin or CSF into the MEA.

Data Preprocessing

Local field potentials (LFP) were derived from the neuronal data recorded at 20 kHz by applying a second-order bandpass Butterworth filter at 0.7–170 Hz to reject frequencies outside the LFP range (Fig. 4). The filtered LFP were then downsampled to 1 kHz for further analysis, yielding a 60,000 time sample series from each electrode. Noisy and saturated electrodes were visually detected and excluded from further analysis.

Earlier observations with the LFP time series revealed that they are nonstationary with respect to the mean, and highly autocorrelated (26). Because our aim was to assess the interaction between LFP time series obtained from each of the 60 electrodes, using cross correlation (CC) functions, it was required that individual series be rendered stationary and non-autocorrelated. Accordingly, by appropriate preprocessing, the time series were converted into white noise (prewhitening) as described earlier (26). The prewhitening was achieved in two stages: model identification and model parameter estimation (26). We used the Auto-Regressive Integrative Moving Average (ARIMA) model to prewhiten the LFP time series. The ARIMA modeling was carried out using MATLAB (R2010b, v. 7.11.0.584, 64 bit). The basic ARIMA equations can be found in an earlier publication (28). Earlier work indicated that the ARIMA model is adequate and yields residuals (innovations) that are stationary and non-autocorrelated, and can be used for cross-correlation analysis (26).

Sleep Studies

Five-month-old male Sprague–Dawley rats (Charles River, Kingston, NY) (n = 6/group) were housed individually in solid bottom cages with corncob bedding and a chewing substrate (Nylabone, natural flavor, BioServ, Frenchtown, NJ). Throughout the study, a 12-h light/12-h dark cycle (lights on at 06:00) in a temperature-controlled environment (21°C–22°C) was followed. Rodent chow (Harlan Teklad 8604) and water were allowed ad libitum, as described in our earlier study (29). Studies were approved by the Institutional Animal Care and Use Committee at the Minneapolis VA Health Care System.

Surgery

As described in our earlier study (29), rats were anesthetized with a ketamine/xylazine mixture (30 mg/kg; 3 mg/kg), implanted with a radiotelemetric transmitter and EEG/electromyogram (EMG) electrodes to record vigilance states [F40-EET, Data Sciences International (DSI), St. Paul, MN]. Stereotaxic coordinates for the EEG electrodes were as follows: 3.1 mm posterior and 1.5 mm lateral to bregma; incisor bar set at 3.3 mm below ear bars. Electrodes were inserted to pre-drilled holes to touch the dura. The EMG leads were secured in the nuchal musculature. During the same procedure animals were implanted with a 26-gauge stainless-steel guide cannula (Plastics One, Roanoke, VA) targeted toward the rostral-lateral hypothalamus (rLH). Coordinates for the cannulae were as follows: −2.0 lateral and 2.1 mm posterior to bregma and 7.3 mm below the skull surface. Animals were allowed to recover from surgery for at least a week before injections and recordings began.

Injections and Recording

Rats were injected with either orexin-A (American Peptides, Sunnyvale, CA) dissolved in artificial cerebrospinal fluid (CSF, Sigma-Aldrich, St Louis, MO) or CSF alone, which served as the vehicle control for orexin-A in a counter-balanced design. Doses were chosen based on our prior studies with other sites where orexin produced significant behavioral effects (13, 29). A volume of 0.5 μL was injected over 30 s with a 33-gauge injector (Plastics One) that extended 1.0 mm beyond the tip of the guide cannula (29). Injections were performed between 0800 and 1000 h (>48 h between injections).

Continuous EEG/EMG recordings were obtained for 2 h postinjection. To record the EEG/EMG signals from the implanted EEG and EMG electrodes and transmitter, a receiver was placed beneath the rat cage. The EEG and EMG data were visualized with the Neuroscore software (v. 2.0.1; Data Sciences International). Consecutive 10-s epochs of EEG and EMG were manually scored to determine wake (W), nonrapid eye movement (NREM) sleep, and rapid eye movement (REM) sleep, in accordance with previously described criteria (29).

Statistical Analyses

We assessed the synchronicity between neuronal populations by calculating all pairwise zero-lag cross correlations between pre-whitened LFP time series (ARIMA innovations, see *Data Preprocessing*). For statistical analyses, we took the absolute value of CC (since its sign is not relevant) and z-transformed it (30) to normalize its distribution: zCC = atanh (CC). Statistical comparisons were tested using ANCOVA where group (control, CSF, CSF + orexin) was a fixed factor and DIV was a covariate. MATLAB R2010b, v. 7.11.0.584 (64 bit), the IMSL statistical library in FORTRAN [Intel Fortran Compiler Classic 2021.1.2 (Intel 64)], and the IBM-SPSS statistical package (v. 26) were used to analyze the data.

For the sleep/wake parameters, data were analyzed with Prism 6.0 (GraphPad Software Inc., San Diego, CA). Data are expressed as means ± SE. Alpha was 0.05 for all statistical tests. Data were analyzed with repeated-measures analysis of variance followed by Fischer's tests to determine differences between individual treatments. All assumptions for repeated-measures analysis of variance were met. Sample size was based on power calculations from previous report (29). The data were analyzed in the 0–1 h and 1–2 h postinjection time periods, which will be referred to as the 0–1 and 1–2 h postinjection.

RESULTS

As there was no change in network activity (strength of cross correlations) or the magnitude of orexin effect across recordings days, all the recording days were pooled. Application of both CSF and orexin dissolved in CSF (CSF + orexin) increased synchronicity with respect to untreated cultures: untreated (means ± SE), 0.010514 ± 0.000032, n = 52,628; CSF, 0.011313 ± 0.000019, n = 78116; CSF + Orexin, 0.011428 ± 0.000019, n = 76920. The overall effect of the Group factor was highly significant [$F(2,207,660) = 239.011$, $P < 0.001$, ANCOVA]. Compared with the untreated group (ANCOVA), the magnitude of synchronicity was significantly higher in both the CSF ($P < 0.001$) and the CSF + Orexin group ($P < 0.001$); synchronicity was significantly higher in the CSF + Orexin than the CSF group (Fig. 1; $P < 0.001$), indicating enhanced synchronization following orexin application. The increased synchronization following CSF application is unexpected and might be partly resulting from the transient mechanical disturbance, membrane potential

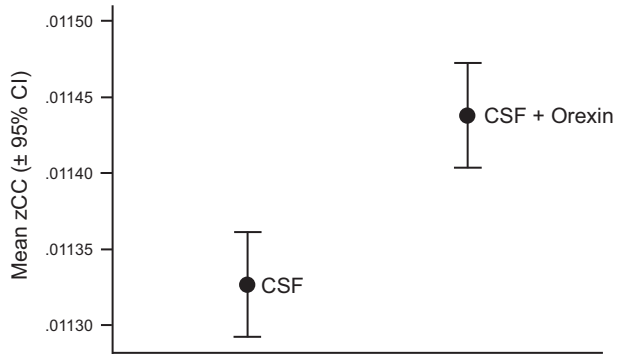

Figure 1. Mean z-transformed zero-lag cross correlation (zCC) [±95% confidence intervals (CI)] for control (cerebrospinal fluid, CSF) and CSF + orexin-treated adult hypothalamic multi-electrode-array (MEA) cultures. $n = 32$, MEA × day in vitro for control, and orexin treatments.

change, and resulting release of neuromodulators from the cultured neurons.

Our immunocytochemistry labeling showed that adult neurons are viable in vitro, for up to 85 days after plating. As shown in Fig. 2, labeling revealed that the adult hypothalamic cultures exhibited a mixture of neurons, glia, and immature neurons. In addition, we found mature neurons in the hypothalamic culture developing in the MEA, with intact neuronal processes, and clusters of neurons forming networks with neuronal processes (Fig. 3). In addition, spikes were visible following application of orexin into the MEA containing adult hypothalamic cultures (Fig. 4).

Sleep Modulation Studies

To test whether rLH injected OXA increases time awake, 5-mo-old male Sprague–Dawley rats were injected (into the LH) with either orexin-A (250 pmol), dissolved in artificial cerebrospinal fluid (CSF) or CSF alone that served as the vehicle control for orexin-A in a counter-balanced design. The dose of 250 pmol was chosen based on our prior studies with other sites where orexin produced significant behavioral effects (13, 29). A volume of 0.5 μL was injected for 30 s with a 33-gauge injector (Plastics One) that extended 1.0 mm beyond the tip of the guide cannula (29). Injections were performed between 0800 and 1000 h (>48 h between injections) as described earlier, and sleep/wake parameters were performed using continuous EEG/EMG recordings for 2-h postinjection.

There was a stimulating effect of rLH-injected OXA on vigilance states (Fig. 5). Administration of OXA increased wakefulness, reduced nonrapid eye movement (NREM) sleep, and REM sleep time. There was a main effect of treatment on wakefulness (Fig. 5, *A* and *B*, $P < 0.001$), NREM sleep (Fig. 5, *A* and *B*, $P < 0.001$), and REM sleep (Fig. 5, *A* and *B*, $P < 0.001$) during the 0–1 h and 0–2 h postinjection time period.

DISCUSSION

In the current study, we investigated network synchronization in adult hypothalamic neural cultures using the MEA technique. Our results showed that adult hypothalamic tissue is viable in vitro up to 85 days, and that good quality neuronal activity can be recorded from these neurons. In addition, we found that addition of OXA into the culture medium enhances synchronization in these neurons compared with the application of CSF. Our in vivo study in rats showed increased time awake and reduced time in both NREM and REM sleep following administration of OXA into the LH.

These results are novel in that for the first time we showed orexin responsiveness in neural cultures obtained from adult rat hypothalamus. Supporting an earlier study, here we demonstrated that neurons from adult animals can be isolated and maintained long-term in vitro (27, 31). An earlier study from our laboratory showed higher synchronization following orexin application in embryonic hypothalamic cultures obtained from rats (15), and the current findings are an extension of these earlier findings into adult hypothalamic cultures. The results presented here along with our study in embryonic hypothalamic cultures indicate that exogenous orexin enhances neural synchronization, and beneficial effects of exogenous orexin might partly arise from its ability to enhance neural synchronization. Our results also indicate that synchronizing effect of orexin observed in embryonic hypothalamus persists even in adult animals. Current results are in agreement with earlier electrophysiological studies, which demonstrated the involvement of orexin in enhancing synchronization between neural structures (25, 32), which might results partly from orexin's ability to modulate

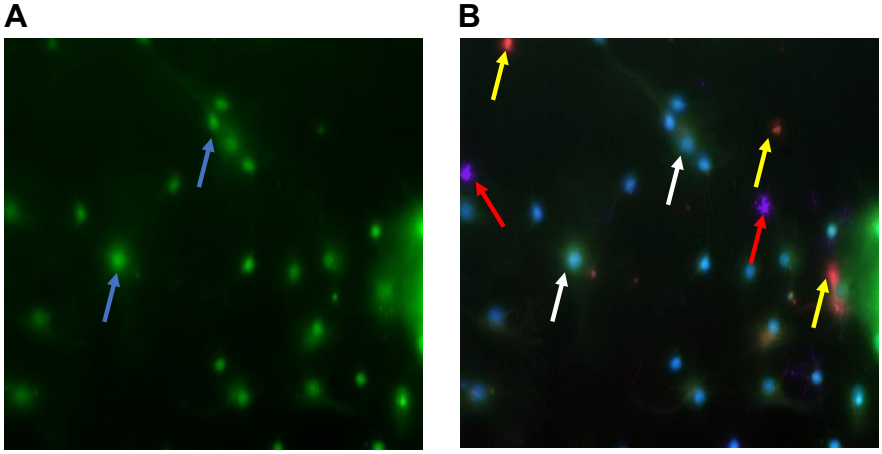

Figure 2. Photomicrographs of slides containing adult hypothalamic culture. Immunofluorescent neurons (*A*, blue arrow) showing green fluorescence, stained for neuronal nuclear marker (NeuN). *B*: a mixture of neurons and glia in the adult hypothalamic culture. Merged blue (DAPI, a nuclear stain) and green (NeuN) fluorescence showing mature neurons (*B*, white arrows), astrocytes (*B*, yellow arrows), stained in red for glial fibrillary acidic protein (GFAP), and immature neurons (*B*, red arrow), stained in purple for doublecortin (DCX), a marker for immature/migrating neurons.

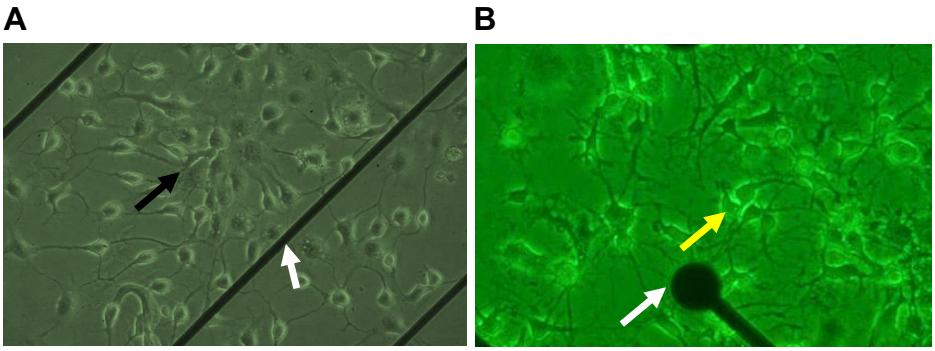

Figure 3. Hypothalamic neurons in multi-electrode-array (MEA) culture after isolation from a 5-mo-old Sprague–Dawley rat. *A*: well-connected neurons (black arrow) and electrode track (white arrow). *B*: MEA cultures with neural networks (yellow arrow) and the electrode disk (white arrow).

membrane voltage oscillations (25), via modulation of Ca (2 +) currents (33, 34).

The hypothalamus plays an important role in integrative functions including the regulation of energy balance (35, 36). Numerous neuronal subtypes with many neurotransmitters, neuromodulators, and humoral factors in the hypothalamus integrate peripheral and environmental signals and in turn modulate activity in extrahypothalamic structures to maintain behavioral and physiological homeostasis, including energy balance. This integrative function of the hypothalamus depends on the synchronized output of highly differentiated hypothalamic neural networks (15, 37–41). Lateral hypothalamic orexin is critical for the regulation of the sleep/wake cycle, feeding, motivated behavior, movement initiation, and energy expenditure (35). Orexin neurons are extensively interconnected with other hypothalamic areas and extrahypothalamic structures important for multiple integrative functions (5, 16–18, 42–45). Orexin modulation of these target structures may be important to the regulation of energy homeostasis. The current data suggest that orexin neurons may achieve this function by synchronizing hypothalamic network activity, thereby modulating efferent signals to extrahypothalamic structures with temporal coherence to regulate behavior and bodily homeostasis. In support of this concept are results from a recent study showing that orexin likely controls behavior via output to other hypothalamic and extra hypothalamic structure, as within LH connectivity is demonstrated to be sparse (46).

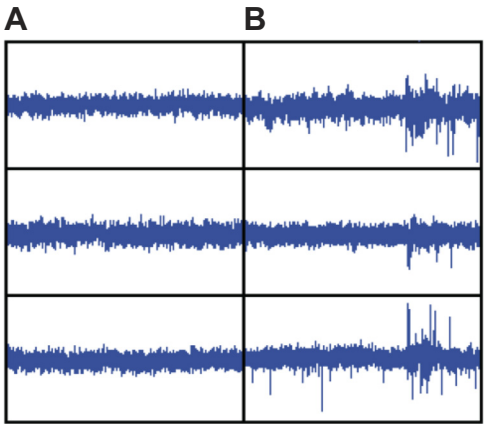

Figure 4. Raw local field potentials (LFP) and spikes from cerebrospinal fluid (*A*) and orexin-treated hypothalamic cultures (*B*) obtained from three multi-electrode-array channels each.

Normal hypothalamic function is important for integrative functions, and neural synchrony appears to be a feature of normal brain function (and to be required for memory formation) (47). Obesity and metabolic disease cause structural and functional changes in the hypothalamus (5), and various neurological diseases and neurodegeneration are associated with altered neural synchronization (48). In addition, activity during the resting phase of the sleep/wake cycle results in hypothalamic desynchrony as measured by c-Fos expression in rats (49). Earlier work in our laboratory showed improvements in behavioral outcomes following activation of orexin neurons in a mouse model of Parkinson's disease (50–52). Given that neurological disorders are associated with altered neural synchronization, the observed improvements in behavioral outcomes in the Parkinson's mouse model may be partly due to enhanced neural synchrony following orexin neuron activation.

In our in vivo study using rats, OXA in rLH enhanced the amount of time awake. The mechanism(s) underlying the physiological effect (wake promotion) of orexin-A in the LH remain unclear. Orexin-A excites and enhances firing rates of arousal-promoting brain areas such as the locus coeruleus (53), the tuberomammillary nucleus (54), cholinergic neurons in the pedunculopontine and latero-dorsal tegmental nuclei (55), and the dorsal raphe neurons (56). These neural structures promote wakefulness to some extent by inhibiting sleep promoting neurons in the ventrolateral preoptic area (VLPO) (57). Thus, it is possible that orexin-induced synchronized output to these sleep/wake regulatory areas is partly responsible for orexins' role in wake promotion.

The finding of wake promotion by LH OXA agrees with previous studies, which show that central orexin and peripheral orexin agonists enhance wakefulness. It has been previously shown that peripheral administration of orexin 2 receptor (OX2R) agonists (Tak-925 and YNT-185) enhances wake time in mice (58, 59). We showed increased wakefulness and reduced NREM and REM sleep following ventrolateral preoptic area (VLPO) injection of orexin-A in rats (29, 57). Similarly, intracerebroventricular administration of orexin-A (3 nmol) enhanced wakefulness and suppressed both NREM and REM sleep in mice up to 3 h following treatment (60). Central administration (intra-cerebro-ventricular, icv) of TAK-925 (30, 100, and 300 nmol doses) during the light phase decreased the percent of time in NREM sleep and increased time in wakefulness up to 3 h postinjection (61). Conversely, dysfunction of orexins, orexin receptors, or orexin neurons results in the sleep disorder narcolepsy, which is characterized by excessive active period sleep (60, 62).

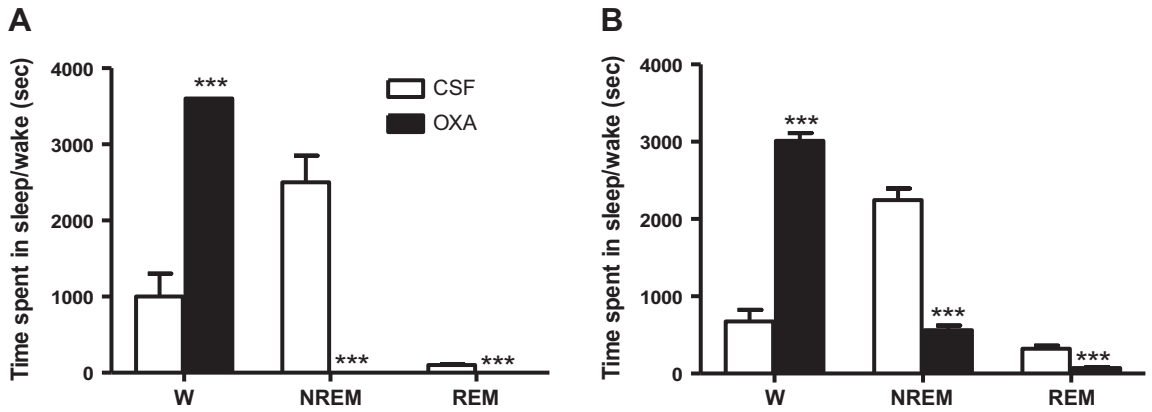

Figure 5. Orexin-A infusion (250 pmol) into the lateral hypothalamus (LH) significantly increases time spent awake (W) and decreases time spent in non-rapid eye movement (NREM) sleep and rapid eye movement (REM) sleep compared with cerebrospinal fluid (CSF) injections (*A* and *B*). Intra-LH orexin increases wake and decreases NREM and REM sleep 0–1 h postinjection period (*A*). Similar wake promoting and sleep suppressing effect (both NREM and REM sleep) was also observed during the 1–2 h postinjection period (*B*). $n = 6$. Data represented as means ± SE. ***$P < 0.001$.

The rapid postorexin increase in wake and neural synchrony in the current study is in agreement with recent electrophysiological data showing that the speed of orexin cell excitation by external stimuli can be as short as 34 ms, and orexin cell electrical excitation can cause physical activity within 300 ms (63). Adequate background arousal and behavioral stabilization is necessary for motor behavior. Given that orexin is critical for behavioral stabilization (64), motor control (65), and wake promotion, it is possible that orexin-induced neural synchrony simultaneously sends excitatory signals to both sleep/wake and motor regulatory areas to promote wake and physical activity. In contrast, sleep disruption during the resting phase results in reduced orexin immunoreactivity (66), neural desynchrony, and excessive active period sleep (49).

There are a few limitations of the current study, one of which is the single orexin dose (300 nmol), which was based on our earlier study using embryonic hypothalamic culture (15). Future studies will be designed to assess the dose-dependent effect of orexin on adult hypothalamic neural synchrony and sleep/wake parameters, to better understand the physiological relevance of orexin-induced neural synchrony. Another limitation is that the primary cultures used were heterogeneous in nature and contained a mixture of various neuronal and glial cell populations. Future studies using cultures of specific hypothalamic neurons (anatomically and neurochemically distinct) are warranted to understand the specific role of these neuronal networks in orexin function. Similarly, studies with slice preparations are required to address neural location-specific effects of orexin on neural synchronicity. In addition, future studies will compare the same MEA network before and after orexin application, where same MEA serves as its own control. The final limitation is the lack of studies with orexin antagonists as well as other neuromodulators such as MCH and NPY, and future studies are needed to address the effect of orexin antagonists and other neuromodulators to identify the role of specific receptors and neuromodulators in neural synchronization and sleep/wake modulation.

In conclusion, energy balance and wake promotion requires simultaneous activation of discrete neural structures including the hypothalamus (67). The current studies indicate that orexin action enhances temporal integration of hypothalamic neural activation and results in higher synchronous output to extrahypothalamic structures important for energy balance and sleep/wake regulation. The current study, coupled with our earlier findings in embryonic hypothalamic structures (15), suggests that enhancing synchrony in the hypothalamus may have therapeutic value in treating obesity and sleep disorders such as narcolepsy.

DATA AVAILABILITY

Based on the Institutional guidelines, data will be made available to interested parties upon written request to the authors.

ACKNOWLEDGMENTS

The authors thank Martha K. Grace, Dale V. Boeff, and Curtis D. Evans of Minneapolis VAHCS for technical help during the experiments, data collection, and analysis.

GRANTS

This work was supported by the Department of Veterans Affairs Grants 5I01BX003004-01A2 and 1I01BX003687-02 (to C. M. Kotz); 5IO1CX001045-03 (to A. P. Georgopoulos), the National Institute of Health Grant 5R01DK100281-03 (to C. M. Kotz), Award Number T32DK083250 from the National Institute of Diabetes and Digestive and Kidney Diseases (to C. M. Kotz), the University of Minnesota McKnight Presidential Chair of Cognitive Neuroscience, the American Legion Brain Sciences Chair (to A. P. Georgopoulos), and the U.S. Department of Defense Award Number W81XWH-15-1-0520 (to A. P. Georgopoulos).

DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

AUTHOR CONTRIBUTIONS

V.M., A.P.G., and C.M.K. conceived and designed research; V.M. performed experiments; V.M. and A.P.G. analyzed data; V.M.,

A.P.G., and C.M.K. interpreted results of experiments; V.M. and A.P.G. prepared figures; V.M., A.P.G., and C.M.K. drafted manuscript; V.M., A.P.G., and C.M.K. edited and revised manuscript; V.M., A.P.G., and C.M.K. approved final version of manuscript.

REFERENCES

1. **Kotz C**, **Nixon J**, **Butterick T**, **Perez-Leighton C**, **Teske J**, **Billington C.** Brain orexin promotes obesity resistance. *Ann NY Acad Sci* 1264: 72–86, 2012. doi:10.1111/j.1749-6632.2012.06585.x.
2. **Kotz CM**, **Perez-Leighton CE**, **Teske JA**, **Billington CJ.** Spontaneous physical activity defends against obesity. *Curr Obes Rep* 6: 362–370, 2017. doi:10.1007/s13679-017-0288-1.
3. **Knutson KL.** Does inadequate sleep play a role in vulnerability to obesity? *Am J Hum Biol* 24: 361–371, 2012. doi:10.1002/ajhb.22219.
4. **Tregellas JR**, **Wylie KP**, **Rojas DC**, **Tanabe J**, **Martin J**, **Kronberg E**, **Cordes D**, **Cornier M-A.** Altered default network activity in obesity. *Obesity (Silver Spring)* 19: 2316–2321, 2011. doi:10.1038/oby.2011.119.
5. **Williams LM.** Hypothalamic dysfunction in obesity. *Proc Nutr Soc* 71: 521–533, 2012. doi:10.1017/s002966511200078x.
6. **Delgado JMR**, **Anand BK.** Increase of food intake induced by electrical stimulation of the lateral hypothalamus. *Am J Physiol* 172: 162–168, 1953. doi:10.1152/ajplegacy.1952.172.1.162.
7. **Moruzzi G**, **Magoun HW.** Brain stem reticular formation and activation of the EEG. *Electroencephalogr Clin Neurophysiol* 1: 455–473, 1949. doi:10.1016/0013-4694(49)90219-9.
8. **Anand BK**, **Chhina GS**, **Sharma KN**, **Dua S**, **Singh B.** Activity of single neurons in the hypothalamic feeding centers: effect of glucose. *Am J Physiol* 207: 1146–1154, 1964. doi:10.1152/ajplegacy.1964.207.5.1146.
9. **de Lecea L**, **Kilduff TS**, **Peyron C**, **Gao X**, **Foye PE**, **Danielson PE**, **Fukuhara C**, **Battenberg EL**, **Gautvik VT**, **Bartlett FS**, **Frankel WN**, **van den Pol AN**, **Bloom FE**, **Gautvik KM**, **Sutcliffe JG.** The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci USA* 95: 322–327, 1998. doi:10.1073/pnas.95.1.322.
10. **Levin BE**, **Richard D**, **Michel C**, **Servatius R.** Differential stress responsivity in diet-induced obese and resistant rats. *Am J Physiol Regul Integr Comp Physiol* 279: R1357–R1364, 2000. doi:10.1152/ajpregu.2000.279.4.R1357.
11. **Kotz CM**, **Teske JA**, **Billington CJ.** Neuroregulation of nonexercise activity thermogenesis and obesity resistance. *Am J Physiol Regul Integr Comp Physiol* 294: R699–R710, 2008. doi:10.1152/ajpregu.00095.2007.
12. **Mavanji V**, **Teske JA**, **Billington CJ**, **Kotz CM.** Elevated sleep quality and orexin receptor mRNA in obesity-resistant rats. *Int J Obes (Lond)* 34: 1576–1588, 2010. doi:10.1038/ijo.2010.93.
13. **Teske JA**, **Levine AS**, **Kuskowski M**, **Levine JA**, **Kotz CM.** Elevated hypothalamic orexin signaling, sensitivity to orexin A, and spontaneous physical activity in obesity-resistant rats. *Am J Physiol Regul Integr Comp Physiol* 291: R889–R899, 2006. doi:10.1152/ajpregu.00536.2005.
14. **Mavanji V**, **Teske JA**, **Billington CJ**, **Kotz CM.** Partial sleep deprivation by environmental noise increases food intake and body weight in obesity-resistant rats. *Obesity (Silver Spring)* 21: 1396–1405, 2013. doi:10.1002/oby.20182.
15. **Mavanji V**, **Georgopoulos AP**, **Kotz CM.** Synchronous neuronal interactions in rat hypothalamic culture: a novel model for the study of network dynamics in metabolic disorders. *Exp Brain Res* 239: 755–764, 2021. doi:10.1007/s00221-020-05977-7.
16. **Burt J**, **Alberto CO**, **Parsons MP**, **Hirasawa M.** Local network regulation of orexin neurons in the lateral hypothalamus. *Am J Physiol Regul Integr Comp Physiol* 301: R572–R580, 2011. doi:10.1152/ajpregu.00674.2010.
17. **Spinazzi R**, **Andreis PG**, **Rossi GP**, **Nussdorfer GG.** Orexins in the regulation of the hypothalamic-pituitary-adrenal axis. *Pharmacol Rev* 58: 46–57, 2006. doi:10.1124/pr.58.1.4.
18. **Yoshida K**, **McCormack S**, **Espana RA**, **Crocker A**, **Scammell TE.** Afferents to the orexin neurons of the rat brain. *J Comp Neurol* 494: 845–861, 2006. doi:10.1002/cne.20859.
19. **Butterick TA**, **Nixon JP**, **Billington CJ**, **Kotz CM.** Orexin A decreases lipid peroxidation and apoptosis in a novel hypothalamic cell model. *Neurosci Lett* 524: 30–34, 2012. doi:10.1016/j.neulet.2012.07.002.
20. **Sokołowska P**, **Urbańska A**, **Biegańska K**, **Wagner W**, **Ciszewski W**, **Namiecińska M**, **Zawilska JB.** Orexins protect neuronal cell cultures against hypoxic stress: an involvement of Akt signaling. *J Mol Neurosci* 52: 48–55, 2014. doi:10.1007/s12031-013-0165-7.
21. **Xiong X**, **White RE**, **Xu L**, **Yang L**, **Sun X**, **Zou B**, **Pascual C**, **Sakurai T**, **Giffard RG**, **Xie XS.** Mitigation of murine focal cerebral ischemia by the hypocretin/orexin system is associated with reduced inflammation. *Stroke* 44: 764–770, 2013. doi:10.1161/STROKEAHA.112.681700.
22. **Butterick TA**, **Billington CJ**, **Kotz CM**, **Nixon JP.** Orexin: pathways to obesity resistance? *Rev Endocr Metab Disord* 14: 357–364, 2013. doi:10.1007/s11154-013-9259-3.
23. **Selbach O**, **Doreulee N**, **Bohla C**, **Eriksson KS**, **Sergeeva OA**, **Poelchen W**, **Brown RE**, **Haas HL.** Orexins/hypocretins cause sharp wave- and theta-related synaptic plasticity in the hippocampus via glutamatergic, gabaergic, noradrenergic, and cholinergic signaling. *Neuroscience* 127: 519–528, 2004. doi:10.1016/j.neuroscience.2004.05.012.
24. **Yamanaka A**, **Tabuchi S**, **Tsunematsu T**, **Fukazawa Y**, **Tominaga M.** Orexin directly excites orexin neurons through orexin 2 receptor. *J Neurosci* 30: 12642–12652, 2010. doi:10.1523/jneurosci.2120-10.2010.
25. **van den Pol AN**, **Ghosh PK**, **Liu RJ**, **Li Y**, **Aghajanian GK**, **Gao XB.** Hypocretin (orexin) enhances neuron activity and cell synchrony in developing mouse GFP-expressing locus coeruleus. *J Physiol* 541: 169–185, 2002. doi:10.1113/jphysiol.2002.017426.
26. **Christopoulos VN**, **Boeff DV**, **Evans CD**, **Crowe DA**, **Amirikian B**, **Georgopoulos A**, **Georgopoulos AP.** A network analysis of developing brain cultures. *J Neural Eng* 9: 046008, 2012. doi:10.1088/1741-2560/9/4/046008.
27. **Brewer GJ**, **Torricelli JR.** Isolation and culture of adult neurons and neurospheres. *Nat Protoc* 2: 1490–1498, 2007. doi:10.1038/nprot.2007.207.
28. **Christova P**, **Lewis SM**, **Jerde TA**, **Lynch JK**, **Georgopoulos AP.** True associations between resting fMRI time series based on innovations. *J Neural Eng* 8: 046025, 2011. doi:10.1088/1741-2560/8/4/046025.
29. **Mavanji V**, **Perez-Leighton CE**, **Kotz CM**, **Billington CJ**, **Parthasarathy S**, **Sinton CM**, **Teske JA.** Promotion of wakefulness and energy expenditure by orexin-A in the ventrolateral preoptic area. *Sleep* 38: 1361–1370, 2015. doi:10.5665/sleep.4970.
30. **Fisher RA** (Editor)**.** *Statistical Methods for Research Workers*. Edinburgh: Oliver and Boyd, 1958.
31. **Evans MS**, **Collings MA**, **Brewer GJ.** Electrophysiology of embryonic, adult and aged rat hippocampal neurons in serum-free culture. *J Neurosci Methods* 79: 37–46, 1998. doi:10.1016/s0165-0270(97)00159-3.
32. **Chen H**, **Wang YJ**, **Yang L**, **Sui JF**, **Hu ZA**, **Hu B.** Theta synchronization between medial prefrontal cortex and cerebellum is associated with adaptive performance of associative learning behavior. *Sci Rep* 6: 20960, 2016. doi:10.1038/srep20960.
33. **Ishibashi M**, **Gumenchuk I**, **Kang B**, **Steger C**, **Lynn E**, **Molina NE**, **Eisenberg LM**, **Leonard CS.** Orexin receptor activation generates gamma band input to cholinergic and serotonergic arousal system neurons and drives an intrinsic Ca^{2+}-dependent resonance in LDT and PPT cholinergic neurons. *Front Neurol* 6: 120, 2015. doi:10.3389/fneur.2015.00120.
34. **Ishibashi M**, **Gumenchuk I**, **Miyazaki K**, **Inoue T**, **Ross WN**, **Leonard CS.** Hypocretin/orexin peptides alter spike encoding by serotonergic dorsal raphe neurons through two distinct mechanisms that increase the late afterhyperpolarization. *J Neurosci* 36: 10097–10115, 2016. doi:10.1523/jneurosci.0635-16.2016.
35. **Bonnavion P**, **Mickelsen LE**, **Fujita A**, **de Lecea L**, **Jackson AC.** Hubs and spokes of the lateral hypothalamus: cell types, circuits and behaviour. *J Physiol* 594: 6443–6462, 2016. doi:10.1113/JP271946.
36. **van Galen KA**, **Ter Horst KW**, **Serlie MJ.** Serotonin, food intake, and obesity. *Obes Rev* 22: e13210, 2021. doi:10.1111/obr.13210.
37. **Gautron L**, **Elmquist JK**, **Williams KW.** Neural control of energy balance: translating circuits to therapies. *Cell* 161: 133–145, 2015. doi:10.1016/j.cell.2015.02.023.
38. **Saper CB**, **Fuller PM**, **Pedersen NP**, **Lu J**, **Scammell TE.** Sleep state switching. *Neuron* 68: 1023–1042, 2010. doi:10.1016/j.neuron.2010.11.032.

39. **Saper CB**, **Lu J**, **Chou TC**, **Gooley J.** The hypothalamic integrator for circadian rhythms. *Trends Neurosci* 28: 152–157, 2005. doi:10.1016/j.tins.2004.12.009.
40. **Sternson SM**, **Nicholas Betley J**, **Cao ZF.** Neural circuits and motivational processes for hunger. *Curr Opin Neurobiol* 23: 353–360, 2013. doi:10.1016/j.conb.2013.04.006.
41. **Williams RH**, **Alexopoulos H**, **Jensen LT**, **Fugger L**, **Burdakov D.** Adaptive sugar sensors in hypothalamic feeding circuits. *Proc Natl Acad Sci USA* 105: 11975–11980, 2008. doi:10.1073/pnas.0802687105.
42. **Espana RA**, **Reis KM**, **Valentino RJ**, **Berridge CW.** Organization of hypocretin/orexin efferents to locus coeruleus and basal forebrain arousal-related structures. *J Comp Neurol* 481: 160–178, 2005. doi:10.1002/cne.20369.
43. **Mahler SV**, **Smith RJ**, **Moorman DE**, **Sartor GC**, **Aston-Jones G.** Multiple roles for orexin/hypocretin in addiction. *Prog Brain Res* 198: 79–121, 2012. doi:10.1016/b978-0-444-59489-1.00007-0.
44. **Vittoz NM**, **Berridge CW.** Hypocretin/orexin selectively increases dopamine efflux within the prefrontal cortex: involvement of the ventral tegmental area. *Neuropsychopharmacology* 31: 384–395, 2006. doi:10.1038/sj.npp.1300807.
45. **Yang L**, **Zou B**, **Xiong X**, **Pascual C**, **Xie J**, **Malik A**, **Xie J**, **Sakurai T**, **Xie XS.** Hypocretin/orexin neurons contribute to hippocampus-dependent social memory and synaptic plasticity in mice. *J Neurosci* 33: 5275–5284, 2013. doi:10.1523/jneurosci.3200-12.2013.
46. **Burdakov D**, **Karnani MM.** Ultra-sparse connectivity within the lateral hypothalamus. *Curr Biol* 30: 4063–4070.e2, 2020. doi:10.1016/j.cub.2020.07.061.
47. **Fell J**, **Axmacher N.** The role of phase synchronization in memory processes. *Nat Rev Neurosci* 12: 105–118, 2011. doi:10.1038/nrn2979.
48. **Sorrentino P**, **Rucco R**, **Baselice F**, **De Micco R**, **Tessitore A**, **Hillebrand A**, **Mandolesi L**, **Breakspear M**, **Gollo LL**, **Sorrentino G.** Flexible brain dynamics underpins complex behaviours as observed in Parkinson's disease. *Sci Rep* 11: 4051, 2021. doi:10.1038/s41598-021-83425-4.
49. **Salgado-Delgado R**, **Nadia S**, **Angeles-Castellanos M**, **Buijs RM**, **Escobar C.** In a rat model of night work, activity during the normal resting phase produces desynchrony in the hypothalamus. *J Biol Rhythms* 25: 421–431, 2010. doi:10.1177/0748730410383403.
50. **Stanojlovic M**, **Pallais JP**, **Kotz CM.** Chemogenetic modulation of orexin neurons reverses changes in anxiety and locomotor activity in the A53T mouse model of Parkinson's disease. *Front Neurosci* 13: 702, 2019. doi:10.3389/fnins.2019.00702.
51. **Stanojlovic M**, **Pallais JP**, **Lee MK**, **Kotz CM.** Pharmacological and chemogenetic orexin/hypocretin intervention ameliorates Hipp-dependent memory impairment in the A53T mice model of Parkinson's disease. *Mol Brain* 12: 87, 2019. doi:10.1186/s13041-019-0514-8.
52. **Stanojlovic M**, **Pallais Yllescas JP Jr**, **Vijayakumar A**, **Kotz C.** Early sociability and social memory impairment in the A53T mouse model of Parkinson's disease are ameliorated by chemogenetic modulation of orexin neuron activity. *Mol Neurobiol* 56: 8435–8450, 2019. doi:10.1007/s12035-019-01682-x.
53. **Hagan JJ**, **Leslie RA**, **Patel S**, **Evans ML**, **Wattam TA**, **Holmes S**, **Benham CD**, **Taylor SG**, **Routledge C**, **Hemmati P**, **Munton RP**, **Ashmeade TE**, **Shah AS**, **Hatcher JP**, **Hatcher PD**, **Jones DN**, **Smith MI**, **Piper DC**, **Hunter AJ**, **Porter RA**, **Upton N.** Orexin A activates locus coeruleus cell firing and increases arousal in the rat. *Proc Natl Acad Sci USA* 96, 10911–10916, 1999. doi:10.1073/pnas.96.19.10911.
54. **Yamanaka A**, **Tsujino N**, **Funahashi H**, **Honda K**, **Guan JL**, **Wang QP**, **Tominaga M**, **Goto K**, **Shioda S**, **Sakurai T.** Orexins activate histaminergic neurons via the orexin 2 receptor. *Biochem Biophys Res Commun* 290: 1237–1245, 2002. doi:10.1006/bbrc.2001.6318.
55. **Takahashi K**, **Koyama Y**, **Kayama Y**, **Yamamoto M.** Effects of orexin on the laterodorsal tegmental neurones. *Psychiatry Clin Neurosci* 56: 335–336, 2002. doi:10.1046/j.1440-1819.2002.00967.x.
56. **Brown RE**, **Sergeeva OA**, **Eriksson KS**, **Haas HL.** Convergent excitation of dorsal raphe serotonin neurons by multiple arousal systems (orexin/hypocretin, histamine and noradrenaline). *J Neurosci* 22: 8850–8859, 2002. doi:10.1523/JNEUROSCI.22-20-08850.2002.
57. **Coborn JE**, **DePorter DP**, **Mavanji V**, **Sinton CM**, **Kotz CM**, **Billington CJ**, **Teske JA.** Role of orexin-A in the ventrolateral preoptic area on components of total energy expenditure. *Int J Obes* 41: 1256–1262, 2017. doi:10.1038/ijo.2017.92.
58. **Irukayama-Tomobe Y**, **Ogawa Y**, **Tominaga H**, **Ishikawa Y**, **Hosokawa N**, **Ambai S**, **Kawabe Y**, **Uchida S**, **Nakajima R**, **Saitoh T**, **Kanda T**, **Vogt K**, **Sakurai T**, **Nagase H**, **Yanagisawa M.** Nonpeptide orexin type-2 receptor agonist ameliorates narcolepsy-cataplexy symptoms in mouse models. *Proc Natl Acad Sci USA* 114: 5731–5736, 2017. doi:10.1073/pnas.1700499114.
59. **Nagahara T**, **Saitoh T**, **Kutsumura N**, **Irukayama-Tomobe Y**, **Ogawa Y**, **Kuroda D**, **Gouda H**, **Kumagai H**, **Fujii H**, **Yanagisawa M**, **Nagase H.** Design and synthesis of non-peptide, selective orexin receptor 2 agonists. *J Med Chem* 58: 7931–7937, 2015. doi:10.1021/acs.jmedchem.5b00988.
60. **Willie JT**, **Chemelli RM**, **Sinton CM**, **Tokita S**, **Williams SC**, **Kisanuki YY**, **Marcus JN**, **Lee C**, **Elmquist JK**, **Kohlmeier KA**, **Leonard CS**, **Richardson JA**, **Hammer RE**, **Yanagisawa M.** Distinct narcolepsy syndromes in Orexin receptor-2 and Orexin null mice: molecular genetic dissection of Non-REM and REM sleep regulatory processes. *Neuron* 38: 715–730, 2003. doi:10.1016/s0896-6273(03)00330-1.
61. **Yukitake H**, **Fujimoto T**, **Ishikawa T**, **Suzuki A**, **Shimizu Y**, **Rikimaru K**, **Ito M**, **Suzuki M**, **Kimura H.** TAK-925, an orexin 2 receptor-selective agonist, shows robust wake-promoting effects in mice. *Pharmacol Biochem Behav* 187: 172794, 2019. doi:10.1016/j.pbb.2019.172794.
62. **Suno R**, **Kimura KT**, **Nakane T**, **Yamashita K**, **Wang J**, **Fujiwara T**, **Yamanaka Y**, **Im D**, **Horita S**, **Tsujimoto H**, **Tawaramoto MS**, **Hirokawa T**, **Nango E**, **Tono K**, **Kameshima T**, **Hatsui T**, **Joti Y**, **Yabashi M**, **Shimamoto K**, **Yamamoto M**, **Rosenbaum DM**, **Iwata S**, **Shimamura T**, **Kobayashi T.** Crystal structures of human orexin 2 receptor bound to the subtype-selective antagonist EMPA. *Structure* 26: 7–19.e5, 2018. doi:10.1016/j.str.2017.11.005.
63. **Karnani MM**, **Schöne C**, **Bracey EF**, **González JA**, **Viskaitis P**, **Li H-T**, **Adamantidis A**, **Burdakov D.** Role of spontaneous and sensory orexin network dynamics in rapid locomotion initiation. *Prog Neurobiol* 187: 101771, 2020. doi:10.1016/j.pneurobio.2020.101771.
64. **Mochizuki T**, **Crocker A**, **McCormack S**, **Yanagisawa M**, **Sakurai T**, **Scammell TE.** Behavioral state instability in orexin knock-out mice. *J Neurosci* 24: 6291–6300, 2004. doi:10.1523/JNEUROSCI.0586-04.2004.
65. **Concetti C**, **Burdakov D.** Orexin/hypocretin and MCH neurons: cognitive and motor roles beyond arousal. *Front Neurosci* 15: 639313, 2021. doi:10.3389/fnins.2021.639313.
66. **Nobunaga M**, **Obukuro K**, **Kurauchi Y**, **Hisatsune A**, **Seki T**, **Tsutsui M**, **Katsuki H.** High fat diet induces specific pathological changes in hypothalamic orexin neurons in mice. *Neurochem Int* 78: 61–66, 2014. doi:10.1016/j.neuint.2014.09.002.
67. **Cone RD**, **Simerly RB.** Leptin grows up and gets a neural network. *Neuron* 71: 4–6, 2011. doi:10.1016/j.neuron.2011.06.033.