Orexin-driven GAD65 network of the lateral hypothalamus sets physical activity in mice

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Damage to the lateral hypothalamus (LH) causes profound physical inactivity in mammals. Several molecularly distinct types of LH neurons have been identified, including orexin cells and glutamic acid decarboxylase 65 (GAD65) cells, but their interplay in orchestrating physical activity is not fully understood. Here, using optogenetic circuit analysis and cell-type-specific deep-brain recordings in behaving mice, we show that orexin cell activation rapidly recruits GAD65\textsubscript{LH} neurons. We demonstrate that internally initiated GAD65\textsubscript{LH} cells burst precede and accompany spontaneous running bouts, that selective chemogenetic silencing of natural GAD65\textsubscript{LH} cells depresses voluntary locomotion, and that GAD65\textsubscript{LH} cell overactivation leads to hyperlocomotion. These results thus identify a molecularly distinct, orexin-activated LH submodule that governs physical activity in mice.

The lateral hypothalamus (LH) is thought to provide an essential drive for diverse vital behaviors, including locomotion. Peri-LH lesions in mammals disrupt context-appropriate physical activity, and are associated with the human disorder encephalitis lethargica, which has been noted to make humans “sitt motionless...all day” (1–6). The LH is not a homogeneous entity, but contains many molecularly distinct classes of neurons that are thought to have different physiological roles (7). However, the interrelations of distinct LH cell classes in computing and driving context-appropriate physical activity are not fully understood.

For example, LH neurons expressing orexins/hypocretins (8, 9) become activated in diverse stressful contexts, including acute auditory stimulation, hypoglycemia, hypercapnia, and physical capture (3, 9–13). Orexin\textsubscript{LH} cell activity may thus represent an important input variable for computing context-appropriate locomotor outputs (3, 14), and orexin peptides have been proposed to increase arousal and locomotion by actions on extrahypothalamic projections to areas such as the locus coeruleus (15, 16). However, hypocreactivity phenotypes caused by orexin\textsubscript{LH} cell deletion are milder than the hypothesis that orexin excites individual GAD65\textsubscript{LH} cells by activating a current with a reversal potential consistent with a nonselective cationic conductance (Fig. 1C). Overall, >98% of GAD65\textsubscript{LH} cells (64 of 65 cells) were activated by orexin peptide in vitro.

If orexin cells also activate GAD65\textsubscript{LH} cells in vivo, then stress stimuli that elicit orexin\textsubscript{LH} cell activity should also activate GAD65\textsubscript{LH} cells in vivo. Acute immobilization stress rapidly and robustly activates orexin\textsubscript{LH} cells in awake mice (13). We therefore used this paradigm, combined with deep-brain fiber photometry recording of GAD65\textsubscript{LH} cell activity and pharmacological antagonism of orexin receptors, to determine if stress and orexin drive GAD65\textsubscript{LH} cell activity in vivo. We expressed Cre-dependent GCaMP6s, or control protein EGFP, in the LH of GAD65\textsubscript{Ires}-Cre mice, and recorded fluorescence intensity using a fiberoptic probe implanted in the LH (Fig. 2A). We observed bursts of activity in GAD65\textsubscript{Ires}-GCaMP6s cells, but not in GAD65\textsubscript{Ires}-EGFP cells, of freely moving mice (Fig. 2A). These bursts were similar (≈5–30% ΔF/F) to the bursts recorded using photometry in...
Evidence for orexin

Values (italics) are from sign tests of 13 cells, respectively, mean cells (representative example of five brains) (44 and 5 cells). (5/6 cells) were connected. (Network cell. Gray lines are individual trials, and value (regular font) is from a two-tailed Mann–Whitney test comparing response amplitude between groups. (C) Effects of orexin peptide on GAD65 cell electrical activity. (Left) Membrane potential effect of 300 nM orexin-A on a GAD65 cell (representative example of six cells). syn. synaptic. (Center) Example of whole-cell current-voltage relation showing effect of orexin-A (representative example of five cells). (Right) Net orexin-activated current (mean and SEM of n = 5 cells).

other deep networks, and are thought to represent network firing bursts (24–26). Using simultaneous electrical recordings and GCaMP6s imaging from the same GAD65-GCaMP6s cells in vitro, we confirmed that GCaMP6s linearly reports spike firing rate (Fig. 2 B–D), with a mean slope (±1% ΔF/F per hertz; Fig. 2D) similar to the mean slope observed in other LH neurons (12, 13). Upon acute immobilization, we observed rapid activation of GAD65-GCaMP6s cells, but not control GAD65-GFP cells (Fig. 2E; peak amplitude of stress-induced GAD65-GCaMP6s activity transient was 12.3 ± 2.7%; P = 0.0174 by one-sample t test; t, df = 4.782, 3; n = 4 mice). The competitive orexin receptor antagonist SB-334867 (Materials and Methods) reversibly reduced this stress-induced activation of GAD65LH cells (Fig. 2F).

Collectively, these data identify the orexin system as a physiologically relevant upstream activator of GAD65LH neurons.

GAD65LH Cell Activity Drives (and Correlates with) Voluntary Locomotion. We next tested whether the GAD65LH module could act as an LH effector of physical activity. To probe causal links between GAD65LH cell activity and locomotion, we explored locomotor effects of selective silencing or activation of GAD65LH cell activity in freely moving mice.

First, we targeted the neuroinhibitory clozapine-N-oxide (CNO) receptor hM4Di (27) to the LH of GAD65-Ires-Cre transgenic

Fig. 1. Evidence for orexinLH → GAD65LH excitatory circuit. (A) Optogenetic interrogation of orexinLH → GAD65LH signaling. (Left) Targeting strategy. (Center, Left and Right) Localization of orexin-ChR2-mCherry and GAD65-GFP expression (representative example of five brains). (Right) Orexin cell photostimulation (vertical blue line) evokes a CNQX/D-AP5-sensitive (i.e., glutamate receptor-mediated) inward current in a GAD65LH cell. Gray lines are individual trials, and colored lines are trial averages [representative example of five cells; n = 5/6 cells] were connected. (B) Effects of orexin peptide on GAD65LH network activity. Scheme for targeting GCaMP6s (Left), example of GCaMP6s expression in GAD65LH cells (representative example of five brains) (Center Left), GaMP6s response of GAD65LH cells to 300 nM orexin-A with corresponding mean ± SEM plot (n = 44) (Center Right), and data summary without (C) and with (D) synaptic blockers (n = 44 and n = 13 cells, respectively, mean ± SEM responses of cells during 2–20 min after orexin-A infusion) (Right). P values (italics) are from sign tests of whether the response within each group is different from zero, and P value (regular font) is from a two-tailed Mann–Whitney test comparing response amplitude between groups. (C) Effects of orexin peptide on GAD65LH cell electrical activity. (Left) Membrane potential effect of 300 nM orexin-A on a GAD65LH cell (representative example of six cells). syn. synaptic. (Center) Example of whole-cell current-voltage relation showing effect of orexin-A (representative example of five cells). (Right) Net orexin-activated current (mean and SEM of n = 5 cells).
**Discussion**

Despite the long-recognized importance of the LH for maintaining normal physical activity in mammals, including humans (1–6, 29, 30), the functional relations of molecularly distinct LH subnetworks remained poorly understood. Our study identifies several properties of the GAD65LH subnetwork that reveal a previously overlooked interplay between molecularly distinct LH signals relevant for locomotion control. First, GAD65LH cells are activated by orexin neurons, and are rapidly recruited by stress in vivo in an orexin receptor-dependent manner. Second, natural locomotion bursts and movement initiation cells act as mediators of locomotion driven by natural orexin signaling. These multiple lines of evidence demonstrate a critical role for GAD65LH cells in driving normal locomotion, and in acting as effectors of the orexin system.

In known brain controllers of voluntary action, such as the basal ganglia, neural signals implicated in movement control appear several seconds before action initiation (e.g., figure 3E and F of ref. 24). These delays are comparable to the delays we observed between the natural GAD65LH signal rise and locomotion initiation, which we defined as the peak in the derivative of smoothed locomotion signals (4D). The steepest rise of the perilocomotion GAD65LH signal occurred approximately at movement onset, whereas the onset of GAD65LH signal rise occurred ∼2 s before movement onset (Fig. 4E and Discussion).

Collectively, these data provide correlative (Fig. 4) and causal (Fig. 3) evidence that GAD65LH cell activity drives locomotion in mice.

**Materials and Methods**

Despite the long-recognized importance of the LH for maintaining normal physical activity in mammals, including humans (1–6, 29, 30), the functional relations of molecularly distinct LH subnetworks remained poorly understood. Our study identifies several properties of the GAD65LH subnetwork that reveal a previously overlooked interplay between molecularly distinct LH signals relevant for locomotion control. First, GAD65LH cells are activated by orexin neurons, and are rapidly recruited by stress in vivo in an orexin receptor-dependent manner. Second, natural locomotion bursts and movement initiation cells act as mediators of locomotion driven by natural orexin signaling. These multiple lines of evidence demonstrate a critical role for GAD65LH cells in driving normal locomotion, and in acting as effectors of the orexin system.

In known brain controllers of voluntary action, such as the basal ganglia, neural signals implicated in movement control appear seconds before action initiation (e.g., figure 3E and F of ref. 24). These delays are comparable to the delays we observed between the onset of the GAD65LH network bursts and movement initiation (∼2 s; Fig. 4E). These long delays presumably imply multiple downstream processing stages between GAD65LH cell signals and...
motor commands that drive muscle activity underlying locomotion. When we attempted to determine downstream targets of GAD65<sub>LH</sub> neurons by tracing their axon distribution in brains of GAD65<sub>LH</sub>-ChR2-YFP mice, we found projections to multiple neural systems, extending from the LH to the thalamus, ventral tegmental area, cortical sensory areas, brainstem, and many hypothalamic nuclei (Fig. S1). A comprehensive analysis of these downstream effectors of GAD65<sub>LH</sub> cells will be included in future studies, because it is a large task requiring the relative locomotor impacts of GAD65<sub>LH</sub> cell targets to be deciphered. A specific question for such future work would be whether the locomotion drivers described here, and the classical basal ganglia pathways, operate in series or in parallel to promote physical activity.

Related questions for future studies are what neurotransmitters GAD65<sub>LH</sub> cells release at their many projection targets, and what regulatory receptors they express. GAD65<sub>LH</sub> cells do not express orexin or MCH neurotransmitters (21); note that this finding does not contradict previous reports of GAD67 expression in MCH cells, because GAD65 and GAD67 are not always coexpressed in the hypothalamus (e.g., refs. 31, 32). We also found that the GAD65<sub>LH</sub> cells are distinct from the previously studied LH neurons expressing neuropeptide Y (NPY) or leptin receptor (33, 34) (Fig. S2 A and B). It remains to be determined whether GAD65<sub>LH</sub> cells release excitatory and/or inhibitory transmitters, because vesicular GABA transporter (VGAT) is not always expressed in GAD65 neurons, and some hypothalamic GAD65 neurons contain glutamatergic markers (31, 32, 35). Indeed, we found that only 50% of the GAD65<sub>LH</sub> cells contain VGAT, and that most (~80%) VGAT<sub>LH</sub> cells are distinct from GAD65<sub>LH</sub> cells (Fig. S2 C and D). Thus, the previous studies of VGAT<sub>LH</sub> cells (36–39) likely targeted larger and more heterogeneous cell populations than our study, which was selective for GAD65<sub>LH</sub> cells. This fact may explain why the VGAT<sub>LH</sub> cell stimulation drove different behaviors [increased eating and insignificant effect on locomotion (36, 39)] than the GAD65<sub>LH</sub> cell stimulation (increased locomotion and no significant effect on eating; Fig. 3 and Fig. S3). The latter findings presumably imply that GAD65<sub>LH</sub>-stimulated locomotion is unlikely to be specifically motivated by hunger or directed toward a food goal. We note that in some contexts, it may be evolutionarily advantageous to develop a locomotion drive not directed toward a specific goal (e.g., food), but toward a more general goal (e.g., change of place, physical escape). It is thus tempting to speculate that the orexin-activated GAD65<sub>LH</sub> cells may provide such a general locomotion drive that serves, for example, to move the animal away from stresses that activate orexin neurons (10, 11, 13). Such an escape-like behavioral drive would also be consistent with the observed GAD65<sub>LH</sub> cell dynamics during capture, when the mouse is immobilized but the drive is high.

In summary, we found that physiological activity of GAD65<sub>LH</sub> cells is vital for normal locomotion and identified orexin as a physiological upstream regulator of GAD65<sub>LH</sub> activity. These findings establish the GAD65<sub>LH</sub> cells as key players in intra-LH information processing. Together with recent findings on roles of defined LH neurons in eating and arousal (7, 36, 38), our results substantiate specific LH circuits as orchestrators of key voluntary actions.

Materials and Methods

Gene Transfer. All procedures followed United Kingdom Home Office regulations and were approved by the Animal Welfare and Ethical Review Panel of the Francis Crick Institute. Adult male and female mice (at least 8 wk old) were used for in vitro experiments. Male mice were used for behavioral experiments, and kept on a standard 12-h light/dark cycle and on standard mouse chow and water ad libitum. Mice were anesthetized with isoflurane and injected with meloxicam (2 mg/kg of body weight, s.c.) for analgesia. After placement into a stereotaxic frame (David Kopf Instruments), a craniotomy was performed and a borosilicate glass pipette was used to inject viral vectors bilaterally into the LH. Two injections (each 75 nL, delivered at 75 nl min<sup>−1</sup> were made into the LH in each hemisphere (bregma: −1.30 mm, midline: ±1 mm, from brain surface: 5.20 mm and 5.25 mm). Mice were allowed to recover for at least 1 wk after surgery while single-housed.

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**Fig. 3.** Effect of natural and evoked GAD65<sub>LH</sub> cell activity on locomotion. (A, Left) Targeting scheme for hM4Di-mCherry. (A, Right) Localization of hM4Di-mCherry. (B) Group data, raw values, and means; the on-plot P value is from a one-tailed, ratio-paired t test (t, df = 3.251, 4; n = 5 cells). APs, action potentials. (C) Effect of CNO on locomotion of GAD65<sub>LH</sub>-mCherry. APs are truncated at 0 mV (representative example of five brains). (D) Effect of CNO on locomotion of GAD65<sub>LH</sub>-mCherry. Raw data and means are shown in red (Left), and the same data are shown as % change (Right). On-plot P values are from a one-tailed paired t test (t, df = 3.001, 4; Right) (n = 5 mice). Corresponding negative control data are described in Results. (E) Effect of CNO on GAD65<sub>LH</sub>-mCherry cell firing. Spikes are truncated at 0 mV (representative example of five brains). (F) Effect of CNO on GAD65<sub>LH</sub>-mCherry cell firing. Spikes are truncated at 0 mV (representative example of n = 5 cells).
Cell Type-Specific Deep-Brain Recordings in Awake Behaving Mice. After injection of Cre-dependent GCaMP6s into Gad65-Ires-Cre mice, the fiberoptic implants were stereotaxically installed with the fiber tip above the LH (1.38 mm caudal from bregma, 0.95 mm lateral from midline, and 5 mm ventral from brain surface) and fixed to the skull as previously described (12, 13); this method is expected to capture signals only from within 500 μm of the fiber tip (12). Fiber tip locations were verified in each mouse by examining slices with a visible fiber tract. For locomotion experiments and immobilization, mice also had a custom-built, stainless-steel head-restraint implant glued to their skulls using tissue-friendly cyanoacrylate (Histoacyl; TissueSeal). Fiber photometry was performed as described by Gonzalez et al. (13). Fluorescence signals were converted to ΔF/F (%) values as follows: ΔF/F = 100 × (F−F0)/F0, where F is the raw signal and F0 is the trial mean.

In Fig. 2E (immobilization stress experiments), mice were placed in a recording cage and left for 10 min; then, a trial consisting of 10 s of restraint stress (holding the mouse head stationary inside the cage by clamping the head-fixed restraint) was performed, based the method of Gonzalez et al. (13). For each mouse, four trials per day were performed (with intertrial intervals >5 min). Trial blocks for drug condition (control, SB-334867, and recovery) were separated by 48 h. The competitive orexin receptor antagonist SB-334867 (or 0.9% NaCl and 10% DMSO as a control vehicle) was given (i.p. 1 h before immobilization stress). This antagonist has a higher affinity for orexin type-1 receptor than for orexin type-2 receptor, but at higher concentrations, it antagonizes orexin binding to both receptors (47). We used a single high dose of SB-334867 (30 mg/kg), based on previous studies with this compound (48, 49). To control for circadian factors, all experiments were performed during the dark phase.

Quantifying Physical Activity. Locomotor activity was assessed after a single i.p. dose of CNO or saline during the dark phase in open home cages using either video-tracking (Anymaze or Ethovision) or a rotary treadmill. Mice of the same genotype were grouped according to vectors injected into their brains, and all groups had a similar composition based on sex (male), age, and body weight. For treadmill locomotion quantification, mice were head-restrained in a sound-attenuated chamber where they could run on a disk treadmill with a rotary encoder. The center of the animal’s head was such that 1° of rotation equated 1.8 mm of forward locomotion. The encoder generated a transistor-transistor logic (TTL) pulse every time the animal moved 1.8 mm. The pulses were collected by the same analog-to-digital (A/D) card as the photodetector signals for accurate time-stamping of Gad65-GCaMP6s signals and locomotion. Locomotion bout onset was defined as the time of crossing a velocity threshold of 0.17 cm s⁻¹, and locomotion bouts of at least 5 s in duration from this onset are illustrated in the analyses shown in Fig. 4C.

Brain Slice Optogenetics, Electrophysiology, and Imaging. Patch-clamp recordings combined with optogenetics were carried out as reported by Schöne et al. (50). Briefly, LH slices were prepared at least 2 months after virus injection. Coronal brain slices of 250-μm thickness containing the LH were cut while immersed in ice-cold slicing solution. Slices were incubated for 1 h in artificial cerebrospinal fluid (ACSF) at 35 °C, and then transferred to a submerged-type recording chamber. Neurons containing fluorescent markers were visualized with an Olympus BX61WI microscope with an oblique condenser and fluore-escence filters. Excitation light for ChR2 was delivered from a Sutter S 5 beam switcher (Sutter) with a xenon lamp and ET470/40-nm bandpass filter. A 40× 0.8-N.A. objective was used to deliver 1-ms flashes of blue light (−10 mW/mm²) onto ChR2-containing axons around the recorded cell, and postynaptic responses were recorded at a range of potentials (0 to −90 mV) in each cell. For studying effects of orexin on membrane current (Fig. 1C), voltage-clamp ramps from 0 to −120 mV were performed at 0.1 mV ms⁻¹, and orexin-evoked current was then derived in each cell by subtracting the baseline current from the orexin current at each potential and plotted at 10-mV increments for visual clarity (Fig. 1C, Right). Functional ChR2 expression was confirmed by recording light-activated action potentials in the target cells (n = 3 cells per group). For calcium imaging, brain slices were placed in a recording chamber of a BX61WI Olympus microscope controlled by Olympus Fluoview software (FV10-ASV version 4.0), perfused at 35 °C with ACSF. Confocal imaging was performed at 0.5 Hz with an Olympus 20× 0.50-N.A. objective, 488-nm argon laser excitation, and 500- to 545-nm emission collection. Motion and bleach corrections were applied if needed (StackReg plug-in, Image); NIH. A region of interest (ROI) containing each GaCaMP6s-positive neuron was selected via ROI manager in ImageJ. The mean fluorescence of each ROI was extracted for each frame. These raw fluorescence (F0) values were normalized to 0% F/F values, where F is the mean baseline before orexin application and ΔF/F = (F − F0)/F0. Simultaneous brain slice cell-attached recordings and calcium imaging (Figs. 2B and C) were performed as reported by Gonzalez et al. (13). Because the cell-attached experiments, which were necessary to preserve
cytosolic integrity and thus in vivo-like GCaMP signals, do not measure transmembrane potential, it is possible that the GCaMP signal is increased by interstrip depolarization as well as by the depolarization during the spikes.

**Chemicals and Solutions.** For brain slice recordings, ACSF and ice-cold slicing solution were gassed with 95% O₂ and 5% CO₂. The slicing solution contained the following: 120 mM K-gluconate, 21 mM NaHCO₃, 2.0 mM MgCl₂, 2.0 mM CaCl₂, 2.0 mM MgCl₂, 21 mM NaHCO₃, 2.0 mM HEPES, 0.1 mM Na-pyruvate, and 0.4 mM ascorbic acid. The slicing solution contained 2.5 mM KCl, 1.3 mM NaH₂PO₄·H₂O, 2.6 mM KCl, 12.3 mM sucrose, 10.0 mM HEPES, 2.0 mM MgCl₂, and 2.0 mM CaCl₂. For standard whole-cell recordings, pipettes were filled with intracellular solution containing the following: 120 mM K-gluconate, 10 mM KCl, 10 mM Hepes, 0.1 mM EGTA, 4 mM KCl, 2 mM NaATP, 0.3 mM Na-GTP, and 2 mM MgCl₂ (pH 7.3) with KOH. Concentrations used were as follows: 300 nM orexin-A; 5 μM CRF; synaptic blocker mix: 50 μM D-AP5, 10 μM picrotoxin, 10 μM CNQX, and 10 μM CGP 35348; and 3 μM gabazine. For in vivo chemogenetic manipulations, CRF was injected i.p. at 5 mg/kg (experiments involving HMD or) and at 0.5 mg/kg (experiments involving HMD3q). All chemicals were from Sigma or Tocris Bioscience.

**Histochemistry.** For the immunolabeling of pSTAT3 in Fig. S2, 30-μm cryosections of leptin-injected GAD65-GFP mice were pretreated with H2O2, NaOH, glycine, and SDS. Staining for pSTAT3 was done with rabbit anti-pSTAT3 IgG (no. 9131, 1:500; Cell Signaling Technology) sera overnight as the primary antibody and Alexa 555-conjugated donkey to rabbit IgG (a. 1:500; Life Technologies) as the secondary antibody (St). In chemogenetic experiments, actuator targeting to LH (Fig. S4) was verified in fixed sections prepared as reported by González et al. (12).

**Statistical Analysis.** Statistical tests and descriptive statistics were performed as specified in the figure legends. In each experimental dataset at the cellular level, each n was a different cell (no repeated trials from the same cell were used as n values) and cells from at least three mice were analyzed. Before performing parametric tests, data were assessed for normality with a D’Agostino–Pearson omnibus test or Kolmogorov–Smirnov test for small sample sizes, and variances were assessed for homogeneity with a Brown–Forsyth test. To compare interactions within normally distributed data with repeated measurements, repeated measures ANOVA was used, with multiple comparison tests where appropriate. Analysis was performed with GraphPad Prism, MATLAB (The MathWorks, Inc.), and ImageJ.

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