

Repeated Morphine Exposure Reversibly Impairs Spike Generation and Repetitive Firing in a Functionally Distinct Subpopulation of Orexin Neurons

Elizabeth A. Berry,¹ Ellen N. Huhulea,¹ Masaru Ishibashi,² Ronald McGregor,^{3,4} Jerome M. Siegel,^{3,4} and Christopher S. Leonard¹

¹Department of Physiology, New York Medical College, Valhalla, New York 10595, ²Department of Neurophysiology, Hamamatsu University School of Medicine, Hamamatsu 431-3192, Japan, ³Neuropsychiatric Institute, University of California, Los Angeles, California 90095, and ⁴Veterans Administration, Greater Los Angeles Healthcare System, Los Angeles, California 91343

Orexin (hypocretin) neuropeptides regulate numerous essential functions including sleep/wake state stability and reward processing. Orexin synthesizing neurons respond to drug cues and undergo structural changes following persistent drug exposure. Postmortem brains from opioid users and opioid-treated rodents have orexin somata that become ~20% smaller and ~50% more numerous and are postulated to promote hypermotivation for drug seeking through increased orexin release. Biophysical considerations suggest that the decreased soma size should increase cellular excitability; however, the impact of chronic opioids on firing ability, which drives peptide release, has not been explored. To test this, we assessed the intrinsic electrophysiological properties of orexin neurons by whole-cell recordings in slices from male orexin-EGFP mice treated for 2 weeks by daily morphine or saline injections. Paradoxically, we found that while daily morphine decreased the average soma size, it impaired excitability in a subpopulation of orexin neurons identified by electrophysiological criteria as “H-type” while entirely sparing “D-type” neurons. This impairment was manifest by smaller, broader action potentials, variable firing, and a downscaling of firing gain. These adaptations required more than a single morphine dose and recovered, along with soma size, after 4 weeks of passive withdrawal. Taken together, these observations indicate that daily opioid exposure differentially impacts H-type orexin neurons and predicts that the ability of these neurons to encode synaptic inputs into spike trains and to release neuropeptides becomes impaired in conjunction with opioid dependence.

Key words: drug-induced adaptation; intrinsic properties; opioid use disorder; patch clamp; reward; substance use disorder

Significance Statement

Orexin peptide signaling sustains motivation for opioid and cocaine seeking, and chronic use upregulates orexin peptides and induces structural changes consistent with enhanced orexin release. However, the impact of chronic drug exposure on the ability of orexin neurons to fire action potentials which drive peptide release has not been explored. Paradoxically, we found that 2 weeks of daily morphine exposure selectively and reversibly impaired action potential firing in a distinct subpopulation of orexin neurons (H-cells). These findings further support a functional dichotomy among orexin neurons and imply that the ability of H-cells to encode input signals and release neuropeptides becomes impaired during development of opioid dependence.

Introduction

Orexin (hypocretin) neuropeptides are synthesized by neurons located around the fornix in the hypothalamus (de Lecea et al., 1998; Sakurai et al., 1998) that have widespread central

projections and regulate numerous functions including arousal, feeding, and reward (Peyron et al., 1998; Ohno and Sakurai, 2008; Mahler et al., 2014; Li et al., 2017). Orexin peptides act through two G-protein-coupled receptors, orexin 1 (Ox1R)

Received March 20, 2025; revised Oct. 8, 2025; accepted Oct. 12, 2025.

Author contributions: E.A.B., M.I., R.M., J.M.S., and C.S.L. designed research; E.A.B., E.N.H., and M.I. performed research; E.A.B., E.N.H., M.I., and C.S.L. analyzed data; E.A.B., M.I., R.M., J.M.S., and C.S.L. wrote the paper.

This work was supported by National Institutes of Health Grants R01DA034748 and R01NS027881. We thank Drs. Luis De Lecea and William Joseph Giardino for generously providing Hypocretin/Orexin-EGFP founder mice and Dr. C. I. Thompson for the helpful statistics advice.

The authors declare no competing financial interests.

Correspondence should be addressed to Christopher S. Leonard at chris_leonard@nymc.edu.

<https://doi.org/10.1523/JNEUROSCI.0563-25.2025>

Copyright © 2025 the authors

and 2 (Ox2R) to excite and modulate their target neurons (Kukkonen and Leonard, 2014; Leonard and Kukkonen, 2014).

Intact orexin signaling is critical for stabilizing sleep and wake states since genetic disruption results in narcolepsy with cataplexy (Chemelli et al., 1999; Lin et al., 1999; Willie et al., 2003; Kalogiannis et al., 2011), and patients with this sleep disorder have little to no orexin peptide (Peyron et al., 2000; Thannickal et al., 2000; Scammell, 2003; Seifinejad et al., 2023).

Strong evidence also links the orexin system to motivation and reward processing. Many orexin neurons are located in the lateral hypothalamic area (LHA), a region classically linked to feeding, drinking, and reinforcement (Stuber and Wise, 2016). Moreover, ~50% of orexin neurons are immunopositive for mu-opioid receptors and respond to chronic morphine exposure with increased activity of the cAMP response element (cAMPRE) and to naloxone-precipitated withdrawal with increased cAMPRE and c-Fos (Georgescu et al., 2003). In brain slice experiments, approximately half of mouse orexin neurons are directly inhibited and show decreased EPSC frequency following acute morphine application (Li and van den Pol, 2008), and Ox1R signaling in the VTA is necessary for induction of excitatory synaptic plasticity in dopamine neurons following single cocaine or morphine injections (Borgland et al., 2006; Baimel and Borgland, 2015).

Orexin neurons are also c-Fos-responsive to drug-related cues (Harris et al., 2005; Harris and Aston-Jones, 2006), and orexin-A can reinstate extinguished drug seeking (Boutrel et al., 2005; Harris et al., 2005), while the genetic absence of orexin dampens reward-related behavior (Georgescu et al., 2003; McGregor et al., 2011; Shaw et al., 2017) and orexin receptor antagonists attenuate drug-induced behavioral changes in rodent substance use disorder models (Harris et al., 2005; Hutcheson et al., 2011; Brown et al., 2013; Gentile et al., 2018; James and Aston-Jones, 2020).

Orexin neurons undergo structural and synaptic plasticity in response to both environmental challenges and repeated exposure to drugs of abuse. Orexin neurons show increased numbers of asymmetric somatic synapses and increased mEPSP frequency after overnight fasting (Horvath and Gao, 2005) but show decreased synaptic integration following diet-induced obesity (Tan et al., 2020). Sleep deprivation decreases perisomatic astrocytic processes resulting in presynaptic inhibition of glutamatergic inputs (Briggs et al., 2018) while apparently increasing the AMPA/NMDA ratio (Rao et al., 2007)—a change also observed following 3 d of cocaine injections which facilitated high-frequency LTP (Rao et al., 2013).

Strikingly, brains from human heroin addicts and mice treated with 2 weeks of daily morphine have ~50% more immunodetectable orexin neurons with a decrease in mean soma size of ~20% (Thannickal et al., 2018) and an increase in orexin fiber density and TH levels in mouse locus ceruleus (McGregor et al., 2022) and VTA (McGregor et al., 2024). Comparable increases in orexin immunodetectable neurons were observed in rats following self-administration of cocaine (James et al., 2019) and fentanyl (Fragale et al., 2021) suggesting dysregulation of an “orexin reserve” that might enhance orexin release and drive hypermotivation for drug taking (James and Aston-Jones, 2022).

However, orexin output depends not only on peptide availability but also on orexin neuron excitability. Since two electrically distinct subtypes of orexin neurons have been identified (Williams et al., 2008; Schöne et al., 2011) and since the effects of repeated morphine exposure on orexin neuron excitability

were unknown, we used brain slices and whole-cell recordings to investigate the intrinsic electrical properties of orexin neurons following 2 weeks of daily morphine or saline injections.

Materials and Methods

All procedures complied with NIH guidelines and were approved by New York Medical College Institutional Animal Care and Use Committee, and all efforts were directed at minimizing the number of animals used for this study.

Mice

Male orexin-EGFP mice, which express enhanced green fluorescence protein (EGFP) under the control of the human prepro-orexin promoter (Yamanaka et al., 2003), were used for all experiments. These mice were bred in our animal facility from founder mice generously provided by Dr. Luis De Lecea and Dr. William J Giardino (Adamantidis et al., 2007). Mice were singly housed in standard mouse cages (10.5 × 6 × 5.75 in; 22 ± 1°C) throughout the experiment and kept on a 12–12 light/dark cycle with lights on at 5:00 A.M. Mice had *ad libitum* access to water and food (Purina rodent laboratory diet 5001, protein 23%, fat 4.5%, fiber 6%). In these experiments we used only male mice in order to limit the number of mice needed for the study and to limit possible biological variability, since it is known that the orexin cell number (Brownell and Conti, 2010) and reactivity (James et al., 2014; Dawson et al., 2023) differ by sex. While male and female human opioid users show similar changes in the orexin cell number and size (Thannickal et al., 2018; McGregor et al., 2024), opioid use disorder manifests differently in males and females (Becker, 2016; Towers et al., 2023) so future experiments will also be necessary to examine how excitability of orexin neurons from female mice is altered by these treatments.

Treatment cohorts

Experiments used three treatment cohorts that we termed the “14 d,” “4 week recovery,” and “1 d” cohorts. Mice in each cohort were randomly assigned to one of the two groups that received single, daily subcutaneous injections of either sterile saline (0.9% NaCl) or 50 mg/kg morphine sulfate dissolved in sterile saline between 9:30 and 10:30 A.M. Mice were weighed daily, just before injection, to determine the volume (~0.1 ml) of morphine/saline to be administered. Mice were then killed for brain slice electrophysiology or for immunohistochemistry (IH) according to the schedule illustrated in Figure 1A. Mice in the 14 d cohort received daily saline or morphine injections for 14 d and were killed between 11:30 A.M. and 12:30 P.M. on the day of their last injection. Mice in the 4 week recovery cohort received injections as did the 14 d cohort but then received no injections during a 4 week recovery period. During this recovery period, mice were checked and weighed a few times per week to monitor for overall health and weight change and received weekly cage changes by veterinary staff. On the last day of the 4 week period, mice were killed for slice electrophysiology or IH, at the same time as mice in the 14 day cohort. Mice in the 1 d cohort received a single subcutaneous injection of saline or 50 mg/kg morphine sulfate and were killed 2 h later for slice electrophysiology.

Brain slice preparations and in vitro electrophysiological recordings

Following deep anesthesia with isoflurane (>5% in O₂), mice were decapitated, and their brains were quickly removed into ice-cold artificial cerebrospinal fluid (ACSF) which contained (in mM) 126 NaCl, 3 KCl, 1.2 NaH₂PO₄, 2 CaCl₂, 2 MgSO₄, 24 NaHCO₃, 10 D-glucose, osmolarity 295–300 mOsm, and were oxygenated with carbogen (95% O₂ and 5% CO₂). The brain was blocked by first resting the brain on its ventral surface and making coronal cuts caudally through the midbrain-pons junction and rostrally just behind the NAc to leave a block containing the hypothalamus. This block was then affixed with cyanoacrylate to the stage of a Leica VT1000S vibratome, caudal side down with the ventral side of the brain facing the blade. Brain slices (250 μm) were then cut in oxygenated ice-cold ACSF and then incubated at room temperature in oxygenated NMDG recovery solution containing (in mM) 115 NMDG, 2.5 KCl, 1.2 NaH₂PO₄, 0.5 CaCl₂, 10 MgSO₄, 25 NaHCO₃, 25 D-glucose, osmolarity 295–300, for 10 min (Ting et al., 2014). Slices

were then rinsed five times in ACSF and transferred to continuously oxygenated, room-temperature ACSF for 1 h before starting recordings.

We recorded EGFP-positive orexin neurons in brain slices submerged and perfused (1–2 ml/min) with room temperature ($22^{\circ} \pm 2^{\circ}\text{C}$) ACSF in a chamber mounted on a fixed stage microscope (Olympus BX-50WI). EGFP-positive neurons were selected for recording using epifluorescence illumination with a white LED light source (UHP-T-W50-SR; Prizmatix) filtered with an EGFP filter cube (#49002; Chroma Technology) and imaged with a CCD camera (Qimaging Retiga Electro; Teledyne Photometrics). Patching the selected neuron was guided using infrared differential interference contrast optics with the same camera operated with Ocular software (Teledyne Photometrics). We recorded orexin neurons in whole-cell voltage and current-clamp (I-clamp fast) configurations using an Axopatch 200B amplifier (Molecular Devices) with the output filter set at 5 kHz. Membrane currents and voltages were sampled at 10 kHz and controlled using a Digidata 1550B and pCLAMP11 software (Molecular Devices). Recording pipettes (4–7 M Ω ; Sutter Instruments Item Number BF150-86-10HP) were made using a horizontal puller (model P-97, Sutter Instruments). Pipettes were filled with an internal solution containing (in mM) 144 K-gluconate, 0.2 EGTA, 3 MgCl₂, 10 HEPES, 0.3 NaGTP, 4 Na₂ATP of pH 7.2, and osmolarity 295–305 mOsm. Alexa Fluor 568 biocytin or Alexa Fluor 594 biocytin (0.2 mg/ml Thermo Fisher Scientific) was added to the patch solution to identify the recorded cell and confirm that it was EGFP-positive.

The formation of a giga seal in a voltage-clamp mode was monitored using the membrane test routine in Clampex 11. Upon seal formation ($R_{\text{seal}} = 3.50 \pm 0.26 \text{ G}\Omega$; $n = 51$), the fast and slow pipette capacitance compensation was adjusted to minimize stray pipette capacitance. Seal resistance was not different for recordings obtained in slices from saline-treated ($R_{\text{seal}} = 3.2 \pm 0.37 \text{ G}\Omega$; $n = 24$) or morphine-treated mice ($R_{\text{seal}} = 3.75 \pm 0.37 \text{ G}\Omega$; $n = 27$; $t_{(48)} = 1.01$; $p = 0.360$; t test). Following breakthrough, the membrane test routine was used to estimate and monitor series (access) resistance (R_a), total capacitance (C_m), and input resistance (R_m) in response to a 50 ms duration -10 mV voltage jump from a holding potential of -60 mV . These values were determined by Clampex 11 from real-time exponential fitting of the average transient current decay ($n = 20$) and a measure of the average baseline and steady-state current following the decay as described in the membrane test algorithms support page (<https://support.moleculardevices.com/s/article/Membrane-Test-Algorithms>). R_a was tracked for 3 min to be sure it remained low and stable ($17.1 \pm 1.0 \text{ M}\Omega$; $n = 68$). If it was unstable or exceeded $40 \text{ M}\Omega$, the recording was terminated. In principle, these values of R_a should not affect our estimates of R_m and C_m since this algorithm estimates C_m from the total charge delivered during the capacitive transient. Delivery of charge was not limited by R_a or the amplifier since peak currents were $<2 \text{ nA}$ and the Axopatch 200B is capable of sourcing 20 nA . If R_a , C_m , and R_m were stable, we sampled five times over this period, and the average of these values was used as the estimate of these parameters for that cell. Since specific membrane capacitance ($\sim 1 \mu\text{F}/\text{cm}^2$) is considered a constant (Gentet et al., 2000), membrane capacitance is proportional to the membrane surface area. Capacitance estimated by this method is most influenced by somatic and proximal dendritic membrane (Taylor, 2012) since these compartments experience most of the voltage change produced by our clamp step.

The holding current was then recorded at -60 mV for 30 s, and the amplifier was put in the $I = 0$ mode to measure the resting membrane potential or spontaneous firing of the neuron. The amplifier was then switched into I-clamp fast mode to execute a series of current-clamp protocols. Series resistance was generally uncompensated unless it was $>20 \text{ M}\Omega$, since the voltage error from our injected currents were small.

Mouse orexin neurons can be identified as either D-type or H-type based on their membrane potential trajectory following strong hyperpolarizing current pulses delivered while the neurons were spontaneously firing. D-cells show a depolarizing recovery response and rapid resumption of spiking while H-cells show a delayed recovery produced by an A-current mediated hyperpolarization that delayed resumption of spiking (Williams et al., 2008; Schöne et al., 2011). We therefore identified whether the recorded cell was D- or H-type by delivering a series of increasing amplitude, 1 s duration current pulses from baseline firing.

We then computed a “spike ratio” and measured the time to first spike after the end of the pulse, to compare with previously identified D- and H-cells (Schöne et al., 2011). Time to the first spike was measured starting 50 ms after pulse offset to the time of the following spike. The “spike ratio” was measured as the number of spikes in the 1 s starting 50 ms after the end of the pulse divided by the number of spikes in the 1 s before the pulse (Fig. 2B,C).

We next determined the action potential properties by measuring the rheobase and the shape of single spikes. Rheobase was measured as the amount of current required to elicit a single action potential from a holding potential of -60 mV using current pulses of 80 ms duration. Action potential shape was measured by spikes evoked by a current strength just above rheobase. We then measured the firing rate–current (F – I) relation with firing evoked by 2 s duration constant current pulses that were incremented in amplitude in 10 pA steps. Reported membrane potentials were not corrected for the liquid junction potential which we previously measured for these solutions as about -15 mV .

Tissue preparation for histology

Mice were overdosed with a ketamine (100 mg/kg)/xylazine (10 mg/kg) cocktail and perfused through the heart with 0.01 M phosphate-buffered saline (PBS) followed by 4% PFA, 2 h following their last injection. Brains were removed and postfixed in 4% PFA for 24 h, after which they were cryoprotected in 30% sucrose for at least 72 h. For sectioning, brains were flash frozen in -80°C methylbutane and then frozen into a block of OCT freezing compound (Fisher HealthCare Tissue-Plus, catalog #23-730-571) at -20°C . Brain blocks were then sectioned into $40 \mu\text{m}$ coronal sections using a Leica Cryostat CM1850. Sections were collected and stored in 0.01 M PBS solution prior to immunofluorescence (IF) or VIP IH labeling.

Brain sections ($40 \mu\text{m}$) through the LHA were taken $160 \mu\text{m}$ apart (every fourth section) and immunostained to visualize orexin-A and/or GFP. Sections were incubated for 30 min in 1% BSA/0.2% Triton X-100/0.01 M PBS solution to block nonspecific binding. For IF identification of orexin-A and/or GFP, sections were incubated in rabbit polyclonal anti-orexin-A (1:10,000, Phoenix Pharmaceuticals, H-003-3 lot#01651-8) and/or sheep polyclonal anti-GFP antibody (1:1,000, Novus Biologicals, #NB100-62622, lot#1710). Following incubation overnight in 1% BSA/0.2% Triton X-100/0.01 M PBS, antibodies were removed, and sections were washed in 0.01 M PBS. Sections were then incubated for 45 min in 1:200 donkey-anti-rabbit-Alexa Fluor 594 and/or donkey-anti-sheep-Alexa Fluor 488 in 1% BSA/0.2% Triton X-100/0.01 M PBS. Sections were washed in 0.01 M PBS a final time, and then sections were mounted on charged microscope slides and coverslipped with antifade VECTASHIELD (H-1700) aqueous mounting media.

Sections destined for VIP IH for GFP were first incubated for 30 min with 1% H₂O₂ to block endogenous peroxidase activity and then for 30 min in 1% BSA/0.2% Triton X-100/0.01 M PBS solution to block nonspecific antibody binding. Sections were then incubated in the same sheep polyclonal anti-GFP antibody (1:1,000 in 1% BSA/0.2% Triton X-100/0.01 M PBS) at room temperature on a shaker overnight. The following morning, sections were washed in 0.01 M PBS and then incubated with biotinylated rabbit anti-sheep IgG (1:200, Vector BA-6000) for 30 min, washed with PBS and then incubated in avidin-biotin horseradish peroxidase complex (Vectastain Elite ABC Kit; PK-6100 lot#ZF0802) for 30 min. Sections were then washed in 0.01 M PBS and reacted with Vector VIP peroxidase substrate kit (SK-4600) for enough time to obtain optimal purple staining (2–10 min). Sections were then mounted onto charged microscope slides (Thermo Fisher Scientific, Superfrost plus) and dehydrated in graded ethanol's (80, 90, 100%) for $\sim 2 \text{ min}$ each to achieve optimal removal of background VIP and dehydrate the tissue. Sections were then cleared with xylene and coverslipped using Permount mounting media.

Mapping recorded orexin neurons

Low-power digital images of the brain slice containing the fornix, mammillothalamic tract, and patch pipette at the location of the last patched neuron were acquired using a $4\times$ objective following each whole-cell recording. These images were imported into the Fiji distribution of ImageJ (Schindelin et al., 2012) calibrated, and the distance of the tip

of the patch pipette to both the fornix and mammillothalamic tract was measured using the line tool and the ROI manager. Corresponding atlas pages were selected from the Paxinos Mouse Atlas (Franklin and Paxinos, 2008) and opened in Adobe Illustrator. The measurements taken in ImageJ were then used to plot the location of each recorded neuron onto the atlas page in Adobe Illustrator. A vertical line was drawn through the center of the fornix to separate the nucleus into medial and lateral regions.

Experimental design and statistical analyses

All investigators were blinded to the mouse treatment conditions for data collection and analyses.

Electrophysiological analysis. Analysis of electrophysiological waveforms was performed using the Igor Pro software (versions 8 and 9; WaveMetrics). Action potential shape and repetitive firing parameters were measured using custom-programmed routines in Igor Pro (Erisir et al., 1999). Action potentials were evoked from a holding potential of -60 mV by injecting incrementing current steps until reaching threshold to generate one spike. Spike amplitude was measured offline as the difference between the action potential peak and its threshold. Spike threshold was determined as the potential at which the second derivative of the voltage waveform exceeded three times its standard deviation in the period preceding spike onset. The afterhyperpolarization (AHP) was measured as the difference between the spike threshold and the voltage minimum following the action potential peak. Maximum rates of rise and decay of the action potential were computed from the maximum and minimum of the smoothed first derivative of the voltage waveform. Spike width was measured at half the spike amplitude.

Instantaneous frequency (1/interspike interval) versus time curves were computed from trains of action potentials evoked by 2 s duration current pulses from the times after pulse onset at which each spike crossed 0 mV. The initial firing rate was the instantaneous frequency of the first interval, and the final rate or steady-state rate was computed as the average of instantaneous frequency for the last five intervals of a train. Instantaneous frequency for the first, second, fourth, and the last intervals along with the steady-state firing rate were plotted as a function of the injected current strength to construct F – I curves. In the repetitive firing protocol, current strength was increased at 10 pA increments until spike failure occurred within the 2 s duration pulse. Firing rates between 10 and 120 pA were used to compare F – I relations between cells and across treatment conditions and did not include firing at current strengths that produced spike failures. For analysis of firing rate variability, we computed the coefficient of variation (CV; standard deviation of firing rate/mean firing rate) for the period of firing following early accommodation for each firing rate trace at a midcurrent strength (80 pA). To estimate the firing rate gain, average F – I curves were fit with a second-order polynomial as follows:

$$\text{Firing Rate} = K_0 + K_1(x - x_0) + K_2(x - x_0)^2,$$

where K_0 is the firing rate for the minimal current ($x_0 = 10$ pA) and K_1 and K_2 are the coefficients derived from the best fit using the built-in curve fitting function of Igor Pro 9 which makes use of a singular value decomposition algorithm. Firing gain was then computed in Igor Pro 9 by taking the first derivative of the best-fit polynomial.

Histological data collection and analysis. Every fourth section containing EGFP and orexin-A immunopositive neurons were imaged with a Keyence microscope (BZ-X800) using a 4× (Nikon CFI Plan Apo Lambda, NA 0.2) or a 40× (Nikon CFI Plan Apo Lambda, NA 0.95) objective. On average, seven sections per brain (160 μ m apart) covered the rostrocaudal extent of the orexin field. Efficacy and specificity of EGFP expression in the orexin-EGFP mice were determined using double IF and counting the number of neurons single and double-labeled for orexin-A (Alexa Fluor 594) and GFP (Alexa Fluor 488) in untreated mice. Efficacy was calculated as the percentage of orexin-A-positive neurons that were also GFP positive for each mouse. Specificity was calculated as the percentage of GFP-positive cells that were also orexin-A positive for each mouse.

For analysis of the soma size, GFP-immunopositive neurons visualized by VIP ($n = 49$ –133 per mouse) were measured from bright-field image Z-stacks acquired with the 40× objective using 0.3 μ m Z-steps. Z-stack locations were chosen randomly from throughout the GFP-positive neuron field. A typical Z-stack had 30 images to encompass the entire soma. GFP-positive neurons were only measured if the soma was completely encompassed by the Z-stack, and dendritic/axon projections were visible coming off the soma. Soma measurements were done using the polygon tool in ImageJ to trace the outline of each soma in the Z-stack. Measurements of the somatic cross-sectional area and other shape parameters were collected in the region of interest manager in ImageJ.

Statistical analyses. Statistical analyses were conducted using DataDesk RP (v8.2.1; Data Description), Excel, Igor Pro 8 and 9, or R (v4.5.0) in R Studio. Data are presented as the mean $\pm$ standard error of the mean (SEM). For all statistical tests, $p < 0.05$, $p < 0.01$, and $p < 0.001$ were considered significant, and $p \geq 0.05$ was considered not significant (ns). For histological data, the average soma size per mouse was compared between treatment groups by T test, and cumulative distributions of the individual cell size were compared with a Kolmogorov–Smirnov test implemented in Igor Pro. For electrophysiological data, D- and H-cell comparisons of the spike ratio versus latency to first spike were analyzed using the Fasano–Franceschini Test: A Multivariate Kolmogorov–Smirnov Two-Sample Test (v2.2.2) implemented in R (Puritz et al., 2023). Analysis of C_m , R_m , rheobase, spontaneous firing, and action potential shape parameters were compared between treatments groups with a two-way ANOVA. Since we were specifically focused on testing the hypothesis that morphine treatment had differential effects on D- and H-cells, we further analyzed the results of these ANOVAs using two planned, pairwise contrasts (saline-treated D-cells vs morphine-treated D-cells and saline-treated H-cells vs morphine-treated H-cells) using the estimated marginal means (emmeans) package (version 1.11.2) in R. The resulting p values for these planned contrasts are given in Tables 1–3. Analysis of different firing categories (continuous, cluster, reluctant) was done via a χ^2 test or Fisher's exact test both of which were implemented in R. We then tested the a priori hypothesis that morphine would have a differential effect on the F – I curves for D-cells and H-cells from mice in the 14 d cohort (Figs. 5, 6), the 4 week recovery cohort (Fig. 7), and the 1 d cohort (Fig. 8) using two-way repeated-measure ANOVAs (rmANOVA) with the firing rate as the dependent variable and treatment, current strength (repeat factor), and interaction (treatment $\times$ current strength) as factors with neurons nested in the treatment factor, using the DataDesk application. Planned contrasts were then conducted for pairwise differences between the firing rate at each current strength with Bonferroni's correction. We then tested the a priori hypothesis that there would be within-treatment differences in F – I curves that would vary by cohort using a two-way rmANOVA with the firing rate as the dependent variable and cohort, current strength (repeat factor), and interaction (cohort $\times$ current strength) as factors with neurons nested in the cohort factor, using the DataDesk application. Planned contrasts were then conducted for pairwise differences between the firing rate at each current strength with Bonferroni's correction.

Results

Repeated morphine exposure reduced the average soma size of orexin-EGFP neurons (14 d cohort)

To study the effects of repeated morphine on excitability, we first confirmed the efficacy and specificity of EGFP expression in orexin neurons and then determined whether EGFP-expressing orexin neurons reflect the decrease in the soma size previously reported for the orexin immunoreactive population (Thannickal et al., 2018).

Using double IF, we found that that efficacy was variable, but specificity was very high (Fig. 1B–D). Efficacy ranged between 41 and 63% across animals with an average of $49.1 \pm 3.5\%$ ($n = 6$) of orexin-A immunopositive neurons expressing EGFP (Fig. 1D, left), while the specificity of orexin-A expression

Table 1. Cell type properties from the 14 d cohort

	D-type (saline)		D-type (morphine)		<i>p</i>	H-type (saline)		H-type (morphine)		<i>p</i>
	Mean ± SEM	<i>n</i>	Mean ± SEM	<i>n</i>		Mean ± SEM	<i>n</i>	Mean ± SEM	<i>n</i>	
Passive properties										
Capacitance (pF)	26.89 ± 2.05	17	28.16 ± 2.42	17	0.700	25.55 ± 1.68	15	33.93 ± 3.35	13	0.025
Input resistance (GΩ)	1.14 ± 0.17	17	1.01 ± 0.09	17	0.467	1.06 ± 0.18	15	0.75 ± 0.07	13	0.135
Active properties										
Rheobase (pA)	15.88 ± 1.50	17	18.24 ± 1.76	17	0.471	22.86 ± 3.22	14	33.33 ± 3.55	12	0.007
Spontaneous firing rate (Hz)	8.60 ± 1.02	17	9.41 ± 1.39	17	0.650	8.50 ± 1.30	15	7.73 ± 1.51	13	0.690
Threshold (mV)	−36.97 ± 0.59	16	−37.64 ± 0.66	17	0.431	−38.19 ± 0.48	15	−36.70 ± 0.73	13	0.112
Rising slope max (mV/ms)	203.11 ± 8.06	16	198.08 ± 10.3	17	0.738	245.59 ± 14.53	15	203.22 ± 10.10	13	0.012
AP max (mV)	51.35 ± 1.01	16	49.67 ± 1.32	17	0.396	52.87 ± 1.78	15	46.73 ± 1.67	13	0.006
AP amplitude (mV)	88.32 ± 1.22	16	87.31 ± 1.34	17	0.640	91.06 ± 2.08	15	83.43 ± 1.65	13	0.002
Falling slope max (mV/ms)	−88.91 ± 3.64	16	−87.25 ± 4.49	17	0.797	−102.08 ± 5.88	15	−82.15 ± 4.57	13	0.006
Width at half max (ms)	1.11 ± 0.02	16	1.14 ± 0.03	17	0.475	1.03 ± 0.03	15	1.13 ± 0.04	13	0.035
AHP min (mV)	−67.01 ± 0.92	16	−67.14 ± 0.75	17	0.912	−68.31 ± 0.80	15	−64.89 ± 0.83	13	0.007

Passive and active properties of D-cells (left columns) and H-cells (right columns) from mice treated for 14 d with a single daily injection of saline or morphine (50 mg/kg). Measured parameters are displayed as mean ± SEM. Columns show cell type data from mice treated with saline [D- or H-type (saline)] or morphine [D- or H-type (morphine)]. Each measurement is followed by the number of cells (*n*) measured. The value in the column labeled “*p*” is the probability of rejecting the null hypothesis when it is true that the saline and morphine values were drawn from the same distribution. The *p* values were computed by planned comparisons using the estimated marginal means package in R following a two-way ANOVA.

Table 2. Cell type properties from the 4 week recovery cohort

	D-type (saline)		D-type (morphine)		<i>p</i>	H-type (saline)		H-type (morphine)		<i>p</i>
	Mean ± SEM	<i>n</i>	Mean ± SEM	<i>n</i>		Mean ± SEM	<i>n</i>	Mean ± SEM	<i>n</i>	
Passive properties										
Capacitance (pF)	25.31 ± 2.26	21	33.47 ± 3.00	12	0.061	28.06 ± 2.17	14	26.74 ± 2.85	14	0.642
Input resistance (GΩ)	1.41 ± 0.18	21	1.46 ± 0.25	12	0.252	1.46 ± 0.33	14	1.18 ± 0.19	14	0.466
Active properties										
Rheobase (pA)	11.71 ± 1.27	21	11.9 ± 1.11	20	0.935	14.78 ± 2.56	14	14.21 ± 2.47	14	0.836
Spontaneous firing rate (Hz)	7.75 ± 1.14	19	7.72 ± 1.09	19	0.987	5.48 ± 1.28	13	8.46 ± 1.33	14	0.114
Threshold (mV)	−38.98 ± 0.79	22	−37.36 ± 0.68	22	0.102	−37.79 ± 0.80	14	−39.44 ± 0.73	14	0.186
Rising slope max (mV/ms)	180.45 ± 7.70	22	173.31 ± 6.46	22	0.506	203.53 ± 11.14	14	202.46 ± 9.08	14	0.937
AP max (mV)	49.16 ± 1.35	22	48.78 ± 0.66	22	0.803	51.17 ± 1.26	14	50.22 ± 1.49	14	0.624
AP amplitude (mV)	88.15 ± 1.46	22	86.14 ± 0.77	22	0.239	88.96 ± 1.46	14	86.43 ± 1.62	14	0.741
Falling slope max (mV/ms)	−80.02 ± 3.23	22	−78.22 ± 2.49	22	0.708	−89.92 ± 5.38	14	−89.24 ± 4.69	14	0.910
Width at half max (ms)	1.26 ± 0.04	22	1.26 ± 0.03	22	0.960	1.17 ± 0.06	14	1.17 ± 0.03	14	0.953
AHP min (mV)	−68.11 ± 0.99	22	−65.80 ± 0.60	22	0.080	−67.90 ± 1.42	14	−68.16 ± 1.21	14	0.877

Passive and active properties of D-cells (left columns) and H-cells (right columns) from mice treated for 14 d with a single daily injection of saline or morphine (50 mg/kg) and then allowed to recover for 4 weeks. Measured parameters are displayed as mean ± SEM. Columns show the cell type data from mice treated with saline [D- or H-type (saline)] or morphine [D- or H-type (morphine)]. Each measurement is followed by the number of cells (*n*) measured. The value in the column labeled “*p*” is the probability of rejecting the null hypothesis when it is true that the saline and morphine values were drawn from the same distribution. The *p* values were computed by planned comparisons using the estimated marginal means package in R following a two-way ANOVA.

Table 3. Cell-type properties from the 1 d cohort

	D-type (saline)		D-type (morphine)			H-type (saline)		H-type (morphine)		
	Mean ± SEM	<i>n</i>	Mean ± SEM	<i>n</i>	<i>p</i>	Mean ± SEM	<i>n</i>	Mean ± SEM	<i>n</i>	<i>p</i>
Passive properties										
Capacitance (pF)	22.52 ± 2.07	13	22.29 ± 1.42	28	0.918	20.83 ± 1.03	16	20.77 ± 1.65	15	0.979
Input resistance (GΩ)	1.25 ± 0.23	13	1.25 ± 0.19	28	0.992	1.2 ± 0.21	16	1.55 ± 0.21	15	0.293
Active properties										
Rheobase (pA)	13.17 ± 2.51	12	11.08 ± 1.2	26	0.334	12.38 ± 1.52	16	8 ± 2.24	13	0.061
Spontaneous firing rate (Hz)	8.50 ± 1.07	12	7.96 ± 0.81	24	0.744	10.43 ± 1.74	14	8.75 ± 1.13	14	0.345
Threshold (mV)	−40.05 ± 1.39	11	−39.6 ± 0.61	26	0.718	−39.74 ± 0.86	16	−38.62 ± 0.76	13	0.384
Rising slope max (mV/ms)	160.47 ± 9.3	11	152.84 ± 5.42	26	0.504	186.71 ± 6.55	16	196.18 ± 12.17	13	0.426
AP max (mV)	52.49 ± 2.05	11	50.72 ± 1.15	26	0.403	53.42 ± 1.57	16	54.96 ± 1.17	13	0.484
AP Amplitude (mV)	92.54 ± 1.42	11	90.32 ± 1.19	26	0.296	93.16 ± 1.51	16	93.59 ± 1.67	13	0.848
Falling slope max (mV/ms)	−72.14 ± 3.62	11	−71.85 ± 2.99	26	0.962	−83.43 ± 4.34	16	−94.27 ± 6.14	13	0.090
Width at half max (ms)	1.49 ± 0.05	11	1.49 ± 0.04	26	0.957	1.31 ± 0.04	16	1.22 ± 0.05	13	0.218
AHP min (mV)	−67.51 ± 1.16	11	−67.88 ± 0.60	26	0.777	−70.04 ± 1.10	16	−70.79 ± 0.91	13	0.579

Passive and active properties of D-cells (left columns) and H-cells (right columns) from mice treated with a single injection of saline or morphine (50 mg/kg). Measured parameters are displayed as mean ± SEM. Columns show cell type data from mice treated with saline [D- or H-type (saline)] or morphine [D- or H-type (morphine)]. Each measurement is followed by the number of cells (*n*) measured. The value in the column labeled “*p*” is the probability of rejecting the null hypothesis when it is true that the saline and morphine values were drawn from the same distribution. The *p* values were computed by planned comparisons using the estimated marginal means package in R following a two-way ANOVA.

within the GFP-positive population was $99.7 \pm 0.1\%$ ($n = 6$; Fig. 1D, right).

We next determined if chronic morphine treatment altered the soma size of EGFP-expressing neurons. Male orexin-EGFP mice in our 14 d cohort were injected with 50 mg/kg morphine or saline once daily for 2 weeks prior to VIP IHC for GFP (Fig. 1E,F). We found that the average soma area was reduced by $\sim 16\%$, decreasing from $277.5 \pm 6.2 \mu\text{m}^2$ in saline-treated mice ($n = 4$) to $232.5 \pm 6.7 \mu\text{m}^2$ in morphine-treated mice

($n = 4$; $t_{(6)} = 4.907$; $p = 0.003$; Fig. 1G). Pooling the soma measurements from all mice in each treatment (saline-treated, $n = 356$; morphine-treated, $n = 440$) indicated a leftward shift in the size distribution for morphine-treated mice and suggested that the average size was decreased due to a modest increase in small somata and a large decrease in large somata (Fig. 1H). This leftward shift was apparent by comparing the cumulative size distributions which were significantly different based on a Kolmogorov–Smirnov test ($p < 0.0001$; Fig. 1I). Thus, these

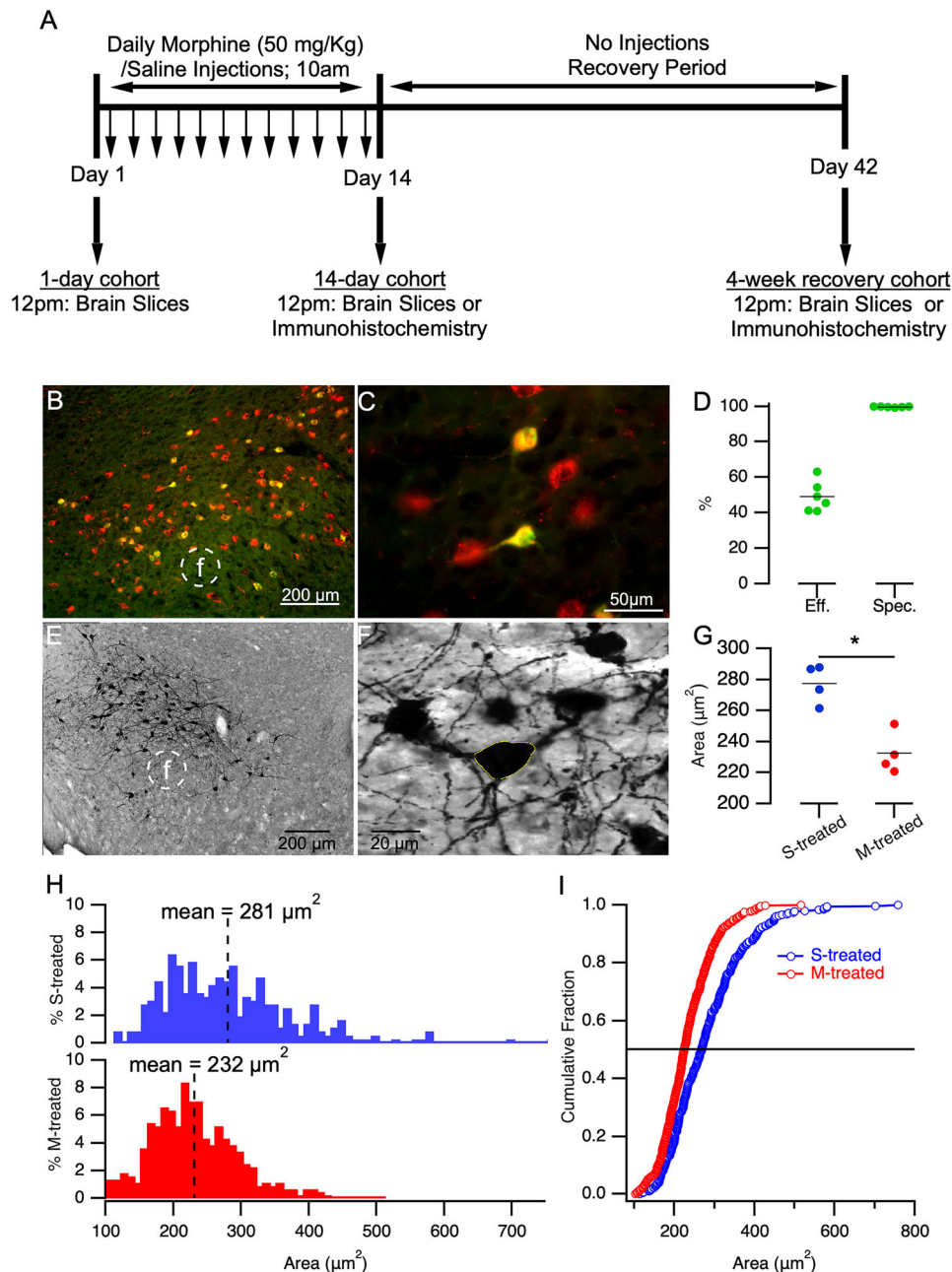


Figure 1. The orexin-EGFP soma size was reduced in 14 d cohort mice that received repeated morphine treatment. **A**, Schematic of experimental cohorts, injection timing, and tissue preparation. **B**, Low power (4× objective) and **C** higher power (40× objective) epifluorescence images of GFP-positive neurons within the orexin neuron field. Anti-orexin-A IF (red), anti-GFP IF (green). **D**, Efficacy (Eff.; percentage of orexin immunopositive neurons also expressing GFP IF) and specificity (Spec.; percentage of GFP-immunopositive neurons also expressing orexin IF). **E**, **F**, Bright-field low power (**E**) and higher power (**F**) of GFP-immunopositive neurons visualized with VIP. Yellow polygon delineates the soma outline (**F**) used for measurements in **G–I**. **G**, The GFP-immunopositive soma area per mouse from saline-treated (S-treated; blue symbols) and morphine-treated (M-treated; red symbols) 14 d cohort mice. **H**, Histogram (10 μm^2 bins) of all measured GFP-immunopositive soma sizes from each treatment group as the percentage of cells in group (S-treated, blue, $n = 356$ cells, top; M-treated, red, $n = 440$ cells, bottom). Dashed lines mark the mean area. **I**, Cumulative fraction of soma sizes from each treatment group (S-treated, blue symbols; M-treated, red symbols) from the 14 d cohort. f, fornix; Eff., efficacy; Spec., specificity; S-treated, saline-treated; M-treated, morphine-treated.

GFP-positive neurons undergo a similar reduction in the soma size to that previously found for the overall population of orexin immunopositive neurons following chronic morphine exposure.

Repeated morphine exposure did not alter the passive electrical properties or spontaneous firing of orexin neurons and did not alter the fraction of D- or H-type orexin neurons (14 d cohort)

We next assessed the passive electrical properties of orexin-EGFP neurons in brain slices from mice in the 14 d cohort by whole-cell recording (Fig. 2*A*, left). Total membrane capacitance (C_m) and input resistance (R_m) were measured from a holding potential of -60 mV using the Clampex membrane test measurements of capacitive currents resulting from -10 mV voltage pulses (Fig. 2*A*, right). Since orexin-EGFP neurons had smaller somata following 14 d of morphine exposure, we expected that estimates of C_m might be lower and R_m might be higher following morphine treatment; however, this was not the case. There were no significant differences between EGFP-positive cells from saline- and morphine-treated mice in either C_m (saline, 26.262 ± 1.330 pF; $n = 32$; morphine, 30.664 ± 2.104 pF; $n = 30$; $t_{(49)} = -1.768$; $p = 0.083$; t test) or R_m (saline, 1.105 ± 0.121 G Ω , $n = 32$; morphine, 0.893 ± 0.062 G Ω ; $n = 30$; $t_{(46)} = 1.551$; $p = 0.128$; t test) although there was a trend toward a higher average C_m among neurons from the morphine-treated mice.

Orexin neurons are in an intrinsic state of depolarization and typically fire spontaneously in brain slices (Li et al., 2002; Eggermann et al., 2003; Cvetkovic-Lopes et al., 2010). We found that 64/68 neurons fired spontaneously, but there was no statistical difference in the average rate between EGFP-positive cells from mice treated with saline (8.55 ± 0.80 Hz; $n = 32$) and morphine (8.68 ± 1.02 Hz, $n = 30$; $t_{(55)} = -0.10$, $p = 0.92$, t test). Moreover, we found no statistical difference in variability of spontaneous firing as measured by the CV (saline, 0.13 ± 0.03 ; $n = 32$; morphine, 0.13 ± 0.02 ; $n = 30$; $t_{(55)} = -0.10$; $p = 0.92$; t test).

We next characterized each neuron as D- or H-subtypes (Williams et al., 2008; Schöne et al., 2011) by delivering hyperpolarizing current-clamp pulses, while they were spontaneously firing (Fig. 2*B,C*). Out of 68 cells tested in the 14 d cohort, 34 were classified as D-type, with a rapid return to firing after offset of the current pulse (arrow), and 28 were classified as H-type, with a delayed return to firing after offset of the current pulse (arrow). Six neurons were not classifiable (four of which were not spontaneously firing). D- and H-cells were identified in slices from both saline- and morphine-treated mice. In slices from saline-treated mice, D-cells made up 53% ($n = 17$) and H-cells made up 47% ($n = 15$) of those identified, while in slices from morphine-treated mice, this percentage was 57% ($n = 17$, D-cells) and 43% ($n = 13$, H-cells) indicating the encounter

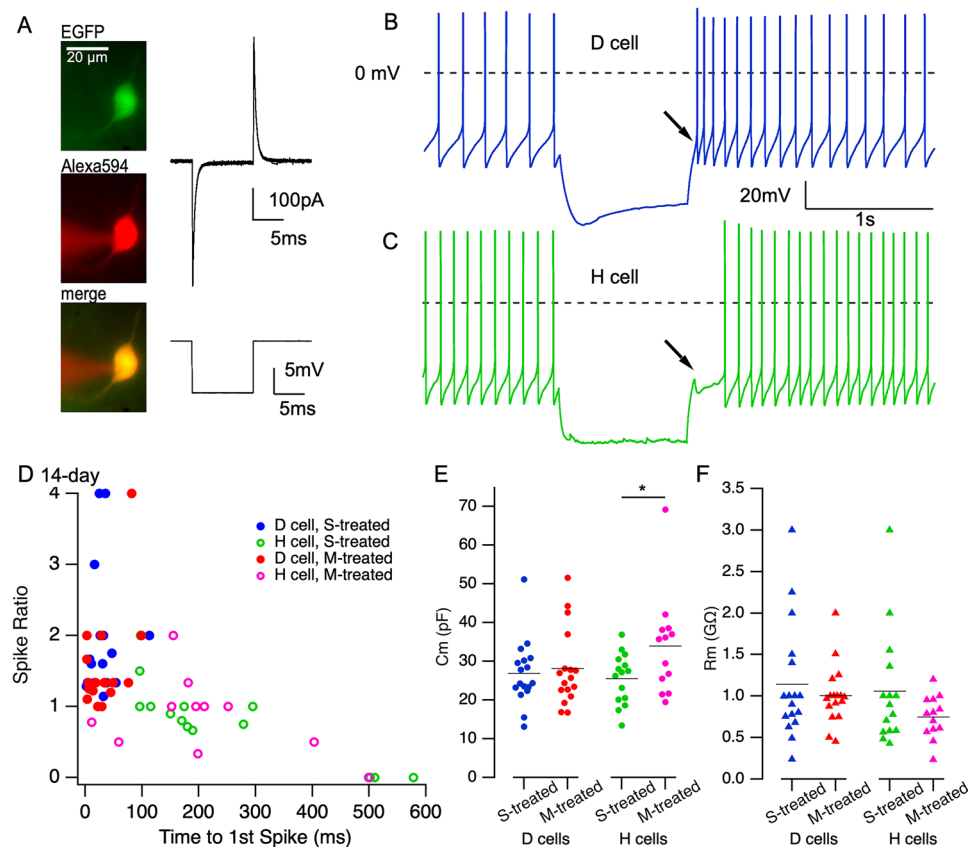


Figure 2. Identification of D and H orexin neurons and the effect of repeated saline and morphine treatment on C_m and R_m for 14 d cohort mice. **A**, Recorded orexin neuron visualized by EGFP fluorescence (left, top) and Alexa Fluor 594-biotin in the pipette solution (left, middle; images merged left, bottom). Corresponding membrane current (right, top) and voltage (right, bottom) traces from this recorded cell used to estimate C_m and R_m . **B**, **C**, Example voltage traces from a D-cell (**B**) and H-cell (**C**). Arrows point to the characteristic depolarizing (D-cells) or hyperpolarizing (H-cells) membrane responses following termination of a hyperpolarizing current pulse. The dashed line indicates 0 mV and the scale bars apply to **B** and **C**. **D**, The spike ratio versus time to first spike following the hyperpolarizing current pulse for D- (filled blue symbols; D cell, S-treated) and H- (open green symbols; H cell, S-treated) neurons from saline-treated (S-treated) mice and D- (red filled symbols; D cell, M-treated) and H- (pink open symbols; H cell, M-treated) neurons from morphine-treated (M-treated) mice. **E**, Distribution of C_m for D-cells (D cells) and H-cells (H cells). C_m from D-cells in slices from saline- (blue symbols) or morphine (red symbols)-treated mice, or from H-cells in slices from saline- (green symbols) or morphine (pink symbols)-treated mice. **F**, Distributions of R_m for D- and H-cells from saline- and morphine-treated mice (colors as in **E**).

frequency of D- and H-cells was not different by treatment ($\chi^2_{(1, n=62)} = 0.0006$; $p = 0.98$).

We also found orexin neurons showed a similar relation between the spike ratio and time to first spike, as previously described (Schöne et al., 2011). D-type neurons had higher spike ratios and shorter times to first spike compared with H-type orexin neurons in both slices from saline-treated and morphine-treated mice (Fig. 2D). Using the Fasano–Franceschini test, which is a generalization of the Kolmogorov–Smirnov test for multidimensional data, we compared whether D- and H-cells were drawn from the same underlying two-dimensional distribution of these parameters. We could reject the null hypothesis that D- and H-cells were drawn from the same distribution in slices from both saline- and morphine-treated mice (saline-treated D-cells $n = 16$ vs saline-treated H-cells $n = 14$; $d = 404$; $p = 0.009$; morphine-treated D-cells $n = 17$ vs morphine-treated H-cells $n = 11$; $d = 307$; $p = 0.007$), indicating these parameters remained distinct for D- and H-cells following morphine treatment. We also found we could not reject the null hypotheses that saline-treated D-cell and morphine-treated D-cell parameters were drawn from the same distribution or that saline-treated H-cell and morphine-treated H-cell parameters were drawn from the same distribution (D-cells from saline-treated $n = 16$ vs D-cells from morphine-treated mice $n = 17$; $d = 244$; $p = 0.108$; H-cells from saline-treated $n = 14$ vs H-cells from morphine-treated mice $n = 11$; $d = 99$; $p = 0.605$), indicating that these D- and H-cell parameters remained stable across saline and morphine treatments.

Analyzing spontaneous firing for each cell type separately by a two-way ANOVA did not reveal an effect of treatment (saline or morphine), cell type (D- or H-), or interaction (treatment $\times$ cell type) on the firing rate or CV (see Table 1 for firing rate means $\pm$ SEMs; firing rate ANOVA, treatment $F_{(1,58)} = 0.00023$; $p = 0.99$; cell type $F_{(1,58)} = 0.467$; $p = 0.497$; interaction $F_{(1,58)} = 0.366$; $p = 0.548$; CV ANOVA, treatment $F_{(1,58)} = 0.093$; $p = 0.762$; cell type $F_{(1,58)} = 3.505$; $p = 0.066$; interaction $F_{(1,58)} = 1.930$; $p = 0.170$). There were also no significant effects on the planned comparisons of the firing rate, mean firing rate for saline- and morphine-treated H-cells ($t_{(58)} = 0.398$; $p = 0.69$) and saline- and morphine-treated D-cells ($t_{(58)} = -0.462$; $p = 0.65$), or planned comparison of CV saline- and morphine-treated H-cells ($t_{(58)} = -1.142$; $p = 0.258$) and saline- and morphine-treated D-cells ($t_{(58)} = 0.808$; $p = 0.422$).

Analyzing C_m and R_m separately for each cell type by two-way ANOVA did not reveal an effect of treatment, cell type, or interaction on these parameters (Fig. 2E,F; Table 1; C_m , treatment $F_{(1,58)} = 3.873$; $p = 0.054$; cell type $F_{(1,58)} = 0.820$; $p = 0.369$; interaction $F_{(1,58)} = 2.100$; $p = 0.153$; R_m , treatment $F_{(1,58)} = 2.606$; $p = 0.112$; cell type $F_{(1,58)} = 1.469$; $p = 0.230$; interaction $F_{(1,58)} = 0.400$; $p = 0.530$). However, our planned comparisons indicated that C_m was elevated in H-cells from morphine-treated slices ($t_{(58)} = -2.305$; $p = 0.025$) compared with saline-treated slices, while no significant effect was observed for D-cells ($t_{(58)} = -0.386$; $p = 0.701$). The planned comparisons of R_m did not reveal significant changes (H-cell R_m , $t_{(58)} = 1.515$; $p = 0.135$; D-cell R_m , $t_{(58)} = 0.732$; $p = 0.467$). Thus, despite the soma size reduction after 2 weeks of daily morphine exposure, electrical measurement did not reveal a corresponding decrease in C_m or increase in R_m . In fact, C_m in H-cells was elevated, although this likely depended on a single high C_m value H-cell, since following removal of this case, the planned comparison was not significant ($t_{(57)} = -1.679$; $p = 0.099$). Several factors may contribute to this apparent discrepancy between the soma

size and C_m . First, cell sampling differences between fixed sections and brain slices may have masked differences in their size distributions. Second, the soma area is only $\sim 15\%$ of the total membrane area (Schöne et al., 2011), so the effect of a decrease in the soma size alone could be obscured by the greater dendritic area, which itself might change. Finally, small membrane infoldings may contribute to soma size decrease without reducing or even increasing the total membrane area and hence capacitance (Matovic et al., 2020).

Repeated morphine exposure altered the action potential shape of H- but not D-type orexin neurons (14 d cohort)

We next assessed the consequences of 14 d of morphine exposure on the active membrane properties underlying spike generation in orexin neurons by measuring rheobase and action potential shape parameters. Orexin neurons from morphine-treated mice had a rheobase that trended higher (24.48 ± 2.25 pA; $n = 29$) than those from saline-treated mice (19.03 ± 1.76 pA; $n = 31$; $t_{(58)} = 1.922$; $p = 0.060$). Comparing treatment and cell type effects on rheobase by two-way ANOVA revealed a significant effect of treatment ($F_{(1,56)} = 6.75$; $p = 0.012$) and cell type ($F_{(1,56)} = 19.99$; $p < 0.0001$) but no significant effect of treatment $\times$ cell type ($F_{(1,56)} = 2.7$; $p = 0.106$). Planned comparisons revealed that the H-cell rheobase was $\sim 45\%$ higher in slices from morphine-treated mice compared with those from saline-treated mice (Table 1; $t_{(56)} = -2.815$, $p = 0.007$), while no difference was found among D-cells ($t_{(56)} = -0.725$; $p = 0.471$). Thus, daily morphine exposure selectively increased the amount of current required to elicit an action potential in H-cells.

We next analyzed the threshold and shape of single spikes produced by just-suprathreshold current injections from a membrane potential of -60 mV (Fig. 3, Table 1). This revealed that while the thresholds were unaffected by treatment, the action potentials in H-cells from morphine-treated mice were, on average, smaller and slower than action potentials from saline-treated mice (Fig. 3A, right). Moreover, there were no corresponding changes to the action potential shape in D-cells recorded from the same brain slices (Fig. 3A, left). In addition to having action potentials of lower amplitude (Fig. 3B) and longer half-widths (Table 1), morphine-treated H-cells had action potentials with significantly slower maximal rising (Fig. 3D; Table 1) and falling rates (Fig. 3E; Table 1) with a significantly less negative average peak AHP (Fig. 3C; Table 1), while there were no corresponding differences between these parameters for D-cells (Table 1). These conclusions are based on two-way ANOVAs examining the effects of treatment, cell type, and interaction on each spike-shape parameter followed by our planned, pairwise comparisons.

For thresholds, there were no significant effects of treatment ($F_{(1,57)} = 0.273$; $p = 0.603$), cell type ($F_{(1,57)} = 0.079$; $p = 0.795$), or interaction ($F_{(1,57)} = 2.981$; $p = 0.090$). Planned comparisons did not reveal a significant effect of morphine treatment on thresholds from D- ($t_{(57)} = 0.794$; $p = 0.431$) or H-cells ($t_{(57)} = -1.616$; $p = 0.112$; Table 1).

In contrast, there was a significant effect on the action potential amplitude of treatment ($F_{(1,57)} = 6.457$; $p = 0.014$, but not cell type $F_{(1,57)} = 0.096$; $p = 0.758$), and there was a significant interaction between treatment and cell type ($F_{(1,57)} = 4.337$; $p = 0.042$). Planned comparisons further revealed that action potential amplitude was significantly reduced in H-cells from morphine-treated slices ($t_{(57)} = 3.261$; $p = 0.002$) compared with saline-treated slices, while there was no significant effect of treatment on D-cells ($t_{(57)} = 0.471$; $p = 0.640$; Fig. 3B; Table 1).

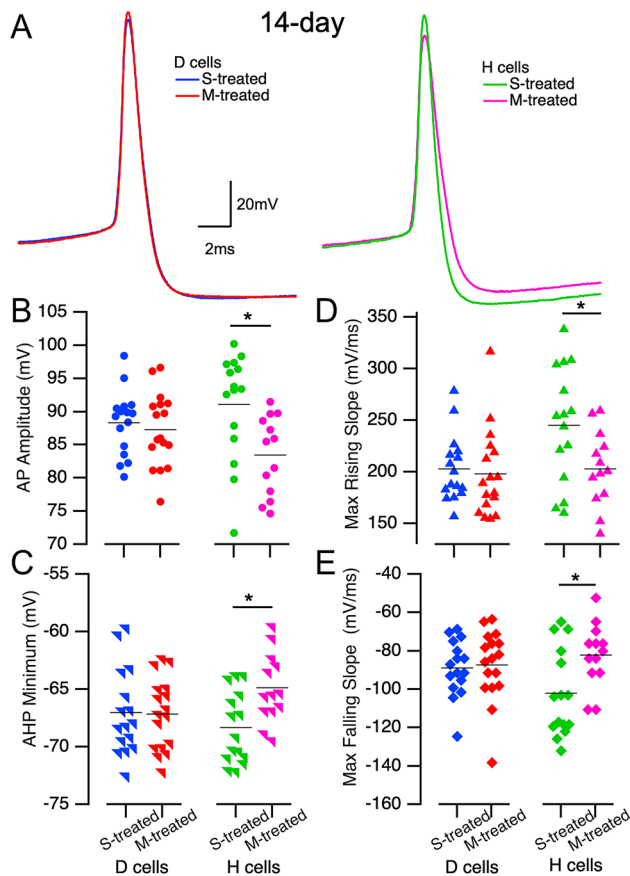


Figure 3. H-type, but not D-type orexin neurons have smaller, wider action potentials following repeated morphine treatment in 14 d cohort mice. **A**, Superimposed single action potentials from representative D-cells following repeated saline (blue; S-treated) or morphine (red; M-treated; left) and from H-cells following repeated saline (green; S-treated) or morphine (pink; M-treated; right). **B–E**, Distributions of spike shape parameters for D-cells (left) and H-cells (right) following either saline (S-treated) or morphine (M-treated) treatment (colors as in **A**). The average spike amplitude (**B**; AP Amplitude), minimum value of the AHP (**C**; AHP minimum), maximum rate of rise (**D**; Max rising slope), and maximal falling rate (**E**; Max Falling Slope) were all altered for H-cells, but not D-cells from morphine-treated mice compared with saline-treated mice. **B–E**, Horizontal lines indicate mean values. Means and p values in Table 1.

This reduction in amplitude of H-cell action potential was accompanied by a significant effect of treatment ($F_{(1,57)} = 6.521$; $p = 0.013$) but not cell type ($F_{(1,57)} = 0.203$; $p = 0.654$) or interaction ($F_{(1,57)} = 2.358$; $p = 0.130$) on the peak membrane potential reached by action potentials. Planned comparisons showed that action potential peak was significantly reduced in H-cells from morphine-treated slices ($t_{(57)} = 2.872$; $p = 0.006$) compared with saline-treated slices, while there was no significant effect of treatment on D-cells ($t_{(57)} = 0.855$; $p = 0.396$; Table 1).

For action potential half-widths, there was also a significant effect of treatment ($F_{(1,57)} = 4.281$; $p = 0.043$), while neither the effect of cell type ($F_{(1,57)} = 2.322$; $p = 0.133$) nor the interaction between treatment and cell type ($F_{(1,57)} = 1.212$; $p = 0.276$) were significant. Planned comparisons revealed that action potential half-width was significantly longer in H-cells from morphine-treated slices ($t_{(57)} = -2.157$; $p = 0.035$) than saline-treated slices. In contrast, no significant effect of treatment was observed between D-cells ($t_{(57)} = -0.719$; $p = 0.475$; Table 1).

For the maximal rising rate (Fig. 3D; Table 1), there were significant main effects of both treatment ($F_{(1,57)} = 4.531$; $p = 0.038$) and cell type ($F_{(1,57)} = 4.849$; $p = 0.032$), indicating that maximal

rising slope differed between both treatment groups and cell types. The interaction between treatment and cell type did not reach significance ($F_{(1,57)} = 2.868$; $p = 0.096$). However, our planned comparisons revealed that the action potential maximal rising rate was significantly slower in H-cells from morphine-treated slices ($t_{(57)} = 2.610$; $p = 0.012$) than saline-treated slices. In contrast, no significant effect of morphine treatment on the maximal rising rate was observed for D-cells ($t_{(57)} = 0.337$; $p = 0.738$).

For the maximal falling rate (Fig. 3E; Table 1), there were significant main effects of treatment ($F_{(1,57)} = 4.742$; $p = 0.034$) but not the cell type ($F_{(1,57)} = 0.816$; $p = 0.370$), while the interaction between treatment and cell type approached, but did not reach, statistical significance ($F_{(1,57)} = 3.741$; $p = 0.058$). Planned comparisons revealed that the action potential maximal falling rate was also significantly slower in H-cells from morphine-treated slices ($t_{(57)} = -2.865$; $p = 0.006$) than saline-treated slices. In contrast, there was no significant effect of morphine treatment on D-cells ($t_{(57)} = -0.259$; $p = 0.797$).

For the peak AHP (Fig. 3C; Table 1), the main effect of treatment did not reach statistical significance ($F_{(1,57)} = 3.180$; $p = 0.080$), nor did the main effect of the cell type ($F_{(1,57)} = 0.266$; $p = 0.608$). However, there was a significant interaction between treatment and cell type ($F_{(1,57)} = 4.539$; $p = 0.037$). Planned comparisons indicated that for H-cells, the peak AHP was significantly less negative in slices from morphine-treated mice than those from saline-treated mice ($t_{(57)} = -2.791$; $p = 0.007$). There was no significant difference observed between D-cells from saline or morphine-treated mice ($t_{(57)} = 0.111$; $p = 0.912$).

Despite these changes in spike shape, there were no indications that H-cells from morphine-treated mice were more depolarized than their counterparts from saline-treated mice. Neither their spontaneous firing rates were different (Table 1), nor was a greater negative current required to hold H-cells from the morphine-treated mice at -60 mV (holding current from saline-treated = -24.9 ± 5.2 pA; $n = 15$; from morphine-treated = -30.1 ± 10.4 pA; $n = 13$; $t_{(24)} = 0.61$; $p = 0.65$). These data indicate that 14 d of daily morphine exposure results in a selective impairment in the spike generating mechanisms of H-cells while sparing D-cells.

Repeated morphine exposure impaired repetitive firing in H-type but not D-type orexin neurons (14 d cohort)

We next examined the ability of orexin neurons to fire repetitively in response to constant current pulses. During this protocol, steady current was injected to hold the baseline membrane potential at -60 mV, and 2 s duration positive current pulses from 10 up to 120 pA in 10 pA steps were injected with enough time between each pulse to allow the membrane potential to recover to the baseline. Across the injected current steps, we noticed that cells displayed different firing patterns that were reflected in their spike-rate variability. Three patterns were discerned: the largest proportion of cells fired continuously with a regular pattern throughout the current pulse (termed “continuous”); a smaller portion fired irregularly with pauses in spiking punctuating the train, which we termed “cluster firing”; and some cells we termed “reluctant”, only fired one or a few spikes no matter how much current was delivered (Fig. 4A). To capture the variability differences between continuous and cluster cells, we measured the CV of the instantaneous firing after early adaptation at a midcurrent strength of 80 pA for cells in each treatment group (Fig. 4B). Based on the distribution of CVs across groups, we chose a CV of 0.22 to separate cells into continuous

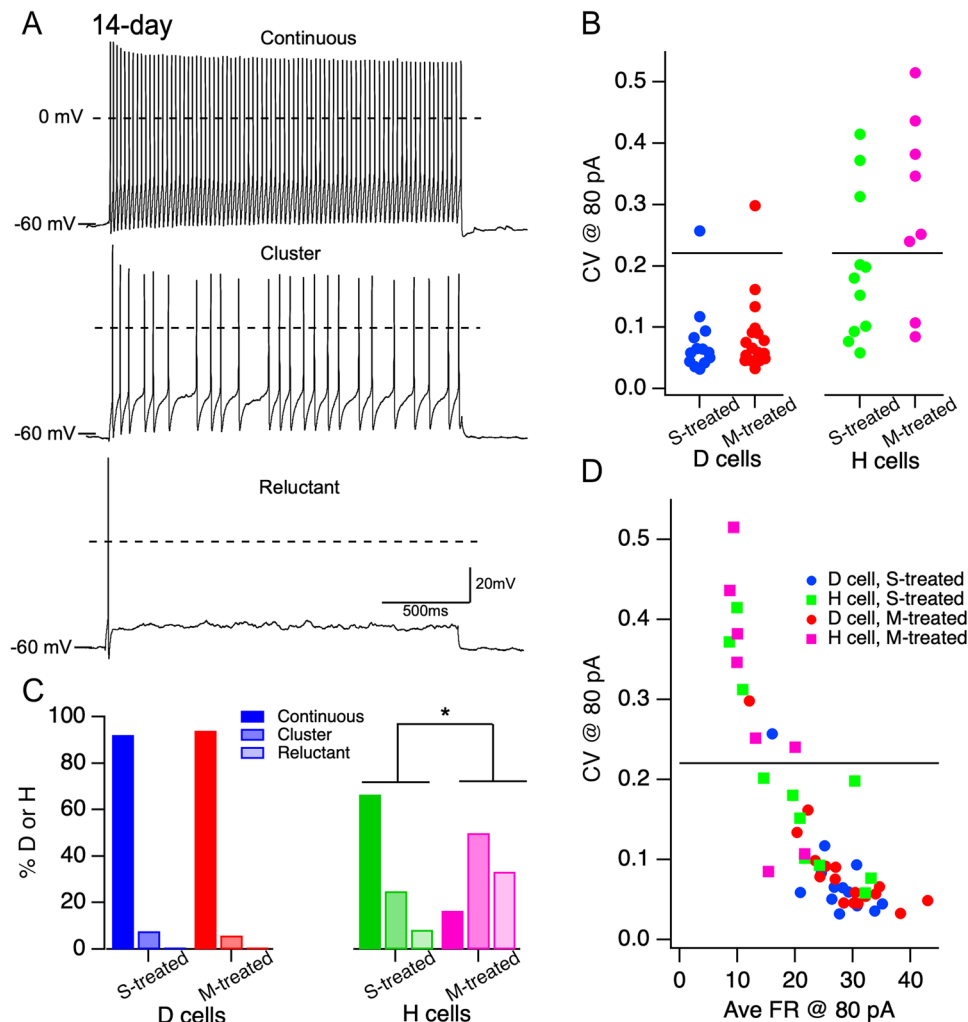


Figure 4. Repeated morphine treatment in 14 d cohort mice increased spike-rate variability in H-type, but not D-type orexin neurons. **A**, Repetitive firing examples from three orexin neurons in response to midrange depolarizing currents illustrating “continuous” (top), “cluster” (middle), and “reluctant” (bottom) firing patterns. **B**, Distribution of firing rate CVs resulting from an 80 pA current step for D-cells (left) from saline- (S-treated; blue symbols) and morphine (M-treated; red symbols)-treated mice and H-cells (right) from saline- (S-treated; green symbols) and morphine (M-treated; pink symbols)-treated mice. A CV of 0.22 at 80 pA of current injection (horizontal lines) was selected to distinguish continuous (<0.22) and cluster (>0.22) firing types. **C**, Percentage of D- (left) and H- (right)-cells identified with each firing pattern. Colors indicate cell-type and treatment group as in **B**. Firing pattern indicated by bar saturation (continuous, 100%; cluster, 50% and reluctant, 25%). **D**, CV at 80 pA versus the average firing rate at 80 pA of current injection (Ave FR at 80 pA), horizontal line indicates a CV of 0.22. As indicated in the legend, colors indicate the cell type and treatment group.

(<0.22) and cluster (>0.22) categories. Using this criterion, we observed D-cells to have a high percentage of continuous firing cells with only a few neurons showing cluster or reluctant firing patterns. This distribution did not change the following morphine exposure ($\chi^2_{(1, n=30)} = 0.039$; $p = 0.844$; Fig. 4C, left). However, assessing the firing patterns of H-type cells revealed that those from morphine-treated mice had significantly more cluster and reluctant firing cells than H-cells from saline-treated mice ($\chi^2_{(2, n=24)} = 6.400$; $p = 0.041$; Fig. 4C, right). CV was inversely related to the average firing rate, with H-cells from mice treated with morphine comprising most of the cells with high CVs and low firing rates for the same current strength (Fig. 4D). These data indicate that 14 d of daily morphine increases the variability in firing of H-cells but not D-cells and may impair the ability of H-cells to repetitively fire.

To examine repetitive firing ability, we compared the initial and final firing rates achieved in response to 2 s current pulses from 10 to 120 pA in cells from mice treated with either saline or morphine. Sample D-cell recordings are shown in Figure 5A (saline-treated, left; morphine-treated, right), and sample H-cell recordings are

shown in Figure 6A (saline-treated, left; morphine-treated, right). We computed instantaneous firing versus time curves for each current strength (D-cells, Fig. 5B; H-cells, Fig. 6B) and then constructed average $F-I$ curves. Since continuous and cluster firing neurons showed spike frequency adaptation, we constructed $F-I$ curves for both the initial firing rate, measured between the first two spikes, and the final firing rate, measured as the average rate of the last seven spikes, or the average over 2 s when there were fewer than seven spikes. $F-I$ curves were then averaged across cells, and the average curves ($\pm$ SEM) for D-cells and H-cells from each treatment condition are illustrated in Figures 5C and 6C, respectively.

To compare the $F-I$ curves, we used a two-way rmANOVA. Examining the initial firing rate for D-type cells, we found that the rmANOVA was significant for current strength ($F_{(11,260)} = 513.47$; $p < 0.0001$) but not significant for treatment ($F_{(1,260)} = 0.468$; $p = 0.500$) or for the interaction between treatment and current strength ($F_{(11,260)} = 0.594$; $p = 0.833$). Also, none of the planned pairwise contrasts were different between treatment and current strength (all $p > 0.97$; Fig. 5C, top). Similarly, for the D-cell final firing rate, the rmANOVA was

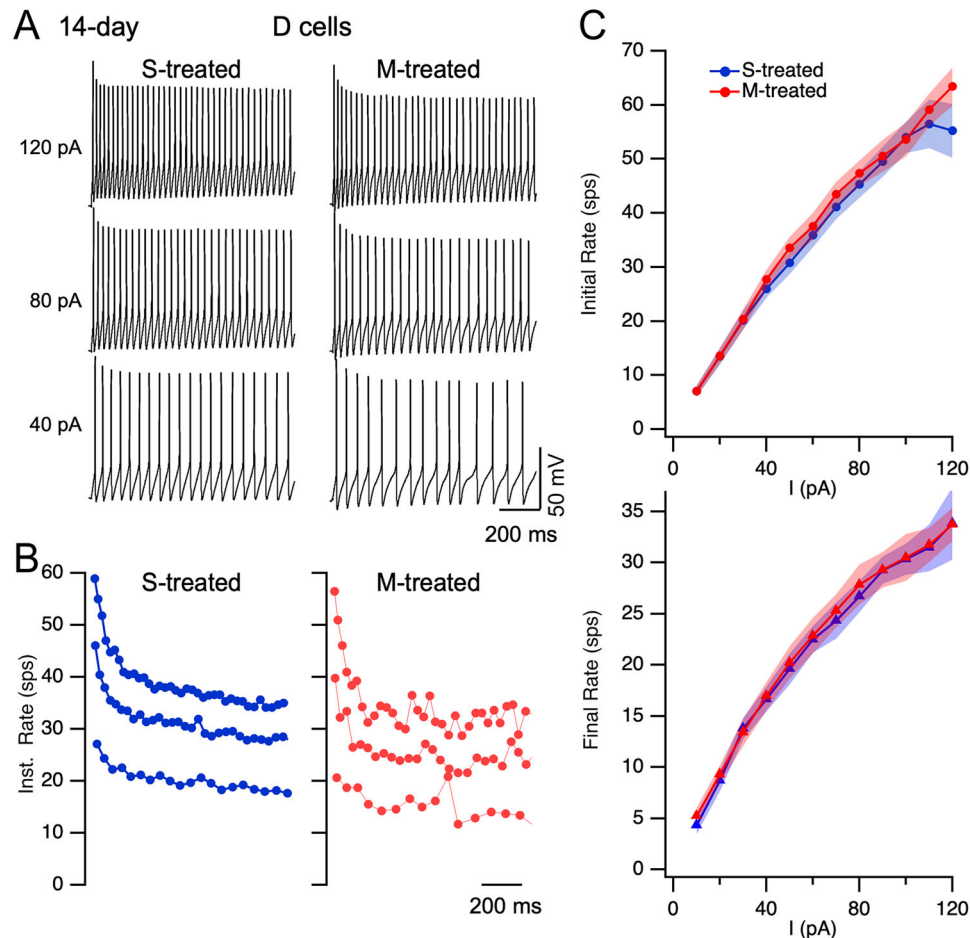


Figure 5. Repeated morphine treatment did not alter the repetitive firing capability of D-type orexin neurons in 14 d cohort mice. **A**, Example repetitive firing of D-cells with continuous firing patterns evoked by 40, 80, and 120 pA current pulses from a saline- (left; S-treated) and a morphine-treated (right; M-treated) mouse. **B**, Corresponding instantaneous firing rate curves for the traces in **A** (saline-treated, S-treated; blue symbols; morphine-treated, M-treated; red symbols). **C**, Initial rate (mean ± SEM) versus current (I) curves (top) and final rate (mean ± SEM) versus current (I) curves (bottom) for D-cells. Blue symbols indicate firing rate from saline-treated (S-treated) mice; red symbols indicate the firing rate from morphine-treated (M-treated) mice. No pairwise differences between treatments were found by planned contrasts with Bonferroni's correction.

significant for current strength ($F_{(11,240)} = 294.08$; $p < 0.0001$) but not significant for treatment ($F_{(1,240)} = 0.0001$; $p = 0.991$) or for the interaction between treatment and current strength ($F_{(11,240)} = 0.161$; $p = 0.999$), nor were there any planned pairwise contrasts different between treatment and current strength (all $p = 1.0$; Fig. 5C, bottom). Thus, 2 weeks of daily morphine treatment did not alter the repetitive firing ability of D-cells compared with 2 weeks of daily saline treatment.

In contrast, the average F - I curves for initial and final firing rates for H-cells from mice treated with morphine were largely not overlapping with the F - I curves obtained from mice treated with saline. H-cells from morphine-treated mice had on average a $35.0 \pm 5.3\%$ and $40.9 \pm 4.1\%$ decrease in the average firing rate across 10–120 pA of current injection for the initial and final firing rates, respectively (Fig. 6C). The rmANOVA for the initial firing rate was significant for current strength ($F_{(11,165)} = 125.76$; $p < 0.0001$) but was not significant for treatment ($F_{(1,165)} = 3.268$; $p = 0.088$) or interaction ($F_{(11,165)} = 1.176$; $p = 0.308$). However, planned pairwise contrasts between treatment and current strength were different at most current strengths (Fig. 6C, top). A similar pattern was apparent for the final firing rates. The rmANOVA for the final firing rate was significant for current strength ($F_{(11,166)} = 65.40$; $p < 0.0001$) but was not significant for treatment ($F_{(1,166)} = 3.788$; $p = 0.069$) or interaction ($F_{(11,166)} = 1.385$; $p = 0.184$). Nevertheless, planned pairwise

contrasts between treatment and current strength were different at most current strengths (Fig. 6C, bottom). Thus, chronic morphine altered H-cell firing by increasing the numbers of H-cells with cluster and reluctant firing patterns and diminishing the ability of continuous and cluster firing H-cells to repetitively fire while having no detectable effects on the firing ability of D-cells recorded from the same brain slices.

It was also apparent from these recordings that both D- and H-cells showed changes in spike shape during repetitive firing. To determine if our morphine treatment impacted these changes, we measured how spike amplitude, maximal rising rate, and half-width varied with the spike number. Since the firing rate can influence these shape changes, successive spike shapes were evaluated from traces where the final firing rate reached ~ 10 spikes per second. This revealed that successive action potentials from both D- and H-cells decreased in amplitude, decreased in the maximal rising rate, and increased in half-width. We found that between the 1st and 10th spike, action potentials from D-cells recorded in slices from saline-treated mice ($n = 13$) decreased in amplitude by $25.6 \pm 2.3\%$, while the maximal rising rate decreased by $53.7 \pm 3.6\%$ and half-width increased by $44.5 \pm 4.2\%$. None of these changes were statistically different in D-cells recorded in slices from mice treated with morphine ($n = 17$). These action potentials decreased in amplitude by $24.5 \pm 1.1\%$ ($t_{(28)} = 0.462$; $p = 0.65$), the maximal rising rate decreased by $54.8 \pm 1.4\%$ ($t_{(28)} = 0.308$; $p = 0.76$), and half-

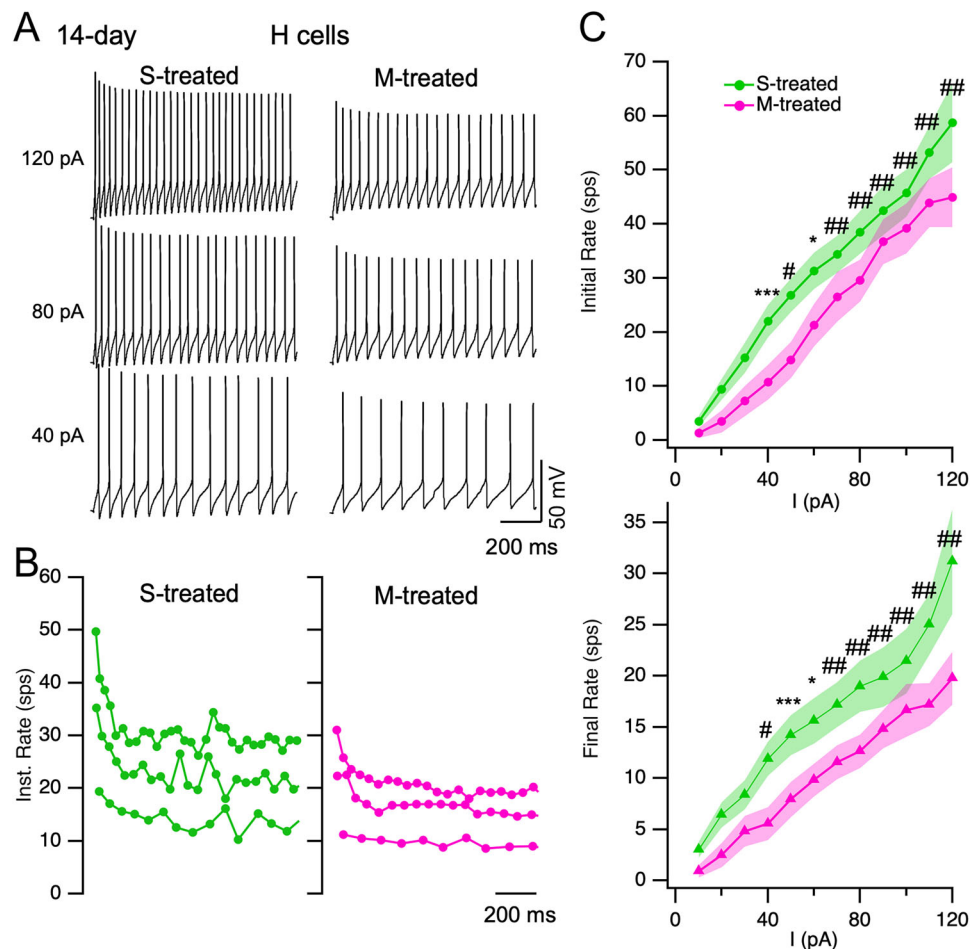


Figure 6. Repeated morphine treatment impaired the repetitive firing capability of H-type orexin neurons in 14 d cohort mice. **A**, Example repetitive firing of H-cells with continuous firing patterns evoked by 40, 80, and 120 pA current pulses from a saline- (left; S-treated) and a morphine-treated (right; M-treated) mouse. **B**, Corresponding instantaneous firing rate curves for the traces in **A** (saline, green symbols; morphine, pink symbols). **C**, Initial rate (mean $\pm$ SEM) versus current (*I*) curves (top) and final rate (mean $\pm$ SEM) versus current (*I*) curves (bottom) for H-cells. Green symbols indicate the firing rate from saline-treated (S-treated) mice; pink symbols indicate the firing rate from morphine-treated (M-treated) mice. Pairwise differences in the firing rate between treatments were found for most current strengths by planned contrasts with Bonferroni's correction. * $p < 0.05$; *** $p < 0.005$; # $p < 0.001$; ## $p < 0.0001$.

width increased by $46.0 \pm 3.7\%$ ($t_{(28)} = 0.267$; $p = 0.79$). Over the first 10 spikes, action potentials from H-cells recorded in slices from saline-treated mice ($n = 11$) decreased in amplitude by $20.8 \pm 2.0\%$, while the maximal rising rate decreased by $47.1 \pm 3.6\%$ and half-width increased by $35.3 \pm 4.8\%$. In contrast to D-cells, H-cells recorded in slices from mice treated with morphine ($n = 8$) had action potentials that showed statistically greater changes in these parameters. Action potential amplitude decreased by $30.0 \pm 3.9\%$ ($t_{(17)} = 2.26$; $p = 0.04$), the maximal rising rate decreased by $59.7 \pm 5.1\%$ ($t_{(17)} = 2.09$; $p = 0.05$), and the half-width increased by $56.0 \pm 8.5\%$ ($t_{(17)} = 2.26$; $p = 0.038$). Thus, 14 daily injections of morphine exaggerated the processes underlying spike amplitude decrease and spike slowing during repetitive firing in H-cells but not in D-cells.

Four weeks of recovery was sufficient to reverse the soma size decrease of orexin-EGFP neurons and impaired excitability of H-cells produced by repeated morphine treatment

The decrease in the orexin neuron soma size produced by 2 weeks of morphine exposure was shown previously to recover by 4 weeks (Thannickal et al., 2018). We therefore determined whether a 4 week recovery time was sufficient for the orexin-EGFP soma size to recover and if the impaired excitability of H-cells persisted or recovered by this time point.

We measured the soma size of orexin-EGFP neurons from mice in the 4 week recovery cohort and found that the per-animal soma size averages were not different between groups 4 weeks after the end of the 14 d of saline or morphine treatment (Fig. 7A; saline, $191.3 \pm 12.89 \mu\text{m}^2$; $n = 5$; morphine, $195.56 \pm 5.16 \mu\text{m}^2$; $n = 5$; $t_{(5)} = -0.307$; $p = 0.771$). The similarity of cell sizes for the two treatment groups after 4 weeks of recovery was also clearly reflected in the overlapping distributions of individual cell sizes (Fig. 7B) which were not statistically different based on the Kolmogorov–Smirnov test ($d = 0.007$; $p = 1.0$).

Comparing the electrical properties of D- and H-cells after 4 weeks of recovery from 14 d of saline or morphine treatment by two-way ANOVA and planned comparisons revealed that H-cell passive properties and spike parameters that differed between morphine and saline-treated mice in the 14 d cohort were no longer different among H-cells from the 4 week recovery cohort (Table 2). Moreover, spike-shape parameters that were not different in neurons from the 14 d cohort were also not different in neurons from the 4 week recovery cohort (e.g., spontaneous firing and threshold) suggesting no overall changes emerged during this period. Thus, following 4 weeks of recovery, H-cell C_m and rheobase were no longer larger in slices from mice treated with morphine. Similarly, spike parameters like amplitude, maximal rate of rise, maximal falling rate, width at half

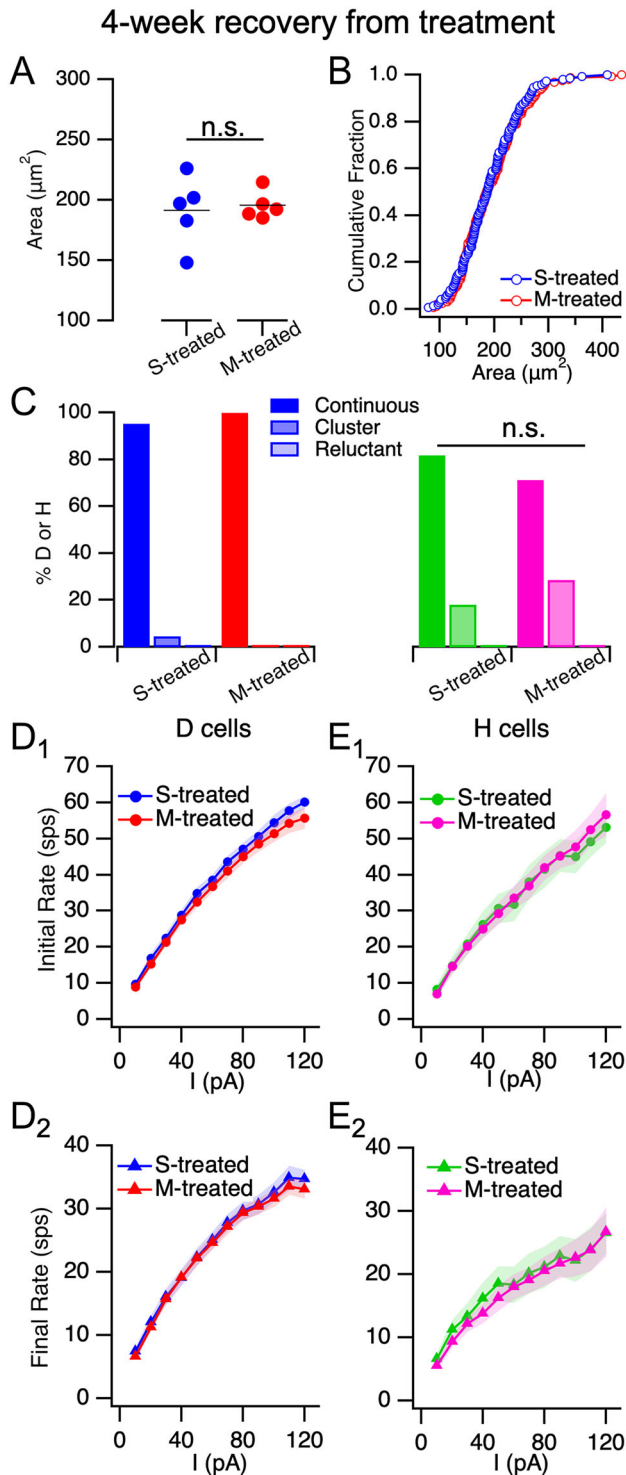


Figure 7. After 4 weeks of recovery, differences in the soma size and intrinsic excitability were no longer apparent. **A**, The average soma size per mouse for saline-treated (S-treated, blue symbols, $n = 5$) and morphine-treated mice (M-treated, red symbols, $n = 5$) were not significantly (n.s.) different 4 weeks after the end of treatment. **B**, Cumulative distribution of individual soma sizes from saline-treated (S-treated, open blue symbols, $n = 148$) and morphine-treated mice (M-treated, open red symbols, $n = 156$) measured 4 weeks after the end of treatment. **C**, Distribution of recorded D- (left) and H-cells (right) with "continuous," "cluster," and "reluctant" firing pattern from slices made following 4 weeks of recovery after saline treatment (S-treated, blue for D-cells; green for H-cells) and morphine treatment (M-treated, red for D-cells; pink for H-cells). The distribution of H-cells from morphine-treated mice was no longer significantly (n.s.) different from that of saline-treated mice. **D**, **E**, Firing frequency (mean $\pm$ SEM)—current (I) curves for initial rates (**D₁**, **E₁**) and final rates (**D₂**, **E₂**) for D-cells (**D**)

max, and AHP minimum in morphine-treated H-cells were no longer different from those measured in saline-treated H-cells following 4 weeks of recovery. This indicates that the impaired spike-generation mechanisms which resulted in smaller and slower spikes in H-cells following repeated morphine treatment, either recovered or were compensated for by alternative mechanisms following 4 weeks of recovery.

A similar picture emerged from analysis of the repetitive firing ability of D- and H-cells from mice in the 4 week recovery cohort. After 4 weeks of recovery, H-cells from morphine-treated mice no longer showed an increase in the fraction of cells with cluster or reluctant firing patterns compared with H-cells from saline-treated mice (Fig. 7C; $\chi^2_{(1, n=25)} = 0.365$; $p = 0.546$). Moreover, neither the initial (Fig. 7D₁, E₁) nor final $F-I$ relations (Fig. 7D₂, E₂) of D- and H-cells from morphine-treated mice were different compared with those from saline-treated mice after 4 weeks of recovery. This was determined using a two-way rmANOVA analyses as above, for the initial firing rate (D-cell treatment, saline vs morphine, $F_{(1,373)} = 1.118$; $p = 0.297$; H-cell treatment, saline vs morphine, $F_{(1,229)} = 0.025$; $p = 0.876$) and final firing rates (D-cell treatment, saline vs morphine, $F_{(1,373)} = 0.143$; $p = 0.707$; H-cell treatment, saline vs morphine, $F_{(1,229)} = 0.046$; $p = 0.832$). None of the planned pairwise contrasts between treatment and current strength were different for D- or H-cells for the initial or final firing rate (all p value between 0.564 and 1).

Thus, neither the decrease in the soma size nor the decrease in excitability of H-type orexin-EGFP neurons following 2 weeks of daily morphine treatment was still apparent following 4 weeks of passive recovery.

A single injection of morphine was insufficient to impair excitability of H-type orexin neurons (1 d cohort)

On the last treatment day, 14 d cohort mice received a final injection of either saline or morphine 2 h prior to brain slice preparation. To test for possible lingering effects of morphine from this dose, we injected a different cohort of orexin-EGFP mice (1 d cohort) with a single injection of either saline or morphine (50 mg/Kg) and prepared brain slices 2 h later.

We found that there were no significant differences in the passive properties or action potential shape between orexin neurons from saline- and morphine-treated 1 d cohort mice, for either D- or H-cells (Table 3) based on a two-way ANOVA and planned comparisons.

Most of both D- and H-cells displayed a "continuous" firing pattern following a single saline or morphine injection (Fig. 8A) suggesting there was no association between this treatment and the firing pattern distribution. This conclusion was supported by Fisher's exact tests (D-cells, $n = 36$; $p = 1.000$; H-cells, $n = 30$; $p = 0.236$). There was also no evidence that a single dose of morphine impaired repetitive firing of H-cells (Fig. 8C). Rather, we found that the initial, but not final, firing rates were modestly increased (10–15%) at a few current strengths for D-cells (Fig. 8B₁) and H-cells (Fig. 8C₁) as determined by planned contrasts with Bonferroni's correction after two-way rmANOVAs of D- and H-cell firing rates, with treatment (saline vs morphine), current strength, and their

←

and H-cells (**E**). Colors as in **C**. No pairwise differences between treatments for D- or H-cells were found by planned contrasts with Bonferroni's correction. S-treated, saline-treated; M-treated, morphine-treated; sps, spikes/second.

interaction as factors. For H-cells, neither treatment nor the interaction had a significant effect on the initial (treatment, $F_{(1,255)} = 2.345$; $p = 0.139$; interaction, $F_{(11,255)} = 0.614$; $p = 0.816$) or final firing rate (treatment, $F_{(1,255)} = 0.680$; $p = 0.418$; interaction, $F_{(11,255)} = 0.734$; $p = 0.706$). Nevertheless, our planned pairwise contrasts identified the Initial firing rate of H-cells to be higher following a single morphine injection at 40, 50, 70, 90 and 100 pA (Fig. 8B, right). For D-cells, treatment had no significant effect on the initial rate ($F_{(1,336)} = 1.478$; $p = 0.232$; Fig. 8B, left) or final rate ($F_{(1,336)} = 0.936$; $p = 0.340$; Fig. 8C, left), while the interaction term had a significant effect on both (initial

rate, $F_{(11,336)} = 2.863$; $p = 0.001$; final rate, $F_{(11,336)} = 1.852$; $p = 0.045$). Our planned pairwise contrasts found that the initial firing rate was higher following morphine treatment at 110 and 120 pA but that there were no differences at any current strength for the final firing rate.

Thus, a single morphine treatment was insufficient to impair spike generation or the repetitive firing ability of H-cells. This indicates that the reduced excitability after 2 weeks of daily morphine injections does not result from the acute effects of morphine lingering from the last injection and that more than a single morphine dose is necessary to induce these changes. Instead, a single injection of morphine appeared to produce a modest increase in excitability of D- and H-cells.

Repeated morphine treatment downscaled the F - I gain of H-type orexin neurons

To compare the within-treatment effects of saline or morphine on the firing ability of D- and H-cells, we superimposed the average F - I curves from the 1 d, 14 d, and 4 week recovery cohorts for each treatment. F - I curves were then compared by two-way rmANOVAs with cohort, current, and their interaction (cohort $\times$ current) as factors. We then conducted planned contrasts between cohort at each current strength with Bonferroni's correction. This analysis underscored that while there were only a few pairwise differences between F - I curves for D-cells from all cohorts (data not shown), there were substantial differences between F - I curves for H-cells from cohorts treated with saline and morphine. Figure 9A illustrates these differences for the final firing rate F - I curves following saline (left) and morphine (right) treatment, although initial firing F - I curves showed similar differences (data not shown). The rmANOVA was not different for the saline-treated cohort factor ($F_{(2,343)} = 0.886$; $p = 0.422$) or for the interaction term ($F_{(22,343)} = 0.878$; $p = 0.625$) but planned pairwise contrasts found there was a significant 5–6 sps lower average final firing rate for current strengths between 60 and 100 pA for saline-treated H-cells from the 14 d cohort compared with those from the 1 d cohort (1 d; $p < 0.005$; Fig. 9A, left). This suggests that repeated daily saline injections also modestly reduced the firing ability of H-cells.

For the morphine-treated H-cell cohorts (Fig. 9A, right), the final firing rate rmANOVA was significant for the cohort factor ($F_{(2,307)} = 8.585$; $p = 0.001$), but not for the cohort $\times$ current interaction ($F_{(22,307)} = 1.245$; $p = 0.208$). However, planned pairwise contrasts between the 1 and 14 d cohorts revealed large reductions in the mean firing rate of ~6–13 sps at each current strength ($p = 0.007$ for 10 pA; $p < 1 \times 10^{-6}$ for each larger current). Planned contrasts also revealed that the mean firing rates of H-cells in the 14 d cohort were 6–8 sps lower than that from the 4 week recovery cohort over most current strengths (30–110 pA; $p < 0.01$). There were also smaller (~5–6 sps) but still significant differences between 1 d and 4 week recovery cohorts over a range of currents (40–70 pA and 100 pA; $p < 0.05$ for each). Thus, the most depressed F - I curves were expressed by H-cells from mice treated with daily morphine injections for 2 weeks. In contrast, the F - I curves were most robust for cells from mice in the 1 d cohort who received a single morphine injection, while F - I curves achieved intermediate levels following 4 weeks of recovery from 2 weeks of daily morphine treatment.

To estimate how the shape of these F - I curves differed between treatments, we fit these average curves with second-order polynomials (Fig. 9A, solid lines). We then estimated the average firing rate gain for each condition by taking

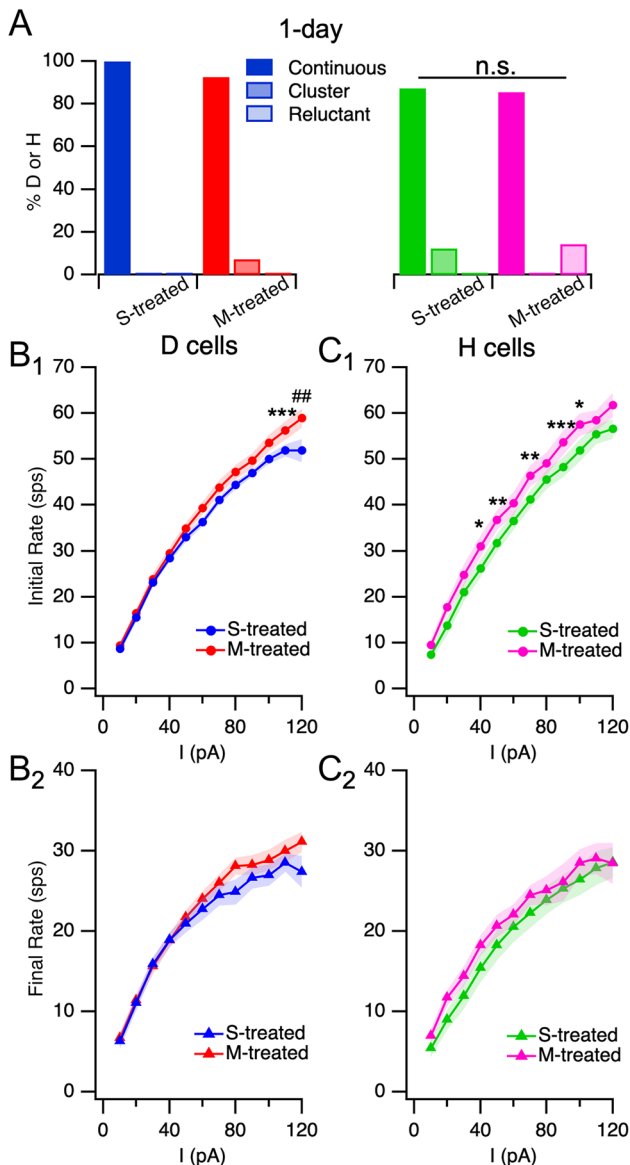


Figure 8. A single morphine injection did not impair firing of D- or H-type orexin neurons from 1 d cohort mice. **A**, Distribution of recorded D- (left) and H-cells (right) with "continuous," "cluster," and "reluctant" firing patterns from slices made following a single injection of saline (S-treated, blue for D-cells; green for H-cells) or morphine (M-treated, red for D-cells; pink for H-cells). The percentage of D- and H-type orexin neurons with each repetitive firing pattern. **B**, **C**, Firing frequency (mean $\pm$ SEM)–current (I) curves for initial (**B**₁, **C**₁) and final (**B**₂, **C**₂) firing rates following a single saline or morphine injection for D-cells (**B**) and H-cells (**C**). Planned contrasts with Bonferroni's correction identified several pairwise increases in the firing rate following single morphine injections, especially for initial firing rate for H-cells (* $p < 0.05$; ** $p < 0.01$; *** $p < 0.005$; # $p < 0.0001$). n.s., not significant; S-treated, saline-treated; M-treated, morphine-treated; 1-Day, 1 d cohort; sps, spikes/second.

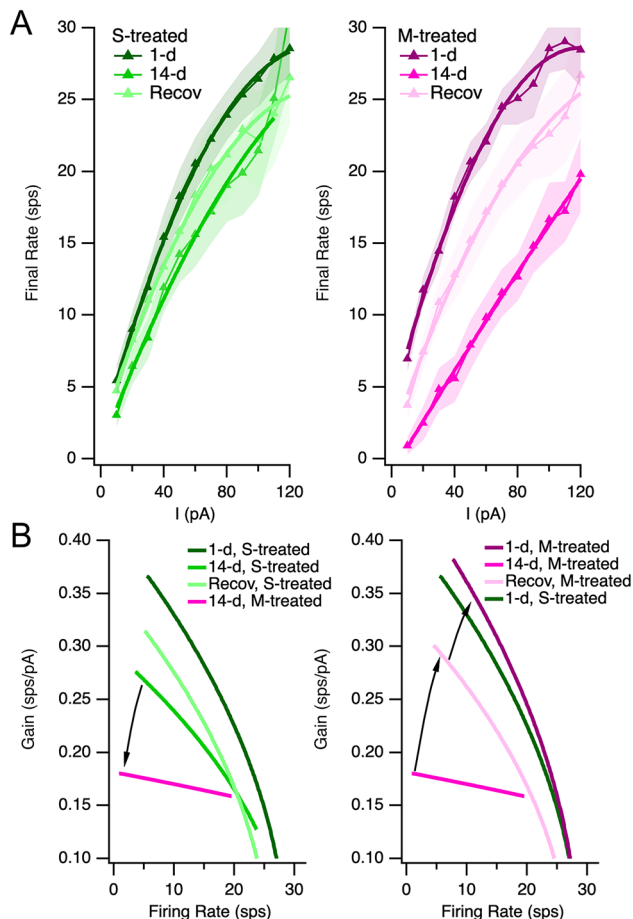


Figure 9. Repeated morphine treatment downscaled the gain of H-type orexin neurons. **A**, The final firing rate (mean $\pm$ SEM) versus current (I) curves for H-cells are superimposed for saline-treated cohorts of mice (left, S-treated, green symbols) and morphine-treated mice (right, M-treated, pink symbols). The 1 d cohort (1-d) is darkest, the 14 d cohort (14-d) is lighter, and the 4 week recovery cohort (Recov) is lightest. Corresponding solid lines are best-fit second-order polynomials. **B**, Comparison of the mean firing rate gain versus mean firing rate for each cohort. The left graph compares the gain–firing relation for morphine-treated H-cells from the 14 d cohort (14-d, M-treated) to those from the saline-treated H-cells from the 1 d (1-d), 14 d (14-d), and 4 week recovery (Recov.) cohorts (1-d, S-treated; 14-d, S-treated; Recov, S-treated). The downward arrow connects the gain–firing curve from saline-treated and morphine-treated cells from the 14 d cohort to illustrate the large downscaling produced by morphine treatment. Right graph compares the gain–firing relation for morphine-treated H-cells from the 14 d cohort (14-d, M-treated) to those from the morphine-treated H-cells from the other cohorts (1-d, M-treated; Recov, M-treated). The long upward arrow connects the curve from the morphine-treated 14 d cohort to that of the morphine-treated 4 week recovery cohort to illustrate the recovery from downscaling following 4 weeks of morphine abstinence. The shorter arrow connects the 4 week recovery cohort curve to the 1 d morphine cohort curve (1-d, M-treated) to illustrate that after 4 weeks of morphine abstinence, the gain–firing relation is still reduced. The curve from the saline-treated mice from the 1 d cohort (1-d, S-treated) is shown in each graph for reference.

the derivative of these best-fit polynomials and compared the gain curves by plotting them against their corresponding firing rates (Fig. 9B). In this representation, purely additive or subtractive changes to the F – I curve, like those resulting from shunting inhibition, which translate the F – I curve along the current axis, result in gain–firing rate curves that are superimposed (Chance et al., 2002). Rather than producing a subtractive effect, 2 weeks of morphine treatment resulted in a striking downscaling in firing gain. Compared with H-cells from mice treated with saline for 2 weeks, the lowest firing rate for neurons from morphine-

treated mice (K_0) was reduced by 76%, and their coefficients for the linear (K_1) and squared current terms (K_2) of the best-fit polynomial were reduced by 35 and 87%, respectively (Fig. 9B, left panel arrow). These reduced coefficients reflect the lower and nearly constant gain–firing curve for morphine-treated neurons. This is a divisive reduction in firing gain of $\sim 55\%$ for small inputs, but the magnitude of this gain reduction decreases at higher firing rates where it approaches the firing gain of H-cells from saline-treated mice at their highest firing rates. This predicts that H-cells from morphine-treated mice will have a reduced ability to encode input signals into firing rate changes and that the impairment will be worse for smaller inputs. This representation also illustrates the lower gain–firing relation for H-cells from the saline-treated 14 d cohort compared with the saline-treated H-cells from the 1 d cohort. As noted above, this is consistent with the repeated subcutaneous injections themselves partly attenuating the firing gain of H-type orexin neurons.

Following 4 weeks of recovery, neurons from morphine-treated mice have gain–firing rate relations that correspond well to that of cells recorded from saline-treated mice after 4 weeks of recovery. This indicates that the decreased encoding capability of H-type orexin neurons after chronic morphine exposure is mostly reversed following 4 weeks of morphine abstinence. Nevertheless, peak gain remains lower than that for neurons recorded after a single dose of morphine or saline (Fig. 9B, right, short arrow).

D- and H-type orexin neurons were intermingled throughout the orexin field

It has been proposed that the orexin cell field is organized into a functional topography (Harris and Aston-Jones, 2006) based, in part, on the finding that orexin neurons lateral to the fornix increase c-Fos labeling following the development of a conditioned place preference to morphine (Harris et al., 2007). We therefore examined the distribution of our sampled D- and H-cells with respect to the fornix. We plotted the locations of all neurons we identified from the 14 d, 4 week recovery, and 1 d cohorts onto four corresponding atlas pages (Franklin and Paxinos, 2008) from the bregma -1.58 to -1.94 mm according to their distances from the fornix and mammillothalamic tract (Fig. 10A). We then bisected the orexin field into medial and lateral parts with a line through the fornix to determine their distribution on either side. This showed that while $\sim 60\%$ of our recorded cells were lateral to the fornix, both cell types were distributed throughout the orexin field (Fig. 10B). A similar proportion of D- and H-cells were sampled on either side of the fornix, with $\sim 57\%$ D- and $\sim 43\%$ H-cells on both the medial (DMH/PFA) and lateral (LH) sides (Fig. 10C). When we examined the anterior–posterior distribution of D- and H-cells (Fig. 10D), we found that H-cells were enriched lateral to the fornix in the most rostral section while D-cells were enriched lateral to the fornix in the next two more posterior sections. Thus, while D- and H-cells are present throughout the orexin field, H-cells are over-represented in the most rostral LH, and D-cells are over-represented more caudally in the LH.

Discussion

Fourteen days of repeated morphine decreased the orexin-EGFP soma size, but did not decrease C_m or increase R_m . Instead, we found excitability of H-type orexin neurons was impaired while D-type neurons were entirely spared. A single morphine injection did not produce this impairment, and 4 weeks of passive

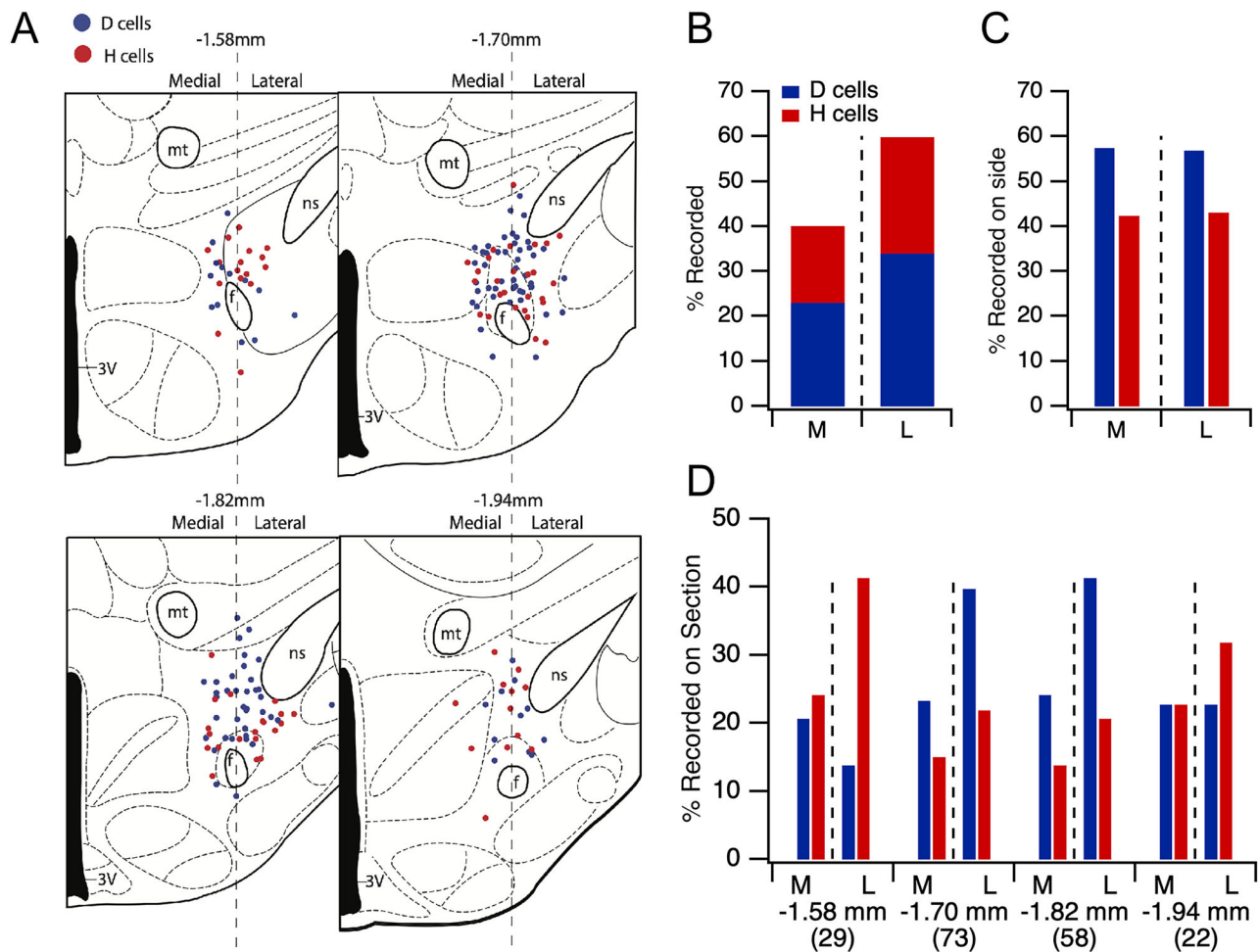


Figure 10. D- and H-type orexin neurons were intermingled throughout the orexin neuron field. **A**, Recorded D- (blue symbols) and H-cells (red symbols) were mapped onto four atlas sections spanning from -1.58 to -1.94 mm from the bregma. The dotted line bisects the fornix to divide each section into medial and lateral regions. **B**, The percentage of recorded cells ($n = 182$) medial (M) and lateral (L) to the fornix. **C**, The percentage of cells recorded on each side that were identified as D- or H-cells. **D**, Anterior to posterior distribution of D- and H-cells medial and lateral to the fornix as a percentage of cells in each section. The number of cells mapped to each section indicated in parentheses. 3V, third ventricle; mt, mammillothalamic tract; f, fornix; ns, nigrostriatal tract.

recovery reversed impaired excitability and the soma size decrease. Finally, D- and H-cells were distributed throughout the orexin cell field, but H-cells were enriched rostrally, while D-cells were enriched more caudally within the LH.

Somatic structural plasticity resulting from chronic morphine exposure

Chronic exposure to drugs of abuse produces well-documented structural changes in reward circuitry neurons (Robinson and Kolb, 2004; Russo et al., 2010). The effect of repeated morphine on the orexin soma size appears similar to its effect on the VTA DA neuron soma size although spontaneous firing is also increased in DA neurons (Sklaire-Tavron et al., 1996; Mazei-Robison et al., 2014; Simmons et al., 2019). The firing rate and soma size are tightly linked in VTA DA neurons, since augmenting firing decreased the soma size, while suppressing firing prevented morphine-induced soma size decreases (Mazei-Robison et al., 2011; Koo et al., 2012). Unlike VTA DA neurons, morphine directly inhibits $\sim 50\%$ of orexin neurons in vitro (Li and van den Pol, 2008), and repeated morphine did not increase their spontaneous firing in vitro, suggesting a different mechanism may be involved. Nevertheless, systemic morphine can induce sustained firing of putative orexin neurons in vivo (Thannickal et al., 2018), which may suffice to drive the soma size decrease in orexin

neurons if similar signaling pathways are engaged. Future experiments should determine if orexin neurons are regulated by such activity-dependent processes.

H- but not D-type orexin neurons showed impaired excitability following repeated morphine treatment

Changes in intrinsic excitability have been described throughout the reward system following both repeated noncontingent and contingent drug exposure (Kourrich et al., 2015; Hearing, 2019). Multiple mechanisms are involved and vary depending on cell type (Kourrich and Thomas, 2009; Kim et al., 2011) and conditions (Mu et al., 2010). The impaired excitability we found in H-cells included changes in spike generation that were consistent with attenuated voltage-gated sodium currents (increased rheobase, reduced spike amplitude, and rise-times that were exacerbated with sequential spikes). These effects are similar to changes reported in ventral subiculum neurons during amphetamine withdrawal (Cooper et al., 2003) and in medium spiny NAc neurons during cocaine withdrawal (Zhang et al., 1998). Numerous other currents have also been linked to impaired repetitive firing (irregular and slower firing) following chronic drug exposure (Ishikawa et al., 2009; Chen et al., 2013; Kourrich et al., 2013; Leyrer-Jackson et al., 2021) and are among the numerous candidates underlying impaired H-cell excitability.

Functional roles and hypothalamic distribution of orexin neurons

Selective excitability impairment of H-cells following repeated morphine may indicate that they are more engaged in reward processing than D-cells. Conversely, D-cells may be more engaged with regulating arousal since 6 h of sleep deprivation selectively increased their excitability (Briggs et al., 2019). Importantly, both findings support the idea that D- and H-cells are functionally specialized, as originally proposed based on differences in glucose sensing (Williams et al., 2008).

It has been proposed that LH orexin neurons lateral to the fornix regulate reward processing and preferentially project to the VTA and NAc, while PFA/DMH orexin neurons medial to the fornix regulate arousal and stress and preferentially project to LC, TMN, and LDT/PPT (Harris and Aston-Jones, 2006). However, D- and H-cells were intermixed throughout the entire orexin field, as previously observed (Williams et al., 2008). H-cells were enriched in the most rostral LH region, while D-cells were enriched more caudally in the LH. This implies that rostral LH orexin neurons expressing increased c-Fos to reward cues (Harris et al., 2005; Fragale et al., 2019; James et al., 2019) would be enriched in H-cells, while more caudal LH orexin neuron may be enriched in D-cells. Nevertheless, while orexin neurons projecting to the LC and/or TMN appear distinct from those projecting to the VTA and/or NAc, these neurons are intermingled without medial–lateral segregation (Iyer et al., 2018). Moreover, both D- and H-cells project to VTA and LC, but 80% of these projections were comprised of D-cells (González et al., 2012), raising the possibility that H-cells preferentially project elsewhere. Identifying those targets would be facilitated by molecular markers, but profiling has yet to distinguish D- or H-cells (Yelin-Bekerman et al., 2015; Mickelsen et al., 2017, 2019; Sagi et al., 2021).

Impaired encoding and decoding by H-cells following chronic morphine

Increased firing rate variability and the downscaling of firing gain diminishes the ability of H-cells to encode synaptic inputs into firing rate changes, with the largest impairment occurring for the smallest inputs (Fig. 9). Orexin neurons normally encode both rapid sensory-motor signals (Milevskiy et al., 2005; Hassani et al., 2016; Karnani et al., 2020) and slower signals related to arousal, metabolism, and reward (Lee et al., 2005; Milevskiy et al., 2005; Williams et al., 2008; Karnani et al., 2011). Since both initial and steady-state firing gain was reduced, our findings suggest the fidelity of both fast and slower signals would be degraded following repeated morphine.

Impaired firing would also be expected to attenuate the ability of H-cells to release their coexpressed neuropeptides (for review, see Ma et al., 2018). Orexin and dynorphin appear copackaged into dense-core vesicles (Muschamp et al., 2014) which require higher frequency and longer spike trains for exocytosis than do small vesicles (van den Pol, 2012). Although studies are limited (Sears et al., 2013; Schöne et al., 2014), orexin fibers innervating histaminergic neurons required prolonged firing at >10 Hz to detect an orexin-dependent slow EPSP, while AMPA EPSPs followed at all frequencies (Schöne et al., 2014). Following repeated morphine, H-cells required about twice the input current to achieve firing rates (>10 Hz) consistent with peptide release (Fig. 6; ~30 pA saline-treated vs ~60 pA morphine-treated mice). Since orexin and dynorphin have opposing actions whose relative strengths differ by target (Li and van den Pol, 2006; Baimel et al., 2017), impairment of H-cell peptide release could differentially impact their targets if D- and H-cells have divergent connections. This may be

especially relevant for connections with VTA DA neurons where optical activation of orexin/dynorphin afferents enhances excitation of a majority of lateral shell-projecting neurons but inhibits activity in a majority of medial shell- and amygdala-projecting neurons (Mohammadkhani et al., 2024). If D- and H-cells provide differential innervation, impaired peptide release from H-cells could alter the balance of activity in these DA output circuits which may promote motivation for drug seeking.

Orexin system adaptations following chronic morphine and cocaine, including increased neuron numbers (Thannickal et al., 2018; James et al., 2019; Pantazis et al., 2020; Fragale et al., 2021) with enhanced innervation of LC (McGregor et al., 2022) and VTA (McGregor et al., 2024), are consistent with greater peptide biosynthesis and release. Ox1R signaling sustains motivation for cocaine and fentanyl consumption without altering their reward value (Bernstein et al., 2018; Fragale et al., 2019; James et al., 2019). Intriguingly, enhanced motivation for drug consumption resulting from the use of an intermittent access behavioral economics paradigm further augmented the numbers of orexin neurons and increased the sensitivity of the drug demand curves to the Ox1R antagonist SB-334867, requiring only 30% of the dose needed under lower motivation conditions to attenuate demand (James et al., 2019; Fragale et al., 2021). This was interpreted to mean that high motivation drug seeking had become more orexin-dependent, perhaps due to greater abundance and release of orexin peptide (James and Aston-Jones, 2022). Although we did not measure the orexin cell number or peptide levels in this study, our findings may suggest an alternative interpretation: Perhaps the augmented immunostaining of orexin somata and terminals, representing the “orexin reserve,” results from peptide accumulation in H-cells from decreased release due to impaired excitability. Decreased release might also explain the greater efficacy of antagonists under conditions of high motivation, since there would be lower orexin receptor occupancy and less competition for antagonist. Future research should address this possibility by measuring peptide release, excitation-release coupling, and orexin receptor efficacy.

In summary, our findings demonstrate that daily morphine exposure produces a selective, accumulating, and reversible impairment of intrinsic excitability in H-type orexin neurons while sparing the D-cells recorded from the same slices. Morphine treatment also decreased the orexin soma size, which, like H-cell excitability, recovered over 4 weeks of passive recovery time. Our findings reinforce the idea that orexin neurons with D- and H-response properties are functionally distinct and suggest that the ability of H-cells to encode inputs and transform them into orexin and dynorphin release becomes impaired with morphine dependence. We speculate that these changes contribute to dysregulating the “orexin reserve” and may promote drug seeking.

References

- Adamantidis AR, Zhang F, Aravanis AM, Deisseroth K, de Lecea L (2007) Neural substrates of awakening probed with optogenetic control of hypocretin neurons. *Nature* 450:420–424.
- Baimel C, Borgland SL (2015) Orexin signaling in the VTA gates morphine-induced synaptic plasticity. *J Neurosci* 35:7295–7303.
- Baimel C, Lau BK, Qiao M, Borgland SL (2017) Projection-target-defined effects of orexin and dynorphin on VTA dopamine neurons. *Cell Rep* 18:1346–1355.
- Becker JB (2016) Sex differences in addiction. *Dialogues Clin Neurosci* 18:395–402.
- Bernstein DL, Badve PS, Barson JR, Bass CE, Espana RA (2018) Hypocretin receptor 1 knockdown in the ventral tegmental area attenuates mesolimbic dopamine signaling and reduces motivation for cocaine. *Addict Biol* 23:1032–1045.

- Borgland SL, Taha SA, Sarti F, Fields HL, Bonci A (2006) Orexin A in the VTA is critical for the induction of synaptic plasticity and behavioral sensitization to cocaine. *Neuron* 49:589–601.
- Boutrel B, Kenny PJ, Specio SE, Martin-Fardon R, Markou A, Koob GF, de Lecea L (2005) Role for hypocretin in mediating stress-induced reinstatement of cocaine-seeking behavior. *Proc Natl Acad Sci U S A* 102:19168–19173.
- Briggs C, Hirasawa M, Semba K (2018) Sleep deprivation distinctly alters glutamate transporter 1 apposition and excitatory transmission to orexin and MCH neurons. *J Neurosci* 38:2505–2518.
- Briggs C, Bowes SC, Semba K, Hirasawa M (2019) Sleep deprivation-induced pre- and postsynaptic modulation of orexin neurons. *Neuropharmacology* 154:50–60.
- Brown RM, Khoo SY, Lawrence AJ (2013) Central orexin (hypocretin) 2 receptor antagonism reduces ethanol self-administration, but not cue-conditioned ethanol-seeking, in ethanol-preferring rats. *Int J Neuropsychopharmacol* 16:2067–2079.
- Brownell SE, Conti B (2010) Age- and gender-specific changes of hypocretin immunopositive neurons in C57Bl/6 mice. *Neurosci Lett* 472:29–32.
- Chance FS, Abbott LF, Reyes AD (2002) Gain modulation from background synaptic input. *Neuron* 35:773–782.
- Chemelli RM, et al. (1999) Narcolepsy in orexin knockout mice: molecular genetics of sleep regulation. *Cell* 98:437–451.
- Chen BT, Yau HJ, Hatch C, Kusumoto-Yoshida I, Cho SL, Hopf FW, Bonci A (2013) Rescuing cocaine-induced prefrontal cortex hypoactivity prevents compulsive cocaine seeking. *Nature* 496:359–362.
- Cooper DC, Moore SJ, Staff NP, Spruston N (2003) Psychostimulant-induced plasticity of intrinsic neuronal excitability in ventral subiculum. *J Neurosci* 23:9937–9946.
- Cvetkovic-Lopes V, Eggermann E, Uschakov A, Grivel J, Bayer L, Jones BE, Serafin M, Mühlethaler M (2010) Rat hypocretin/orexin neurons are maintained in a depolarized state by TRPC channels. *PLoS One* 5:e15673.
- Dawson M, Terstege DJ, Jamani N, Tsutsui M, Pavlov D, Bugescu R, Epp JR, Leininger GM, Sargin D (2023) Hypocretin/orexin neurons encode social discrimination and exhibit a sex-dependent necessity for social interaction. *Cell Rep* 42:112815.
- de Lecea L, et al. (1998) The hypocretins: hypothalamus-specific peptides with neuroexcitatory activity. *Proc Natl Acad Sci U S A* 95:322–327.
- Eggermann E, Bayer L, Serafin M, Saint-Mieux B, Bernheim L, Machard D, Jones BE, Mühlethaler M (2003) The wake-promoting hypocretin-orexin neurons are in an intrinsic state of membrane depolarization. *J Neurosci* 23:1557–1562.
- Erisir A, Lau D, Rudy B, Leonard CS (1999) Function of specific K(+) channels in sustained high-frequency firing of fast-spiking neocortical interneurons. *J Neurophysiol* 82:2476–2489.
- Fragale JE, Pantazis CB, James MH, Aston-Jones G (2019) The role of orexin-1 receptor signaling in demand for the opioid fentanyl. *Neuropsychopharmacology* 44:1690–1697.
- Fragale JE, James MH, Aston-Jones G (2021) Intermittent self-administration of fentanyl induces a multifaceted addiction state associated with persistent changes in the orexin system. *Addict Biol* 26:e12946.
- Franklin K, Paxinos G (2008) *The mouse brain in stereotaxic coordinates*, Ed 3. Amsterdam: Academic Press.
- Gentet LJ, Stuart GJ, Clements JD (2000) Direct measurement of specific membrane capacitance in neurons. *Biophys J* 79:314–320.
- Gentile TA, Simmons SJ, Barker DJ, Shaw JK, Espana RA, Muschamp JW (2018) Suvorexant, an orexin/hypocretin receptor antagonist, attenuates motivational and hedonic properties of cocaine. *Addict Biol* 23:247–255.
- Georgescu D, Zachariou V, Barrot M, Mieda M, Willie JT, Eisch AJ, Yanagisawa M, Nestler EJ, DiLeone RJ (2003) Involvement of the lateral hypothalamic peptide orexin in morphine dependence and withdrawal. *J Neurosci* 23:3106–3111.
- González JA, Jensen LT, Fugger L, Burdakov D (2012) Convergent inputs from electrically and topographically distinct orexin cells to locus coeruleus and ventral tegmental area. *Eur J Neurosci* 35:1426–1432.
- Harris GC, Aston-Jones G (2006) Arousal and reward: a dichotomy in orexin function. *Trends Neurosci* 29:571–577.
- Harris GC, Wimmer M, Aston-Jones G (2005) A role for lateral hypothalamic orexin neurons in reward seeking. *Nature* 437:556–559.
- Harris GC, Wimmer M, Randall-Thompson JF, Aston-Jones G (2007) Lateral hypothalamic orexin neurons are critically involved in learning to associate an environment with morphine reward. *Behav Brain Res* 183:43–51.
- Hassani OK, Krause MR, Mainville L, Cordova CA, Jones BE (2016) Orexin neurons respond differentially to auditory cues associated with appetitive versus aversive outcomes. *J Neurosci* 36:1747–1757.
- Hearing M (2019) Prefrontal-accumbens opioid plasticity: implications for relapse and dependence. *Pharmacol Res* 139:158–165.
- Horvath TL, Gao XB (2005) Input organization and plasticity of hypocretin neurons: possible clues to obesity's association with insomnia. *Cell Metab* 1:279–286.
- Hutcheson DM, Quarta D, Halbout B, Rigal A, Valerio E, Heidbreder C (2011) Orexin-1 receptor antagonist SB-334867 reduces the acquisition and expression of cocaine-conditioned reinforcement and the expression of amphetamine-conditioned reward. *Behav Pharmacol* 22:173–181.
- Ishikawa M, Mu P, Moyer JT, Wolf JA, Quock RM, Davies NM, Hu XT, Schluter OM, Dong Y (2009) Homeostatic synapse-driven membrane plasticity in nucleus accumbens neurons. *J Neurosci* 29:5820–5831.
- Iyer M, Essner RA, Klingenberg B, Carter ME (2018) Identification of discrete, intermingled hypocretin neuronal populations. *J Comp Neurol* 526:2937–2954.
- James MH, Aston-Jones G (2020) Introduction to the special issue: “making orexin-based therapies for addiction a reality: what are the steps from here?”. *Brain Res* 1731:146665.
- James MH, Aston-Jones G (2022) Orexin reserve: a mechanistic framework for the role of orexins (hypocretins) in addiction. *Biol Psychiatry* 92:836–844.
- James MH, Campbell EJ, Walker FR, Smith DW, Richardson HN, Hodgson DM, Dayas CV (2014) Exercise reverses the effects of early life stress on orexin cell reactivity in male but not female rats. *Front Behav Neurosci* 8:244.
- James MH, Stopper CM, Zimmer BA, Koll NE, Bowrey HE, Aston-Jones G (2019) Increased number and activity of a lateral subpopulation of hypothalamic orexin/hypocretin neurons underlies the expression of an addicted state in rats. *Biol Psychiatry* 85:925–935.
- Kalogiannis M, Hsu E, Willie JT, Chemelli RM, Kisanuki Y, Yanagisawa M, Leonard CS (2011) Cholinergic modulation of narcoleptic attacks in double orexin receptor knockout mice. *PLoS One* 6:1–14.
- Karnani MM, Apergis-Schoute J, Adamantidis A, Jensen LT, de Lecea L, Fugger L, Burdakov D (2011) Activation of central orexin/hypocretin neurons by dietary amino acids. *Neuron* 72:616–629.
- Karnani MM, Schone C, Bracey EF, Gonzalez JA, Viskaitis P, Li HT, Adamantidis A, Burdakov D (2020) Role of spontaneous and sensory orexin network dynamics in rapid locomotion initiation. *Prog Neurobiol* 187:101771.
- Kim J, Park BH, Lee JH, Park SK, Kim JH (2011) Cell type-specific alterations in the nucleus accumbens by repeated exposures to cocaine. *Biol Psychiatry* 69:1026–1034.
- Koo JW, et al. (2012) BDNF is a negative modulator of morphine action. *Science* 338:124–128.
- Kourrich S, Thomas MJ (2009) Similar neurons, opposite adaptations: psychostimulant experience differentially alters firing properties in accumbens core versus shell. *J Neurosci* 29:12275–12283.
- Kourrich S, Hayashi T, Chuang JY, Tsai SY, Su TP, Bonci A (2013) Dynamic interaction between sigma-1 receptor and Kv1.2 shapes neuronal and behavioral responses to cocaine. *Cell* 152:236–247.
- Kourrich S, Calu DJ, Bonci A (2015) Intrinsic plasticity: an emerging player in addiction. *Nat Rev Neurosci* 16:173–184.
- Kukkonen JP, Leonard CS (2014) Orexin/hypocretin receptor signalling cascades. *Br J Pharmacol* 171:314–331.
- Lee MG, Hassani OK, Jones BE (2005) Discharge of identified orexin/hypocretin neurons across the sleep-waking cycle. *J Neurosci* 25:6716–6720.
- Leonard CS, Kukkonen JP (2014) Orexin/hypocretin receptor signalling: a functional perspective. *Br J Pharmacol* 171:294–313.
- Leyrer-Jackson JM, Hood LE, Olive MF (2021) Drugs of abuse differentially alter the neuronal excitability of prefrontal layer V pyramidal cell subtypes. *Front Cell Neurosci* 15:703655.
- Li SB, Giardino WJ, de Lecea L (2017) Hypocretins and arousal. *Curr Top Behav Neurosci* 33:93–104.
- Li Y, van den Pol AN (2006) Differential target-dependent actions of coexpressed inhibitory dynorphin and excitatory hypocretin/orexin neuropeptides. *J Neurosci* 26:13037–13047.
- Li Y, van den Pol AN (2008) Mu-opioid receptor-mediated depression of the hypothalamic hypocretin/orexin arousal system. *J Neurosci* 28:2814–2819.
- Li Y, Gao XB, Sakurai T, van den Pol AN (2002) Hypocretin/orexin excites hypocretin neurons via a local glutamate neuron—a potential mechanism for orchestrating the hypothalamic arousal system. *Neuron* 36:1169–1181.

- Lin L, Faraco J, Li R, Kadotani H, Rogers W, Lin X, Qiu X, de Jong PJ, Nishino S, Mignot E (1999) The sleep disorder canine narcolepsy is caused by a mutation in the hypocretin (orexin) receptor 2 gene. *Cell* 98:365–376.
- Ma S, Hangya B, Leonard CS, Wisden W, Gundlach AL (2018) Dual-transmitter systems regulating arousal, attention, learning and memory. *Neurosci Biobehav Rev* 85:21–33.
- Mahler SV, Moorman DE, Smith RJ, James MH, Aston-Jones G (2014) Motivational activation: a unifying hypothesis of orexin/hypocretin function. *Nat Neurosci* 17:1298–1303.
- Matovic S, et al. (2020) Neuronal hypertrophy dampens neuronal intrinsic excitability and stress responsiveness during chronic stress. *J Physiol* 598:2757–2773.
- Mazei-Robison MS, et al. (2011) Role for mTOR signaling and neuronal activity in morphine-induced adaptations in ventral tegmental area dopamine neurons. *Neuron* 72:977–990.
- Mazei-Robison MS, Appasani R, Edwards S, Wee S, Taylor SR, Picciotto MR, Koob GF, Nestler EJ (2014) Self-administration of ethanol, cocaine, or nicotine does not decrease the soma size of ventral tegmental area dopamine neurons. *PLoS One* 9:e95962.
- McGregor R, Wu MF, Barber G, Ramanathan L, Siegel JM (2011) Highly specific role of hypocretin (orexin) neurons: differential activation as a function of diurnal phase, operant reinforcement versus operant avoidance and light level. *J Neurosci* 31:15455–15467.
- McGregor R, Wu MF, Holmes B, Lam HA, Maidment NT, Gera J, Yamanaka A, Siegel JM (2022) Hypocretin/orexin interactions with norepinephrine contribute to the opiate withdrawal syndrome. *J Neurosci* 42:255–263.
- McGregor R, Wu MF, Thannickal TC, Li S, Siegel JM (2024) Opioid-induced neuroanatomical, microglial and behavioral changes are blocked by suvorexant without diminishing opioid analgesia. *Nat Mental Health* 2: 1018–1031.
- Mickelsen LE, Kolling FW, Chimileski BR, Fujita A, Norris C, Chen K, Nelson CE, Jackson AC (2017) Neurochemical heterogeneity among lateral hypothalamic hypocretin/orexin and melanin-concentrating hormone neurons identified through single-cell gene expression analysis. *eNeuro* 4: ENEURO.0013-17.2017.
- Mickelsen LE, Bolisetty M, Chimileski BR, Fujita A, Beltrami EJ, Costanzo JT, Naparstek JR, Robson P, Jackson AC (2019) Single-cell transcriptomic analysis of the lateral hypothalamic area reveals molecularly distinct populations of inhibitory and excitatory neurons. *Nat Neurosci* 22:642–656.
- Milevskiy BY, Kiyashchenko LI, Siegel JM (2005) Behavioral correlates of activity in identified hypocretin/orexin neurons. *Neuron* 46:787–798.
- Mohammadkhani A, Qiao M, Borgland SL (2024) Distinct neuromodulatory effects of endogenous orexin and dynorphin corelease on projection-defined ventral tegmental dopamine neurons. *J Neurosci* 44:e0682242024.
- Mu P, Moyer JT, Ishikawa M, Zhang Y, Panksepp J, Sorg BA, Schluter OM, Dong Y (2010) Exposure to cocaine dynamically regulates the intrinsic membrane excitability of nucleus accumbens neurons. *J Neurosci* 30: 3689–3699.
- Muschamp JW, Hollander JA, Thompson JL, Voren G, Hassinger LC, Onvani S, Kamenecka TM, Borgland SL, Kenny PJ, Carlezon WA (2014) Hypocretin (orexin) facilitates reward by attenuating the anti-reward effects of its cotransmitter dynorphin in ventral tegmental area. *Proc Natl Acad Sci U S A* 111:E1648–E1655.
- Ohno K, Sakurai T (2008) Orexin neuronal circuitry: role in the regulation of sleep and wakefulness. *Front Neuroendocrinol* 29:70–87.
- Pantazis CB, James MH, Bentzley BS, Aston-Jones G (2020) The number of lateral hypothalamus orexin/hypocretin neurons contributes to individual differences in cocaine demand. *Addict Biol* 25:e12795.
- Peyron C, Tighe D, Van den Pol AN, De Lecea L, Heller C, Sutcliffe JG, Kilduff TS (1998) Neurons containing hypocretin orexin project to multiple neuronal systems. *J Neurosci* 18:9996–10015.
- Peyron C, et al. (2000) A mutation in a case of early onset narcolepsy and a generalized absence of hypocretin peptides in human narcoleptic brains. *Nat Med* 6:991–997.
- Puritz C, Ness-Cohn E, Braun R (2023) Fasano.franceschini.test: an implementation of a multivariate KS test in R. *R J* 15:159–171.
- Rao Y, Liu ZW, Borok E, Rabenstein RL, Shanabrough M, Lu M, Picciotto MR, Horvath TL, Gao XB (2007) Prolonged wakefulness induces experience-dependent synaptic plasticity in mouse hypocretin/orexin neurons. *J Clin Invest* 117:4022–4033.
- Rao Y, et al. (2013) Repeated in vivo exposure of cocaine induces long-lasting synaptic plasticity in hypocretin/orexin-producing neurons in the lateral hypothalamus in mice. *J Physiol* 591:1951–1966.
- Robinson TE, Kolb B (2004) Structural plasticity associated with exposure to drugs of abuse. *Neuropharmacology* 47:33–46.
- Russo SJ, Dietz DM, Dumitriu D, Morrison JH, Malenka RC, Nestler EJ (2010) The addicted synapse: mechanisms of synaptic and structural plasticity in nucleus accumbens. *Trends Neurosci* 33:267–276.
- Sagi D, de Lecea L, Appelbaum L (2021) Heterogeneity of hypocretin/orexin neurons. *Front Neurol Neurosci* 45:61–74.
- Sakurai T, et al. (1998) Orexins and orexin receptors- a family of hypothalamic neuropeptides and G protein coupled receptors that regulate feeding behavior. *Cell* 92:573–585.
- Scammell TE (2003) The neurobiology, diagnosis, and treatment of narcolepsy. *Ann Neurol* 53:154–166.
- Schindelin J, et al. (2012) Fiji: an open-source platform for biological-image analysis. *Nat Methods* 9:676–682.
- Schöne C, Venner A, Knowles D, Karnani MM, Burdakov D (2011) Dichotomous cellular properties of mouse orexin/hypocretin neurons. *J Physiol* 589:2767–2779.
- Schöne C, Apergis-Schoute J, Sakurai T, Adamantidis A, Burdakov D (2014) Coreleased orexin and glutamate evoke nonredundant sleep outputs and computations in histamine neurons. *Cell Rep* 7:697–704.
- Sears RM, Fink AE, Wigstrand MB, Farb CR, de Lecea L, Ledoux JE (2013) Orexin/hypocretin system modulates amygdala-dependent threat learning through the locus coeruleus. *Proc Natl Acad Sci U S A* 110:20260–20265.
- Seifinejad A, et al. (2023) Epigenetic silencing of selected hypothalamic neuropeptides in narcolepsy with cataplexy. *Proc Natl Acad Sci U S A* 120: e222091120.
- Shaw JK, Ferris MJ, Locke JL, Brodnik ZD, Jones SR, Espana RA (2017) Hypocretin/orexin knock-out mice display disrupted behavioral and dopamine responses to cocaine. *Addict Biol* 22:1695–1705.
- Simmons SC, Wheeler K, Mazei-Robison MS (2019) Determination of circuit-specific morphological adaptations in ventral tegmental area dopamine neurons by chronic morphine. *Mol Brain* 12:10.
- Sklair-Tavron L, Shi WX, Lane SB, Harris HW, Bunney BS, Nestler EJ (1996) Chronic morphine induces visible changes in the morphology of mesolimbic dopamine neurons. *Proc Natl Acad Sci U S A* 93:11202–11207.
- Stuber GD, Wise RA (2016) Lateral hypothalamic circuits for feeding and reward. *Nat Neurosci* 19:198–205.
- Tan Y, et al. (2020) Impaired hypocretin/orexin system alters responses to salient stimuli in obese male mice. *J Clin Invest* 130:4985–4998.
- Taylor AL (2012) What we talk about when we talk about capacitance measured with the voltage-clamp step method. *J Comput Neurosci* 32:167–175.
- Thannickal TC, Moore RY, Nienhuis R, Ramanathan L, Gulyani S, Aldrich M, Cornford M, Siegel JM (2000) Reduced number of hypocretin neurons in human narcolepsy. *Neuron* 27:469–474.
- Thannickal TC, et al. (2018) Opiates increase the number of hypocretin-producing cells in human and mouse brain and reverse cataplexy in a mouse model of narcolepsy. *Sci Transl Med* 10:eaa04953.
- Ting JT, Daigle TL, Chen Q, Feng G (2014) Acute brain slice methods for adult and aging animals: application of targeted patch clamp analysis and optogenetics. *Methods Mol Biol* 1183:221–242.
- Towers EB, Williams IL, Qillawala EI, Rissman EF, Lynch WJ (2023) Sex/gender differences in the time-course for the development of substance use disorder: a focus on the telescoping effect. *Pharmacol Rev* 75:217–249.
- van den Pol AN (2012) Neuropeptide transmission in brain circuits. *Neuron* 76:98–115.
- Williams RH, Alexopoulos H, Jensen LT, Fugger L, Burdakov D (2008) Adaptive sugar sensors in hypothalamic feeding circuits. *Proc Natl Acad Sci U S A* 105:11975–11980.
- Willie JT, et al. (2003) 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.
- Yamanaka A, et al. (2003) Hypothalamic orexin neurons regulate arousal according to energy balance in mice. *Neuron* 38:701–713.
- Yelin-Bekerman L, Elbaz I, Diber A, Dahary D, Gibbs-Bar L, Alon S, Lerer-Goldshtein T, Appelbaum L (2015) Hypocretin neuron-specific transcriptome profiling identifies the sleep modulator Kcnh4a. *Elife* 4: e08638.
- Zhang XF, Hu XT, White FJ (1998) Whole-cell plasticity in cocaine withdrawal: reduced sodium currents in nucleus accumbens neurons. *J Neurosci* 18:488–498.