# Cell

# **Clock-Generated Temporal Codes Determine Synaptic Plasticity to Control Sleep**

# **Graphical Abstract**



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# In Brief

Temporal patterns of *Drosophila* clock neuron firing are sufficient to drive synaptic plasticity, impacting neuronal function and behavior.

# **Highlights**

- The temporal pattern of clock neuron firing controls sleep quality in *Drosophila*
- Cyclical ionic fluxes drive changes in spike waveform to generate temporal codes
- These temporal codes are transformed to rate codes in a downstream arousal circuit
- This transformation is via a temporal pattern-dependent form of synaptic plasticity





# Clock-Generated Temporal Codes Determine Synaptic Plasticity to Control Sleep

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# SUMMARY

Neurons use two main schemes to encode information: rate coding (frequency of firing) and temporal coding (timing or pattern of firing). While the importance of rate coding is well established, it remains controversial whether temporal codes alone are sufficient for controlling behavior. Moreover, the molecular mechanisms underlying the generation of specific temporal codes are enigmatic. Here, we show in Drosophila clock neurons that distinct temporal spike patterns, dissociated from changes in firing rate, encode time-dependent arousal and regulate sleep. From a large-scale genetic screen, we identify the molecular pathways mediating the circadian-dependent changes in ionic flux and spike morphology that rhythmically modulate spike timing. Remarkably, the daytime spiking pattern alone is sufficient to drive plasticity in downstream arousal neurons, leading to increased firing of these cells. These findings demonstrate a causal role for temporal coding in behavior and define a form of synaptic plasticity triggered solely by temporal spike patterns.

# INTRODUCTION

Understanding how the brain represents and processes information is a fundamental goal of neuroscience research. For the first half of the 20<sup>th</sup> century, the dominant neural coding model postulated that simple action potential (spike) counts in the relevant time window encode information about the environment or internal states (i.e., the rate-coding model) (Adrian and Zotterman, 1926a, 1926b; Stein, 1967). However, it has long been recognized that neural coding schemes using temporal codes (timing and/or pattern of spiking) would be computationally more powerful than traditional rate codes (MacKay and McCulloch, 1952). In contrast to rate coding, which comprises a singular mechanism, temporal coding encompasses a diverse repertoire of coding schemes in individual or groups of neurons, ranging from latency to first spike to synchronization of oscillatory activity between spatially segregated neuronal populations (Rieke

A wide variety of temporal codes have now been observed to correlate with specific external stimuli in different settings, including sensory systems, hippocampal place cells, and neocortical circuits (Gollisch and Meister, 2008; Gray et al., 1989; Laurent and Davidowitz, 1994; Skaggs et al., 1996). However, the assessment of whether and how temporal codes embody neurobiologically relevant information is complicated by multiple factors, such as the concomitant presence of changes in firing rate, integration of spatial with temporal information, and the requirement for multiple interacting brain regions in the regulation of behavior (Baranauskas, 2015; Panzeri et al., 2010). The rigorous demonstration of a causal role for temporal codes in representing biological information requires the fulfillment of three criteria (Chong and Rinberg, 2018; Panzeri et al., 2017). First, the temporal code should exist under native conditions or be elicited by naturalistic stimuli. Second, as articulated in the "reader-actuator" model proposed by Buzsáki (Buzsáki, 2010), a meaningful neural code should trigger a distinct response in the downstream neural circuit. Third, the temporal code should have physiological significance and be utilized by the brain to inform behavioral choices (Miesenböck, 2011; Panzeri et al., 2017).

While many studies have identified temporal codes occurring in response to naturally occurring sensory stimuli (Panzeri et al., 2010; Uchida et al., 2014), relatively few studies have shown that induction of specific temporal codes alter the firing of target neural circuits or affect behavior. For instance, in olfactory sensory neurons in mammals, varying the timing of firing relative to other neurons or the sniff cycle impacts firing of downstream neurons (Haddad et al., 2013; Smear et al., 2011). However, other studies have found that temporal coding in olfactory, visual, and somatosensory systems had no effect on the activity of target neurons or behavioral readouts (Blumhagen et al., 2011; Histed and Maunsell, 2014; O'Connor et al., 2013). Thus, the functional relevance for temporal coding alone to represent information about the environment and internal states remains controversial. Moreover, the molecular mechanisms that underlie the generation of different temporal codes within a neural circuit are largely unknown.

Here, in the clock neuron network in Drosophila, we demonstrate the presence of naturally occurring temporal spiking patterns associated with daytime versus nighttime and find that the cycling of these patterns depends on the core clock and wide awake (wake), a recently identified clock output gene required for circadian regulation of sleep (Liu et al., 2014). Using optogenetic approaches in vivo, we show that these distinct patterns of clock neuron firing, in the absence of changes in firing rate, serve as a temporal code to signify time-dependent arousal and directly impact sleep behavior. From a large-scale forward genetic screen, we identify the molecular mechanisms underlying the generation of these clock-dependent temporal codes. We then use electrophysiological and computational analyses to delineate the biophysical processes that rhythmically shape spike morphology and tune the firing patterns of these clock neurons. Remarkably, we demonstrate that the temporal spiking pattern alone drives neural plastic changes, which mediate the transformation of temporal codes in clock neurons to increased firing of a downstream arousal circuit. Together, our data demonstrate a causal role for temporal coding in behavior that is mediated by a distinct form of synaptic plasticity specifically triggered by the pattern of neural spiking.

# RESULTS

# Temporal Regulation of Sleep Quality Is Dependent on CLOCK and WIDE AWAKE

The circadian regulation of physiology and behavior is achieved, in part, by modulating the excitability and activity of clock neurons (Cao and Nitabach, 2008; Flourakis et al., 2015; Liu et al., 1997, 2014). However, studies to date have focused on rate coding of clock neurons, and it is unclear whether the pattern or timing of clock neuron firing is functionally relevant for behavior. To address this question, we focused on the circadian clock network in *Drosophila* and on the posterior dorsal neuron 1 (DN1p) cluster in particular; these cells have been implicated in regulating sleep/wake behavior (Guo et al., 2016; Kunst et al., 2014), and they directly project to a defined arousal circuit (Dilp2+ neurons of the pars intercerebralis) (Barber et al., 2016), thus allowing for detailed analyses of behavioral outputs and circuit signaling mechanisms.

In order to analyze temporal patterns of spike trains, we performed recordings of the DN1p neurons using perforated patch-clamp technique to enhance stability of the recordings by reducing "washout" of DN1p intracellular contents. DN1p neurons exhibited irregular firing during the mid-day (ZT6-8, Zeitgeber Time 6-8), but regular firing at mid-night (ZT18-20). This cycling of the regularity of spike firing was absent in the core clock mutant *Clk*<sup>*irk*</sup> and with loss of *wake*, which we have previously shown impairs circadian-dependent cycling of clock neuron firing rate (Liu et al., 2014) (Figures 1A-1F and S1A-S1D and Table S1) (see STAR Methods for discussion of measures of irregularity). Strikingly, in contrast to the change in spike-timing pattern, the spontaneous and evoked mean firing rates (MFRs) of DN1ps did not differ between day (ZT6-8) and night (ZT18-20) (Figures 1C and S1E).

Because differences in spiking patterns of individual cells within the DN1p cluster could influence analyses of temporal coding, we performed dual patch-clamp recordings. We found clear evidence of both electrical coupling and spike-timing synchronization between pairs of DN1p neurons (Figures S1F-S1H), suggesting that our recordings from single cells accurately reflect the spiking patterns of the group of neurons being studied. In addition, because bursting can represent a form of temporal coding, we asked whether bursting occurred in these neurons at mid-day versus mid-night. As shown in Figure S1I, bursting was not observed in DN1ps at ZT6-8 or ZT18-20 but was observed at ZT0-2. In contrast, bursting was observed in DN1ps at both ZT6-8 and ZT18-20 in Clk and wake mutants (Figure S1I), consistent with the notion that additional sleep/wake phenotypes are observed in these mutants. We also assessed the relative contributions of synaptic versus intrinsic mechanisms underlying the generation of the regular nighttime spike train in the DN1ps (Figures S1J-S1N). Application of synaptic blockers did not significantly alter MFR or local (LV and CV<sub>2</sub>) or global (CV) measures of irregularity, suggesting that CLK- and WAKE-dependent generation of regular nighttime spike trains in these cells largely occurs in a cell-autonomous manner. Similar findings regarding the timing and rate of spike firing were obtained in another clock neuron cluster, the large ventrolateral neurons (I-LNvs) (Figures S2A-S2J). Thus, the Drosophila circadian network presented an unusual opportunity to dissect the role of intrinsic temporal spike patterns, uncoupled from rate coding, in behavior and synaptic signaling.

We next examined the relationship between this cycling pattern of spike timing and sleep behavior. Sleep amount did not differ between mid-day (ZT6–8) or mid-night (ZT18–20) periods in control flies. In contrast, sleep quality, as measured by sleep consolidation (sleep bout duration and number), brief awakenings, and arousal threshold, was greater during the night compared to the day (Figures 1G–1K and S2K). Similar findings were obtained under constant darkness conditions (D:D) (Figures S2L–S2P). This cycling of sleep quality was dependent on both CLK and WAKE (Figures 1G–1K and S2K), indicating that it is under circadian control. Together, these data suggest that CLK- and WAKE-dependent temporal coding in the DN1ps is associated with cycling of sleep quality between the day and the night.

# Optogenetic Activation of DN1ps Using Different Temporal Spike Patterns Regulates Sleep Quality

To demonstrate that the different patterns of spike firing observed in the DN1ps directly control sleep quality, we developed an optogenetic/imaging preparation. In these assays, a single fly expressing CsChrimson (Klapoetke et al., 2014) in DN1ps is tethered and locomotor activity assessed using a frame-subtraction method following optogenetic triggering of regular or irregular firing patterns (Figures 2A and 2B). We first confirmed diurnal cycling of locomotor activity and validated the use of 5-min locomotor inactivity to identify sleep behavior in this preparation (Figures S3A and S3B). Wild-type flies, exposed to "sham" optogenetic stimulation, exhibited an increase in sleep quality, but not amount, at mid-night compared to mid-day in this assay (Figures S3F–S3K).

We next performed optogenetic manipulation of the DN1ps using irregular daytime and regular nighttime rate-matched



# Figure 1. Sleep Quality Is Clock-Dependent and Associated with Distinct Temporal Spike Patterns in DN1p Neurons

(A and B) Representative membrane potential traces and temporal raster plots of spontaneous firing of DN1ps at ZT6–8 (A) and ZT18–20 (B) in control (left), *Clk<sup>irk</sup>* (middle), and *wake<sup>D2</sup>* (right) flies. Membrane potential traces are shown for a 4-s window.

(C–E) Mean firing rate (C), coefficient of variation (CV) (D), and local variation (LV) (E) of spontaneous activity of DN1ps at ZT6–8 and ZT18–20 in *wake-GAL4* > *UAS-CD8::GFP* in control (top; n = 11 for ZT6–8 and n = 12 for ZT18-20),  $Clk^{lrk}$  (middle; n = 10 and n = 9), or *wake<sup>D2</sup>* (bottom; n = 9 and n = 9) backgrounds. Comparisons were made between (not within) cells.

(F) Autocorrelation function of the spike trains shown in (A) and (B).

(G–K) Sleep amount (blue) and brief awakenings (red) profile (G), sleep amount (H), number of brief awakenings (I), sleep bout duration (J), and sleep bout number (K) for control wake-GAL4 > UAS-CD8::GFP in control (top; n = 67),  $Clk^{irk}$  (middle; n = 54), and  $wake^{D2}$  (bottom; n = 63) backgrounds.

In (G), mid-day (ZT6–8) and mid-night (ZT18–20) time windows are denoted by dashed lines. Error bars represent SEM; \*p < 0.05, \*\*p < 0.01, \*\*\*p < 0.001; ns, not significant. (See also Figures S1 and S2 and Table S1).

temporal codes derived from actual patch-clamp recordings of these cells ("playback") at ZT18-20 and found that irregular temporal spike patterns induced a decrease in sleep quality, compared to regular temporal spike patterns, without altering sleep amount (Figures S3L-S3Q). To confirm these findings, we wished to generate synthetic temporal codes that would



allow for exact matching of firing rate. We thus performed mathematical modeling of our spike-timing data by training a fivecomponent Gaussian mixture model (GMM) of second-order spike-timing correlations on day (ZT6–8; Figures 2C and 2D) and night (ZT18–20; Figures 2F and 2G) datasets. To assess whether these synthetic spike patterns are biologically realistic, we generated a sample code from the GMM that was the same size as the training datasets and found that the synthetic temporal spike pattern precisely recapitulated the distribution of normalized spike timing from our DN1p recordings (Figures 2E and 2H). We then optogenetically activated DN1p neurons using these synthetic temporal codes at ZT18–20 and found, as expected, that the day temporal code induced reduced sleep quality, compared to the night temporal code, without affecting sleep amount (Figures 2I–2N).

Because precision of optogenetic control is important for assessing temporal coding, we first examined the correlation of spike firing in response to regular CsChrimson activation and found excellent fidelity at firing rates  $\leq 6$  Hz (Figures S3C and S3D). To directly compare the irregular CsChrimsoninduced patterns with evoked spiking of the DN1ps, we performed simultaneous optogenetic activation of the synthetic irregular daytime pattern with perforated patch-clamp recordings of the DN1ps. As shown in Figure S3E, the latency between the optogenetic stimulus and the evoked DN1p spike was highly consistent (6.3  $\pm$  0.5 ms). Moreover, global (CV) and local (CV<sub>2</sub> and LV) measures of irregularity for the two patterns were very similar (Figure S3E, inset). We next characterized the optogenetic and evoked spiking patterns at the level of individual spikes. Individual optogenetic stimuli were associated with a following DN1p spike 98.9% ± 2.1% of the time, while conversely, each DN1p spike was associated with a preceding optogenetic stimulus 98.4%  $\pm$  4.9% of the time. Together, these data demonstrate a high degree of fidelity between the optogenetically triggered patterns and the resulting DN1p spiking patterns. To confirm that the phenotypes observed with optogenetic activation of irregular temporal patterns localize to the DN1p neurons, we used an independent driver line that labels the DN1p neurons and obtained similar data (Figures S4A-S4G). We also asked whether local rate variations, rather than the irregularity, of the firing pattern influence sleep/wake states, so we analyzed the data in Figure 2I and found no correlations between estimated MFR and awake, brief awakening, or sleep states (Figure S3R). Together, these data demonstrate that optogenetic triggering of specific temporal spike patterns in DN1ps is sufficient to determine sleep quality in behaving animals.

# WAKE Upregulates $K_{Ca}$ Current at Night to Enhance Regularity of Spike Firing and Sleep Quality

To delineate the molecular mechanisms underlying the circadian-dependent temporal coding of sleep quality, we chose to use *wake* as a genetic entry point. Overexpression of *wake* in crustacean cardioactive peptide (CCAP) neurons (which are important for wing expansion) (Luan et al., 2006) resulted in unexpanded wings in ~70% of animals. To identify genes whose knockdown could suppress this WAKE-induced phenotype, we performed an RNAi screen of ~1,200 lines. From this screen, we identified the K<sub>Ca</sub> channel Slowpoke (SLO) (Schopperle et al., 1998), its binding partner SLOB (Schopperle et al., 1998), and an uncharacterized Na<sup>+</sup>/K<sup>+</sup> ATPase  $\beta$  subunit (CG33310, NaK $\beta$ ) as potential WAKE interactors (Figure 3A).

We first focused on the role of SLOB in WAKE-dependent temporal coding of sleep quality and began by investigating the WAKE and SLOB interaction. To address whether WAKE and SLOB biochemically interact, we co-expressed wake-FLAG and slob-HA in cultured HEK cells and found that SLOB coimmunoprecipitated with WAKE, suggesting that they physically interact in a complex (Figure S5A). SLOB has been shown to regulate the K<sub>Ca</sub> channel SLO, which itself was a "hit" in our screen (Schopperle et al., 1998). Thus, we measured  $K_{Ca}$  current amplitude from DN1ps and found that it was significantly increased at night versus the day. This cycling of  $K_{Ca}$  current amplitude was dependent on both WAKE and SLOB (Figures 3B–3D and S4H). Interestingly, increased  $K_{Ca}$  current leads to greater afterhyperpolarization (AHP) amplitude of spikes (Lancaster and Nicoll, 1987); AHP in turn has been proposed to be important for temporal coding (Goldberg et al., 1984; Piotrkiewicz, 1999). Thus, we performed spike waveform analysis of our current-clamp data and found that AHP amplitude was greater at night compared to day in wild-type flies and that this cycling of AHP amplitude was lost in Clk mutants, wake mutants, and flies with knockdown of SLOB in DN1ps (Figures 3E, 3F, and S4I and Table S1). Similar data for WAKE-dependent cycling of  $K_{Ca}$  and AHP amplitude were also obtained from I-LNv neurons (Figures S5B-S5F).

Modeling studies have suggested that greater AHP amplitude leads to more regular firing (Stiefel et al., 2013), so we examined DN1p temporal coding in flies with knockdown of SLOB in these

Figure 2. Temporal Spike Patterns Alone Are Sufficient to Determine Sleep Quality

(A) Schematic illustrating video preparation setup and experimental design.

(K–N) Sleep amount (K), number of brief awakenings (L), sleep bout duration (M), and sleep bout number (N) for the flies shown in (I) and (J). Error bars represent SEM. See also Figures S3 and S4.

<sup>(</sup>B) Example frames from video analysis. Fly movement is defined from the preceding frame (top) and the next consecutive frame (middle) by generating a difference image (bottom) through time (2 frames/s).

<sup>(</sup>C–H) To generate synthetic codes for stimulation, Gaussian mixture models captured second-order temporal structure for day (ZT6–8, C–E) and night (ZT18–20, F–H) spiking activity. Histograms showing relative frequency of pairs of adjacent spike intervals in training data. Histogram axes are log scaled; training data were rate normalized and log transformed (C and F). Joint densities of the mixture models reveal the temporal structure of the neural code learned from the training data (D and G). Marginal distributions of normalized spike timing from a size-matched random sample of the mixture model (green) closely match the training data (blue), suggesting that the synthetic codes are biologically realistic (E and H).

<sup>(</sup>I–J) Sleep/wake state plots of individual tethered *R18H11-GAL4* > UAS-CsChrimson flies during optogenetic stimulation of DN1ps using synthetic codes with irregular (I) (CV<sub>ISI</sub> = 1.2, n = 12) and regular (J) (CV<sub>ISI</sub> = 0.3, n = 12) patterns at ZT18–20. Brief awakenings and awake and sleep states are denoted with red, green, and blue bars, respectively.



# Figure 3. WAKE Acts via SLOB to Regulate DN1p Temporal Coding and Sleep Quality

(A) Histogram showing rescue index of RNAi lines from the *wake* genetic interaction screen (n = 1,167 RNAi lines). Inset: 1 = CCAP-GAL4 > UAS-CD8::GFP, 2 = CCAP-GAL4 > UAS-wake, UAS-slob RNAi.

(B) Representative traces of Ca<sup>2+</sup>-dependent K<sup>+</sup> current (K<sub>Ca</sub>) in DN1ps at ZT6–8 (green) and ZT18–20 (blue) in wake-GAL4 > UAS-CD8::GFP, wake-GAL4 > UAS-CD8::GFP, wake-GAL4 > UAS-CD8::GFP, UAS-slob RNAi flies.

(C and D) *I–V* relation of steady-state activation of  $K_{Ca}$  current (C) and peak  $K_{Ca}$  amplitude at a holding potential of +60 mV (D) from DN1ps at ZT6–8 (green) and ZT18-20 (blue) for wake-GAL4 > UAS-CD8::GFP (n = 8 for ZT6–8 and n = 9 for ZT18-20), wake-GAL4 > UAS-CD8::GFP; wake<sup>D2</sup> (n = 7 and n = 7), and wake-GAL4 > UAS-CD8::GFP, UAS-slob RNAi flies (n = 4 and n = 4).

(E and F) Averaged spike waveforms (E) and peak AHP amplitude (F) of DN1ps at ZT6–8 (green) and ZT18–20 (blue) in *wake-GAL4* > *UAS-CD8::GFP* (n = 11 for ZT6–8 and n = 12 for ZT18–20), *wake-GAL4* > *UAS-CD8::GFP*; *Clk<sup>irk</sup>* (n = 10 and 9), *wake-GAL4* > *UAS-CD8::GFP*; *wake<sup>D2</sup>* (n = 9 and 9), and *wake-GAL4* > *UAS-CD8::GFP*, *UAS-CP8*, *UAS-CP8*, *UAS-CP8*, *UAS-CP8*, *UAS-CP8*, *UAS-CP8* 

cells. As shown in Figures 3G, 3H, and S4J, these flies lose cycling of the regularity of spike timing, exhibiting irregular firing both during the day and during the night. As expected, sleep quality at night versus day was reduced in flies with knockdown of SLOB in DN1ps compared to controls, while sleep amount was unaffected (Figures 3I–3K, S4N–S4P, and S4T–S4V). These data suggest that WAKE acts via SLOB to enhance AHP amplitude and promote regular spike timing and greater sleep quality at night.

# WAKE Acts via a Na<sup>+</sup>/K<sup>+</sup> ATPase Subunit to Increase Spike-Onset Rapidness and Modulate Spike Timing and Sleep Behavior

We next investigated the role of NaK $\beta$  in regulation of temporal spike patterns and sleep.  $\beta$  subunits are modulatory subunits of Na<sup>+</sup>/K<sup>+</sup> ATPases that typically promote pump activity (Horisberger et al., 1991; Jaisser et al., 1994). Spike-onset slope and precision (i.e., the consistency of spike-onset slope time to peak) depend on Na<sup>+</sup>/K<sup>+</sup> pump activity (Zhang et al., 2017), so we examined spike-onset slope dynamics in DN1ps. Spike-onset rapidness was greater at night compared to day in wild-type flies, and this difference was lost in *Clk* mutants, *wake* mutants, and flies with knockdown of *NaK* $\beta$  in the DN1ps (Figures 4A, 4B, and S4K and and Table S1).

Previous computational work has shown that greater spikeonset rapidness helps shape spike timing by enhancing spike-onset precision (llin et al., 2013). Indeed, we found that spike-onset precision, as measured by reliability, was greater at night versus day in controls, and this difference was attenuated in Clk mutants, wake mutants, and flies with knockdown of  $NaK\beta$  in DN1ps (Figures 4C and S4L). Similar data for CLK-, WAKE-, and NaK<sub>β</sub>-dependent cycling of spike-onset rapidness and precision were also obtained from I-LNvs (Figures S5G–S5I). We next examined whether NaKβ regulated temporal coding of sleep quality. Knockdown of NaKβ in DN1ps resulted in irregular spike firing during both the day and the night in contrast to control flies, where regular firing was observed during the night (Figures 4D and S4M). Similarly, sleep quality at night resembles that seen during the day in these animals (Figures 4E-4G, S4Q-S4S, and S4W-S4Y).

We wished to perform computational modeling to address whether changes in  $K_{Ca}$  conductance (via SLOB) and Na<sup>+</sup>/K<sup>+</sup> ATPase activity (via NaK $\beta$ ) could reproduce the effects on spike-train variability and spike morphology seen with our experimental data. To do this, we extended a previous model of clock neuron activity based on diurnal shifts in Na<sup>+</sup> and K<sup>+</sup> leak conductances (Flourakis et al., 2015) (see STAR Methods). To compare variability in the spiking outputs, we provided model neurons with a shared, stochastic, time-varying input current. Enhancing  $K_{Ca}$  current slowed firing during periods of high input, while increasing Na<sup>+</sup>/K<sup>+</sup> ATPase activity maintained firing across pauses in the day spike train (Figure 4H). Each of these manipulations, particularly when combined, improved regularity of the spike train (Figures 4H, 4I, S5J, and S5K) similar to that seen in our experimental data at night. Interestingly, because of the opposing effects of  $K_{Ca}$  and Na<sup>+</sup>/K<sup>+</sup> ATPase on firing rate, the implementation of both factors in the model resulted in a relatively neutral impact on mean firing rate (Figure S5K). To validate our model's predictions, we next analyzed the interspike interval (ISI) distributions during the night from DN1ps in flies where SLOB or NaK $\beta$  were knocked down in these cells. These data reveal that knockdown of SLOB led to a shift toward shorter ISIs, whereas knockdown of NaK $\beta$  resulted in a shift toward longer ISIs (Figure 4J); these experimental findings confirm our model's specific predictions regarding the effects of  $K_{Ca}$  current and Na<sup>+</sup>/K<sup>+</sup> ATPase activity on spike timing.

Finally, we investigated whether our model recapitulated the effects of SLOB and NaK $\beta$  on spike morphology. Indeed,  $K_{Ca}$  and Na<sup>+</sup>/K<sup>+</sup> ATPase modulation, both independently and in combination, deepened the average spike AHP and increased the maximum action-potential slope (Figures 4K and S5L), reminiscent of the spike morphology changes experimentally observed at night. Thus,  $K_{Ca}$  and Na<sup>+</sup>/K<sup>+</sup> pump comodulation are sufficient to qualitatively explain experimentally observed time-dependent changes in DN1p spike shape and regularity.

# CLK and WAKE Regulate Subcellular Localization of SLOB and NaK $\beta$ in a Time-Dependent Manner

Our data suggest that the circadian clock acts via WAKE to regulate  $K_{Ca}$  current and Na<sup>+</sup>/K<sup>+</sup> ATPase activity in order to modulate spike biophysical properties and spike-timing patterns in clock neurons to ultimately control sleep. What are the mechanisms by which WAKE regulates SLOB and NaK $\beta$  in this process? We previously showed that WAKE regulates targeting of the GABAA receptor RDL (resistant to dieldrin) in a time-dependent manner (Liu et al., 2014). To address potential cycling of SLOB localization, we expressed SLOB-HA in I-LNvs in wild-type. Clk, or wake mutant backgrounds. We performed these experiments in the I-LNvs as opposed to the DN1ps because of our greater ability to assess subcellular localization in the larger I-LNvs. As shown in Figures 5A-5C, SLOB expression at the plasma membrane was significantly greater at night compared to day, and this effect was dependent on CLK and WAKE. We next asked whether NaK<sub>β</sub> targeting was also dependent on WAKE. We thus similarly expressed NaK<sub>β</sub>-myc in I-LNvs in wild-type, Clk, or wake mutant backgrounds. NaKß levels at the plasma membrane were greater at night compared to day in wild-type flies, and this difference depended on CLK and WAKE (Figures 5E-5G). As shown in Figures 5D and 5H, loss of CLK and WAKE was also generally associated with a reduction in total levels of SLOB and NaK<sub>β</sub>, which may reflect increased degradation secondary to mistargeting of these proteins. Taken together with our previous work (Liu et al., 2014),

<sup>(</sup>G and H) temporal raster plots (G) and CV (H) of spontaneous firing of DN1ps at ZT6–8 (green) and ZT18–20 (blue) in *wake-Gal4* > UAS-CD8::GFP (n = 5 for ZT6–8 and n = 5 for ZT18–20) and *wake-Gal4* > UAS-CD8::GFP, UAS-slob RNAi flies (n = 10 and 9).

<sup>(</sup>I–K) Sleep amount (I), number of brief awakenings (J), and sleep bout duration (K) during ZT6–8 (green) and ZT18–20 (blue) for *R18H11-GAL4* > UAS-CD8::GFP (n = 32) and *R18H11-GAL4* > UAS-slob RNAi flies (n = 32). For panels (D), (F), and (H), comparisons were made between (not within) cells. Error bars represent SEM. See also Figures S4 and S5 and Table S1.



# Figure 4. NaK $\beta$ Is a Clock- and WAKE-Dependent Molecule that Modulates DN1p Temporal Coding and Sleep Quality

(A–C) Derivatives of the spike wave membrane potential, from spike onset threshold to the peak of the spike, plotted over time (dVm/dt) (A, above) and as heatmaps (A, below), the highest dVm/dt from (A) shown as boxplots (B), and reliability of time from spike onset threshold to highest dVm/dt of spikes (C) from DN1ps at ZT6–8 (green) and ZT18–20 (blue) in *wake-GAL4 > UAS-CD8::GFP*, *wake-GAL4 > UAS-CD8::GFP*; *clk*<sup>*irk*</sup>, *wake-GAL4 > UAS-CD8::GFP*; *wake*<sup>D2</sup>, and *R18H11-GAL4 > UAS-tdTomato*, *UAS-NaK* RNA*i* flies (n = 10 for ZT6–8 and n = 10 for ZT18–20). Data for controls and *Clk*<sup>*irk*</sup>, and *wake*<sup>D2</sup> mutants are derived from the dataset for Figures 1A and 1B. Each trace in (A) represents the average of at least 165 spontaneous spikes from a single DN1p cell.

(D) CV of spontaneous firing of DN1ps at ZT6–8 (green) and ZT18–20 (blue) in wake-GAL4 > UAS-CD8::GFP (n = 5 for ZT6–8 and n = 5 for ZT18–20) and wake-GAL4 > UAS-CD8::GFP, UAS-NaK $\beta$  RNAi flies (n = 9 and 10). For panels (B)–(D), comparisons were made between (not within) cells.

(E–G) Sleep amount (E), number of brief awakenings (F), and sleep bout duration (G) during ZT6–8 (green) and ZT18–20 (blue) for R18H11-GAL4 > UAS-CD8::GFP (n = 33) and R18H11-GAL4 > UAS-NAK $\beta$  RNAi flies (n = 54).

(H and I) A Hodgkin-Huxley-type model qualitatively reproduces changes in DN1p temporal variability seen at ZT18–20. Four neuron models were simulated with a shared random input: day (control, representing ZT6–8 activity), increased  $K_{Ca}$  conductance, increased  $Na^+/K^+$  ATPase activity, and combined enhancement of  $Na^+/K^+$  ATPase and  $K_{Ca}$  function.  $K_{Ca}$  modulation was modeled as a 2-fold increase in channel conductance, and  $Na^+/K^+$  ATPase modulation was modeled as equilibrated day-night shifts in  $Na^+$  and  $K^+$  reversal potentials. Shared noise (top) generated an irregular spike train in the day model; the  $K_{Ca}$  model slowed firing during periods of high input (gold highlight), while the  $Na^+/K^+$  ATPase model maintained firing across pauses in the daytime spike train (green highlight), resulting in greater regularity of the spike train in the combined model (H). ISI distributions for simulated spikes were median normalized (dashed line, median) and log transformed for comparison (I).

(J) ISI histograms of spontaneous activity for DN1ps at ZT18–20 for R18H11-GAL4 > UAS-tdTomato, R18H11-GAL4 > UAS-slob-RNAi, or UAS-tdTomato, R18H11-GAL4 > UAS-NaK $\beta$ -RNAi, UAS-tdTomato.

Note that these data are derived from the same datasets used in Figures 3H and 4D.

(K) Average spike shapes for all spikes in a 5-min simulation, with 200 example spike traces shown in gray.

Error bars represent SEM. See also Figures S4 and S5 and Table S1.



# NaKß myc Localization



# Figure 5. WAKE Regulates Membrane Targeting of SLOB and NaK $\beta$ in a Time-Dependent Manner

(A) Immunostaining of I-LNvs with anti-HA (green) with nuclei labeled with DAPI (magenta) in *PDF-GAL4* > *UAS-slob-HA* flies in control (left), *Clk<sup>irk</sup>* (middle), and *wake<sup>D2</sup>* (right) backgrounds at ZT6–8 (top) versus ZT18–20 (bottom). Scale bar indicates 10  $\mu$ m.

(B–D) Quantification of plasma membrane (B), perinuclear (C), and total (D) SLOB-HA levels in I-LNvs at ZT6–8 versus ZT18–20 in control (n = 7 for ZT6–8 and n = 10 for ZT18–20),  $Clk^{lrk}$  (n = 9 and 9), and  $wake^{D^2}$  (n = 8 and 8) backgrounds.

(E) Immunostaining of I-LNvs with anti-myc (green) with nuclei labeled with DAPI (magenta) in *PDF-GAL4* > *UAS-NaKβ-myc* flies in control (left), *Clk<sup>irk</sup>* (middle), and *wake<sup>D2</sup>* (right) backgrounds at ZT6-8 (top) versus ZT18-20 (bottom). Scale bar indicates 10 µm.

(F–H) Quantification of plasma membrane (F), perinuclear (G), and total (H) NaK $\beta$ -myc levels in I-LNvs at ZT6–8 versus ZT18–20 in control (n = 11 for ZT6–8 and n = 6 for ZT18–20), *Clk<sup>lrk</sup>* (n = 8 and 5), and *wake<sup>D2</sup>* (n = 9 and 9) backgrounds. Error bars represent SEM.

# NMDA Receptor Signaling in Arousal-Promoting Dilp2+ PI Neurons Enhances Sleep Quality

What are the circuit mechanisms by which the diurnal cycling of temporal spike patterns of DN1ps leads to changes in sleep behavior? The DN1p neurons project directly to arousalpromoting non-clock neurons, the Drosophila insulin-like peptide2 (Dilp2+) pars intercerebralis (PI) neurons (Barber et al., 2016). Recent work has shown that the mean firing rates (MFR) of these Dilp2+ PI neurons cycles, with greater firing rates during the day, compared to the night (Barber et al., 2016). We reproduced these findings by performing perforated patch-clamp recordings of Dilp2+ PI neurons at mid-day and midnight. These neurons exhibit a marked difference in firing rate at these time windows, with almost no firing at night, and this cycling of MFR was dependent on WAKE (Figures S6A-S6D). Given these

these data suggest that WAKE is a crucial clock output molecule for sleep behavior that broadly regulates multiple ion channels and pumps in a time-dependent manner by controlling the targeting of the channels themselves or their regulatory partners. While our WAKE/SLOB co-immunoprecipitation data (Figure S5A) suggest that WAKE directly regulates these channels and transporters, we cannot exclude an indirect effect secondary to alteration of neuronal excitability.

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findings, we hypothesized that temporal coding in DN1ps is transformed into rate coding changes in the Dilp2+ PI neurons and that  $Ca^{2+}$  signaling, which is instrumental in integrating temporal signals (Ghosh and Greenberg, 1995), is involved in this process.

To identify the molecular mechanisms involved in this transformation of temporal coding to rate coding, we performed a targeted RNAi screen of Ca<sup>2+</sup>-related signaling molecules in the



Figure 6. NMDA Receptors Act in Dilp2+ PI Neurons to Promote Nighttime Sleep Quality

(A) RNAi knockdown screen for  $Ca^{2+}$ -related genes that act in Dilp2+ PI neurons to promote sleep quality at night. The day/night sleep bout duration ratio (sleep bout duration for ZT6-8/sleep bout duration for ZT18-20) is plotted, and the blue dashed line indicates the ratio for control *dilp2-GAL4 > wt* flies (B–E) Sleep amount (B), brief awakenings (C), sleep bout duration (D), and sleep bout number (E) during ZT6-8 versus ZT18-20 for wt *> UAS-dsNR1* control flies

(n = 32) and dilp2-GAL4 > UAS-dsNR1 (n = 58).

(F–I) Sleep amount (F), brief awakenings (G), sleep bout duration (H), and sleep bout number (I) during ZT6–8 versus ZT18–20 for wt > UAS-dsNR2 control flies (n = 32) and *dilp2-GAL4* > UAS-dsNR2 (n = 64).

Error bars represent SEM.

downstream Dilp2+ PI neurons. We assessed changes in the cycling of daytime versus nighttime sleep quality by calculating the ratio of sleep bout duration during ZT6-8 versus ZT18-20. Knockdown of the two subunits of the NMDA receptor (dNR1 and dNR2) (Wu et al., 2007) led to the greatest increase in the ratio of day-to-night sleep bout duration (Figure 6A). As shown in Figures 6B-6I, flies with knockdown of dNR1 or dNR2 in the Dilp2+ PI neurons lost the diurnal cycling of sleep quality and exhibited reduced nighttime sleep quality. These data suggest that

NMDA receptor signaling in the PI neurons is important for promoting sleep quality at night.

# The Daytime Temporal Spike Pattern Drives Synaptic Plastic Changes to Effectuate Rate Coding Changes and Regulate Sleep Behavior

We next examined the effects of the different DN1p temporal spike patterns on Dilp2+ PI neuron activity. To do this, we performed *in vivo* intracellular current-clamp recordings of the



(legend on next page)

Dilp2+ PI neurons while simultaneously optogenetically activating the DN1ps using different temporal codes (Figure 7A). The MFR of the Dilp2+ PI neurons was not appreciably different when triggering regular firing of the DN1ps using night synthetic temporal spike patterns (Figures 7B and 7D). Strikingly, however, optogenetic activation of DN1ps using rate-matched irregular day synthetic temporal spike patterns led to a significant increase in Dilp2+ PI neuron MFR (Figures 7B and 7C). This potentiation of Dilp2+ PI neuron firing exhibited substantial latency (i.e., required about 5 min of the irregular temporal spike pattern) and persisted following cessation of the optogenetic activation. This effect was also dependent on NMDA receptor signaling, as it was abolished with knockdown of dNR1 in these cells (Figures 7B and 7E). It is worth noting that, given the substantial difference between their firing rates, it is unlikely that DN1p spikes directly trigger firing of the Dilp2+ PI neurons but instead serve to drive potentiation of these synapses. We thus also examined whether spike onset threshold, resting membrane potential, or membrane potential fluctuations in the Dilp2+ PI neurons were altered after potentiation and found that these cells exhibit a significant increase in membrane potential fluctuations (Figures S6I-S6K).

One of the challenges of studying the function of temporal pattern codes as distinct from rate codes is that if shorter time windows are considered for an irregular pattern, specific time windows can be identified with an increase in firing rate (i.e., brief fast spiking intervals). These brief fast spiking intervals (typically <90 ms) could thus represent a hidden rate code within a purported temporal pattern code. To address whether such brief fast spiking intervals are important for DN1p-Dilp2+ PI neuron signaling, we first determined the minimum time-window length that would faithfully reproduce the MFR of the entire spike train. To do this, we analyzed sliding time windows of different durations for our synthetic daytime spike trains and determined

that a 40-s window (and all longer windows) was sufficient to consistently maintain an MFR similar to that for the entire spike train (Figure S6E and STAR Methods). As shown in Figures S6F and S6G, optogenetic activation of the DN1ps using a 40-s window of the irregular daytime synthetic code did not induce an appreciable change in Dilp2+ PI neuron MFR. These data suggest that the brief fast spiking intervals found within a 40-s window are not sufficient for driving potentiation of the Dilp2+ PI neurons and instead argue that persistence of the "day" temporal pattern of DN1p neuron spiking is required.

To investigate the mechanisms underlying these neural plastic changes, we next characterized synaptic properties following optogenetic activation with day versus night temporal codes. The frequency of Dilp2+ PI neuron excitatory postsynaptic potentials (EPSPs) was similar when triggering DN1 firing using day temporal codes in the presence or absence of dNR1 knockdown or night temporal codes (Figure 7N). In contrast, the slope and amplitude of these EPSPs were significantly increased when day synthetic temporal spike patterns were used to optogenetically activate the DN1ps compared to rate-matched night temporal spike patterns, and this effect was suppressed with knockdown of dNR1 in these cells (Figures 7F-7M). These changes in EPSP slope and amplitude in the Dilp2+ PI neurons suggest that postsynaptic plastic processes drive the increase in their firing in response to day temporal codes, although non-synaptic plasticity mechanisms cannot be excluded.

Our data suggest that the observed DN1p-PI synaptic plasticity depends upon the pattern, but not the rate, of DN1p spiking. While the timing of spiking has been shown to influence plasticity (Kumar and Mehta, 2011), our findings, to our knowledge, describe one of the first examples of synaptic plasticity being triggered by changes in the regularity of presynaptic spiking. To further support this notion, we assessed whether the plasticity observed at DN1p-PI synapses simply reflects underlying

Figure 7. DN1p Temporal Codes Are Transformed to Downstream Rate Coding Changes via Plasticity-Dependent Potentiation of DN1p-PI Synapses

(A) Schematic: regular (CV = 0.3) or irregular (CV = 1.2) firing patterns were optogenetically induced in DN1ps using synthetic temporal codes, while intracellular current-clamp recordings were performed from Dilp2+ PI neurons in a tethered fly.

(B) Potentiation of Dilp2+ PI neuron firing rate (calculated in 20-s bins) in response to specific DN1p temporal codes for R18H11-LexA, dilp2-Gal4 > LexAop-CsChrimson, UAS-GCaMP6s with CV = 0.3 (green, n = 3), CV = 1.2 (red, n = 4), or CV = 1.2 in the presence of UAS-dsNR1 (blue, n = 3) at ZT18–20.

(C-E), mean firing rate of Dilp2+ PI neurons for the 1-min bins just prior to (before) and just after (after) LED stimulation for synthetic temporal codes CV = 1.2 (C), CV = 0.3 (D), and CV = 1.2 in the presence of UAS-dsNR1 (E). Comparisons were made within cells.

(F) Time-course plot of the normalized EPSP rising slope for events in 10-s bins for CV = 1.2 (red circles), CV = 0.3 (green circles), and CV = 1.2 in the presence of UAS-dsNR1 (blue circles).

(G–I) Quantification of EPSP rising slope for the 1-min bins just prior to (before) and just after (after) LED stimulation for synthetic temporal codes CV = 1.2 (G), CV = 0.3 (H), and CV = 1.2 in the presence of UAS-dsNR1 (I). Comparisons were made within cells.

(J) Time-course plot of EPSP amplitude for events in 10-s bins for CV = 1.2 (red circles), CV = 0.3 (green circles), and CV = 1.2 in the presence of UAS-dsNR1 (blue circles).

(K–M) Quantification of EPSP amplitude for the 1-min bins before and after LED stimulation for CV = 1.2 (K), CV = 0.3 (L), and CV = 1.2 in the presence of UASdsNR1 (M). Comparisons were made within cells.

(N) Cumulative frequency plots of EPSP events and quantification of EPSP frequency (inset) for CV = 1.2 (red), CV = 0.3 (green), or CV = 1.2 + UAS-dsNR1 (blue). (F–N) Data are from the same flies as in (B)–(E). Red bar indicates duration of DN1p optogenetic stimulation.

(O) Model for how clock-dependent temporal coding drives synaptic plasticity to induce changes in downstream rate coding and enable circadian regulation of sleep behavior. The DN1p clock neurons exhibit irregular firing during the day (ZT6–8). Irregular, but not regular, firing of the DN1ps triggers NMDA-receptor-dependent plastic changes in the downstream arousal-promoting Dilp2+ Pl neurons, leading to an increase in firing rate of these Pl neurons and reduced sleep quality during the day. Note that additional inputs to the Pl neurons are also likely required. At night (right), the circadian clock acts via WAKE to upregulate SLOB and NaK $\beta$  to increase  $K_{Ca}$  current and Na<sup>+</sup>/K<sup>+</sup> ATPase activity to rhythmically alter biophysical properties of spikes to promote regular firing, which leads to reduced firing of the Dilp2+ Pl neurons and greater sleep quality.

Error bars represent SEM. See also Figure S6.

spike-timing-dependent plasticity (STDP). We examined the relative spike timing of the DN1p and Dilp2+ PI neurons and did not identify a noticeable peak in the relative spike-timing distribution (Figure S6H), making it unlikely that STDP underlies the plastic changes in the PI neurons. Together, these data demonstrate that daytime temporal codes in the DN1p neurons are transformed into rate coding changes in the arousal-promoting Dilp2+ PI neurons via NMDA receptor-dependent postsynaptic plastic changes to modulate sleep quality (Figure 7O).

# DISCUSSION

The molecular mechanisms underlying the generation of different temporal codes are largely unknown. Here, we show that the circadian clock drives distinct temporal spiking patterns, as defined by the second-order temporal structure of interspike intervals, by adjusting ionic flux in clock neurons in a time-dependent manner. These changes are mediated by the clock output molecule WAKE, which controls the membrane targeting of SLOB and a Na<sup>+</sup>/K<sup>+</sup> ATPase  $\beta$  subunit. This dynamic regulation of ionic flux leads to cycling of specific aspects of spike waveforms, which in turn induces the temporal spiking patterns seen during the day versus the night.

From a broader perspective, our work addresses a central issue in neuroscience: the functional importance of temporal codes in encoding information and impacting behavior. One challenge in demonstrating a causal role of temporal coding is identifying systems with a defined neural circuit where changes in the pattern or timing of spiking occur naturally, lead to measurable effects in target neurons, and regulate a specific behavior. An additional confounding factor is that information can be coded in a multiplexed manner with concurrent spatial, temporal, and rate-related features (Panzeri et al., 2010). Here, we show that the Drosophila clock network fulfills these criteria and find that time is encoded unidimensionally by the spiking patterns of these neurons in the absence of changes in firing rate or network timing (due to synchronization of neural firing within a cluster). Moreover, using computational, in vivo optogenetic, and electrophysiological approaches in these clock neurons, we demonstrate that this temporal coding has functional consequences on the firing of a target arousal circuit and on sleep behavior. While our findings suggest that the irregular second-order spiking pattern is critical for this process, it is also possible that the temporal code consists of brief periods of faster spiking that are repeated over a >40-s time frame.

We previously demonstrated that WAKE is critical for clockdependent regulation of sleep onset at dusk and that it upregulates and properly targets  $GABA_A$  receptor to mediate this process by markedly suppressing the firing rate of clock neurons (Liu et al., 2014). Why would multiple neural coding mechanisms (rate coding changes at dusk and temporal coding at mid-day and mid-night) evolve to underlie circadian clock regulation of sleep at different times? One possibility relates to the dynamics of sleep onset versus sleep quality. Transitions between sleep and wake are major changes in brain state occurring on a relatively short timescale and hence may require dramatic changes in firing rate (i.e., rate coding) that are energetically costly (Levy and Baxter, 1996; Harris et al., 2012). In contrast, maintenance of sleep quality occurs over hours; thus, it may be more energetically favorable for the relevant neurons to alter the pattern, instead of the rate, of their firing. Because of these potential energy savings, we speculate that the use of temporal spiking patterns to encode information could be a broadly used mechanism for representing persistent internal states, such as hunger or emotion.

Finally, we demonstrate that changes in the pattern of spike firing in the DN1ps, independent of changes in firing rate, triggers NMDA-receptor-dependent postsynaptic plasticity in the Dilp2+ PI neurons. Importantly, these data suggest a specific mechanism for inducing synaptic plasticity distinct from previously described processes that are dependent on changes in rate coding (e.g., long-term potentiation) (Bailey et al., 2000) or relative timing of individual spike events (e.g., STDP) (Markram et al., 1997). To our knowledge, these data represent one of the first examples of synaptic plasticity being induced specifically by the intrinsic temporal pattern of spiking, expanding the repertoire by which neural codes can generate plasticity. Together, our findings suggest that temporal patterns of spike firing are a crucial mechanism for driving neural plastic changes that mediate how internal states modulate behavior.

# **STAR**\*METHODS

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# SUPPLEMENTAL INFORMATION

Supplemental Information includes six figures and one table and can be found with this article online at https://doi.org/10.1016/j.cell.2018.09.016.

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## AUTHOR CONTRIBUTIONS

M.T. and M.N.W. conceived the project. M.T. performed all electrophysiological and most behavioral experiments and analyzed the data. Q.L. and M.T. performed immunostaining experiments. S.L. and B.B. performed behavioral, molecular biology, and/or immunoprecipitation experiments. M.T. and G.D. performed genetic screening. J.D.M. and K.Z. performed computational modeling for generation of synthetic temporal codes and neuron modeling. M.T. and M.N.W. wrote the manuscript with input from all authors.

#### **DECLARATION OF INTERESTS**

The authors declare no competing interests.

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# **STAR\*METHODS**

# **KEY RESOURCES TABLE**

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Antibodies		
Normal goat serum	Jackson Immunoresearch	Cat#005-000-121
rabbit anti-FLAG	MilliporeSigma	Cat#F7425; RRID: AB_439687
rat anti-HA	Roche	Cat#3F10; RRID: AB_2314622
rat anti-WAKE-Ab2	Wu Lab	N/A
rat anti-GFP	Santa Cruz	Cat#101536; RRID: AB_1124404
Rabbit anti-GFP	Thermo Fisher	Cat#A-11122; RRID: AB_221569
goat anti-rat HRP	Thermo Fisher	Cat#31470; RRID: AB_228356
mouse anti-MYC	Developmental Studies Hybridoma Bank	Cat#9E10; RRID: AB_2266850
Mouse anti-BRP	Developmental Studies Hybridoma Bank	Cat#nc82; RRID: AB_2314866
Goat anti-rat IgG, Alexa488	Thermo Fisher	Cat#A-11006; RRID: AB_2534074
Goat anti-mouse IgG, Alexa488	Thermo Fisher	Cat#A32723; RRID: AB_2633275
Goat anti-rabbit IgG, Alexa488	Thermo Fisher	Cat#A27034; RRID: AB_2536097
Goat anti-mouse IgG, Alexa568	Thermo Fisher	Cat#A-11004; RRID: AB_141371
Bacterial Strains		
Тор10	Thermo Fisher	Cat#C404003
Chemicals, Peptides, and Recombinant Proteins		
Dulbecco's Modified Eagle Media	Thermo Fisher	Cat#11965-092
L-glutamine	Thermo Fisher	Cat#25030081
Fetal bovine serum	Thermo Fisher	Cat#16140071
Penicillin-Streptomycin	Thermo Fisher	Cat#15140122
Effectene Transfection Reagent	QIAGEN	Cat#301425
0.5x RIPA Buffer	MilliporeSigma	Cat#R0278
Protein G-coated magnetic Dynabeads	Thermo Fisher	Cat#1003D
Pierce ECL Western Blotting Substrate	MilliporeSigma	Cat#32016
DAPI	MilliporeSigma	Cat#10236276001
Paraformaldehyde, EM grade	Polysciences	Cat#00380-250
all-trans-retinal	MilliporeSigma	Cat#R2500
Nutri-Fly Instant food flakes	Genesee Scientific	Cat#66-117
Neurobiotin	Vector Labs	Cat#SP-1120
Picrotoxin	MilliporeSigma	Cat#P1675
Mecamylamine HCI	Tocris Bioscience	Cat#2843
Escin	Santa Cruz	Cat#SC-221596
Collagenase	MilliporeSigma	Cat#C5138
Protease XIV	MilliporeSigma	Cat#P5147
Dispase	MilliporeSigma	Cat#04942078001
Streptavidin, Alexa488-conjugate	Thermo Fisher	Cat#S11223
Streptavidin, Alexa568-conjugated	Thermo Fisher	Cat#S11226
4-Aminopyridine	MilliporeSigma	Cat#A78403
Tetrodotoxin	Tocris Bioscience	Cat#1078
Cadmium chloride	MilliporeSigma	Cat#655198
Experimental Models: Cell Lines		
HEK293T	American Type Culture Collection	Cat#CRL-3216; RRID: CVCL_0063

(Continued on next page)

Continued		
REAGENT or RESOURCE	SOURCE	IDENTIFIER
Experimental Models: Organisms/Strains		
wake-GAL4	Wu Lab	N/A
UAS-CD8::GFP	Bloomington Drosophila Stock Center	BDSC: 5137
wake <sup>D2</sup>	Wu Lab	N/A
Clk <sup>irk</sup>	Bloomington Drosophila Stock Center	BDSC: 24515
PDF-GAL4	Bloomington Drosophila Stock Center	BDSC: 6900
R18H11-GAL4	Bloomington Drosophila Stock Center	BDSC: 48832
R18H11-LexA	Bloomington Drosophila Stock Center	BDSC: 52535
Clk4.1 m-GAL4	Bloomington Drosophila Stock Center	BDSC: 36316
20xUAS-IVS-GCaMP6s	Bloomington Drosophila Stock Center	BDSC: 42749
10xUAS-IVS-myr::tdTomato	Bloomington Drosophila Stock Center	BDSC: 32222
13xLexAop2_IVS-CsChrimson.mVenus	Bloomington Drosophila Stock Center	BDSC: 55139
20xUAS-IVS-CsChrimson.mVenus	Bloomington Drosophila Stock Center	BDSC: 55135
CCAP-GAL4	Bloomington Drosophila Stock Center	BDSC: 25686
UAS-slob-HA	FlyORF	FlyORF: F003557
UAS-slob RNAi	Vienna Drosophila Research Center	VDRC: 100987
UAS-slob RNAi #2	Vienna Drosophila Research Center	VDRC: 30674
UAS-NaKβ RNAi	Vienna Drosophila Research Center	BDRC: 105863
UAS-NaKβ RNAi #2	Vienna Drosophila Research Center	VDRC: 48300
UAS-dsNR1 RNAi	CL. Wu	N/A
UAS-dsNR2 RNAi	CL. Wu	N/A
dip2-GAL4	A. Sehgal	N/A
dip2-mCherry	A. Sehgal	N/A
UAS-wake	Wulab	N/A
Recombinant DNA	The Lab	
nl IAS-NaKß-myc	Wulab	N/Δ
pl/AST-6xmvc	Wulab	N/A
pl/AST-WAKE-T-Elea	Wulab	N/A
pCMV-Slob65-HA		N/A
pCMV-actin-GAL4	Wulab	N/A
pomv-acuir-ace	Wulab	
	Wu Lab	
	MillingueGierreg	N1/A
		N/A
	MilliporeSigma	N/A
Software and Algorithms		
MATLAB R2017b	Mathworks	RRID: SCR_001622
Fiji (ImageJ)	NIH	RRID: SCR_002285
pClamp 10.3	Molecular Devices	RRID: SCR_011323
Prism 6	GraphPad	SCR_002798
Other		
Dental wax	GC Corporation	Cat#27B2X00008000016
Vectashield	Vector Labs	Cat#H-1000
fiber optic cannula	Thorlabs	Cat#CFML21L10
Arduino Uno board	Arduino	Cat#A000066
collimated LED light source (625nm)	Thorlabs	Cat#M625F2
quartz glass with filament (OD/ID: 1.2/0.6mm)	Sutter Instruments	Cat#QF120-60-7.5
Borosilicate glass (OD/ID: 1.2/0.68mm)	A-M Systems	Cat#627500
syringe filter with 0.02 $\mu$ m pore size, Anotop10	Thermo Fisher	Cat#09-926-3

# **CONTACT FOR REAGENT AND RESOURCE SHARING**

Further information and requests for resources and reagents should be directed to and will be fulfilled by the Lead Contact, Mark N. Wu (marknwu@jhmi.edu).

# EXPERIMENTAL MODEL AND SUBJECT DETAILS

# **Drosophila**

All fly lines used, including GAL4 and UAS transgenic lines, were generated in the *iso*<sup>31</sup> background or outcrossed into the *iso*<sup>31</sup> genetic background at least 5 times. Flies were reared on standard food containing molasses, commeal, and yeast at room temperature or 25°C. 4-8 day old female flies were used for all experiments. Transgenic animals were generated using standard techniques (Rainbow Transgenics). We used two different GAL4 drivers to label and manipulate the DN1p neurons. *R18H11-GAL4* labels ~6 DN1p neurons, and also exhibits expression in a few cells in the lateral brain, as well as the thoracic ganglia (Kunst et al., 2014 and see HHMI Janelia Flylight project http://flweb.janelia.org/cgi-bin/flew.cgi). *Clk4.1 m-GAL4* specifically labels ~8-10 DN1p neurons in the central brain (Zhang et al., 2010a; Zhang et al., 2010b) and labels only 4 cells in the thoracic ganglion (see Figure S4A)

## **HEK293T Cells**

*HEK293T* cells (female origin) (ATCC) were cultured in Dulbecco's Modified Eagle Media (Thermo Fisher) with 2 nM L-glutamine (Thermo Fisher), 10% fetal bovine serum (FBS) (Thermo Fisher), and 100 units/mL of Penicillin-Streptomycin (Thermo Fisher). Cells were grown to  $\sim$ 80% confluency in culture flasks in an incubator at 37°C, with 95% O<sub>2</sub> and 5% CO<sub>2</sub>. Experiments using cultured cells were performed using cells passaged 7 or fewer times, and fresh media was made each week.

# **METHOD DETAILS**

## **Molecular Biology**

To generate UAS-NaK $\beta$ -myc, the UAS-NaK $\beta$ /CG33310 open reading frame was PCR amplified using the following primers: 5'- GGC GCG GCC GCA TGT CGG ACG ATG ATA AAA ATC- 3' and 5'- GCC CTC GAG TAA TGA GTA GTC GGT TCG GAG TAA TTT TTC -3' and then digested with Notl and Xhol and subcloned into *pUAST-6xmyc*. For the co-immunoprecipitation experiments, we used *pUAST-WAKE-T-Flag* (Liu et al., 2014) and *pCMV-Slob65-HA* (Jaramillo et al., 2006), which were previously described.

#### **Co-immunoprecipitation**

pUAST-WAKE-T-FLAG, pCMV-Slob-HA, pCMV-actin-GAL4, and/or pCMV-eGFP (as a negative control) were co-transfected singly or in combination into HEK293T cells using Effectene Transfection Reagent (QIAGEN). Cells were harvested, and total protein extracted using 0.5x RIPA Buffer (MilliporeSigma) with 1 mM PMSF and 0.5 mM

DTT. Rabbit anti-FLAG antibodies (MilliporeSigma) were bound to Protein G-coated magnetic Dynabeads (Thermo Fisher) overnight and then mixed with cell extracts for 2 hr with shaking at 4°C. Beads were magnetically collected and then washed 4 times with extraction buffer. Immunoprecipitates were analyzed by western blotting, using rat anti-HA (1:2000, Roche), rat anti-WAKE-Ab2 (1:2000); rat anti-GFP (1:2000, Santa Cruz) in blocking solution (PBST with 5% nonfat powdered milk) overnight at 4°C. Bands were visualized by enhanced chemiluminescence following 2 hr incubation at RT with goat anti-rat HRP secondary antibodies (1:10,000).

#### wake genetic interaction screen

Expression of WAKE in CCAP (crustacean cardioactive peptide) neurons, which are required for wing expansion (Luan et al., 2006), results in unexpanded wings in ~70% of flies, likely due to alterations in excitability. To identify novel WAKE interactors, we conducted an RNAi screen to find suppressors of this phenotype, by crossing *CCAP-GAL4* > *UAS-wake* flies to 1,167 different RNAi lines and examining wing expansion of their progeny. We scored wing phenotypes as either fully expanded or unexpanded (which included partially expanded), and calculated a rescue index based on the % unexpanded wings (UE%) relative to the index line *CCAP-GAL4* > *UAS-wake* (70% - UE% / 70%).

# Immunostaining and subcellular localization

Brains or thoracic ganglion were fixed in 4% PFA for 30 min at room temperature. After several washes with phosphate-buffered saline (137 mM NaCl, 2.7 mM KCl, 10 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.7 mM KH<sub>2</sub>PO<sub>4</sub>) + 0.3% Triton X-100 (PBST), samples were incubated with rat anti-HA at 1:100 (3F10, Roche) mouse anti-MYC (9E10, Developmental Studies Hybridoma Bank) at 1:50, rabbit anti-GFP (Thermo Fisher), and/or mouse anti-BRP (nc82, Developmental Studies Hybridoma Bank) at 4°C overnight. After additional PBST washes, samples were incubated with DAPI (Millipore Sigma, 1:1000) and/or Alexa488 anti-rabbit (Thermo Fisher, 1:1000) for anti-GFP stainings, Alexa568 anti-mouse (Thermo Fisher, 1:1000) for anti-BRP stainings, Alexa488 anti-rat (Thermo Fisher, 1:1000) for anti-HA stainings or Alexa488 anti-mouse (Thermo Fisher, 1:1000) for anti-myc stainings overnight at 4°C. For stainings using the *Clk4.1*  *m*-GAL4 driver, images were taken under 25x magnification using a Zeiss LSM700 and acquired as 512 × 512 pixel images. For subcellular localization experiments, images were taken under 63 × magnification using a Zeiss LSM700 and acquired as 1,024 × 1,024 pixel images. The 1  $\mu$ m slice with the strongest nuclear DAPI signal was used to quantify the appropriate region of interest (ROI) from each cell. ImageJ was used to quantify intensity of total, plasma membrane, and perinuclear signal. Analysis of SLOB-HA and NaK $\beta$ -myc localization (Figure 5) was performed in the I-LNvs, instead of the DN1ps, because changes in subcellular localization were more easily visualized in the former cells, which are larger.

# **Sleep Analysis**

# Standard beam-break analyses

Sleep behavior was measured using consolidated locomotor inactivity as previously described (Liu et al., 2014). Females were loaded into glass tubes containing 5% sucrose/2% agarose and were then monitored using the *Drosophila* Activity Monitoring System (Trikinetics) under 12 hr:12 hr light:dark (LD) or dark:dark (DD) conditions. Activity counts were collected in 1 min bins, and sleep was identified as periods of inactivity lasting at least 5 min. Brief awakenings were calculated essentially as previously described, as a single 1 min bin with either 1 or 2 beam crossings, flanked by 1 min bins with no locomotor activity (Huber et al., 2004). Sleep bout durations were calculated for the 2 hr windows; thus the maximum sleep bout duration possible was 2 hr.

# Single fly video analyses

For experiments involving simultaneous measurement of sleep behavior with optogenetic manipulation, individual flies were anesthetized on ice and glued to a 0.025 mm thick stainless steel shim using dental wax. Flies were given *ad libitum* access to food (rehydrated food flakes, Nutri-Fly Instant, Genesee Scientific), and leg movements were continuously monitored with an IR-sensitive CCD camera (Ailipu Technology) at 2 fps. Inactivity assessed by leg movements was transformed to sleep behavior via a frame-subtraction approach using a noise threshold algorithm, as previously described (van Alphen et al., 2013). Sleep was identified using a 5 min window for this assay was validated by arousal threshold criteria, as determined by administration of varying strengths of air puffs (Figure S3B). Brief awakenings were calculated by detecting "010" sequences (1 = active, 0 = inactive) in the 1 min binned data, where an "active" bin was defined by the presence of any pixel change beyond a 2 SD threshold (van Alphen et al., 2013). 90 min of recording data were used for these analyses, because the first 30 min of the 2 hr window were used to allow the fly to acclimate to the preparation.

# **Optogenetic stimulation**

Flies were fed 1 mM all-trans-retinal (MilliporeSigma), mixed in rehydrated food flakes (Nutri-Fly Instant, Genesee Scientific). A collimated LED light source (625nm, Thorlabs) was used for photostimulation of CsChrimson-expressing DN1p cells. A fiber optic cannula (Thorlabs) was attached to the LED to converge the light. An Arduino Uno board (Arduino) was connected to a computer running MATLAB and used to control the timing of photostimulation. For optogenetic activation of DN1ps using the shorter time window, 40 s windows from 3 of the synthetic daytime spike trains used in Figure 7B were selected. Because our data suggest that the DN1ps are gap junction-coupled (see Figures S1F–S1H), the degree of synchrony of DN1p firing under natural conditions versus triggered by optogenetic methods is likely highly similar, although it remains possible that greater synchrony is observed with optogenetic activation.

## **Electrophysiological recordings**

# ex vivo preparation

Brains were removed and dissected in a *Drosophila* physiological saline solution (101 mM NaCl, 3 mM KCl, 1 mM CaCl<sub>2</sub>, 4 mM MgCl<sub>2</sub>, 1.25 mM NaH<sub>2</sub>PO<sub>4</sub>, 20.7 mM NaHCO<sub>3</sub>, and 5 mM glucose; pH 7.2). To better visualize the recording site and increase the likelihood of a successful recording, the glial sheath surrounding the brain was focally and carefully removed after treating with an enzymatic cocktail, collagenase (0.2 mg/mL), protease XIV (0.4 mg/mL), and dispase (0.6 mg/mL), at 22°C for 1-2 min. The surface of the cell body was briefly cleaned with a small stream of saline that was pressure-ejected from a large-diameter pipette under visualization of a dissecting microscope. The brain was immobilized on the bottom of a recording chamber using a custom-made platinum anchor. *in vivo preparation* 

Flies were mounted as described for single fly video analyses. Then, the cuticle was peeled off to expose the surface of the brain, and the tethered fly was mounted in a chamber containing a *Drosophila* physiological saline solution. The chamber was placed on the stage of an Olympus BX51 microscope, and the PI neurons were first visualized with mCherry or GCaMP6 fluorescence viewed with a 40 × water immersion objective lens and then via infrared-differential interference contrast (IR-DIC) optics and a CCD camera (CV-A50IR; JAI, Japan).

# Perforated patch-clamp recordings

Perforated patch-clamp recordings of I-LNv and DN1p neurons were performed essentially as described (Liu et al., 2016). The use of perforated, as opposed to whole-cell, patch-clamp configuration was important for analysis of temporal coding, because firing patterns recorded were more stable, likely due to reduced cell/electrode solution exchange. Fluorescently labeled neurons were visualized on a fixed-stage upright microscope (BX51WI, Olympus). The cell surface was further cleaned by positive pressure from the recording electrode under visualization of a 40x objective lens before recording (Liu et al., 2016).

Escin was prepared as a 50 mM stock solution in water (stored up to 2 weeks at  $-20^{\circ}$ C) and was added fresh into the internal pipette solution to a final concentration of 50  $\mu$ M. Because escin is light-sensitive, filling syringes were wrapped with aluminum

foil. Pipette tips were dipped briefly for 1 s or less into a small container with escin-free internal pipette solution, and then were backfilled with the escin-containing solution from the filling syringe. Air bubbles were removed by gentle tapping. Escin pipette solutions remained stable for several hr after mixing in the filling syringe, with no evidence of precipitate formation. Recordings were acquired with an Axopatch 200B amplifier (Molecular Devices) or a Model 2400 amplifier with 100 MΩ headstage (A-M systems), and sampled with Digidata 1440A interface (Molecular Devices). These devices were controlled on a computer using pCLAMP 10.3 software (Molecular Devices). The signals were sampled at 20 kHz and low-pass filtered at 2 kHz. Junction potentials were nullified prior to high-resistance (GΩ) seal formation. After establishing a GΩ seal, perforated patches were allowed to develop spontaneously over time (usually ~1-8 min) without any suction pulse applied in the pipette. After breakthrough became evident, as determined by the gradual development of a large capacitance transient in the seal test window of pCLAMP 10 software, access resistance monitoring was initiated employing the membrane test function. After that point, access resistance was monitored continuously during the final completion of perforation process, until it reached a minimal "steady state" (access resistance stably < 40 MΩ). Cells showing evidence of "mechanical" breakthrough, as assessed by the abrupt generation of a large capacitance transient (versus the more progressive, gradual one generated by chemical perforation), were excluded. In addition, cells were also excluded if "inflowing" cytosolic fluorescence into the pipette was visually detected during or after recording. One neuron per brain was recorded. During the recording, the bath solution was continuously perfused with saline by means of a gravity-driven system. Patch pipettes (8-10 MΩ) for perforated patch-clmap were fashioned from borosilicate glass capillary (without filament) using a Flaming-Brown puller (P-1000, Sutter Instrument) and further polished with a MF200 microforge (WPI) prior to filling internal pipette solution (102 mM potassium gluconate, 0.085 mM CaCl<sub>2</sub>, 0.94 mM EGTA, 8.5 mM HEPES, 4 mM Mg-ATP, 0.5mM Na-GTP, 17 mM NaCl; pH7.2). For experiments utilizing synaptic blockers, 50 µM mecamylamine and 250 µM picrotoxin were used to isolate the cells from most excitatory and inhibitory inputs; cells were recorded for 20 s baseline ("control"), followed by 20 s of application of synaptic blockers by gravity perfusion, and then 20 s of recording for the "+ synaptic blockers" condition.

Ca<sup>2+</sup>-dependent K<sup>+</sup> (K<sub>Ca</sub>) currents were pharmacologically isolated with a combination of blockers, appropriate voltage protocols, and ion substitution, as previously described (Tabuchi et al., 2015). Specifically, neurons were held at -70 mV and two series of 200 ms voltage pulses were delivered in 10 mV increments between -80 mV and 60 mV in the presence of 1 x  $10^{-7}$  M tetrodotoxin and 4  $\times$  10<sup>-3</sup> M 4-aminopyridine. The second series was recorded additionally with saline containing 5  $\times$  10<sup>-4</sup> M CdCl<sub>2</sub>, which abolished voltage-activated Ca<sup>2+</sup> currents. The subtracted "Cd<sup>2+</sup>-treated" current series from the untreated current series was defined as  $K_{Ca}$  current.

For the optogenetic/DN1p spiking fidelity experiments, perforated patch-clamp recordings were conducted as above for R18H11-GAL4 > UAS-CsChrimson flies, while simultaneously performing optogenetic activation as described above using irregular daytime (CV = 1.2) synthetic temporal codes.

# **Dual recordings of DN1p neurons**

Dual recordings from pairs of DN1p neurons were performed using two patch-clamp amplifiers-an Axopatch 200B amplifier (Molecular Devices) and a Model 2400 amplifier with 100 MΩ headstage (A-M systems). To examine potential electrical coupling between DN1p cells, hyperpolarizing and subthreshold depolarizing current injections were applied to presynaptic cells, and the membrane potentials of non-injected postsynaptic cells were simultaneously recorded. Electrical coupling recordings were averaged across 30 trials, and the coupling coefficient between two DN1p cells was determined by the ratio of the voltage response in the postsynaptic cell divided by the voltage response in the presynaptic cell under steady-state conditions. The degree of spike-timing correlation of spontaneous activity of two DN1p cells was quantified by computing the cross-correlation function, which represents the probability of encountering a spike in one cell as a function of time before or after a spike in the other cell. Cross-correlograms were normalized by creating shuffled spike trains for each recording and subtracting the mean shuffled cross-correlogram from the raw mean cross-correlogram and then dividing by the standard deviation of the mean shuffled cross-correlogram. The cross-correlation function demonstrates a large peak around 0 ms, suggesting the presence of gap junctional connections between DN1p cells.

# In vivo intracellular recordings

Sharp electrode intracellular (as opposed to patch-clamp) recordings of Dilp2+ pars intercerebralis (PI) neurons were performed in order to clearly dissociate action potential spikes from excitatory post-synaptic potentials (EPSPs). These intracellular recordings were performed essentially as described (Liu et al., 2017), with modifications to enhance signal to noise and stability of the recordings. Sharp electrodes from quartz glass with a filament (OD/ID: 1.2/0.6mm) were fabricated with a laser-based micropipette puller (P-2000, Sutter instrument) and backfilled with 1% Neurobiotin dissolved in 1 M KCl, with resistances of 90–170 MΩ. Solutions were filtered by using a syringe filter with 0.02 µm pore size (Anotop 10, Whatman). The electrode was inserted into the PI neurons expressing mCherry or GCaMP6 (for visualization). Signals were acquired with an Axoclamp 900A, and sampled with an interface, controlled by pCLAMP 10 software. The signals were sampled at 20 kHz and low-pass filtered at 2 kHz. In all cases, one neuron per brain was recorded, and we conducted post hoc single cell staining, to confirm the identity of the recorded cell; data were included only if the cells were positively identified as PI neurons. The duration of the in vivo recordings ranged from 11 to 30 min (mean = 19 min). In all cases, potentiation of Dilp2+ PI neurons shown in Figure 7 lasted for as long as the recordings could be maintained. Single-cell labeling

For intracellular recordings, single-cell labeling was performed by immunostaining of whole-mount brains as previously described (Liu et al., 2017). Intracellular staining was performed by iontophoretic injection of neurobiotin (1.2 nA depolarizing current, 500 ms duration, 1 Hz) for at least 10 min. Brains were then fixed in 4% paraformaldehyde in PBS overnight at 4°C. After several washes in PBST at room temperature over 1 hr, brains were incubated with Alexa488- or Alex568-conjugated streptavidin (Thermo Fisher, 1:100) for 24-40 hr on a shaker at 4°C. After another series of washes in PBST at room temperature over 1 hr, samples were cleared in 70% glycerol in PBS for 5 min at room temperature and then mounted in Vectashield (Vector Labs). Brains were imaged as described in the Immunostaining and subcellular localization. Only samples where a single cell was both GFP-positive and dye-labeled were included in the analyses.

# Synthetic stimulus patterns

For each dataset, ISIs (day, n = 1847; night, n = 2098) were normalized by cell-average firing rates and pooled across cells. Secondorder distributions were created by logarithmic binning of adjacent pairs of normalized intervals into a 2D histogram ( $22 \times 22$  bins; day, natural log range [-2.225, 1.4]; night, [-0.415, 0.555]). Using a Gaussian mixture model (scikit-learn version 0.19.1), we fit 5 Gaussian components with full covariance to the joint second-order distributions; using fewer components did not capture the visually inspected statistical modes of the data. For validation, size-matched random samples from the model produced joint and marginal distributions that were indistinguishable from the training data. To generate a rate-matched pair of day/night stimulus patterns, we first sampled an average firing rate from a beta distribution fit to the day cells. Then, we serially constructed novel spike trains by choosing the next interval in the series based on the current interval and iterating up to the required number of spikes for 1 hr of stimulation. Intervals were chosen by rejection sampling of the continuous conditional probability densities of the GMM after discarding 200 burn-in samples. The resulting log intervals were exponentiated and normalized into spike times that were binned into 10 ms binary signals for stimulation. As was the case for the "playback" temporal codes, no bursts were noted in these synthetic temporal codes.

To identify a shorter time window that maintained a similar MFR, we compared local MFRs and global MFRs for all 100 synthetic spike trains by calculating a moving average for sliding window lengths ranging from 100 ms to the entirety of the spike train (1 hr) using 100 ms steps. The CV of the moving averages was then calculated for each sliding window length and plotted versus window length (Figure S6E). Based on both visual inspection and a criterion of CV  $\leq$  0.05, a time window of 40 s was selected as a shorter time window that maintained a similar MFR compared to the entire 1 hr spike train.

## **Clock neuron models**

We studied parametric variations of a previously published Hodgkin-Huxley-type dynamical model of *Drosophila* circadian neuron excitability (Flourakis et al., 2015) that was based on a prior mammalian suprachiasmatic nucleus model. The model was simulated (0.1 ms timesteps; Runge-Kutta fourth-order method for neurons; Euler-Maruyama method for noise) using the equation-based Brian simulator (Goodman and Brette, 2008) and analyzed with custom Python software. The randomized background input shared by the neuron models was simulated as an Ornstein-Uhlenbeck process with 3 pA bandwidth Gaussian noise; we did not inject voltage noise. The equations and parameters of the Flourakis et al. model (Flourakis et al., 2015) were implemented as described with several variations. The  $K_{Ca}$  conductances were modeled as BK channels. To fix our simulations to the equivalent of mid-day/night (analogous to ZT6–8/ZT18–20 data; *cf.* Figure 7J of Flourakis et al., 2015 (Flourakis et al., 2015)), we set gNALCN = 0.17 nS and gK<sub>leak</sub> = 0.05 nS. To implement the day/night shift due to Na<sup>+</sup>/K<sup>+</sup> modulation, the day/BK neuron models set  $E_{Na} = 40$  mV and  $E_{K} = -85$  mV and the ATPase/ATPase+BK models set  $E_{Na} = 51$  mV and  $E_{K} = -121$  mV. The reversal potentials were tuned by finding the Na<sup>+</sup>/K<sup>+</sup> concentration change (±1.5 mM) that matched the model RMP to the observed range (day to night: -48 to -64 mV). Further, the day neuron set gNa = 150 nS (instead of 229 nS) to reduce hyperexcitability due to the Na<sup>+</sup>/K<sup>+</sup> modulation; the BK/ATPase+BK neurons set gBK = 20 nS (instead of 10 nS); and the ATPase/ATPase+BK neurons set  $I_{app} = 13$  pA (instead of 0 pA), which was the minimal applied current that brought the membrane excitability into the limit cycle regime necessary for spiking.

# **QUANTIFICATION AND STATISTICAL ANALYSIS**

## **Spike train statistics**

To quantify temporal coding, we mainly utilized the coefficient of variation (CV) (Holt et al., 1996) of ISIs, a classical measure of spike time irregularity, which is a global measure defined as the dispersion of the ISIs, ( $CV_{ISI} = ISI SD/ISI$  mean). In addition to  $CV_{ISI}$ , we also calculated the local coefficient of variation ( $CV_2$ ) (Holt et al., 1996) and the local variation (LV) (Shinomoto et al., 2003) as alternative measures of local irregularity, by computing the dispersion of the two adjacent ISIs.

CV<sub>2</sub> is defined as

$$CV_2 = \frac{2|ISI_{i+1} - ISI_i|}{|SI_{i+1} + |SI_i|}$$

LV is defined as

$$Lv = \frac{1}{n-1} \sum_{i=1}^{n-1} \frac{3(ISI_i - ISI_{i+1})^2}{(ISI_i + ISI_{i+1})^2}$$

where  $ISI_i$  is the *ith* ISI and n is the number of ISIs for both. To define the shape of the interspike interval (ISI) histogram, we calculated skewness (which assesses asymmetry) and kurtosis (which assesses "tailedness"). See Kumbhare and Baron (2015) for further discussion of these variables. For identification of bursting events, we used the following criteria: burst onset, ISI < 80 ms; burst offset, ISI > 160 ms (Grace and Bunney, 1984; Liu et al., 2016).

To address whether STDP may underlie the observed DN1p-PI synaptic plasticity, we analyzed the relative spike timing of presynaptic DN1p and the postsynaptic Dilp2+ PI neurons. To do this, we used our intracellular recordings of Dilp2+ PI neuron spikes and our optogenetic stimulus pattern as a surrogate for DN1p spikes and quantified their relative timing for all combinations within  $\pm$  500 ms.

# **General statistical analysis**

Statistical analyses were performed using Prism software (GraphPad). For comparisons of 2 groups of normally or non-normally distributed data, t tests or Mann-Whitney U-tests were performed, respectively. For multiple comparisons, ANOVAs followed by post hoc Tukey or multiple t tests with Holm-Bonferroni correction were performed. For multiple comparisons of non-normally distributed data, Kruskal-Wallis tests were performed, with Bonferroni correction for post hoc comparisons. Reliability of spike onset rapidness (dVm/dt measured from spike onset threshold to peak dVm/dt) was calculated using Cronbach's alpha. For within cells comparisons, paired t tests were used.

# **Supplemental Figures**



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Figure S1. Additional Electrophysiological Data for DN1p Neurons, Related to Figure 1

(A–E) ISI histograms (A), CV<sub>2</sub> (B), skewness (C), and kurtosis (D) of spontaneous activity and *f-I* curves (E) for DN1ps at ZT6-8 and ZT18-20 for *wake-GAL4* > *UAS-CD8::GFP* in control, *Clk<sup>irk</sup>*, and *wake<sup>D2</sup>* background flies. Note that data in (A)–(E) are from the same dataset as for Figures 1A–1F. For (B)–(E), comparisons were made between (not within) cells.

(F) Dual patch-clamp recordings of 2 individual DN1p neurons with injection of subthreshold depolarizing or hyperpolarizing current injection in 1 cell of the pair (presynaptic DN1p neuron) while simultaneously measuring membrane potential responses in the 2<sup>nd</sup> cell (postsynaptic DN1p neuron) (n = 6 pairs). (G) efficiency of coupling between the pairs of DN1ps shown in F.

(H) cross-correlation of spontaneous spike events between 2 individual DN1p neurons. Data in F to H are from the same cells.

(I) Bursting frequency in DN1p neurons at ZT6-8, ZT18-20, and ZT0-2. Data for control flies at ZT6-8 and ZT18-20 are from (A)–(E). Data for  $Clk^{lrk}$  and  $wake^{D2}$  mutant flies at ZT6-8 and ZT18-20 are from the same dataset as for Figures 1C–1E. Data from ZT0-2 are from wake-GAL4 > UAS-CD8::GFP (n = 4).

(J) Representative membrane potential traces of spontaneous DN1p firing at ZT18-20 from *wake-GAL4* > *UAS-CD8::GFP* flies in the presence or absence of 250  $\mu$ M picrotoxin and 50  $\mu$ M mecamylamine.

(K–N) Mean firing rate (K), CV (L), LV (M), and CV<sub>2</sub> (N) for spontaneous DN1p firing at ZT18-20 from *wake-GAL4* > *UAS-CD8::GFP* flies (n = 7) before and after application of 250  $\mu$ M picrotoxin and 50  $\mu$ M mecamylamine. Comparisons were made within cells. Error bars represent SEM.



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Figure S2. CLOCK and WAKE-Dependent Temporal Coding in I-LNv Clock Neurons and Additional Behavioral Data, Related to Figure 1 (A and B) Representative membrane potential traces and temporal raster plots of spontaneous firing of I-LNvs at ZT6-8 (A) and ZT18-20 (B) in PDF-GAL4 > UAS-

CD8::GFP in wild-type (*left*), Clk<sup>/rk</sup> (*middle*), and *wake*<sup>D2</sup> background flies (*right*). Membrane potential traces are shown for an 8 s window. (C-H) Mean firing rate (C), CV (D), LV (E), CV<sub>2</sub> (F), skewness (G), kurtosis (H) of spontaneous activity of I-LNvs at ZT6-8 and ZT18-20 in PDF-GAL4 > UAS-

*CD8::GFP* in control (*top*, n = 12 for ZT6-8, and n = 12 for ZT18-20), *Clk<sup>irk</sup>* (*middle*, n = 12 and 12), and *wake<sup>D2</sup>* background flies (*bottom*, n = 12 and 12). Comparisons were made between (not within) cells.

(I and J) Autocorrelation function (I) and ISI histograms (J) of the spike trains shown in (A) and (B).

(K) % flies awakened to 0.1g, 0.5g, or 1.2g mechanical stimuli at ZT6-8 or ZT18-20 for *wake-GAL4* > *UAS-CD8::GFP* in control (n = 32 for 0.1g, n = 32 for 0.5g, n = 32 for 1.2g), *Clk<sup>trk</sup>* (n = 32, 30, 30), or *wake*<sup>D2</sup> (n = 32, 32, 32) backgrounds.

(L) sleep (blue) and brief awakenings (red) profile for control *wake-GAL4* > *UAS-CD8::GFP* flies in DD, with CT6-8 and CT18-20 time windows highlighted. (M–P) sleep amount (M), number of brief awakenings (N), sleep bout duration (O), and sleep bout number (P) for control *wake-GAL4* > *UAS-CD8::GFP* flies (n = 32)

in DD.

Error bars represent SEM.



Figure S3. Optogenetic Manipulation of DN1p Spiking Patterns Using "Playback" Temporal Codes Alters Sleep Quality and Related Control Data, Related to Figure 2

(A and B) Activity pattern for a sample fly during the day and night (A) and arousal threshold measurements for flies exhibiting < 5 min or > 5 min quiescence (B) in the optogenetic/video imaging setup.

(C) A representative trace showing spiking of a DN1p neuron in response to regular optogenetic triggering of this cell (red lines) in a R18H11-GAL4 > UAS-CsChrimson fly.

(D) Frequency of DN1p spiking relative to regular optogenetic stimulation. The ratio of optogenetically-hijacked action potentials to optical pulses (i.e., the slope) is  $0.97 \pm 0.01$  at driving frequencies from 0.1-6Hz, but the failure rate increased at a driving frequency of 8 Hz (n = 3 cells, 6 trials each).

(E) Relationship between irregular daytime synthetic optogenetic activity patterns and DN1p spiking. Histogram plot of latency between the end of a given optogenetic stimulus and the evoked DN1p spike, with the duration of LED stimulus noted. Inset shows the ratio of CV, LV, and  $CV_2$  for the optogenetic pattern relative to that for the DN1p spike train. Perforated patch-clamp recordings of DN1ps were performed from *R18H11-GAL4* > *UAS-CsChrimson* flies (n = 7) during optogenetic activation using the irregular daytime synthetic code (CV = 1.2).

(F and G) Sleep/wake state plots of individual tethered control *wake-GAL4* > *UAS-CD8::GFP* flies at ZT6-8 (F) or ZT18-20 (G) exposed to "daytime" synthetic codes via IR-LED. Brief awakenings and awake and sleep states are denoted with red, green, and blue bars, respectively.

(H–K) Sleep amount (H), brief awakenings (I), sleep bout duration (J), or sleep bout number (K) for flies shown in F and G.

(L and M) Sleep/wake state plots of individual tethered *R18H11-GAL4* > *UAS-CsChrimson* flies during optogenetic stimulation of DN1p using playback codes with irregular ( $CV_{ISI} = \sim 1.2$ , n = 6) (L) and regular ( $CV_{ISI} = \sim 0.3$ , n = 6) (M) patterns at ZT18-20.

(N–Q) Sleep amount (N), brief awakenings (O), sleep bout duration (P), and sleep bout number (Q) for flies shown in L and M.

(R) Estimated mean firing rates derived from optogenetic activation patterns for the wake, sleep, and brief awakening states for the irregular daytime (CV = 1.2) synthetic codes. Data are derived from the dataset shown in Figure 21.

Error bars represent SEM.



**Figure S4. Electrophysiological and Behavioral Data Using Additional** *GAL4* **and** *UAS-RNAi* **Lines, Related to Figures 2, 3, and 4** (A) Whole-mount brain (left) and thoracic ganglion (right) immunostaining of a *Clk4.1 m-GAL4 > UAS-CsChrimson-Venus* fly with anti-GFP (green) and anti-BRP (magenta). Scale bar indicates 50 μm.

(B and C) Sleep/wake state plots of individual tethered *Clk4.1 m*-*GAL4* > *UAS*-*CsChrimson* flies during optogenetic stimulation of DN1ps using synthetic codes with irregular (B) ( $CV_{ISI} = 1.2$ , n = 12) and regular (C) ( $CV_{ISI} = 0.3$ , n = 12) patterns at ZT18-20. Brief awakenings and awake and sleep states are denoted with red, green, and blue bars, respectively.

(D–G) Sleep amount (D), number of brief awakenings (E), sleep bout duration (F), and sleep bout number (G) for the flies shown in B and C.

Error bars represent SEM.

<sup>(</sup>H) Peak  $K_{Ca}$  amplitude at a holding potential of +60 mV from voltage-clamp recordings of DN1ps at ZT6-8 (green) and ZT18-20 (blue) for *R18H11-GAL4>UAS-slob-RNAi #2, UAS-tdTomato* flies (n = 8 for ZT6-8 and n = 9 for ZT18-20) versus *R18H11-GAL4 > UAS-tdTomato* controls (n = 7 for ZT6-8 and n = 8 for ZT18-20). (I and J) peak AHP amplitude (I) and CV (J) of spontaneous DN1p spiking at ZT6-8 (green) and ZT18-20 (blue) in *R18H11-GAL4 > UAS-tdTomato* flies (n = 7 for ZT6-8 and n = 7 for ZT18-20) versus *R18H11-GAL4 > UAS-tdTomato* flies (n = 7 for ZT6-8 and n = 7 for ZT18-20) versus *R18H11-GAL4 > UAS-tdTomato* flies (n = 7 for ZT6-8 and n = 7 for ZT18-20) versus *R18H11-GAL4 > UAS-tdTomato* flies (n = 7 for ZT6-8 and n = 7 for ZT18-20) versus *R18H11-GAL4 > UAS-tdTomato* controls (n = 5 for ZT6-8 and n = 7 for ZT18-20).

<sup>(</sup>K–M) peak dVm/dt (K), reliability of time from spike onset to peak dVm/dt (L), and CV (M) of spontaneous DN1p spiking at ZT6-8 (green) and ZT18-20 (blue) in R18H11-GAL4 > UAS-NaK $\beta$  RNAi #2, UAS-tdTomato (n = 8 for ZT6-8 and n = 8 for ZT18-20) versus R18H11-GAL4 > UAS-tdTomato (n = 5 for ZT6-8 and n = 6 for ZT18-20) flies. For panels H-M, comparisons were made between (not within) cells.

<sup>(</sup>N–P) Sleep amount (N), number of brief awakenings (O), and sleep bout duration (P) during ZT6-8 (green) and ZT18-20 (blue) for wt > UAS-slob RNAi (n = 30) and Clk4.1 m-GAL4 > UAS-slob RNAi flies (n = 28).

<sup>(</sup>Q-S) Sleep amount (Q), number of brief awakenings (R), and sleep bout duration (S) during ZT6-8 (green) and ZT18-20 (blue) for wt > UAS-NaK $\beta$  RNAi (n = 32) and Clk4.1 m-GAL4 > UAS-NaK $\beta$  RNAi flies (n = 32).

<sup>(</sup>T–V) Sleep amount (T), number of brief awakenings (U), and sleep bout duration (V) during ZT6-8 (green) and ZT18-20 (blue) for wt > UAS-slob RNAi #2 (n = 32) and R18H11-GAL4 > UAS-slob RNAi #2 flies (n = 32).

<sup>(</sup>W–Y) Sleep amount (W), number of brief awakenings (X), and sleep bout duration (Y) during ZT6-8 (green) and ZT18-20 (blue) for wt > UAS-NaKβ RNAi #2 (n = 30) and R18H11-GAL4 > UAS-NaKβ RNAi #2 files (n = 32).



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Figure S5. Biochemical, Electrophysiological, and Computational Analyses Supporting a Role for WAKE Upregulation of SLOB and NaK $\beta$  in Temporal Coding, Related to Figures 3 and 4

(A) western blot analysis of extracts of HEK293T cells transfected with *wake-FLAG*, *slob-HA*, or both, immunoblotted with anti-WAKE and anti-HA antibodies. As a control, immunoprecipitation was also performed from cell extracts transfected with *wake-FLAG* and *eGFP* and immunoblotted with anti-WAKE and anti-GFP antibodies.

(B) Representative current traces of K<sub>Ca</sub> in I-LNvs at ZT6-8 (green) and ZT18-20 (blue) in wake-GAL4 > UAS-CD8::GFP, wake-GAL4 > UAS-CD8::GFP; wake<sup>D2</sup>, and wake-GAL4 > UAS-CD8::GFP, UAS-slob RNAi flies. Scale bars denote 200 pA and 200 ms.

(C and D) *I-V* relation of steady-state activation of  $K_{Ca}$  current (C) and peak  $K_{Ca}$  amplitude at a holding potential of +60 mV (D) from I-LNvs at ZT6-8 (green) and ZT18-20 (blue) for wake-GAL4 > UAS-CD8::GFP (n = 9 at ZT6-8, and n = 7 at ZT18-20), wake-GAL4 > UAS-CD8::GFP; wake<sup>D2</sup> (n = 7 and 11), and wake-GAL4 > UAS-CD8::GFP, UAS-slob RNAi flies (n = 7 and 7).

(E and F) Averaged spike waveforms (E) and peak AHP amplitude (F) of I-LNvs at ZT6-8 (green) and ZT18-20 (blue) in *wake-GAL4* > UAS-CD8::GFP (n = 12 at ZT6-8 and n = 12 at ZT18-20), for *wake-GAL4* > UAS-CD8::GFP; Clk<sup>irk</sup> (n = 12 and 12), wake-GAL4 > UAS-CD8::GFP; wake<sup>D2</sup> (n = 12 and 12), and wake-Gal4 > UAS-CD8::GFP, UAS-slob RNAi flies (n = 10 and 10).

(G–I) Derivatives of the spike wave membrane potential from spike onset threshold to the peak of the spike, plotted over time (dVm/dt) (G, above) and as heatmaps (G, below), the highest dVm/dt from panel G shown as boxplots (H), and reliability of time from spike onset threshold to highest dVm/dt of spikes (I) from I-LNvs at ZT6-8 (green) and ZT18-20 (blue) in *wake-GAL4* > *UAS-CD8::GFP* (n = 12 for ZT6-8 and n = 12 for ZT18-20), *wake-GAL4* > *UAS-CD8::GFP; Clk<sup>Irk</sup>* (n = 12 and 12), *wake-GAL4* > *UAS-CD8::GFP; UAS-CD8::GFP; UAS-CD8::GFP;* 

(J) Spike rasters for 10 s intervals from throughout the simulation illustrate reduction of spike irregularity with increased  $K_{Ca}$  and  $Na^+/K^+$  ATPase activity. For each model, top-to-bottom rows correspond to beginning-to-end time-points of the simulation.

(K) The ISI CV shows additive decreases in spike irregularity (circles) with increased  $K_{Ca}$  and  $Na^+/K^+$  ATPase activity. Enhanced  $K_{Ca}$  and  $Na^+/K^+$  ATPase activity decreased and increased, respectively, the average firing rate (squares) such that the combined  $K_{Ca}$  and  $Na^+/K^+$  ATPase model maintained a similar firing rate as the day model.

(L) Enhanced K<sub>Ca</sub> and Na<sup>+</sup>/K<sup>+</sup> ATPase activity deepened AHP (circles) and increased maximum action potential (AP) slope (squares), both separately and in combination.

Error bars represent SEM.



#### Figure S6. Additional Electrophysiological Data from DN1p-PI Synapses, Related to Figure 7

(A and B) Representative traces (A) and spontaneous MFR (B) for *in vivo* intracellular recordings of Dilp2+ PI neurons from *dilp2::mCherry* flies at ZT6-8 (n = 6) versus ZT18-20 (n = 6).

(C and D) Representative traces (A) and spontaneous MFR (B) for *in vivo* intracellular recordings of Dilp2+ PI neurons from *wake*<sup>D2</sup>; *dilp2::mCherry* flies at ZT6-8 (n = 5) versus ZT18-20 (n = 6). For panels B and D, comparisons were made between (not within) cells.

(E) Relationship between CV of MFR from the daytime synthetic spike train and a given time window length. A 40 s window was selected as exhibiting a MFR similar to that for the entire 1 hr spike train (see STAR Methods for details). The x axis represents window lengths in log-scale. Data are plotted for all 100 synthetic spike trains, and the mean is shown in red.

(F and G) No potentiation of Dilp2+ PI neuron firing rate (calculated in 20 s bins) in response to 40 s DN1p daytime synthetic spike patterns was observed. Time course plot of MFR (F) and MFR of Dilp2+ PI neurons for the 1 min bins just prior to ("before") and after 6 min 20 s ("after") 40 s LED stimulation (G) for *R18H11-LexA*, *dilp2-Gal4* > *LexAop-CsChrimson*, *UAS-GCaMP6s* at ZT18-ZT20 flies (n = 3). Red bar indicates duration of DN1p optogenetic stimulation. For (G), comparisons were made within cells.

(H) Histogram showing distribution of relative spike timing between DN1p and Dilp2+ PI neuron spikes, within  $\pm$  500 ms of the post-synaptic PI neuron spike. (I–K) Spike onset threshold (I), mean resting membrane potential (J), and membrane potential fluctuations (K) of Dilp2+ PI neurons for 1 min bins just prior to ("before") and after ("after") LED stimulation. Data in (I)–(K) are derived from the same dataset shown in Figure 7. Error bars represent SEM.