Dopamine and GPCR-mediated modulation of DN1 clock neurons gates the circadian timing of sleep
Matthias Schlichting1,2,3, Shlesha Richhariya4, Nicholas Herndon5, Dingbang Ma6, Jason Xin5, William Lenh5, Katharine Abruzzi5, and Michael Rosbash1,6,7

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The metronome-like circadian regulation of sleep timing must still adapt to an uncertain environment. Recent studies in Drosophila indicate that neuromodulation not only plays a key role in clock neuron synchronization but also affects interactions between the clock network and brain sleep centers. We show here that the targets of neuromodulators, G Protein Coupled Receptors (GPCRs), are highly enriched in the fly brain circadian clock network. Single-cell sequencing indicates that they are not only enriched but also differentially expressed and contribute to clock neuron identity. We generated a comprehensive guide library to mutagenize individual GPCRs in specific neurons and verified the strategy by introducing a targeted sequencing approach. Combined with a behavioral screen, the mutagenesis strategy revealed a role of dopamine in sleep regulation by identifying two dopamine receptors and a clock neuron subpopulation that gate the timing of sleep.

Significance
Neuromodulation is essential for adaptive animal behaviors among other physiological processes. It is essential to reliably manipulate neuromodulator pathways to understand their functions in animal physiology. In this study, we generated a CRISPR-Cas9-based guide library to target every G-Protein Coupled Receptor (GPCR) in the Drosophila genome and applied it to the well-studied clock neuron network. Notably, these GPCRs are highly enriched and differentially expressed in this small network, making it an ideal candidate to investigate their function. We cell-type specifically mutated GPCRs highly efficiently with no background gene editing detected. Applying this strategy to a specific node of the clock network revealed a role for dopamine in prolonging daytime sleep, suggesting network-specific functions of dopamine receptors in sleep-wake regulation.

Author affiliations: 1Department of Biology, Howard Hughes Medical Institute, Brandeis University, Waltham, MA 02451; and 2Department of Biology, College of Science, Northeastern University, Boston, MA 02136

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fifth sLNv serve a different function and are important for evening activity (14, 15). A pair of dorsal neurons (DN2s) are essential for temperature preference rhythms (19). No function has been assigned to the DN3s, perhaps due to the lack of a specific driver. The DN3s are the most numerous clock neuron group (35 to 40 neurons/hemisphere).

The second most numerous clock neuron group is the DN1 cluster, which consists of ~15 neurons per hemisphere (19). Under DD conditions, accelerating or decelerating the clock in these cells has no effect on rhythmic behavior, and DN1p output is even dispensable for rhythmic DD behavior (20–23). However, changing the speed of these cells under LD cycle conditions shifts the timing of the evening activity peak, indicating DN1p neurons have a conditional role in circadian timing. Moreover, several groups have shown that specific DN1p neurons affect morning as well as evening activity and influence fly sleep, both the amount of sleep and when sleep occurs (24–26). Consistent with a role in sleep promotion and/or maintenance, imaging and tracing experiments identified physiological pathways connecting specific DN1ps to fly brain sleep regions like the ellipsoid body (24).

Despite a general agreement on the variety of functions carried out by DN1ps, there are discrepancies in assigning specific behavioral roles to specific DN1p subgroups. For example, glutamatergic DN1ps have been identified as controlling the morning component of behavior, whereas another study suggests that these same neurons control evening activity (25, 26). A likely explanation is that the DN1ps are even more diverse than previously thought, i.e., there may be multiple glutamatergic DN1p subgroups. Indeed, a recent single-cell clustering study found that there are at least 5 different glutamatergic DN1p subgroups, which is consistent with the notion that different DN1p functions might derive from different neuron subpopulations (27). The physiological and anatomical heterogeneity of the CCNN neuron population is probably due to its striking transcription factor specificity as well as to other differences in gene expression between individual neurons (27).

To address the contribution of DN1ps and neuromodulation to sleep behavior as a specific physiological process, we combined the power of single-cell RNA sequencing (scRNAseq) data with cell-specific CRISPR-Cas9-mediated gene mutagenesis. The scRNAseq results not only show that GPCRs are strongly enriched in the CCNN but also that they are highly differentially expressed. Indeed, clustering the clock neurons only based on GPCR expression shows that each subcluster expresses a unique combination of receptors, suggesting that it enables specific network nodes to integrate and respond to different stimuli. To mutate specific receptors in a cell-specific manner, we generated a CRISPR-Cas9-based guide library for all GPCRs following the pioneering work of Port and Bullock (28). We and others had previously demonstrated that this strategy efficiently removes PER or TIM expression from the clock network in a cell-specific manner (29, 30). Here, we also added an adapted targeted genomic sequencing approach to show that the library effectively mutates GPCRs in a cell-specific manner, indicating that the library and strategy constitute a key asset for investigating neuromodulation. Indeed, we used the library in a behavioral screen that identified several GPCRs that promote sleep from within the CCNN. In addition to already known sleep-promoting GPCRs, we discovered that two dopamine receptors (Dop1R1 and Dop1R2) in the DN1ps prolong daytime sleep. Moreover, combining transynaptic tracing techniques with scRNAseq data shows that different subsets of DN1ps contribute differentially to sleep. The results taken together indicate that dopamine regulates sleep timing via a novel cellular subcircuit within the CCNN that is highly context specific. These findings and methods will facilitate investigating the complex contribution of dopamine as well as other neuromodulators and neurotransmitters to many other physiological processes.

**Results**

**Circadian Neurons Show Enriched Expression of Signaling Molecules.** Recent work implicates clock neuron interactions as essential for molecular and behavioral rhythms. For example, manipulating clock neuron subpopulations can affect behavioral timing, and residual clock protein expression in only a few circadian neurons is sufficient to retain some circadian functions (11, 23, 29–31). To identify molecules within the clock network that contribute to the implied network synchrony, we isolated and sequenced fluorescence-activated cell sorting (FACS)-sorted clock neurons (clk856 > EGFP) under LD conditions at two times, namely, Zeitgeber time 2 (ZT2) and ZT14, and compared the transcriptomes to those from panneuronal adult head samples (nSyb > EGFP).

We first compared clock gene expression between the time points in the clock neurons. Consistent with expectation (32), clk mRNA levels were ~6x higher at ZT2 than at ZT14, whereas tim mRNA levels were 22x higher at ZT14 compared to ZT2 (Fig. 1A). Housekeeping genes such as Act5C and Rpl32 were not different between time points (Fig. 1A). Not surprisingly, clock gene expression was dramatically lower in the nSyb dataset (averaged reads of all nSyb libraries show 1,428x reduced clk and a 27x reduced tim expression compared to sorted clk856-neurons; SI Appendix, Fig. S1), demonstrating highly efficient enrichment of clock neurons by clk856 > EGFP purification.

To address clock neuron gene enrichment more generally, we used edgeR (33) to perform differential gene expression analysis. It identified a total of 1,719 genes as differentially expressed with a false discovery rate of <0.05 between nSyb and clock neurons. Of these, 704 genes were significantly up-regulated, and 305 genes were significantly down-regulated in the clock network with at least a twofold change in amplitude (Fig. 1B). A Gene Ontology (GO)-term analysis of clock-enriched genes resulted in enrichment in expected categories like the circadian regulation of temperature homeostasis (27-fold), positive regulation of circadian sleep/wake cycle (16-fold), and circadian regulation of gene expression (11-fold). Interestingly, genes associated with signaling pathways were also found to be comparably enriched among the clock-enriched genes and include the octopamine and tyramine signaling pathways (16-fold), the serotonin receptor signaling pathway (16-fold), and the GPCR signaling pathway (12-fold). Other unrelated pathways are enriched in the nSyb neurons relative to the clock neurons (for an example, see Fig. 1C). The increased expression of genes involved in intercellular signaling pathways, including serotonin signaling and GPCRs, strongly implicate neuronal communication in clock network function.

**GPCRs Are Differentially Expressed within the Clock Neuron Network.** The enrichment of GPCR signaling pathways inspired a focus on quantitative features of GPCR expression. Surprisingly, more than two-thirds of the 124 GPCR mRNAs encoded by the Drosophila genome are expressed in the clock neurons (reads per million [RP], >3; Fig. 1D) with an almost 100x difference between the least and most expressed of these 86 mRNAs. The nSyb data had similar differences in GPCR expression levels (SI Appendix, Fig. S2). A direct comparison with the nSyb dataset indicates that 22 GPCRs are at least twofold
The RNA expression data suggest that many individual GPCRs are either expressed at significantly higher levels in all clock neurons or are predominantly expressed in a few neuronal subpopulations. To address these alternatives, we analyzed previous scRNAseq data for GPCR expression and focused on the 17 high-confidence clock neuron clusters (Fig. 2A); these clusters are missing most of the enigmatic DN3 clock neurons but include most if not all well-characterized clock neurons including all lateral and most dorsal clock neurons.

The dopamine receptor DopEcR transcript is highly expressed in the nSyb as well as the clk856 dataset with high transcript levels in all clock neuron clusters (Fig. 2B). The differential expression patterns of more poorly expressed GPCR transcripts within the clock neuron population were similarly impressive. For example, CNMaR appears to be almost exclusively expressed in one DN1p and the DN2 cluster, whereas Gaba-B-R3 is highly enriched in the sLNv cluster. sNPFR is expressed more broadly but still mostly in the clusters defining the DN1ps, the INLNs, and the DN3s (Fig. 2B). A differential GPCR expression pattern is also apparent when comparing all GPCR expression using scRNAseq despite dramatic variation in expression levels (SI Appendix, Fig. S3).

To confirm this cell specificity in an independent way, we used GAL4 lines in which the GAL4 sequence was integrated into the enigmatic DN3 clock neurons but include most if not all well-characterized clock neurons including all lateral and most dorsal clock neurons. For example, some GPCRs such as the aforementioned DopEcR appear to be expressed almost panneuronally within the brain, whereas others are not expressed in the brain or only in one to two cells per hemisphere (SI Appendix, Fig. S2).

Fig. 1. Clock neurons show an enriched expression of GPCRs. (A) Plots show normalized expression levels of indicated genes from sorted clock neurons (clk856 > EGFP) at ZT2 and ZT14. As expected, Clk expression is significantly higher at ZT2 than at ZT14, whereas tim expression is significantly higher at ZT14 compared to ZT2. Housekeeping genes such as Rpl32 and Act5C were not significantly different between those timepoints. (B) Heatmap of differentially expressed genes between clock neurons (clk856 > EGFP) and randomly chosen neurons (nSyb > EGFP) at ZT2 (2 repeats, R1 and R2) and ZT14 (2 repeats, R1 and R2) with an at least twofold difference in expression at a false discovery rate of <0.05. Relatively high expression is displayed in red and relatively low expression is displayed in blue (Z-scores indicated on top of the graph). (C) Examples of up- or down-regulated genes representing enriched GO terms. Pdf is significantly up-regulated in the clock network (GO: Circadian control of sleep wake cycle) as are tbh (GO: Octopamine and tyramine signaling pathway) and 5-HT1A (GO: G protein coupled receptors). br is on the other hand is significantly down-regulated in the clock network (GO: Antennal development). Both timepoints were combined for this analysis. (D) Normalized expression (RPM) of GPCR genes in the clock network. GPCR gene expression varies dramatically between receptors from being not expressed (<3 RPM, red line) to being highly expressed. Both timepoints were combined for this analysis. (E) Relative expression of GPCRs in clock neurons (clk856 > EGFP) relative to randomly chosen neurons (nSyb > EGFP). Reads for individual GPCRs were pooled across timepoints and replicates and a ratio was calculated. A total of 22 GPRs are at least twofold higher expressed in the clock cells (red bars) compared to only 2 GPCRs being at least twofold down-regulated in the clock cells (blue bars). The x-axis is log2 scaled. Both timepoints were combined for this analysis.
A Guide Library Allows for Cell-Specific Manipulations of All GPCRs. The likely contribution of GPCRs to clock neuron identity and the neuropeptide requirement for clock neuron clusters is superior to RNA interference (RNAi)-mediated gene expression and the neuropeptide requirement for clock neuron synthesis alone is sufficient to define the identity of most clock neurons and also suggest that each cluster may exhibit a unique neuropeptide response pattern.

Fig. 2. GPCRs are differentially expressed and can define clock neuron identity. (A) scRNAseq of clock neurons (clk856 > EGFP) identifies 17 bona-fide clock neuron clusters. DN1 neurons can be separated into six different clusters (1, 4, 6, 7, 15, and 18, labeled in red). For details on clustering see ref. 27. (B) Expression of different GPCRs within these 17 clusters. DopEcR is highly expressed in all clock neuron clusters, whereas CNMOR, GABA-B-R3, and sNPFR-R are more differentially expressed. For details see “GPCRs are differentially expressed within the clock neuron network” section. (C) Immunohistochemistry of whole-mount brains stained against GFP (green) and PER (magenta). Nuclear GFP (UAS-stinger) was expressed under the control of GAL4-knock-in lines. Dh31-R-GAL4 drives expression in three out of four lLNvs and one sLNv, and does not express in DN1s, DN2, or LNds. sNPFR-GAL4 is expressed in 1 lLNv, 2 DN1s, and the DN2 neurons. (D) Seurat clustering of individual neurons included in 17 high-confidence clusters. Unsupervised clustering (Top Left) recapitulates previously published results. Similarly, clustering only based on GPCR expression generated 17 different clusters (Top Right). Bottom row: Analysis of retained (red) or newly assigned (blue) clock neuron identities when plotted only based on GPCRs. Most neurons in clusters 1 (Bottom Left) and cluster 2 (Bottom Right) are assigned the same clusters (red dots), whereas only a few cells were assigned to different clusters (blue dots) based on GPCR-only clustering of clock cells.

A high-confidence clock neuron clusters (Fig. 2D). We then used GPCR expression alone for clustering, omitting the contribution of all other genes expressed in the individual cells. This unsupervised GPCR-based clustering also identified 17 distinct clusters (Fig. 2D). To our surprise, many of these newly generated clusters could be mapped onto the previously published dataset (27), indicating that the GPCR-generated clusters are of biological and anatomical significance. For example, the newly identified cluster 1 includes ~90% of this previously identified sLNv cluster. This is also the case for other clock clusters like the DN1s (Fig. 2D). The data therefore indicate that GPCR expression alone is sufficient to define the identity of most clock neurons and also suggest that each cluster may exhibit a unique neuropeptide response pattern.

A Guide Library Allows for Cell-Specific Manipulations of All GPCRs. The likely contribution of GPCRs to clock neuron identity and the neuropeptide requirement for clock neuron synchrony inspired the development of a general strategy to eliminate any GPCR in a neuron-specific manner. Relevant to this goal, we and others recently showed that CRISPR-Cas9-based mutagenesis is superior to RNA interference (RNAi)-mediated gene expression knockdown in the fly brain (29, 30). As the former also does not require strongly expressing driver lines, it is much more amenable to highly neuron-specific split-GAL4 lines.

The Drosophila genome encodes 124 GPCRs. They are like mammalian GPCRs and react to a variety of stimuli, including biogenic amines, neurotransmitters, neuropeptides, and even light (Fig. 3A) (4). To mutate these receptors, we generated UAS-guide lines, each of which expresses three guides targeting the coding sequence of an individual GPCR. Three guides have previously been shown to efficiently mutate eye tissue and provide high mutagenesis efficiency by compensating for potential noncutting guides (28); this strategy also worked well to remove PER and TIM from the clock system (29, 30). We used clk856-GAL4 to drive the expression of GFP, Cas9, and the guides of interest to mutate individual GPCRs in most of the clock network. The goal was to generate double-strand breaks within the coding sequences of GPCR genes. Given that repair is error prone, this strategy should generate small deletions of variable sizes and frame shifts, resulting in nonfunctional GPCRs.

As there are no reliable antibodies for many of the receptors, we verified the strategy with a targeted genomic sequencing approach. As the clock neurons were simultaneously labeled with GFP as well as mutated by the CRISPR-Cas9 system, we FACs sorted and analyzed 2,000 GFP-positive cells, which should have been mutated, and 2,000 GFP-negative cells, which should remain wild type (Fig. 3B). We designed three sets of primers flanking each of the guide binding sites to allow for gene-specific amplification. As three guides are being used at the same time, there can either be small deletions in the area of guide binding (Fig. 3C, PCR1 to 3) or larger deletions if multiple guides cut at the same time, resulting in different DNA fragment combinations (Fig. 3C, PCR4 to 6).

We analyzed five randomly chosen target genes on three different chromosomes (PDFR and Tre1 on the X-chromosome, mAchR-A and CG15614 on the second chromosome, and CrzR on the third chromosome) to avoid possible biases from chromosome location. PDFR served as a positive control; guide-mediated mutagenesis of...
the clock network with its guides completely reproduced PDFR full body mutant (han5304) phenotypes (35,36).

All three PDFR guides generated deletions of variable sizes at the predicted cut sites in GFP-positive cell DNA (Fig. 3D). Moreover, there were large deletions as indicated by a genomic fragment representing a deletion of several thousand base pairs (Fig. 3C, PCR5). GFP-negative cells in contrast showed no deletions in the investigated area, suggesting that there is no background mutagenesis due to leaky expression in nontarget cells. We then assayed CG15614. Like for PDFR, all three guides generated deletions, whereas GFP-negative cells were unaffected (Fig. 3C). Tre1 and CrzR had similar results, whereas only two out of the three guides for mAchR-A created deletions (SI Appendix, Fig. S5).

The efficacy of the three individual guide sequences to generate small deletions varied substantially, from between 0.7% (as mentioned above for one guide of mAchR-A) to more than 50%, with no evident chromosome or location bias. When an individual guide failed to generate mutations, the other two guides efficiently mutated the gene of interest; this indicates the importance of using several guides to compensate for potential noncutters. There was also a reduced frequency of bigger deletions of coding sequences. For example, two genes (CrzR and mAchR-A) did not show big deletions, whereas 2% to 6% of the reads from other genes reflected big deletions. It is important to note that these percentage are based on the sequencing of pooled neurons from several animals and therefore do not reflect a single, mutated neuron. Nonetheless, the collective data support the original assertion based on...
eye color essays (28), namely, that combining three guides per gene is an effective strategy to manipulate genes of interest.

**DN1p Modulation Alters the Sleep Structure of Male Flies.** To exploit this functional library, we focused on the DN1ps. This specific group of dorsal neurons influences several aspects of *Drosophila* behavior. This is because manipulating these cells affects activity in the morning and during the siesta as well as in the evening. In addition, DN1ps affect sleep and connect to sleep centers within the central complex (24–26). Recent work also showed that this group of neurons can be subdivided into six independent clusters with functions that are not yet clearly understood. The molecules that affect sleep within these neurons are also mostly unknown. To address this question, we used *clk4.1M-GAL4*, which expresses in 8 to 12 of the 15 DN1ps per hemisphere. We first reproduced previous experiments; activating these neurons significantly altered sleep in the middle of the day, the prominent siesta of male flies (SI Appendix, Fig. S6).

To identify candidate DN1p GPCRs, we turned to our single-cell data and identified 21 GPCRs enriched in DN1ps compared to the other clock cells. We then performed a behavioral screen in which we compared the behavior of control flies with flies harboring mutated GPCRs in their DN1ps. The control flies expressed *Cas9* but no guides in these neurons (*clk4.1M > Cas9*).

As expected, *Cas9* expression in the DN1ps did not affect fly behavior; they showed the canonical sleep pattern with consolidated sleep at night and during the siesta with almost no sleep in the morning and the evening; this reflects the standard bimodal activity pattern (Fig. 3E). We then focused on the siesta and quantified sleep levels between ZT3 and ZT9. Male flies sleep extensively during this time, leading to a median of ~5.5 h of siesta sleep in control flies. Of the 21 mutated strains, 4 significantly reduced their siesta sleep compared to the control group by 30% and reproduced the increased siesta sleep phenotype observed by Pimentel et al. (41) using the same dFB driver 23E10 and RNAi knockdown of *Dop1R2* (SI Appendix, Fig. S8). The results taken together indicate that the target receptors and cells dictate the effect of dopamine, which can promote sleep as well as wake.

The sleep patterns indicate that the siesta is terminated earlier in flies with mutated dopamine receptors compared to controls, suggesting that dopaminergic input contributes to timing the end of the siesta. Notable in this context is the traditional *per* mutant strain; it has a very short free-running period with a similar LD phenotype, e.g., the evening peak occurs during the daytime (41, 42). Yet there was no effect of removing both dopamine receptors from the DN1ps on period length or rhythmicity; experimental groups as well as controls were identical to wild-type flies (Fig. 4E and F), suggesting that changes in clock speed are not responsible for the sleep phenotypes. The data indicate that dopaminergic input onto DN1p neurons enhances sleep during the siesta, likely caused at least in part by delaying the onset of evening activity.

**Subclustering of DN1 Neurons Allows the Differentiation of Neurons Controlling Evening Activity.** How might dopaminergic modulation of DN1ps impact the siesta? To address this question, we first used the *trans-Tango* technique to label target neurons through its anterograde transsynaptic circuit tracing (43). We applied this technique to the dopaminergic system (*TH-GAL4*) and labeled downstream neurons with GFP. Because there were many GFP-positive neurons, we costained with anti-PER and reproduced the increased siesta sleep phenotype observed by Pimentel et al. (41) using the same dFB driver 23E10 and RNAi knockdown of *Dop1R2* (SI Appendix, Fig. S8). The results taken together indicate that the target receptors and cells dictate the effect of dopamine, which can promote sleep as well as wake.

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Examining the 6 DN1p RNA expression clusters in more detail indicated that *Dop1R1* and *Dop1R2* are primarily expressed in 4 clusters, numbers 6, 7, 15, and 18 (Fig. 5A). Intriguingly, four is identical to the number of tyrosine hydroxylase neurons targeting DN1ps. Importantly, the size of these four clusters is rather small, indicating that several of them may only contain a single cell (27). We also considered the two remaining DN1p clusters, namely, clusters 1 and 4. The biggest cluster (#1) is the only one that expresses *AstC*. We showed previously that *AstC* is expressed in four DN1 neurons, which fits well with the size of this cluster (10, 27). Knockdown of *AstC* affected the timing of the evening peak in summer or winter days, suggesting that these neurons also contribute to evening activity, at least as a function of different seasons (10). In addition, this is the only cluster expressing *TrissinR*, which also produced a siesta phenotype in our behavioral screen (SI Appendix, Fig. S7). The data taken together indicate that several and perhaps most DN1 clusters contribute to evening activity.

We exploited the striking difference in *Vglut* expression between morning and evening DN1p cells to further address how the DN1ps regulate evening activity (Fig. 5A). Combining a *per-AD* with *Vglut-DBD* labeled on average seven to eight DN1s per hemisphere, consistent with the expected number of neurons in these clusters (SI Appendix, Fig. S10). *Trans-Tango* experiments with this split-GAL4 revealed that these seven to eight DN1p neurons primarily target neurons in the dorsal part of the brain (Fig. 5B). They include DN1s, DN2s, and DN3s, and this split-GAL4 also targets all LNdS in the lateral part of the brain; they are a major controller of evening activity. These data reinforce previous results (25) indicating that the glutamatergic
subset of DN1ps likely controls evening activity and sleep at least in part through their interactions with other clock neurons. Importantly, the upstream influence of dopamine on these cells and their functions widens the influence of the environment and brain state over the siesta and evening cell timing (see Discussion).

**Discussion**

We show here that GPCRs are strongly expressed in the fly brain CCNN and are capable of identifying individual clock neurons. To identify individual receptors that contribute to specific neuron function, we combined a behavioral-sleep screen with a previously validated CRISPR-Cas9-specific neuron-mutagenesis strategy that exploited comprehensive *Drosophila* GPCR guide library. The strategy was verified with a targeted sequencing approach and revealed a role of dopamine in sleep maintenance during the siesta. Dopamine generally inhibits sleep by stimulating locomotor activity, in flies as well as mammals (38–40, 44). For example, compounds like amphetamine increase synaptic dopamine levels, which enhance fly activity and inhibit sleep (45). However, a specific sleep-promoting subpopulation of DN1ps uses this neurotransmitter for the opposite purpose, namely, to prolong sleep during the siesta. This surprising conclusion resulted from the identification of the two dopamine receptors Dop1R1 and Dop1R2 as well as a clock neuron subpopulation, within which the two receptors gate the timing of daytime sleep.

The CCNN is an ideal platform to study neuromodulation, as genetic studies underscore the importance of neuropeptides to circadian behavior and even to circadian neuron subtype identification (11, 23, 32). This is also true for the mammalian brain and its CCNN, the suprachiasmatic nucleus (46). Notably, most neuropeptides act through GPCRs, and 22 of the GPCRs expressed within the fly brain are at least 2× up-regulated in the CCNN (Fig. 1E). This result is even more striking in light of our scRNAseq data, which show that many GPCRs are differentially expressed among clock neurons (Fig. 2).

To verify expression patterns, we costained relevant GAL4 knock-in lines with an anti-PEL antibody and thereby determined

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**Fig. 4.** Modulation of DN1ps by dopamine enhances sleep. (A) Average sleep profile of male flies in which Dop1R2 was mutated in the DN1ps (red) and controls (gray and black). Error bars represent SEM. Background colors indicate 4× 6-h periods that were quantified in B. (B) Quantification of sleep separated into four different time zones, as follows: morning (ZT21 to ZT3), siesta (ZT3 to ZT9), evening (ZT9 to ZT15), and night (ZT15 to ZT21). Removing Dop1R2 in the DN1ps significantly reduced sleep during the siesta (one-way ANOVA followed by post hoc Tukey test shows significant differences between EXP and both controls, P < 0.01 each), whereas other times of day were not affected. (C) Average sleep profile of male flies in which Dop1R2 and Dop1R1 were mutated in the DN1ps (red) and controls (gray and black). Error bars represent SEM. Background colors indicate 4× 6-h periods that were quantified in D. (D) Quantification of sleep separated into four different time zones. Removing Dop1R2 and Dop1R1 in the DN1ps significantly reduced sleep during the siesta (P < 0.01), whereas other times of day were not affected. n.s., not significant. (E and F) Flies with mutated Dop1R1 and Dop1R2 in the DN1ps (Exp) have rhythmicity the same as controls (E) and no effect in free-running period (F) when recorded in DD compared to both controls (clk4.1M > Cas9 [black] and clk4.1M > Dop1R1-g, Dop1R2-g [gray]).
the overlap between receptor and clock protein gene expression. Despite some minor differences, the knock-in lines were broadly consistent with the RNAseq data and reinforced the notion of cell-specific GPCR expression (Fig. 2C). Importantly, individual GPCRs appeared to be expressed in only some cells of supposedly uniform clusters. For example, only one cell of cluster 1 stains for Dhh31-R (Fig. 2C), suggesting even more clock neuron diversity than previously indicated (27).

The data underscore more generally the importance of GPCR expression to the CCNN. To investigate this further, we analyzed our single-cell data based only on GPCR expression. There were only marginal changes in cluster formation, i.e., we were still able to generate 17 clusters which were similar to those previously published (27). This surprising result indicates that GPCRs can identify individual neuron subpopulations, at least within the clock system, and suggests that they can define functional identity. This likely includes subtle contributions to phenotype beyond defining cell-specific ligand responses.

GPCRs are difficult to study genetically in mammals. This is because there are usually multiple genes encoding a single GPCR. The situation is simpler in flies where there is usually only one gene that encodes each of the 124 GPCRs encoded in the fly genome. Our guide library targets each of these GPCRs and uses three independent guides for each receptor, a strategy shown to be highly efficient in previous studies (28–30). As there are no available antibodies for GPCRs, we validated the strategy with a targeted genomic sequencing approach from isolated neurons. It can directly characterize the molecular consequences of the guide-mediated mutagenesis on the clock network. Like the guide strategy previously used to eliminate PER (29, 30), the GPCR guides can reliably delete GPCR-encoding genomic DNA from GFP-positive cells with no detectable deletions in GFP-negative cells. This further supports the notion that the UAS constructs impact minimally if at all cells outside of the canonical GAL4 expression pattern (Fig. 3).

We focused on DN1ps because of their molecular complexity as well as their known contributions to morning activity, the siesta, and even nighttime sleep (47). Of the 21 DN1p-enriched GPCRs, 4 promote sleep based on the knockdown results. One of them, rh7, reproduced previously published results of whole-body mutations, suggesting that it contributes to the siesta at least in part via DN1p expression (37). To our surprise, two dopamine receptors, namely, Dop1R1 and Dop1R2, also promote siesta sleep via the DN1ps and do so by gating the timing of siesta termination. This role resembles previous results indicating that Vglut and AstC are expressed in the DN1 neurons and influence the siesta and/or evening activity under conditions that mimic seasonal regulation (10, 25, 48).

There are four different clusters of DN1 neurons that show an elevated expression of Dop1R1 or Dop1R2, suggesting that one or more of these clusters are responsible for the siesta phenotype. Unfortunately, the lack of more narrow drivers precludes more precise identification.

How can dopaminergic input influence the circadian gating of siesta sleep? The putative downstream targets of dopaminergic neurons correlate nicely with previously published imaging data; lateral as well as dorsal clock neurons increase their cAMP levels in response to bath-applied dopamine (49). Bath application of the neuropeptide PDF causes a similar cAMP increase (50). Notably, this increase stabilizes PER, which is thought to delay the timing of the molecular clock (51). A similar mechanism might apply to dopamine and the DN1ps, which would then delay clock timing within these cells. Removing dopamine receptors would then lead to a decrease in cAMP levels and a consequent advance in timing, thereby explaining the early termination of the siesta in our experiments. Independent of such mechanistic speculation, our data add to our view of how the clock system works to regulate sleep; dopaminergic input presumably reflects the monitoring by the CCNN of brain and environmental status, which then adjusts circadian timing. This change in CCNN properties likely leads to altered release of neuropeptides and/or neurotransmitters, which will then alter whole animal physiology. (Fig. 6). This complexity could even be part of a resilience and plasticity neuropeptide and neurotransmitter system similar to that described in the crustacean stomatogastric ganglion.
We have found this mutagenesis strategy and guide library to be highly effective and far superior to and more reliable than RNAi. There are no background issues, and weak expression is still sufficient to generate mutations. Given the broad role of neuropeptides, transmitters, and GPCRs in most aspects of brain function and behavior, we anticipate that this GPCR mutagenesis strategy and library will be of use to a broad range of fly brain neuroscientists, well beyond the circadian system and the few other researchers who have already used them (52).

**Materials and Methods**

**Fly Strains and Rearing.** Flies used in this study are listed in SI Appendix, Table S1. All flies were raised at 25 °C in a temperature-controlled incubator in LD 12:12 h.

**Generation of Fly Lines.** To generate UAS-guideRNA flies, we used the pCFD6 vector (addgene #73915, described in ref. 28). In short, we generated three guides targeting the coding sequence of each GPCR. To identify possible target sites and avoid off-target effects, we used the optimal target finder developed by C. Dustin Rubinstein, Ed O'Connor-Giles, and Kate M. O'Connor-Giles (53). Gene-specific guide sequences were then incorporated into the primers (SI Appendix, Table S2), and the protocol described in ref. 28 was followed. Correct clones were identified by colony PCR followed by Sanger sequencing. Plasmids were injected into the attP1 site on the second chromosome (Bloomington Drosophila Stock Center [BDSC]: 8621) by Rainbow Transgenic (Rainbow transgenic Inc.). Individually silencing was done by restricting the variable features to GPCRs by setting the features argument of the ScaleData Seurat function to all genes identified as GPCRs and frozen on dry ice immediately after collection. Two sets of neurons were collected from each dissociated sample.

**cDNA Synthesis and Library Preparation for Bulk Sequencing.** PolyA mRNA was isolated from the frozen cell samples using the Dynabeads mRNA direct kit (Thermo fisher 61011). Subsequently, complementary DNA (cDNA) was prepared using the method described in Picelli et al. (54). cDNA integrity and concentration were assessed using a High Sensitivity D5000 ScreenTape (Agilent 5067-5592). A total of ~500 pg of cDNA was used as the input to make sequencing libraries with the Illumina Nextera XT DNA Library Preparation Kit (FC-131-1096) with nine PCR cycles. Final libraries were quantified on a High Sensitivity D1000 ScreenTape on the TapeStation (5067-5584).

**scRNAseq Analysis.** For details on scRNAseq procedures, refer to Ma et al. (27). In order to identify differentially expressed GPCRs in clock neurons, we first computed all marker genes in each cluster using the FindAllMarkers function. Using a negative binomial generalized linear model, the batch effect from sequencing depth and conditions was regressed out. We next used an adjusted \( P \) value significance of 0.05 and fold change cutoff of 1.25. GPCRs matching these criteria are regarded as differentially expressed in clock neurons. Their expression was plotted by the ComplexHeatmap package.

Annotated single-cell clustering data were used as the basis for GPCR-based re clustering using Seurat version 4 in R (56). For the downstream analysis, only cells from the 17 high-confidence annotated clusters were used. GPCR-based re clustering was done by restricting the variable features to GPCRs by setting the features argument of the ScaleData Seurat function to all genes identified as GPCRs from Hanlon and Andrew (4) that were detectable in all timepoints. For the standard clustering, the FindVariableFeatures function with default settings was used to determine variable genes.

For both approaches, principal components (PCs) were determined using the RunPCA function, and the first 20 PCs were selected for clustering based on visual inspection of the ElbowPlots. Communities were generated using the standard workflow functions FindNeighbors, FindClusters, and RunUMAP with default settings. The resolution argument of the FindClusters argument was set at the default of 0.8 after experimentation with resolutions as low as 0.5. Higher values
for resolution and increased numbers of PCs did not improve clustering results separately or in combination for the GPCR clustering. The cell cluster identities published in Ma et al. (27) were stored in the Seurat metadata and used to assess the correspondence between the annotated clusters and the GPCR-based clustering. In general, the GPCR clusters correspond to a single annotated cluster and vice versa. There are two exceptions, as follows: the fusion of the two LNd clusters 9 and 12 into a single cluster and the fusion of the DN1p cluster 4 into two similarly sized clusters. The split of the DN1p cluster is characterized by a nearly twofold difference in average levels of FMRFaR, which is expressed in subsets of DN1 neurons.

**Targeted Genomic Sequencing.** To analyze the potency of our guide library, we established a targeted genomic sequencing approach similar to the 16S metagenomic sequencing library preparation protocol (Illumina 150444233). In short, we generated three pairs of primers for each gene (SI Appendix, Table S3). Each pair of primers was designed to amplify 230- to 270-bp-long genomic sequences for each gene. For each pair, we synthesized three primers (two short, we generated three pairs of primers for each gene (SI Appendix, Table S3).

**Behavioral Analysis.** Two to 7-d-old male flies were individually placed into glass tubes with food (2% agar, 4% sucrose) on one end and a plug to close the tube on the other end. These tubes were subsequently placed into Drosophila Activity Monitors (DAMs; Trikinetics Inc.), and a computer recorded the number of infrared light beam interruptions in 1-min intervals. Flies were recorded for 1 wk in LD 12:12 followed by another round of cleanup. Final libraries were quantified on a High Sensitivity D1000 ScreenTape on the TapeStation (5067-5584). Libraries were then pooled and sequenced using the miseq platform (Genewiz Inc.). Libraries were analyzed using Crispresso2 (57).

To analyze changes in rhythmicity, we performed a $\chi^2$ analysis for rhythmicity and analyzed the speed of the clock. Individual values were compared using an earlier version of this manuscript. A special thank you to Dr. K. Clement for the help with setting up Crispresso2 on our server. The neuronal network. The PDF neuron is a new functional clock neuropeptide in the fruit fly Drosophila. The neuropeptide is a new functional clock neuropeptide in the fruit fly Drosophila.

Data Availability. RNAseq data have been deposited in GEO (GSE202407) (62). ACKNOWLEDGMENTS. We thank Dr. Y. Rao, Dr. R. Allada, and Dr. C. Helfrich-Förster for their fly lines and antibodies. We also want to thank Dr. P. Garrity, Dr. D. Rogulja, and Dr. E. Levitan for comments on an earlier version of this manuscript. A special thank you to Dr. K. Clement for the help with setting up Crispresso2 on our server. The neuronal network. The PDF neuron is a new functional clock neuropeptide in the fruit fly Drosophila. The neuropeptide is a new functional clock neuropeptide in the fruit fly Drosophila.

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