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Cell type-specific driver lines targeting the *Drosophila* central complex and their use to investigate neuropeptide expression and sleep regulation

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eLife Assessment

This is a **fundamental** body of work reporting anatomical, molecular, and functional mapping of the central complex in *Drosophila*. There were a few concerns of a minor nature, and all were addressed by the authors. The tools generated and the findings, which include characterization of neuromodulators used by different cells, will undoubtedly serve as a foundation for future studies of this brain region. The data are **compelling** and likely to have a major impact.

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Abstract

The central complex (CX) plays a key role in many higher-order functions of the insect brain including navigation and activity regulation. Genetic tools for manipulating individual cell types, and knowledge of what neurotransmitters and neuromodulators they express, will be required to gain mechanistic understanding of how these functions are implemented. We generated and characterized split-GAL4 driver lines that express in individual or small subsets of about half of CX cell types. We surveyed neuropeptide and neuropeptide receptor expression in the central brain using fluorescent *in situ* hybridization. About half of the neuropeptides we examined were expressed in only a few cells, while the rest were expressed in dozens to hundreds of cells. Neuropeptide receptors were expressed more broadly and at lower levels. Using our GAL4 drivers to mark individual cell types, we found that 51 of the 85 CX cell types we examined expressed at least one neuropeptide and 21 expressed multiple neuropeptides. Surprisingly, all co-expressed a small neurotransmitter. Finally, we used our driver lines to identify CX cell types whose activation affects sleep, and identified other central brain cell types that link the circadian clock to the CX. The well-characterized genetic tools and information on neuropeptide and neurotransmitter expression we provide should enhance studies of the CX.

Introduction

The central complex (CX) of the adult *Drosophila melanogaster* brain consists of approximately 2,800 cells that have been divided into 257 cell types based on morphology and connectivity (Scheffer et al., 2020; Hulse et al. 2021 [↗](#); Wol et al., 2015). These cell types are themselves organized into a set of highly structured neuropils (see **Figure 1** [↗](#)). The CX plays a key role in the flow of information between sensory inputs and motor outputs. It is particularly important in orientation and navigation, and much progress has been made in defining the cell types and circuits involved in these behaviors (reviewed in Fisher 2022 [↗](#); Green and Maimon 2018 [↗](#); Keram 2022 [↗](#); Turner-Evans and Jayaraman 2016 [↗](#)). The CX also appears to participate in sleep and/or activity regulation (reviewed in Dubowy and Sehgal 2017 [↗](#); Shafer and Keene 2021 [↗](#)). But these are unlikely to be the only functions performed by the CX.

The connectome has provided a detailed wiring diagram of the CX, information that will be critical for understanding how it performs its functions (Hulse et al., 2021 [↗](#)). However, the functions of most cell types in the CX remain unknown. Gaining this knowledge will likely require measuring and manipulating the activity of individual cell types. Such experiments are greatly facilitated by the availability of cell type-specific genetic driver lines. Such lines also allow biochemical approaches for determining the neurotransmitters and neuropeptides used by individual cells. This is particularly relevant for the CX, which is one of the most peptidergic brain areas (Kahsai & Winther, 2011 [↗](#); Nässel and Zandawala, 2019 [↗](#)). The roles of neuropeptide signaling in the CX are largely unexplored.

In this paper, we present the results of our efforts to develop and characterize genetic driver lines for CX cell types. We also provide a survey of neuropeptide gene expression in the adult central brain as well as determine neurotransmitter (NT) and neuropeptide (NP) gene expression in over 80 CX cell types. Many CX neurons express NPs, with some cell types expressing multiple NPs as well as a fast-acting neurotransmitter. Finally, we demonstrate the use of our collection of driver lines to screen for cell types that influence activity/sleep when activated. In doing so, we uncovered cell types not previously known to play a role in these processes as well as new pathways of communication between the circadian clock and the CX.

Results and Discussion

Generation and analysis of split-GAL4 lines for CX cell types

We generated cell type-specific split-GAL4 lines for CX cell types using the same general approach that we previously applied to the mushroom body (Aso et al., 2014 [↗](#) a; Rubin and Aso, 2023) and the visual system (Tuthill et al., 2013 [↗](#); Wu et al., 2016 [↗](#); Nern et al., 2024 [↗](#)); see methods for details. In a previous report, we described the generation of split-GAL4 lines for cell types innervating the protocerebral bridge (PB), noduli (NO) and asymmetrical body (AB) (Wolff and Rubin 2018) [↗](#). Here we extend this work to the rest of the CX and include some improved lines for the PB, NO and AB.

Figure 2 [↗](#) and **Figure 2—figure supplements 1** [↗](#)–**4** [↗](#) show the expression of 52 new split-GAL4 lines with strong GAL4 expression that is largely limited to the cell type of interest. All lines were imaged in the brain and ventral nerve cord of adult females, and some were also imaged in males; we did not image expression in the peripheral nervous system or in non-neuronal tissues. Together with the other lines generated in this study and our previous work, we generated high-quality lines for nearly one-third of CX cell types that were defined by analysis of the connectome

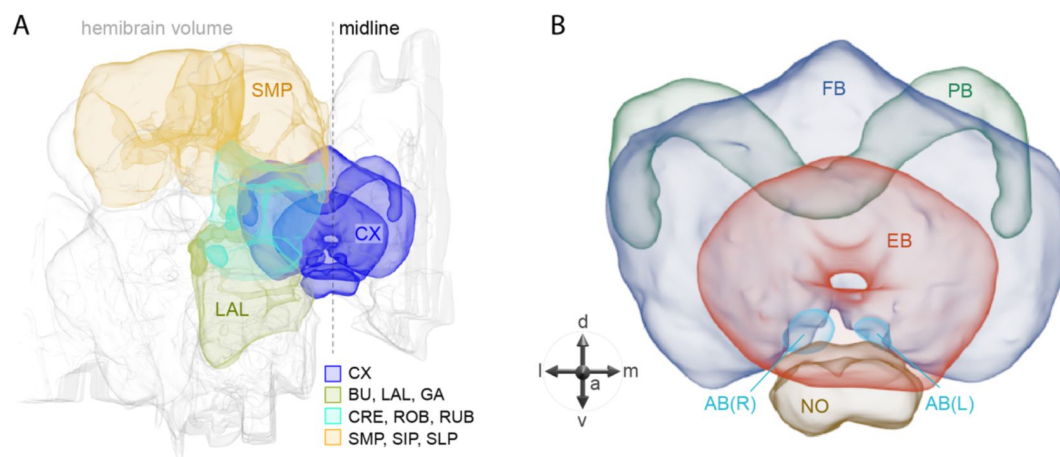


Figure 1.

Schematic diagram of CX neuropils. (A) The brain areas included in the hemibrain connectome are shown with the CX and key connected brain areas highlighted. (B) The neuropils comprising the CX are shown: FB, fan-shaped body; PB, protocerebral bridge; EB, ellipsoid body; NO, noduli; and AB, asymmetrical body. Redrawn from Hulse et al. (2021) <https://doi.org/10.7554/eLife.104764.2>.

(Hulse et al., 2021 [↗](#)). We also generated lines of lesser quality for other cell types that in total bring overall coverage to more than three-quarters of CX cell types. These additional lines often show some combination of expression in more than one CX cell type, unwanted expression in other brain areas, or weak or stochastic expression. Supplementary File 1 lists the two best split-GAL4 lines we generated for each CX cell type with comments about their specificity as well as the enhancers used to construct them. Additional split-GAL4 lines used in the sleep and NP/NT studies are also included in this file. Images of all lines are shown at www.janelia.org/split-GAL4 [↗](#) and the original confocal stacks of key imaging data can be downloaded from that site. For a subset of lines, images revealing the morphology of individual cells using MCFO (Nern et al., 2015 [↗](#)), and higher resolution images, are also available (see for examples, **Figure 3** [↗](#) and **Figure 3—figure supplement 1** [↗](#)). Additional split-GAL4 lines that may be useful for further studies are listed in Supplementary File 2.

Neurotransmitter expression in CX cell types

To determine what neurotransmitters are used by the CX cell types, we carried out fluorescent *in situ* hybridization using EASI-FISH (Close et al., 2024) on brains that also expressed GFP driven from a cell-type-specific split GAL4 line. In this way, we could determine what neurotransmitters were expressed in over 100 different CX cell types based on which members of a panel of diagnostic synthetic enzymes and transporters they expressed: for acetylcholine, ChAT (choline O-acetyltransferase; acetylcholine synthesis) and, in most cases, VAcHT (vesicular acetylcholine transporter); for GABA, GAD1 (glutamate decarboxylase; GABA synthesis); for glutamate, vGlut (vesicular glutamate transporter); for dopamine, ple (tyrosine 3-monooxygenase; dopamine synthesis); for serotonin, SerT (serotonin transporter); for octopamine, Tbh (Tyramine β -hydroxylase; converts tyramine to octopamine); and for tyramine, Tdc2 (Tyrosine decarboxylase 2; converts tyrosine to tyramine) accompanied by lack of Tbh.

Figure 4 [↗](#) shows two examples of this approach. In panels A-D we provide evidence that the PFGs use a less common neurotransmitter, tyramine. Panels E-H show an example of apparent co-transmission. Here, the FB tangential neuron FB4K expresses RNAs suggesting it can release both acetylcholine and glutamate. Cases of co-transmission using two fast acting neurotransmitters have been described in many organisms (reviewed in Svensson et al., 2019 [↗](#)) including *Drosophila*, but are rare and may be post-transcriptionally regulated (Chen et al., 2023 [↗](#)). Our full results are summarized, together with our analysis of neuropeptide expression in the same cell types, in **Figures 5** [↗](#) - **9** [↗](#) and in **Supplemental File 1** [↗](#).

Methods for using machine learning to predict neurotransmitter from EM images show great promise (Eckstein et al., 2024 [↗](#)). However, they are unlikely to fully replace the need for experimental determination and validation for three reasons. First, rarely used transmitters such as tyramine are problematic due to limited training data. Second, accurate prediction of co-transmission is challenging for current computational approaches. Third, FISH remains the gold standard for inferring gene expression.

Survey of neuropeptide and neuropeptide receptor expression in the adult central brain

Neuropeptides provide a parallel mode of communication to wired connections and can act over larger distances using volume transmission (reviewed in Nässel 2009; Bargmann and Marder, 2013 [↗](#)). Neuropeptides are widely expressed in the CX (Kahsai et al., 2011; Nässel and Zandawala, 2019 [↗](#)) and are likely to play important roles in its function. However, information on the expression of neuropeptides and their receptors is not provided by the connectome. To look for expression of neuropeptides in the CX, we took a curated list of 51 neuropeptide-encoding genes from FlyBase (FB2024_02, released April 23, 2024; Öztürk-Çolak et al., 2024 [↗](#)) and eliminated 12 genes based on their not having been detected in RNA profiling studies of the adult brain. Trissin

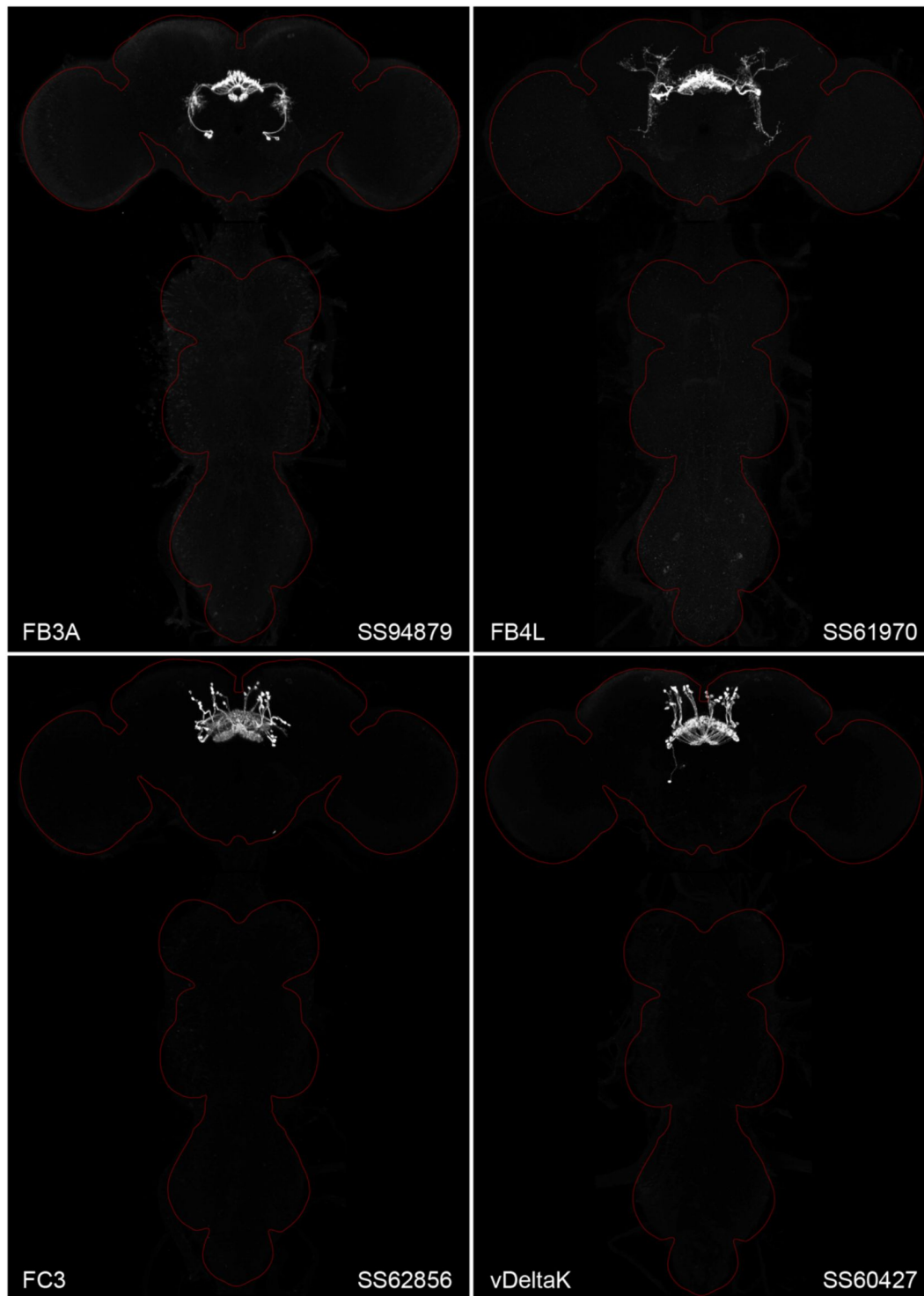


Figure 2.

Maximum intensity projections (20X confocal images) of the expression patterns driven by four stable split-GAL4 lines for the indicated cell types. The brain and VNC are outlined in red. Original confocal stacks that include a neuropil reference channel can be downloaded from www.janelia.org/split-gal4. Similar images for 48 additional lines generated as part of this study are shown in **Figure 2**—figure supplements 1-4.

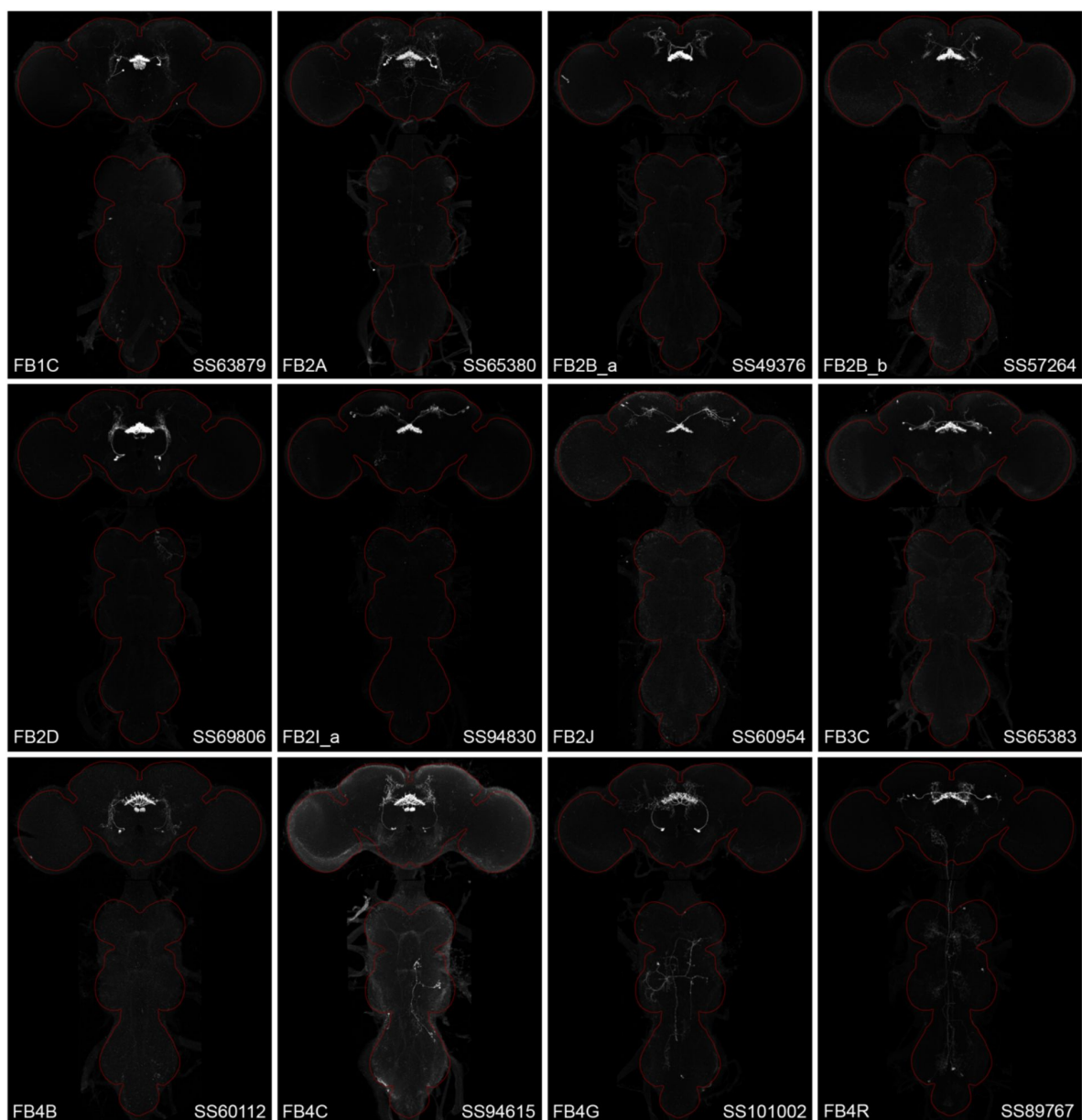


Figure 2—figure supplement 1.

Maximum intensity projections (20X confocal images) of the expression patterns driven by stable split-GAL4 lines for the indicated twelve cell types. The brain and VNC are outlined in red. Original confocal stacks that include a neuropil reference channel can be downloaded from www.janelia.org/split-gal4.

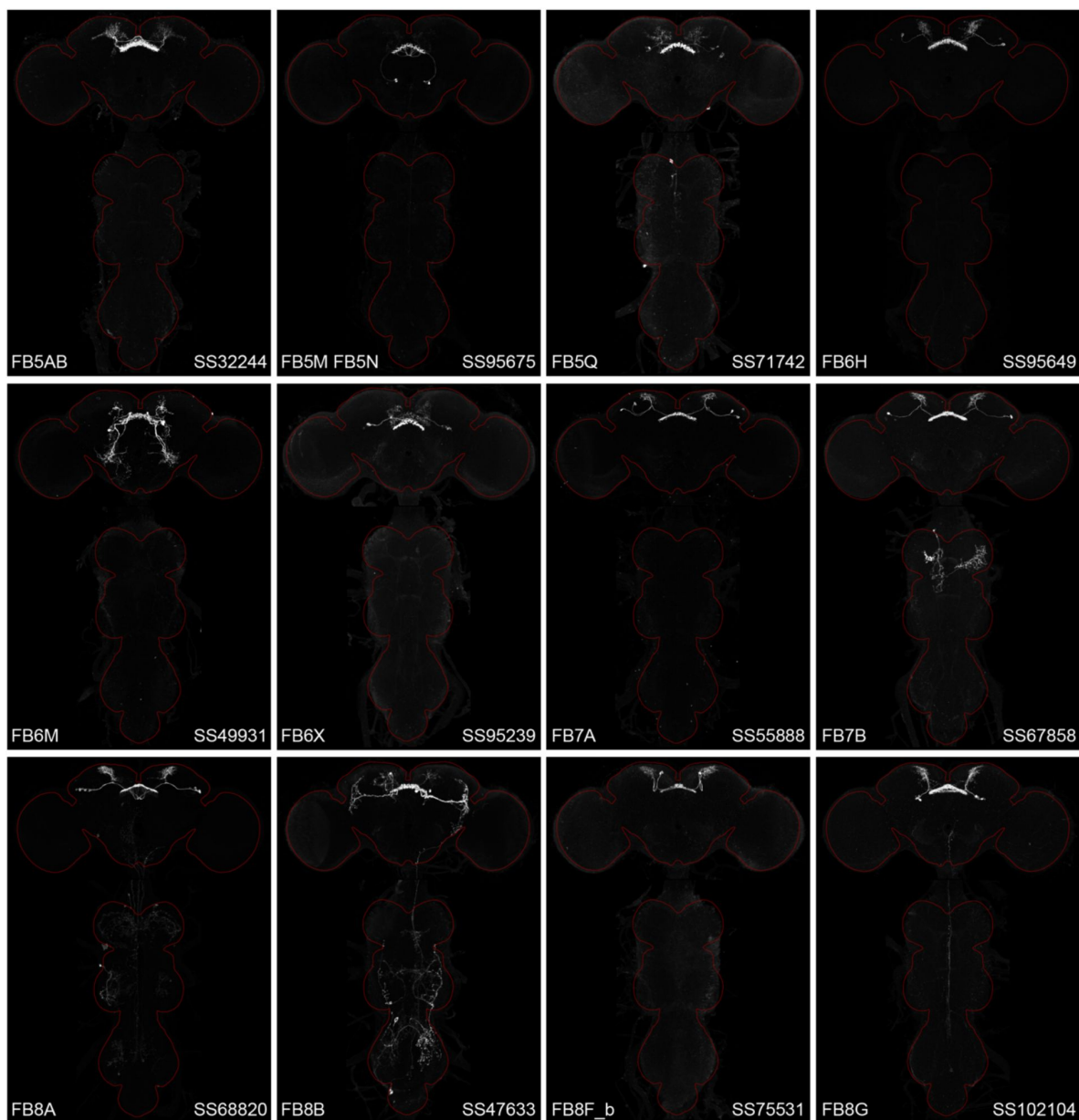


Figure 2—figure supplement 2.

Maximum intensity projections (20X confocal images) of the expression patterns driven by stable split-GAL4 lines for the indicated twelve cell types. The brain and VNC are outlined in red. Original confocal stacks that include a neuropil reference channel can be downloaded from www.janelia.org/split-gal4.

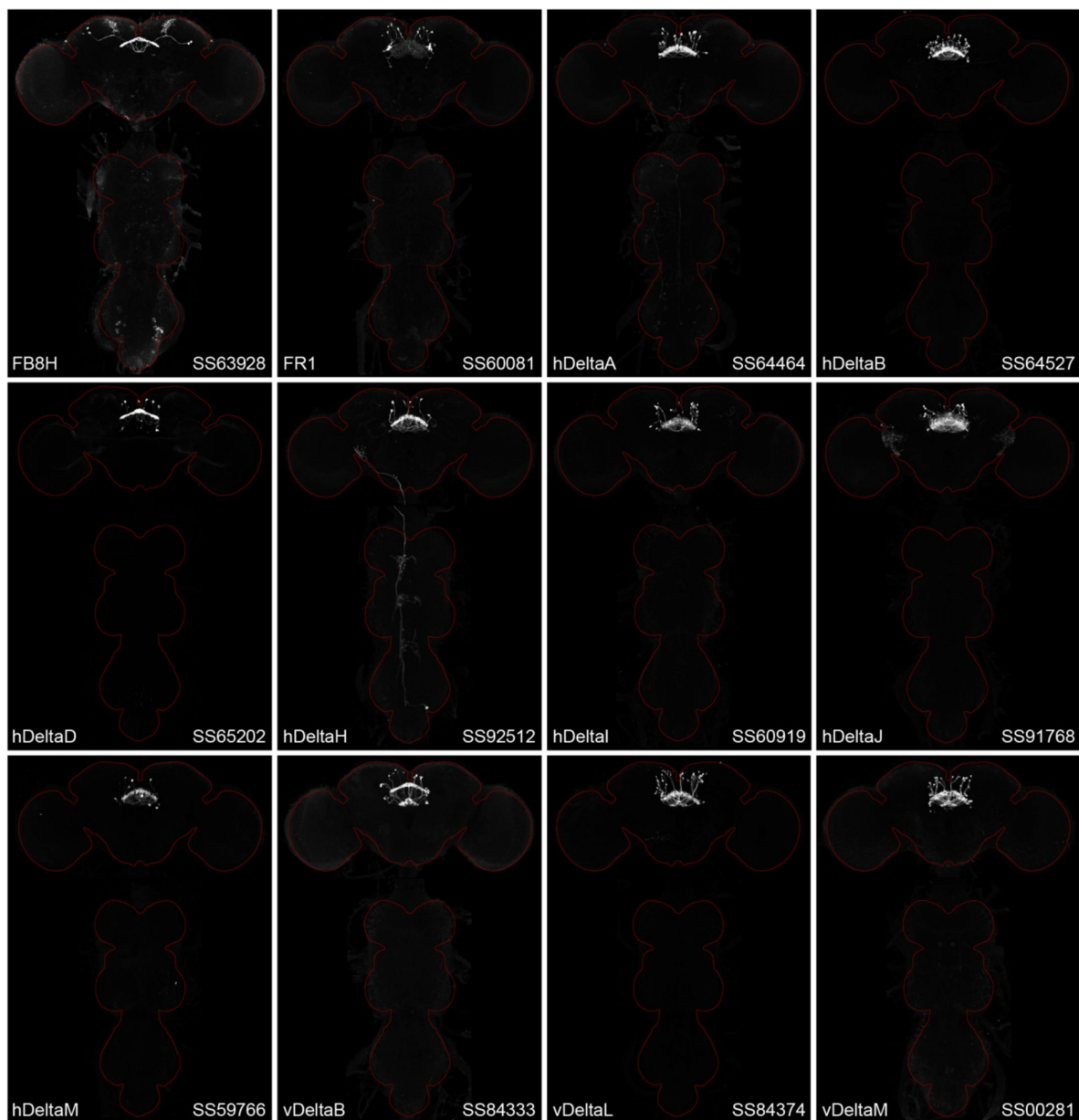


Figure 2—figure supplement 3.

Maximum intensity projections (20X confocal images) of the expression patterns driven by stable split-GAL4 lines for the indicated twelve cell types. The brain and VNC are outlined in red. Original confocal stacks that include a neuropil reference channel can be downloaded from www.janelia.org/split-gal4.

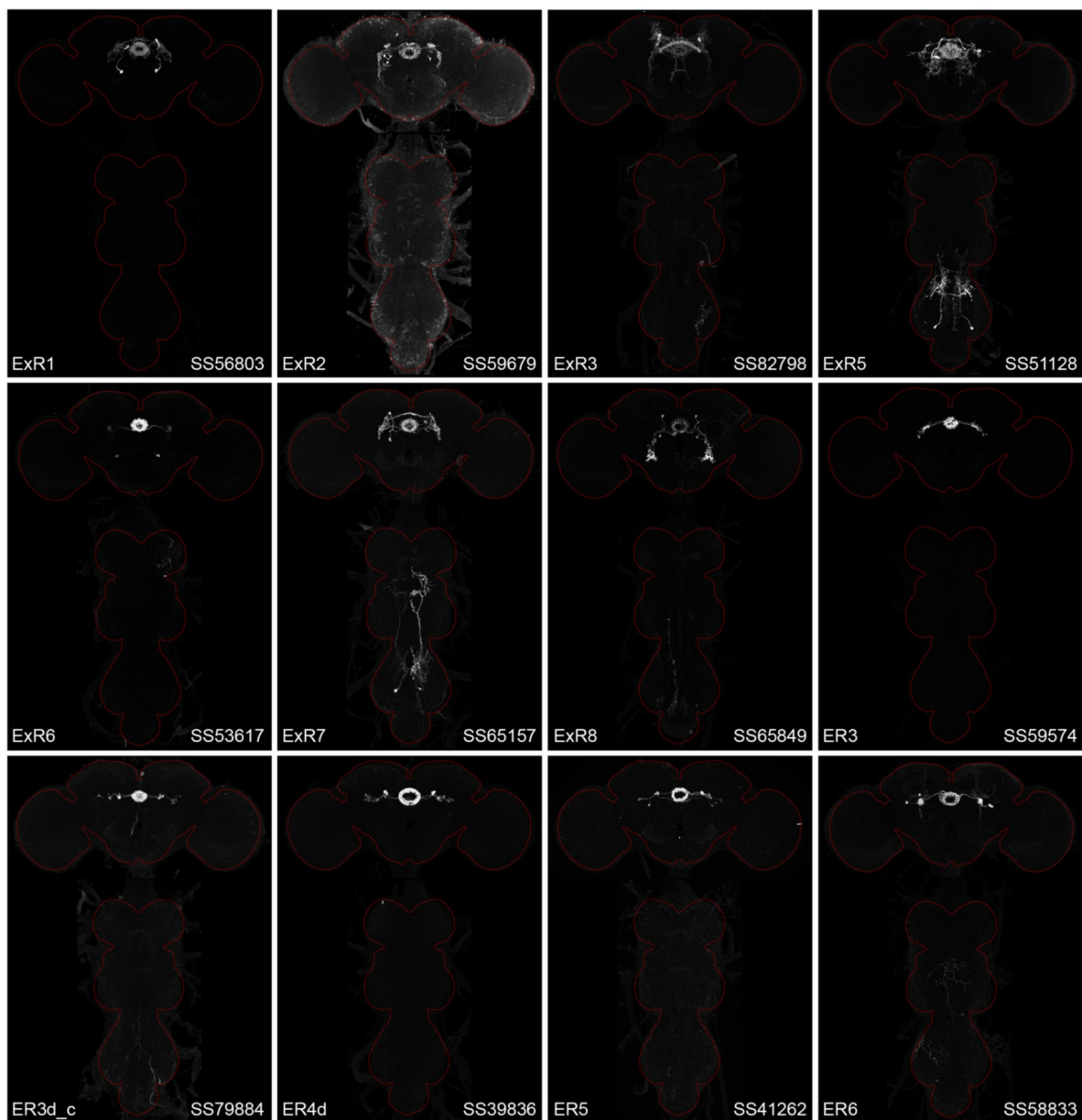


Figure 2—figure supplement 4.

Maximum intensity projections (20X confocal images) of the expression patterns driven by stable split-GAL4 lines for the indicated twelve cell types. The brain and VNC are outlined in red. Original confocal stacks that include a neuropil reference channel can be downloaded from www.janelia.org/split-gal4.

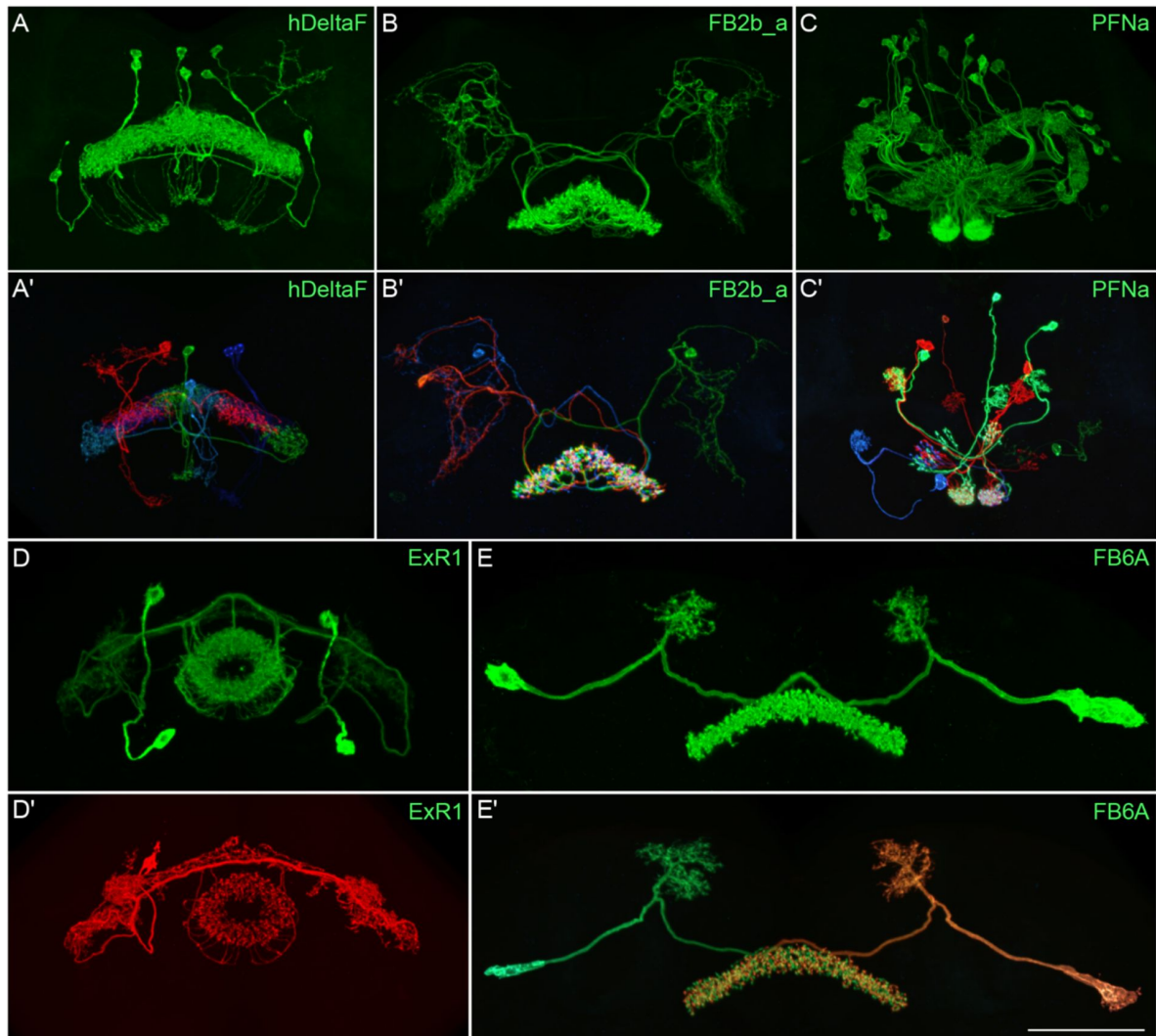


Figure 3.

Visualization of the entire expression pattern of a split-GAL4 line for the indicated five cell types revealed using UAS-myrGFP (A-E). A subset of individual cells within those same cell types (A'-E') are revealed by stochastic labeling using the MCFO method (Nern et al. 2015). The scale bar in E' refers to all panels and = 50 μ m. Images are maximum intensity projections (MIPs). Stable split lines used were as follows: A, SS54903; A', SS53683; B and B', SS49376; C and C', SS02255; D, SS56684; D', SS56803; E and E', SS57656. The original confocal image stacks from which these images were taken are available at www.janelia.org/split-GAL4.

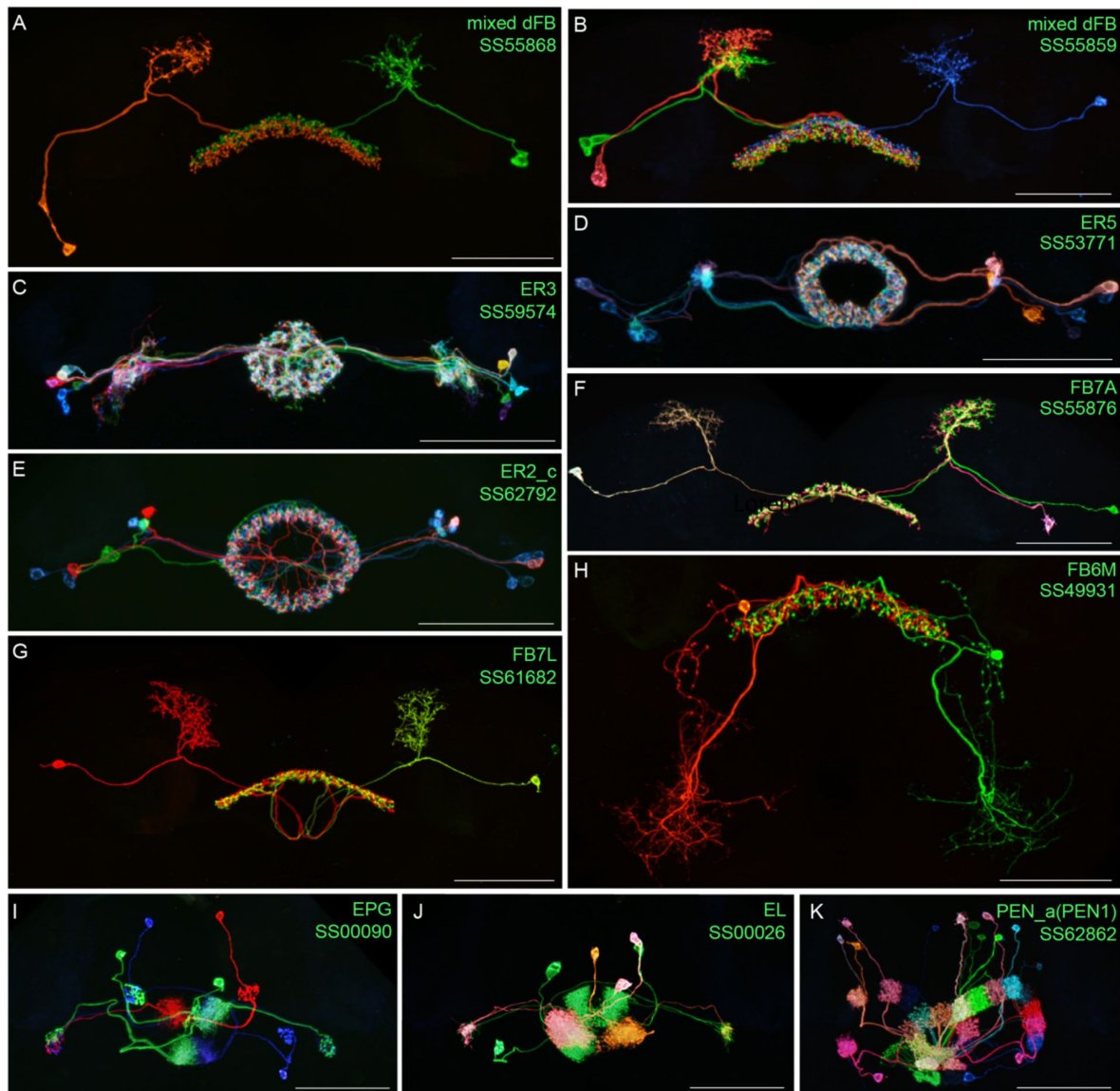


Figure 3—figure supplement 1.

Individual cells within the indicated cell types revealed by stochastic labeling using the MCFO method (Nern et al. 2015) with the indicated split-GAL4 lines. Images are MIPs. Scale bars = 50 μ m.

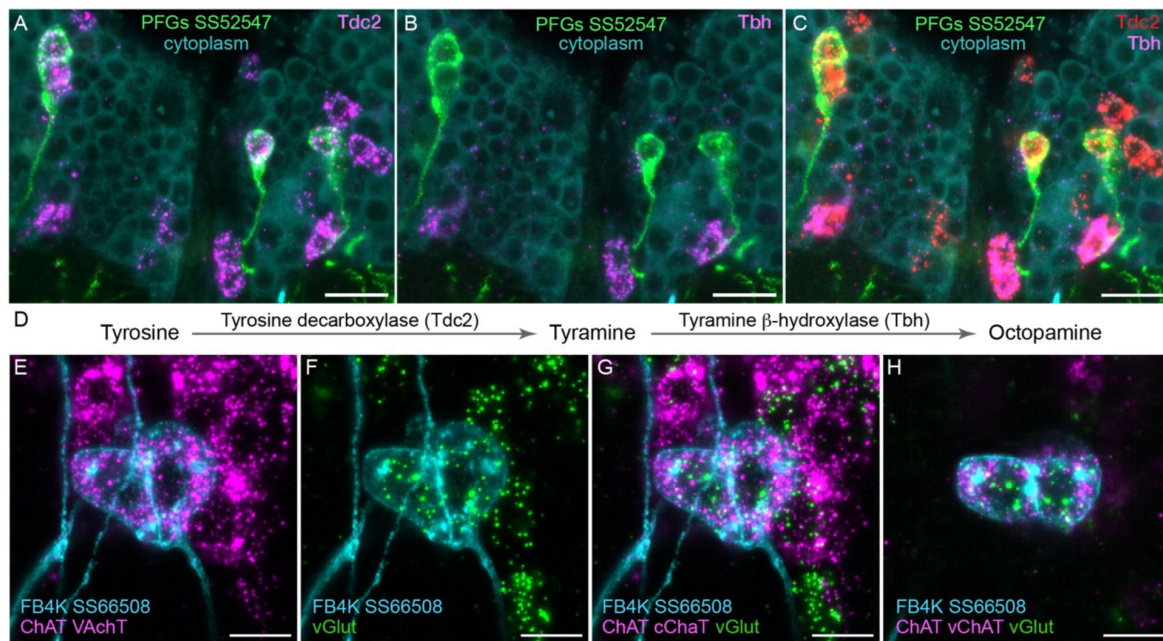


Figure 4.

Using EASI-FISH to assess neurotransmitter expression. (A-C) Expression of the transcripts encoding key enzymes required to synthesize tyramine (Tdc2) and octopamine (Tbh) has been examined as indicated. PFG neurons were marked by the split-GAL4 line SS52547 together with UAS-myr-GFP and visualized by anti-GFP antibody staining. Brains were treated with DNase1 and counterstained with high concentrations of DAPI to reveal total RNA in the cytoplasm. PFG cell bodies show expression of Tdc2 but not Tbh indicating that the PFG neurons use tyramine as a neurotransmitter. Maximum intensity projections (MIPs) of substacks are shown. (D) Biochemical pathway for synthesis of tyramine and octopamine from tyrosine. (E-H) Evidence for co-expression of the neurotransmitters acetylcholine and glutamate in FB4K. EASI-FISH was carried out using probes against choline acetyltransferase (ChAT) and vesicular acetylcholine transporter (VAcHT) and vesicular glutamate transporter (vGlut) as indicated. The Fan-shaped body tangential neuron FB4K has been visualized using the split-GAL4 line SS66508. Panels G and H show two diherent substacks at diherent Z-depths through the same neurons. Scale bar in each panel = 10 μm.

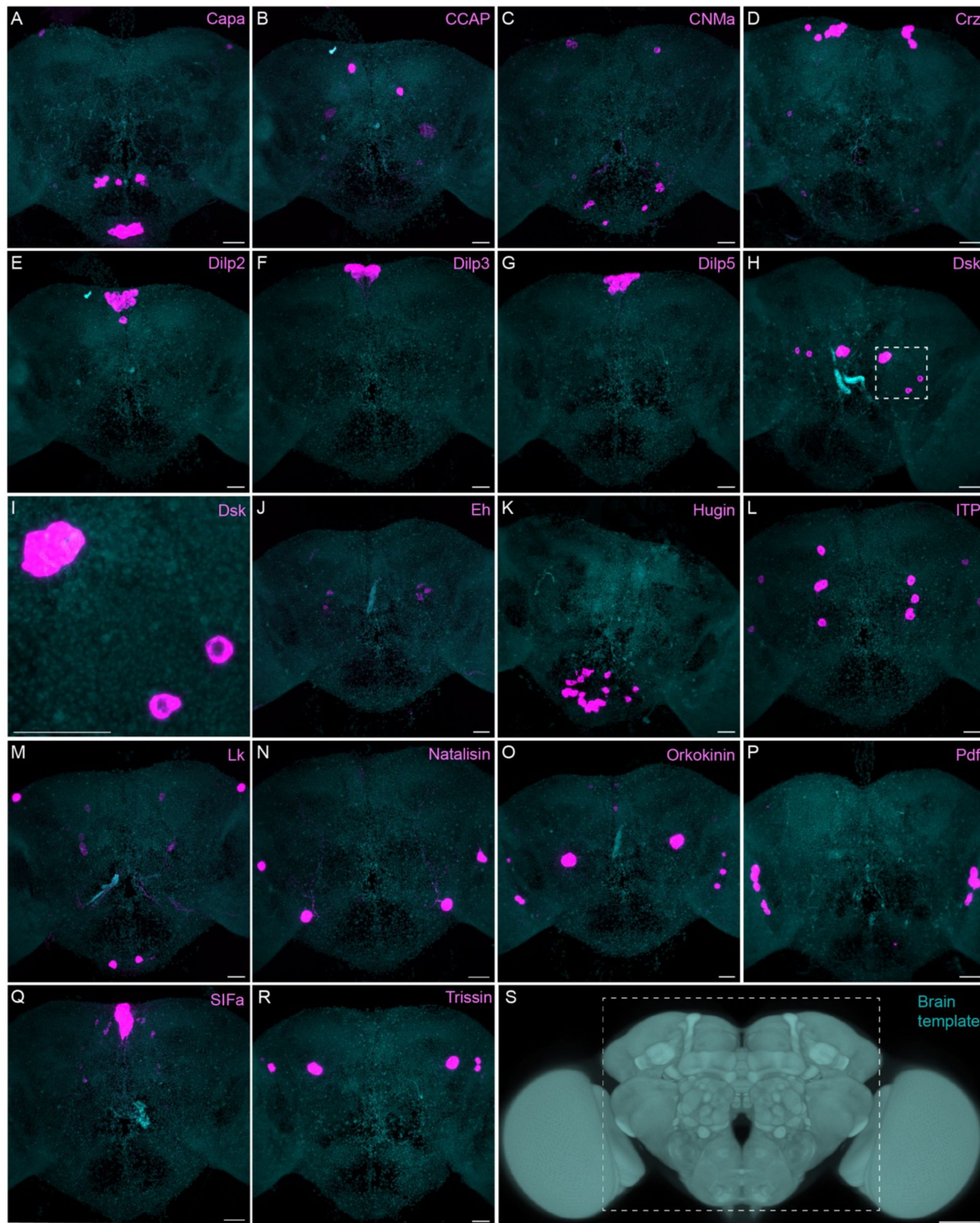


Figure 5.

Sparsely expressed neuropeptide genes. EASI-FISH was used to examine the expression of the indicated neuropeptide genes in the central brain (dashed box in S). Samples were counterstained with DAPI to visualize the outline of brain tissue. Images are MIPs. A higher magnification view of a region of the brain showing Dsk expression (indicated by dashed box in H) is shown in I. Scale bar in each panels A-R = 50 μ m; note that the images shown are from brains that were expanded by about a factor of two during the EASI-FISH procedure. Scale bar for the unexpanded brain in S = 50 μ m.

Neuropeptides				Neuropeptides (cont.)			
Symbol	Name	Figures	Notes	Symbol	Name	Figures	Notes
Akh	Adipokinetic hormone		negative	Nplp3	Neuropeptide-like precursor 3		not tested
amn	amnesiac		negative	Nplp4	Neuropeptide-like precursor 4		not tested
AstA	Allatostatin A	6A, 8C	CX candidate	Orcokinin	Orcokinin	50	
AstC	Allatostatin C	6B, 8D	CX candidate	Pburs	Partner of Bursicon		not tested
AstCC	Allatostatin double C		not tested	Pdf	Pigment-dispersing factor	5P	
Burs	Bursicon		not tested	Proc	Proctolin	6M, 8M	CX candidate
Capa	Capability	5A		Ptth	Prothoracicotropic hormone		not tested
CCAP	Crustacean cardioactive peptide	5B	CX candidate	RYa	RYamide		not tested
CCHa1	CCHamide-1	6C	CX candidate	SIFa	SIFamide	5Q, 8O	CX candidate
CCHa2	CCHamide-2	6D	CX candidate	sNPF	short neuropeptide F precursor	6N, 8L	CX candidate
CNMa	CNMamide	5C		SP	Sex Peptide		not tested
Crz	Corazonin	5D		spab	space blanket	6O, 8K	CX candidate
Dh31	Diuretic hormone 31	6E, 8G, 8N, video 1	CX candidate	Tk	Tachykinin	6P, 8E, 8I, 8J	CX candidate
Dh44	Diuretic hormone 44	6F, 8P	CX candidate	Trissin	Trissin	5R	
Dsk	Drosulfakinin	5H, 5I					
Eh	Ecdysis hormone	5J					
ETH	Ecdysis triggering hormone		not tested				
FMRFa	FMRFamide	6G, 8Q	CX candidate				
Gpa2	Glycoprotein hormone alpha 2		not tested				
Gpa5	Glycoprotein hormone beta 5		negative				
Hug	Hugin	5K					
Iip1	Insulin-like peptide 1		negative				
Iip2	Insulin-like peptide 2	5E					
Iip3	Insulin-like peptide 3	5F					
Iip4	Insulin-like peptide 4		negative				
Iip5	Insulin-like peptide 5	5G					
Iip6	Insulin-like peptide 6		negative				
Iip7	Insulin-like peptide 7		not tested				
Iip8	Insulin-like peptide 8		not tested				
ITP	Ion transport peptide	5L					
Lk	Leucokinin	5M					
Mip	Myoinhibiting peptide precursor	6H, 8F, 8H	CX candidate				
Ms	Myosuppressin	6I	CX candidate				
Natalisin	Natalisin	5N					
NPF	neuropeptide F	6J, 6K, 8A, 8B	CX candidate				
Nplp1	Neuropeptide-like precursor 1	6L, 8R	CX candidate				
Nplp2	Neuropeptide-like precursor 2		negative				

Figure 5—figure supplement 1.

Neuropeptides and neuropeptide receptor genes whose expression patterns were characterized by the EASI-FISH experiments shown in **Figures 5**–**8**. Relevant figure panels are listed. Some predicted neuropeptides were not tested because of lack of evidence for their expression in adult brain and are shown in grey font and listed as “not tested” in the Notes column. Neuropeptides shaded in green were tested against split-GAL4 lines with expression in CX cell types (see **Figures 8** and **9**) based on their cell body positions being in the same brain areas as those of CX cell types. These are listed as “CX candidate” in the Notes column. Those neuropeptides shown in blue font were tested in adult brains, but expression was not detected.; these are listed as “negative” in the Notes column. Lines for which the Notes column is blank were detected in the adult brain but were not chosen for screening against split-GAL4 lines based on the position of their cell bodies.

and Natalisin were added to the FlyBase list based on evidence summarized in [Nässel and Zandawala \(2019\)](#). We only examined a small subset of neuropeptide receptors, selecting those whose cognate neuropeptides we thought might play a role in the CX. We used EASI-FISH to determine the expression patterns of these genes in the adult central brain of females (**Figures 5–7**). The list of 41 neuropeptides and 18 neuropeptide receptors we explored is presented in **Figure 5**—figure supplement 1.

The neuropeptide expression patterns we observed fell into two broad categories. Some neuropeptides, like those whose expression patterns are shown in **Figure 5**, appeared to be highly expressed in only a few relatively larger cells. Such large neurosecretory cells often express the transcription factor DIMM ([Park et al. 2008](#)). Several of these neuropeptides—for example, SIFa ([Terhzaz et al., 2007](#)) and Dsk ([Wu et al., 2020](#))—are expressed in broadly arborizing neurons that appear to deliver them to large areas of the brain and ventral nerve cord. In contrast, neuropeptides like those shown in **Figure 6** are expressed in dozens to hundreds of cells and appear poised to function by transmission to nearby cells in multiple distinct circuits. NPF and SIFa appear to act in both these modes. As we show below, most of the neuropeptides shown in **Figure 6** are expressed in the CX, each in distinct subsets of cell types.

Neuropeptide receptors (**Figure 7**) are more broadly, but not uniformly, expressed. In cases where more than one receptor has been identified for a given neuropeptide, such as Dh44 (**Figure 7G**; reviewed in [Lee et al., 2023](#)) and Tk (**Figure 7T**; see [Wohl et al., 2023](#)), the different receptors have distinct, but overlapping, expression patterns.

Neuropeptide expression in CX cell types

We selected 17 neuropeptides whose transcripts were observed in cell bodies located in the same general brain areas as those of the intrinsic cells of the CX (see **Figure 5**—figure supplement 1). We used probes for these 17 genes to perform EASI-FISH on brains that also expressed GFP in a specific cell type. In this way, we could score neuropeptide expression in individual cell types as we had done for neurotransmitters. **Figure 8** shows examples of this approach.

Figure 9 presents a summary table of neurotransmitter and neuropeptide use by individual cell types based on our EASI-FISH results. Supplementary File 1 contains a list of the individual stable split lines that were used for each cell type and how they were scored. We also characterized six CX cell types by RNA profiling (**Figure 9**—figure supplements 1 and 2) and additional RNA profiling of CX cell types is provided in [Epiney et al. \(2024\)](#). These RNA profiling results are largely congruent with the EASI-FISH data and allow a comparison of transcript number and *in situ* signal strength.

A few general features emerge from these data. First, more than half of the cell types assayed express a neuropeptide. This frequency is perhaps not surprising given that the CX is considered one of the most peptidergic regions of the adult brain ([Kahsai et al., 2011](#); [Nässel and Zandawala, 2019](#)); nevertheless, the fraction of cells expressing an NP appears to be several fold higher in the CX than observed in the adult brain as a whole, as judged by single cell RNA profiling studies ([Davies et al., 2018](#)). Second, every cell type that expressed a neuropeptide also expressed a small molecule neurotransmitter (see [Nässel 2018](#) for a discussion of other cases of co-expression). This co-transmitter was most often acetylcholine or glutamate, but we observed cases of GABA, dopamine, tyramine, octopamine, and serotonin. Third, co-transmission of two small molecule, fast-acting transmitters does occur but is rare. Conversely, co-transmission of a fast-acting transmitter and a modulatory transmitter such as serotonin is common: nine of ten cell types expressing the serotonin transporter also appear to express another small transmitter, most often glutamate or acetylcholine. Octopamine is often, but not always, co-expressed with glutamate (see also [Sherer et al., 2020](#)).

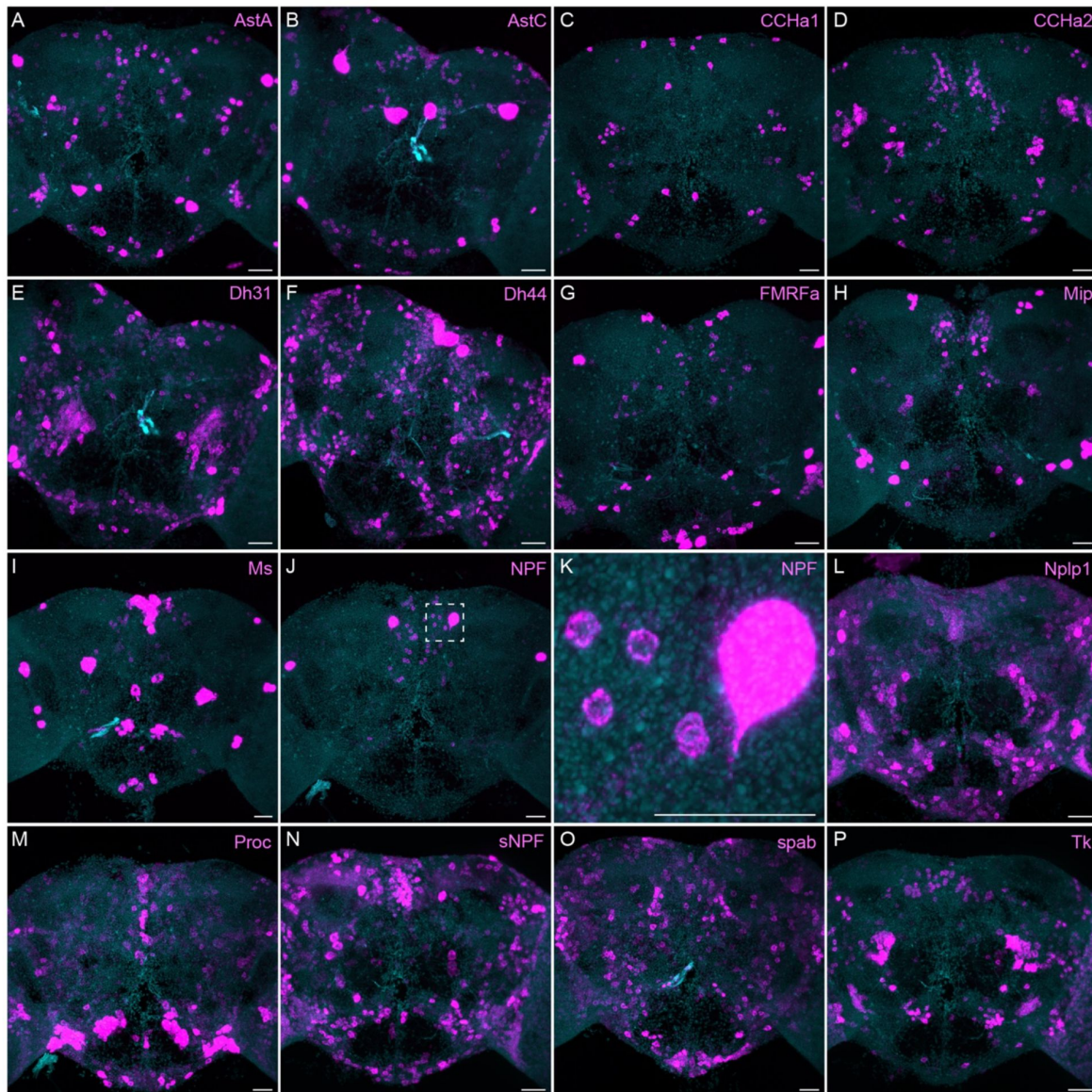


Figure 6.

More broadly expressed neuropeptide genes. EASI-FISH was used to examine the expression of the indicated neuropeptide genes in the central brain. Samples were counterstained with DAPI to visualize the outline of brain tissue. Images are MIPs. We included spab and Nplp1 in our screening although it is unclear whether these are indeed neuropeptides (M. Zandawala, pers. comm.). A higher magnification view of a region of the brain showing NPF expression (indicated by dashed box in J) is shown in K. Scale bar in each panel = 50 μ m; note that the images shown are from brains that were expanded by about a factor of two during the EASI-FISH procedure.

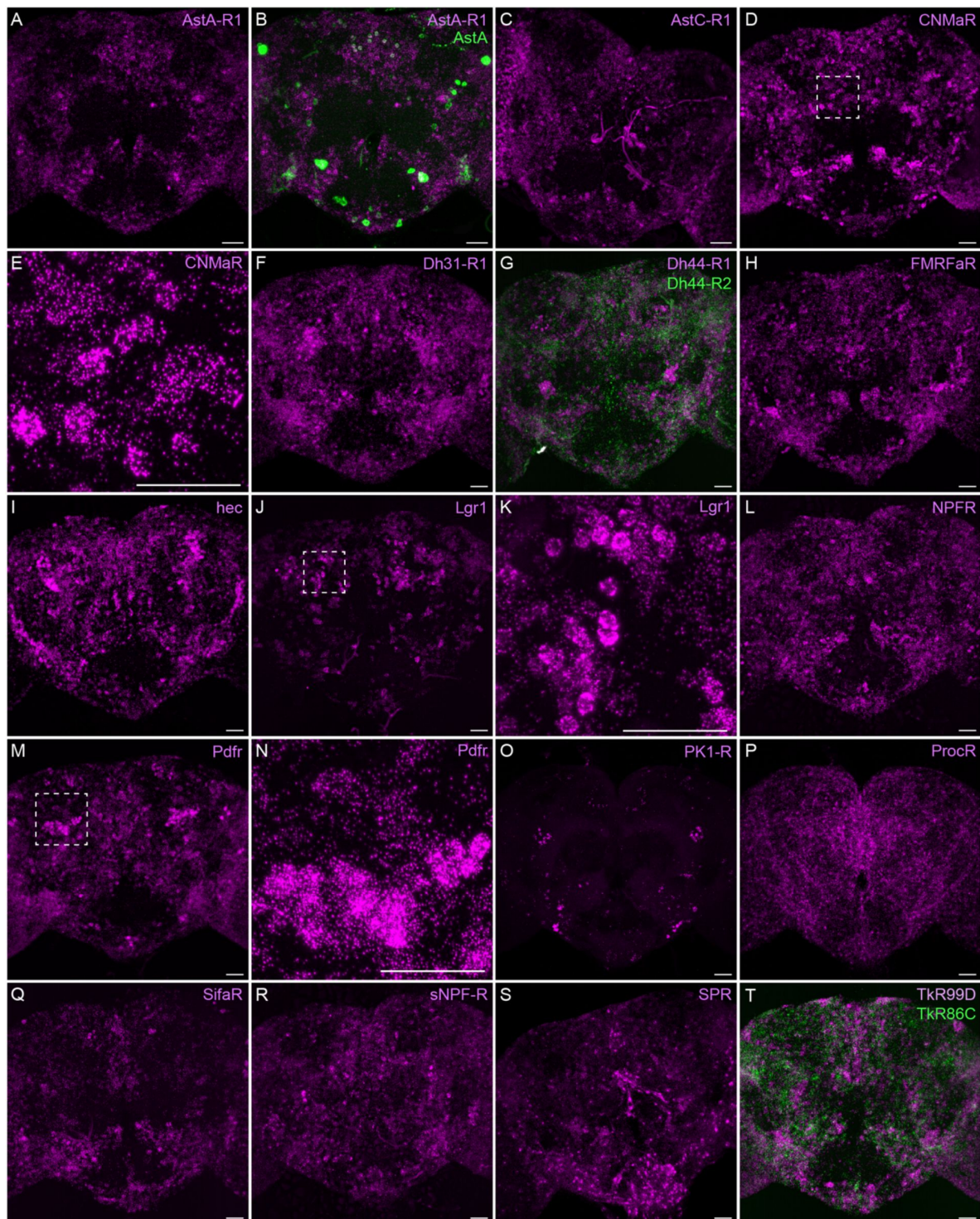


Figure 7.

Neuropeptide receptor gene expression. EASI-FISH was used to examine the expression of the indicated neuropeptide genes in the central brain. Higher magnification views of regions of the brains shown in D, J and M (indicated by dashed boxes) are shown in E, K and N. Images are MIPs. Scale bar in each panel = 50 μ m; note that the images shown are from brains that were expanded by about a factor of two during the EASI-FISH procedure.

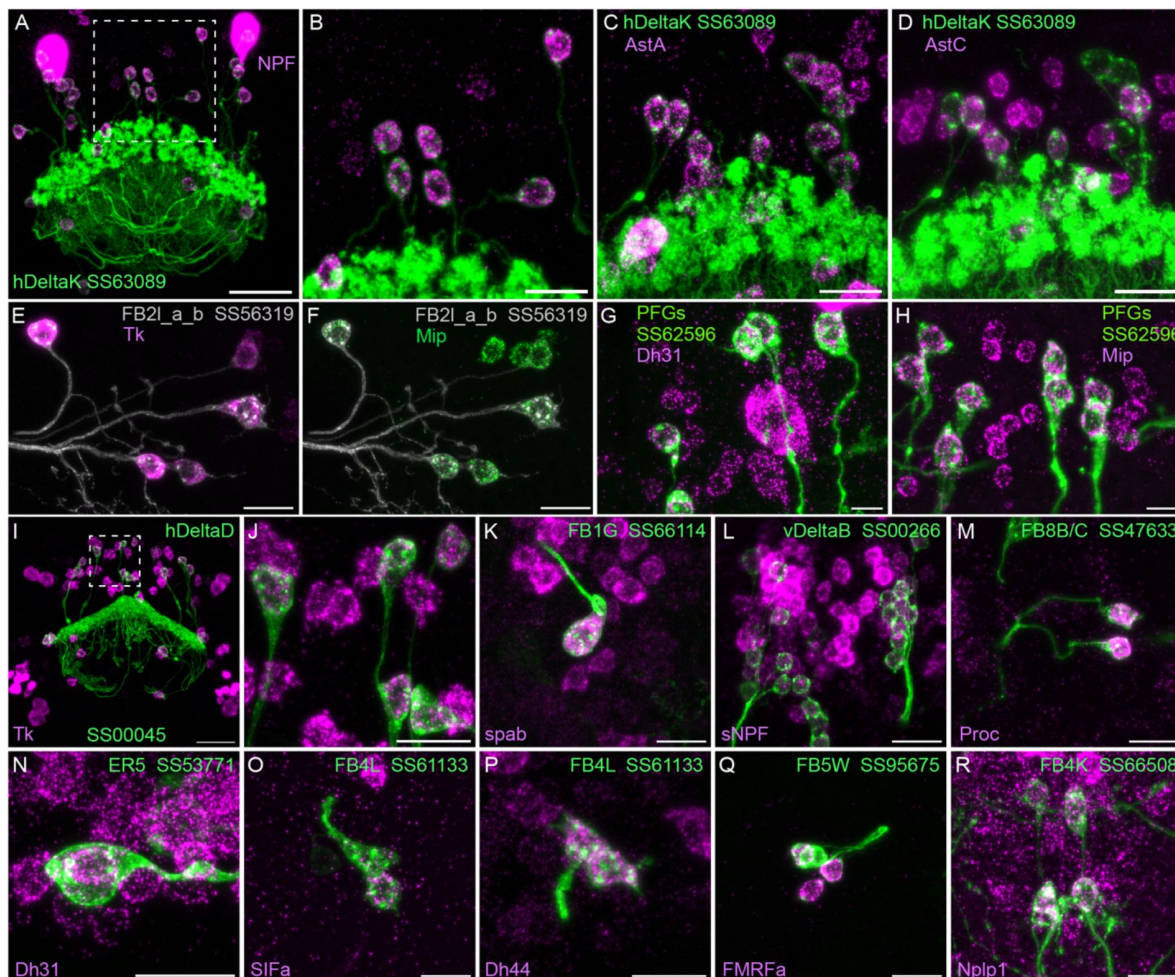


Figure 8.

Neuropeptide gene expression in specific cell types. EASI-FISH was used to examine the expression of the indicated neuropeptide genes in the brains also expressing myr-GFP driven by a cell-type-specific split-GAL4 line. The line name and labelled cell type are indicated. GFP was visualized by anti-GFP antibody staining. Hamid et al. (2024) demonstrated expression of Tk in ventral FB neurons likely to correspond to the cell type shown in panel E. Higher magnification view of regions of the brain in A and I (indicated by dashed box) are shown in B and J, respectively. Scale bar in panels A and I = 50 μ m and for all other panels = 20 μ m; note that the images shown are from brains that were expanded by about a factor of two during the EASI-FISH procedure.

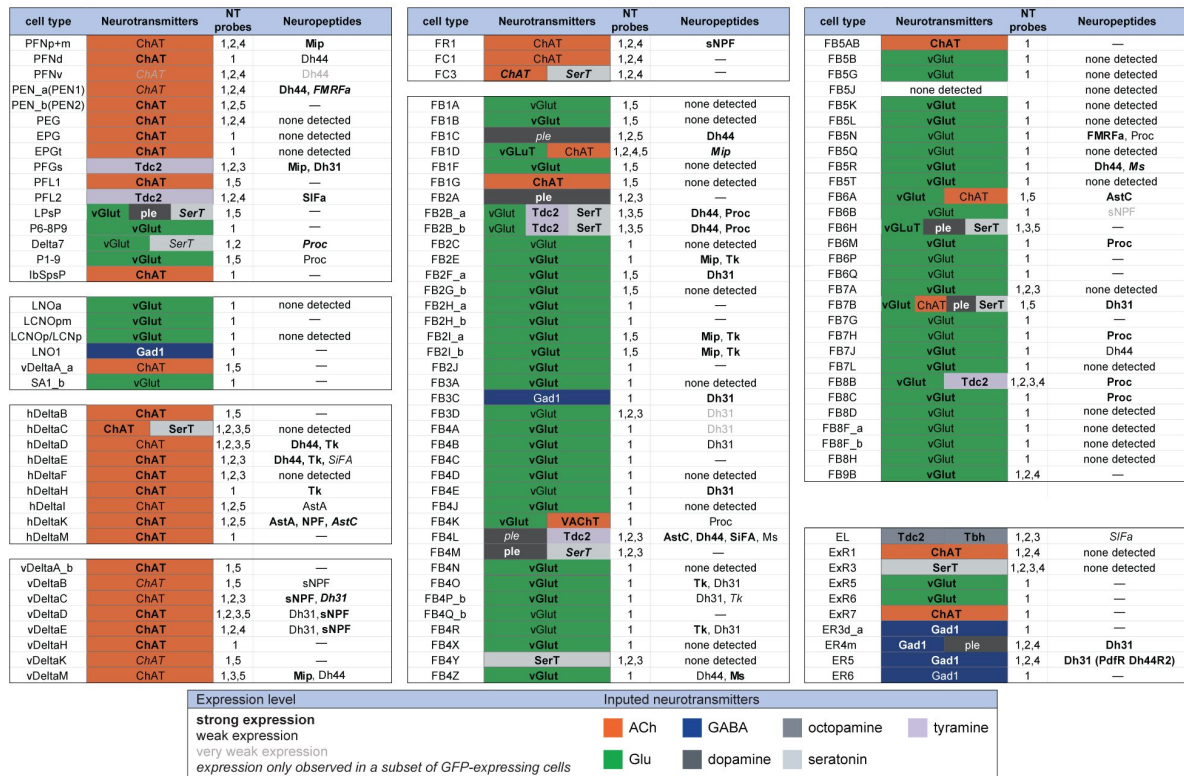


Figure 9.

Summary of neurotransmitter and neuropeptide expression as determined by EASI-FISH performed on adult brains in which selected cell types were marked by a split-GAL4 lines driving GFP expression. About half of all CX cell types were examined using probes for the following 15 NPs: AstA, AstC, CCAP, CCHA1, CCHA2, Dh31, Dh44, FMRFa, Mip, Ms, NPF, Proc, SIFA, sNPF and Tk. All NPs except CCAP, CCHA1, and CCHA2 showed expression in at least one cell type. We excluded spab and Nplp1 from the genes in this Figure, as it is unclear whether these are bonafide neuropeptides (M. Zandawala, pers. comm.); however, data on their expression in the listed cell types is given in [Supplemental File 1](#). See [Figures 4](#) and [8](#) for examples of the experimental data supporting these conclusions. The specific split-GAL4 driver(s) used for each cell type and other information are given in [Supplemental File 1](#). Results are coded for signal strength by typeface as indicated. The color shading used for neurotransmitters indicates what we believe to be the most likely transmitter used by each cell type. “None detected” indicates that an experiment was performed, whereas a “—” indicates no experimental data. To determine neurotransmitter expression various combinations of probe sets were used as indicated: 1, ChAT and VAcHT (choline O-acetyltransferase, and vesicular acetylcholine transporter); GAD1 (glutamate decarboxylase), vGlut (vesicular glutamate transporter); 2, ple (tyrosine 3-monooxygenase), SerT (serotonin transporter), Tbh (Tyramine β hydroxylase); 3, Tdc2 (Tyrosine decarboxylase 2), Tbh; 4, Tdc2; and 5, SerT, ple, Tdc2. In general, all lines were first probed with probe set 1 which reveals expression of genes involved in transmission by acetylcholine (ChAT), GABA (GAD) and glutamate (vGlut). Then a subset of lines was probed for genes involved in transmission by dopamine (ple), serotonin (SerT), octopamine (Tbh and Tdc2) and tyramine (Tdc2).

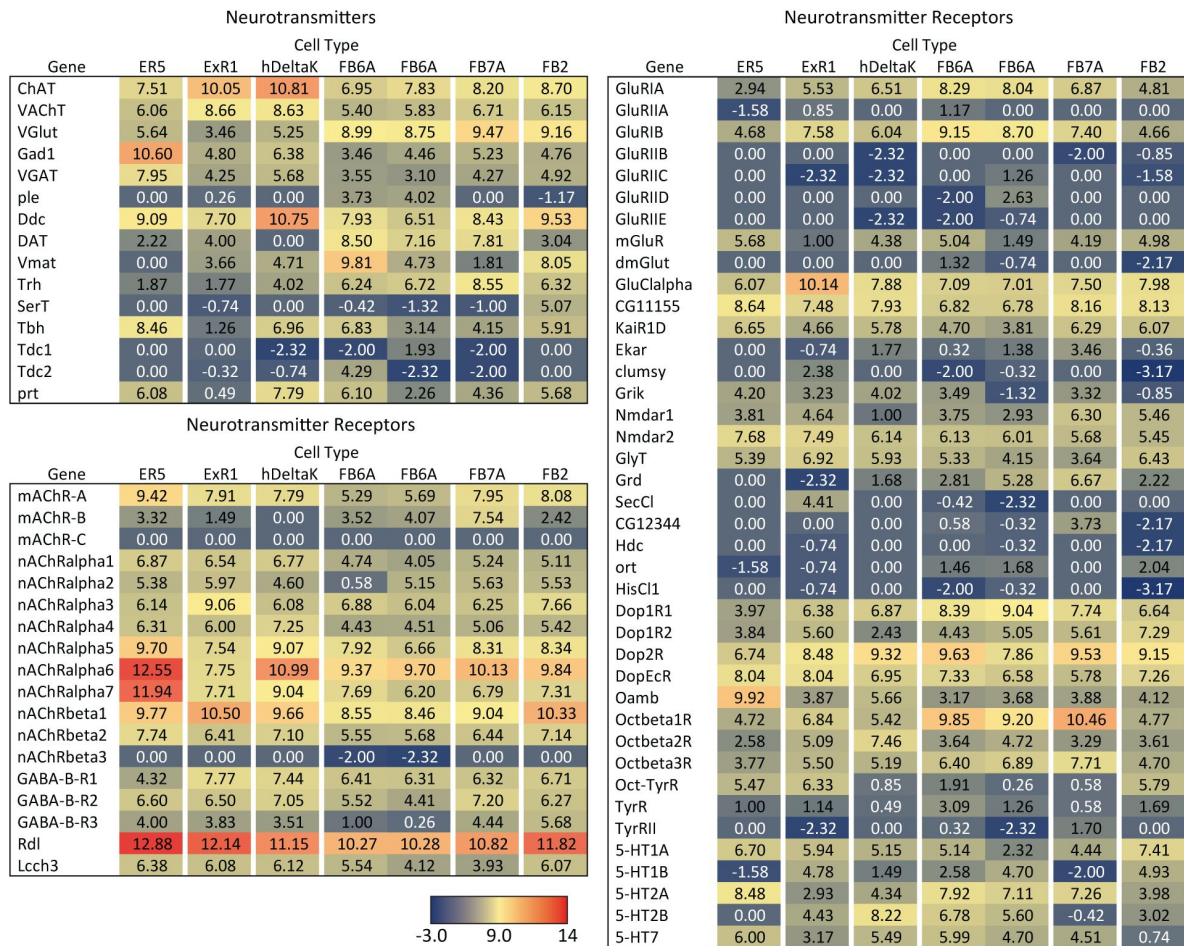


Figure 9—figure supplement 1.

RNA-seq data for genes related to neurotransmitter synthesis, transport, and receptors in the indicated cell types. Mean log₂ of TPM values for each gene are listed. See NCBI Gene Expression Omnibus (accession number GSE271123) for the raw data. The split-GAL4 drivers used for each cell type were as follows: ER5, SS00070; ExR1, SS56684; hDeltaK, SS02748; FB6A, SS57656 and SS54343; FB7A, SS55888; FB2, SS56319.

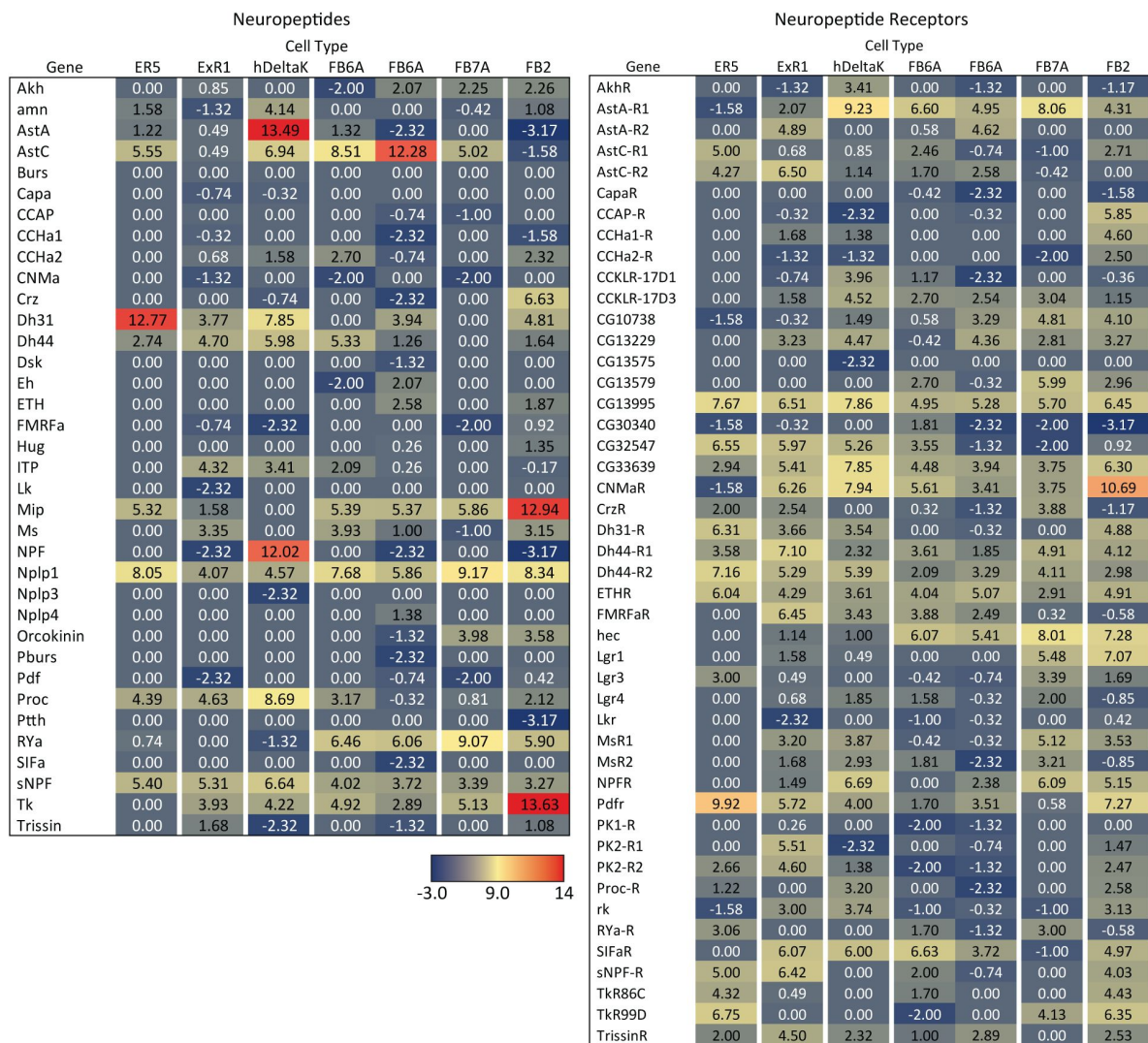


Figure 9—figure supplement 2.

RNA-seq data for genes related to neuropeptides and their receptors in the indicated cell types.

Screen for cell types whose activation modifies sleep

Sleep is a behavior widely studied in *Drosophila* (reviewed in Dubowy and Sehgal 2017 [↗](#); Shafer and Keene 2021 [↗](#)). The phenotypic description of sleep and its relationship to activity, as well as the cell types that play a role in sleep regulation are under active study in many labs. The CX has been documented to be a significant brain region for sleep regulation. But many cell types in the CX have never been assayed for a role in sleep due to the lack of suitable genetic reagents. Therefore, we used our genetic drivers for CX cell types to screen for those whose activation by thermogenetics or optogenetics strongly influenced sleep or activity. As described in methods, we used three metrics: sleep duration; P(Doze), the probability that an active fly will stop moving; and P(Wake), the probability that a stationary fly will start moving. These assays were carried out over several years in parallel with our building the collection of lines, so many of the lines we assayed did not make it into our final collection of selected lines. Conversely, we did not assay all our best lines as many only became available after our behavior experiments were completed.

Our screen identified several cell types not previously associated with sleep and/or activity regulation. For example, hDeltaF was found to be strongly wake promoting (**Figure 10** [↗](#)). We also identified PEN_b (Figure 1—figure supplement 1), PFGs (Figure 1—figure supplement 2), EL (Figure 1—figure supplement 3) and hDeltaK (Figure 1—figure supplement 4) as likely to play a role. In most of these cases, we were able to assay multiple independent driver lines for the cell type. We also assayed several lines that each contained a mixture of dorsal FB cell types (Figure 1—figure supplement 5) but were otherwise free of contaminating brain or VNC expression. In addition to intrinsic components of the CX, we evaluated several cell types that, based on the connectome, we thought likely to convey information from the circadian clock to the CX. **Figure 11** [↗](#) (SMP368) and **Figure 11—figure supplement 1** [↗](#) (SMP531) present two such cases of strongly wake promoting cell types.

Results for lines not discussed in detail in the main paper are provided as Supplementary Files. Supplementary Files 3 and 4 give results for 600 split-GAL4 lines assayed by thermogenetic activation with TRPA1 in both males and females, respectively. Supplementary Files 5 (males) and 6 (females) present results on over 200 lines, selected based on the results of thermogenetic activation, that were also assayed by optogenetic activation with CsChrimson. Images of the expression patterns of these lines and their genotypes can be found at <https://flylight-row.janelia.org/> [↗](#). The set of lines is anatomically biased with the dorsal FB overrepresented, and many lines contain multiple cell types and non-CX expression. Nevertheless, we believe these data might be useful as a starting point for further exploration.

The goal of our screen was to identify candidate cell types that warranted further study. While we identified several new potential sleep regulating cell types within CX, we did not perform the additional characterization needed to elucidate the roles of these cell types. For example, we did not examine the effects of inhibiting their function. Nor did we examine parameters such as arousal thresholds or recovery from sleep deprivation. Finally, by using only a 24-hour activation protocol we might have missed features only observable in shorter activation protocols. On the other hand, we assayed all lines in both males and females with identical genetic and environmental manipulations and many lines were evaluated by both optogenetic and thermogenetic activation. We observed that many lines showed phenotypes that differed between sexes, even though the expression patterns of the split-GAL4 lines did not obviously differ across sexes. Lines that showed strong effects generally did so with both activation modes and with both beam crossing-based activity measures and video-based locomotion tracking.

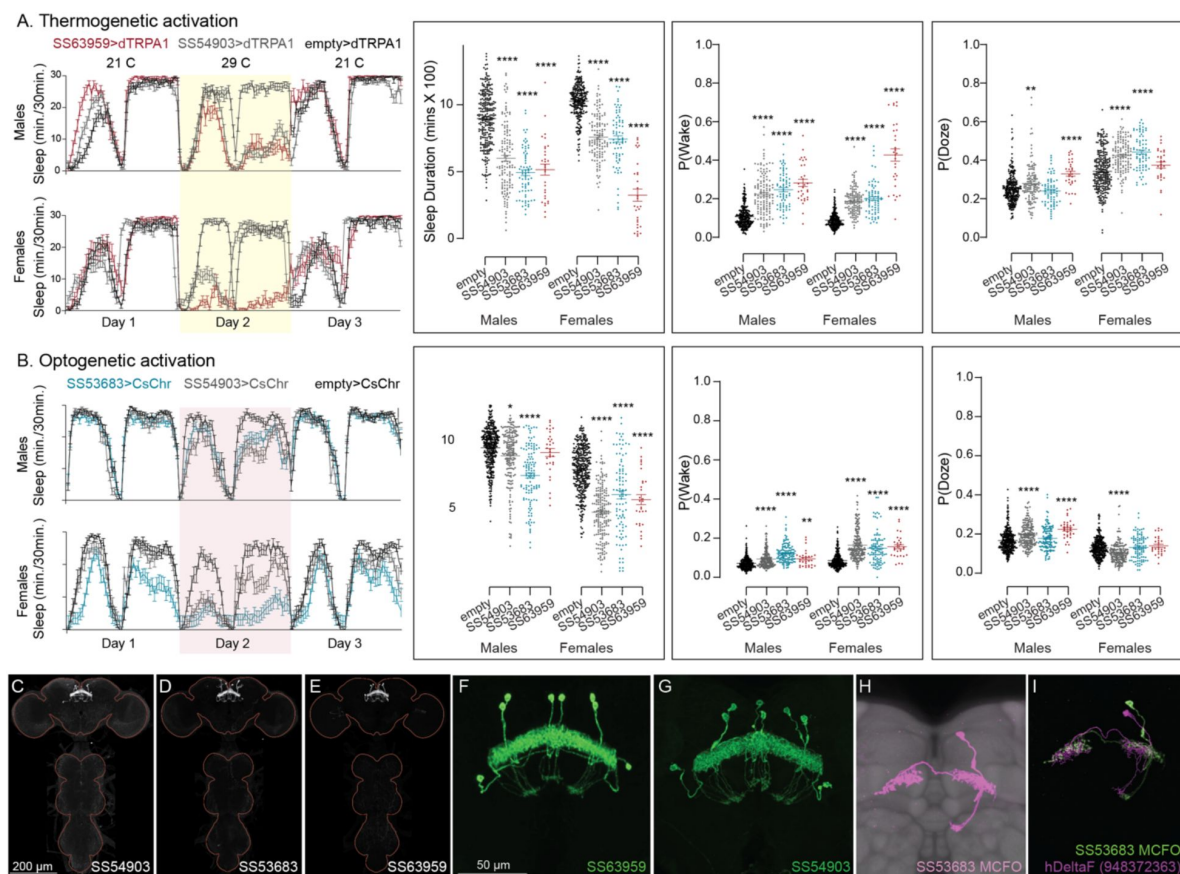


Figure 10.

Activation of hDeltaF, comprised of eight intrinsic FB columnar neurons (see Hulse et al., 2021) decreases sleep. (A) Thermogenetic activation and DAM based monitoring using the split-GAL4 lines SS54903, SS53683 and SS63959 produced decreased sleep duration and increased P(Wake) in all three lines as compared to the control (empty-GAL4). The sleep profile indicated a stronger suppression of sleep during nighttime in males and reduced sleep during both day and nighttime in females. (B) Optogenetic activation and video-based tracking showed that two split-GAL4 lines (SS54903 and SS53683) have decreased sleep duration, and all three tested lines have increased P(Wake) in male flies during optogenetic activation. All three lines showed decreased sleep and increased P(Wake) in female flies. As observed with thermogenetic activation, these phenotypes were more pronounced for nighttime sleep in males. In addition to sleep duration and P(Wake) we also measured activity by beam counts/waking minute in the DAM assays and pixel movements/waking minute in video tracking. We found that activation of hDeltaF does not increase these measures, showing that observed changes are not attributable to hyperactivity (see Supplementary Files 3-6). (C-E) MIP images of GFP-driven expression in the brain and VNC of the three split-GAL4 lines. The brain and VNC are outlined in red. (F,G) Higher resolution images of the relevant brain area of two of the lines. (H) Morphology of a single neuron revealed by stochastic labelling. (I) Comparison of LM and EM cell morphologies. Original confocal stacks for panels C-H can be downloaded from www.janelia.org/split-gal4. The full genotypes of the driver lines are given there and in Supplementary File 1. Statistical comparisons were made by Kruskal-Wallis and Dunn's post hoc test. Asterisk indicates significance from 0: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.0001$.

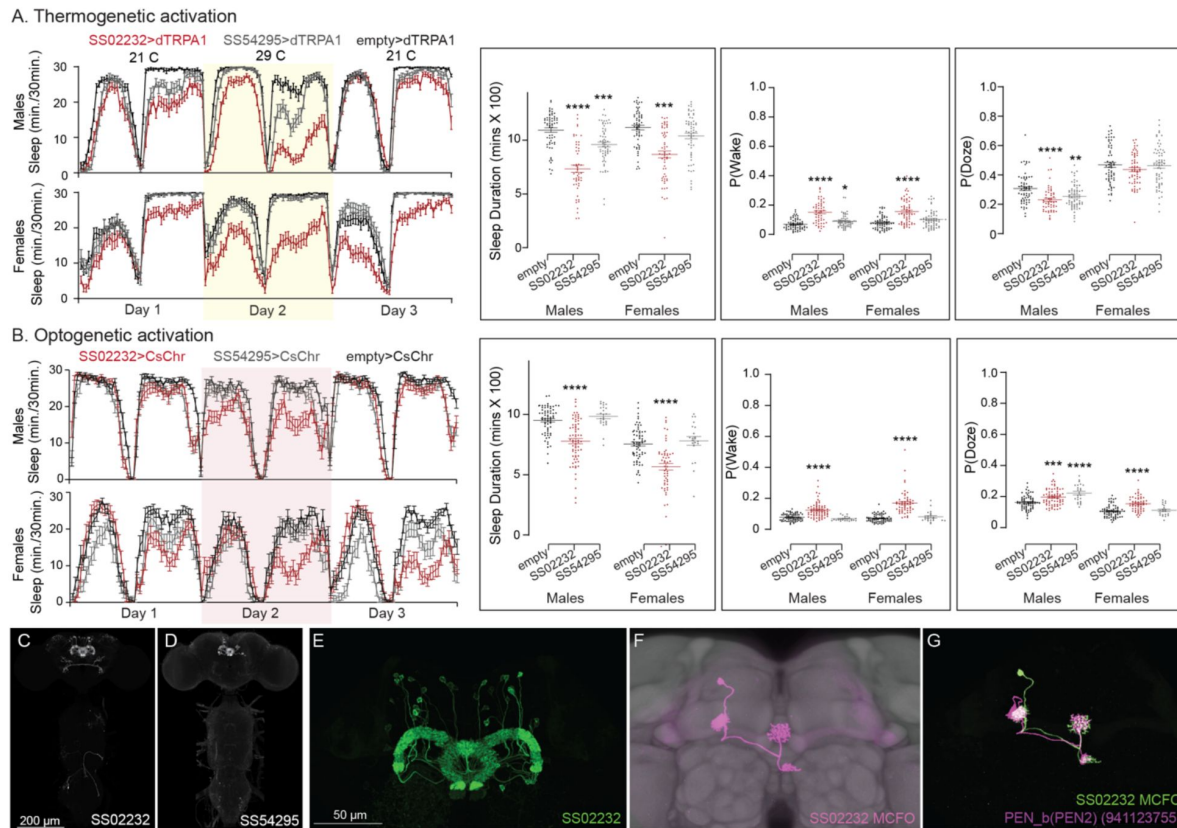


Figure 10—figure supplement 1.

Activation phenotype of PEN_b, comprised of 22 columnar neurons linking the EB, PB and noduli (Wolh and Rubin 2018 [\[1\]](#); Turner-Evans et al., 2020; Green et al., 2017 [\[2\]](#); Hulse et al., 2021 [\[3\]](#)). Effects on activity of thermogenetic (A) and optogenetic (B) activation of PEN_b using split-GAL4 lines SS02232 and SS54295 is shown. Specifically, thermogenetic activation of these split-GAL4 lines decreases sleep duration and increases P(Wake) in males but only SS02232 is wake promoting in females. Optogenetic activation of SS02232 suppresses sleep and increases P(Wake) in both males and females supporting the general function of these neurons as wake-promoting. While both lines show high cell type specificity and minimal VNC expression, SS54295 has stochastic expression that might explain the weaker wake promoting effect seen with this line. (C-D) MIP images of GFP-driven expression in the brain and VNC of the two split-GAL4 lines. The brain and VNC are outlined in red. (E) Higher resolution images of the relevant brain area of SS02232. (F) Morphology of a single neuron revealed by stochastic labelling shown with neuropil reference. (G) Comparison of LM and EM cell morphologies. Original confocal stacks for panels C-F can be downloaded from www.janelia.org/split-gal4 [\[4\]](#). The full genotypes of the driver lines are given there and in Supplementary File 1. Statistical comparisons were made by Kruskal-Wallis and Dunn's post hoc test. Asterisk indicates significance from 0: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

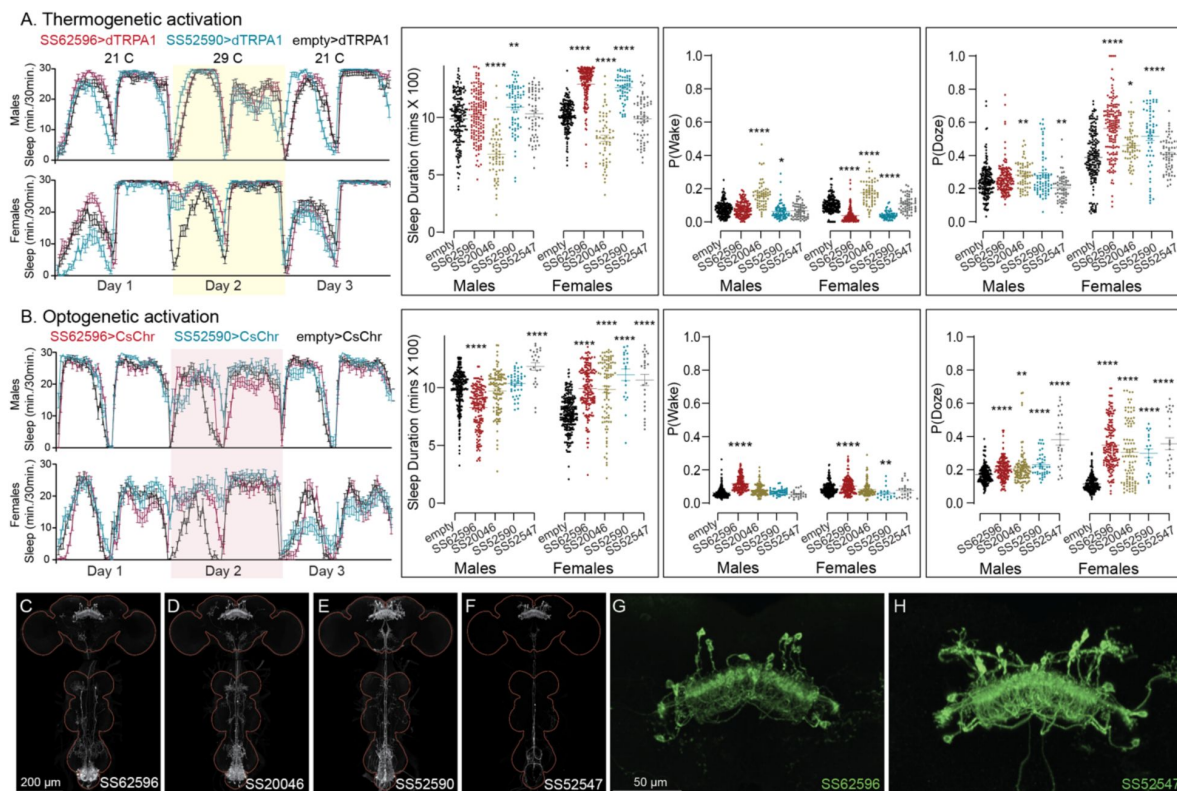


Figure 10—figure supplement 2.

Activation phenotype of PFGs. Thermogenetic (A) and optogenetic (B) activation of PFGs reveals sexually dimorphic sleep behavior. We tested four diherent split-GAL4 lines for this cell type. All four lines display expression in the VNC complicating interpretation of the results. To mitigate this issue, we used lines with diherent hemidriver parents and visually distinct VNC expression patterns (C-F). The brain and VNC are outlined in red. Optogenetic activation (Day 2) in female flies showed increased sleep in all four driver lines and a mild increase in sleep duration in one of lines for male flies (SS52547). However, P(Doze) was consistently higher in male and female flies for all tested PFGs lines. Thermogenetic activation (Day 2) also shows sex specific phenotypes and two of the four lines (SS62596 and SS52590) have increased sleep. SS20046 has decreased sleep in females and male flies with dTRPA1 based activation but shows increased sleep with CsChrimson in females. Despite these inconsistencies, the data suggest a sex specific sleep promoting effect for this cell type. (G-H) Higher resolution images of the relevant brain area of SS62596 and SS52547. Original confocal stacks for panels C-H can be downloaded from www.janelia.org/split-gal4. The full genotypes of the driver lines are given there and in Supplementary File 1. Statistical comparisons were made by Kruskal-Wallis and Dunn's post hoc test. Asterisk indicates significance from 0: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

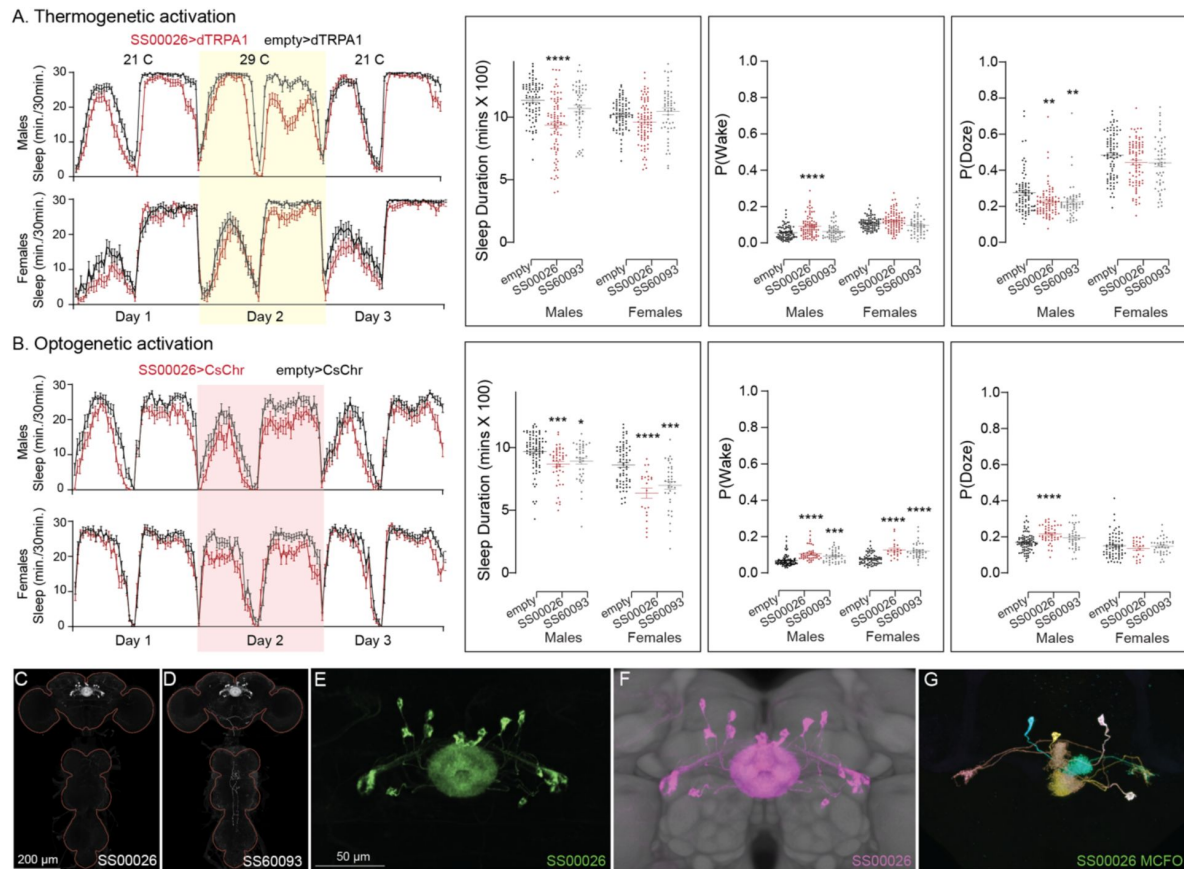


Figure 10—figure supplement 3.

Thermogenetic activation (A) of EL neurons decreases sleep in one of the split-GAL4 lines (SS00026), while optogenetic activation (B) of both lines decreases sleep and increases P(Wake) on Day 2. (C,D) MIP images of GFP-driven expression in the brain and VNC of the three split-GAL4 lines. The brain and VNC are outlined in red. (E) Higher resolution images of the relevant brain area of in SS00026. (F) Same as panel E, but with neuropil reference. (G) Morphology of individual neurons revealed by stochastic labelling. Original confocal stacks for panels C-G can be downloaded from www.janelia.org/split-gal4. The full genotypes of the driver lines are given there and in Supplementary File 1. Statistical comparisons were made by Kruskal-Wallis and Dunn's post hoc test. Asterisk indicates significance from 0: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

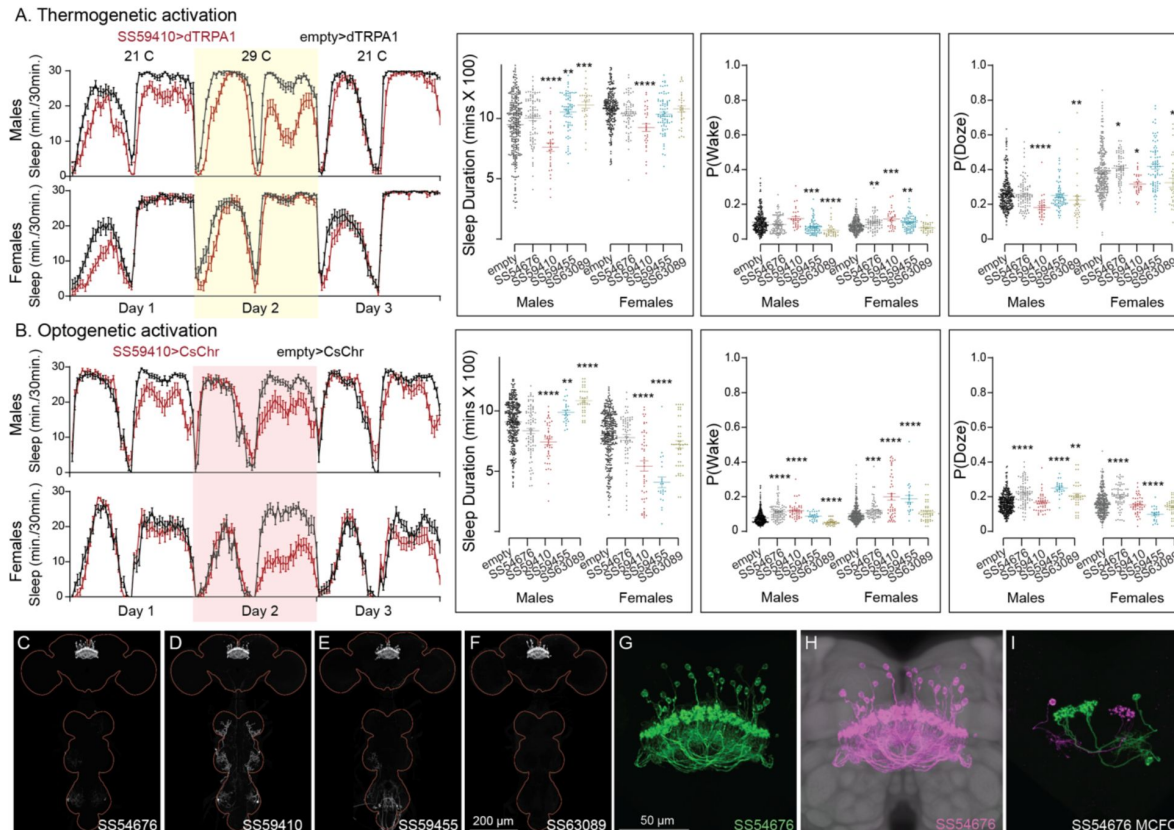


Figure 10—figure supplement 4.

Activation phenotype of hDeltaK. This group of intrinsic columnar neurons is highly peptidergic and has been implicated in chronic social isolation-induced sleep loss (Li et al., 2021). We tested four split-GAL4 lines (SS59455, SS63089, SS54676 and SS59410). Our results do not give a consistent view of the role of this cell type in sleep regulation. (A) Thermogenetic activation of one of the four lines (SS59410) was wake-promoting in both sexes, while two (SS59455 and SS63089) were sleep-promoting in males, but not females. (B) Consistent with what was observed with thermogenetic activation, optogenetic activation of SS59410 also suppressed sleep in males and females, while SS59410 and SS59455 were sleep promoting in males, but not in females. (C-F) MIP images of GFP-driven expression in the brain and VNC of the four split-GAL4 lines. The brain and VNC are outlined in red. (G) Higher resolution images of the relevant brain area of in SS54676 (H) Same as panel G, but with neuropil reference. (I) Morphology of individual neurons revealed by stochastic labelling. We do not have any insight into the diherences in behaviors between lines and cannot make an overall conclusion of their role in sleep regulation. Such line-to-line inconsistencies argue for caution in the interpretation of results, especially when only one line for a cell type has been assayed. Original confocal stacks for panels C-I can be downloaded from www.janelia.org/split-gal4. The full genotypes of the driver lines are given there and in Supplementary File 1. Statistical comparisons were made by Kruskal-Wallis and Dunn's post hoc test. Asterisk indicates significance from 0: * $p < 0.05$; ** $p < 0.01$; *** $p < 0.001$; **** $p < 0.0001$.

A. Thermogenetic activation

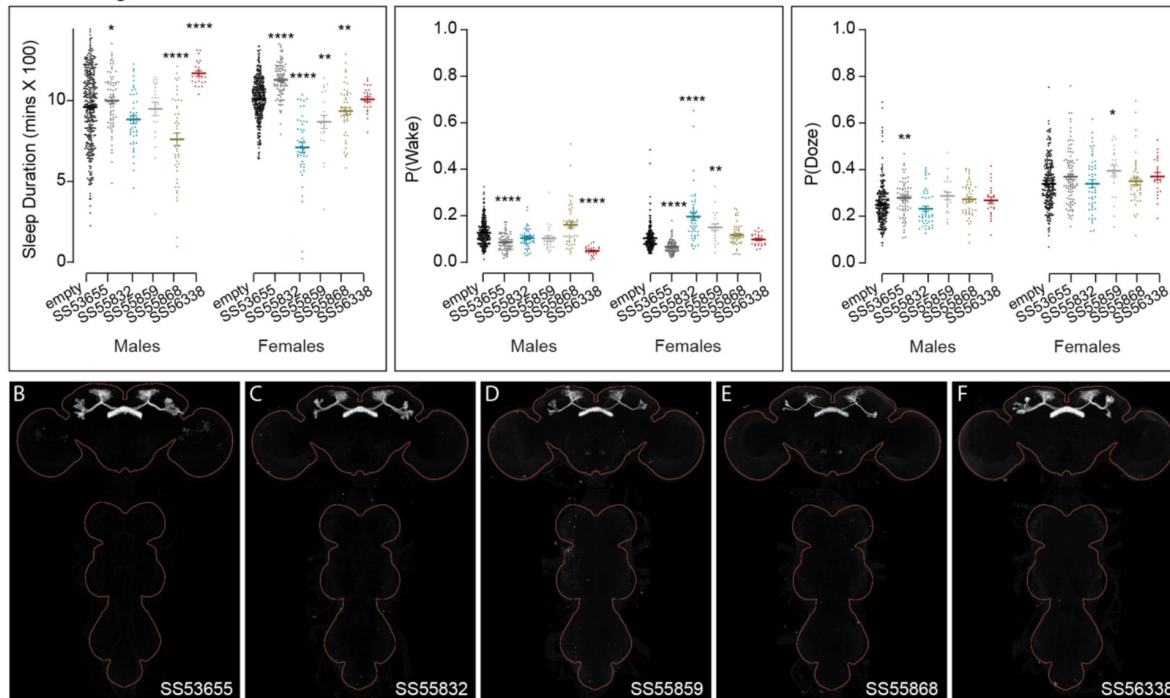


Figure 10—figure supplement 5.

Activation phenotypes of combinations of dFB cell types. The five split-GAL4 lines used here each utilize R84C10 as one of their hemidrivers. They represent diherent (but largely overlapping subsets of layer 6 and 7 FB tangential cell types and have no detectable VNC expression. Jones et al. (2024) reported analysis of additional lines that used R84C10 as a hemidriver in split-GAL4 lines. We did not examine expression in other parts of the peripheral nervous system or muscle. (A) Thermogenetic activation revealed that some lines were sleep promoting and others wake promoting. (B-F) MIP images of GFP-driven expression in the brain and VNC of the four split-GAL4 lines. The brain and VNC are outlined in red. Original confocal stacks can be downloaded from www.janelia.org/split-gal4. The full genotypes of the driver lines are given there and in Supplementary File 1. Statistical comparisons were made by Kruskal-Wallis and Dunn's post hoc test. Asterisk indicates significance from 0: *p<0.05; **p<0.01; ***p<0.001; ****p<0.0001.

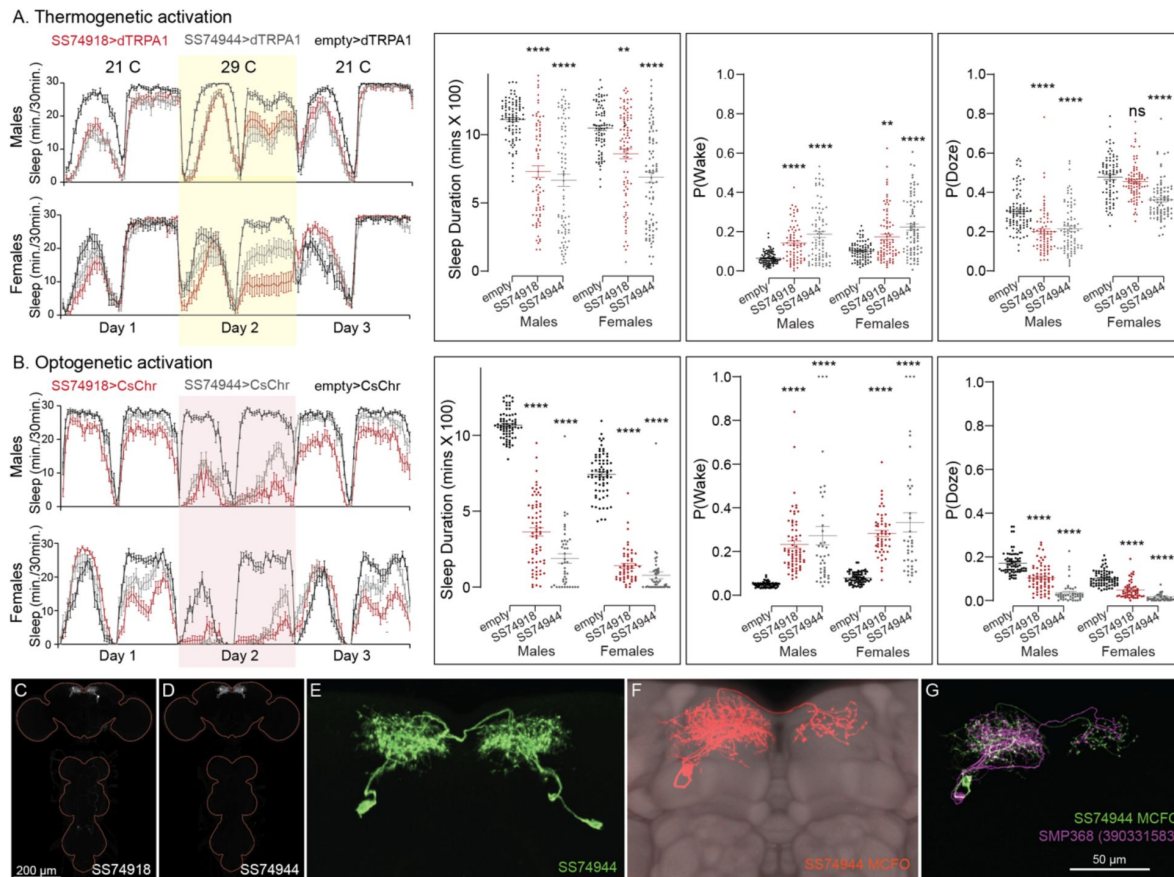


Figure 11.

Activation of SMP368 decreases sleep. SMP368 connects central clock outputs to the CX (see [Figure 12](#) and [Figure 12](#)—figure supplement 1). Both split-GAL4 lines for SMP368 that we tested (*SS74918* and *SS74944*) are strongly wake-promoting in both thermogenetic (A) and optogenetic (B) activation and the phenotypes are consistent across sexes. Further, these lines show increase P(Wake) and decreased P(Doze) indicative of decreased sleep pressure and altered sleep depth. (C-D) MIP images of GFP-driven expression in the brain and VNC of the two split-GAL4 lines. The brain and VNC are outlined in red. (E) Higher resolution images of the relevant brain area of the two split-GAL4 lines. (F) Morphology of a single neuron revealed by stochastic labelling shown with neuropil reference. (G) Comparison of LM and EM cell morphologies. Original confocal stacks for panels C-F can be downloaded from www.janelia.org/split-gal4. The full genotypes of the driver lines are given there and in Supplementary File 1. Statistical comparisons were made by Kruskal-Wallis and Dunn's post hoc test. Asterisk indicates significance from 0: ** $p < 0.01$; **** $p < 0.0001$.

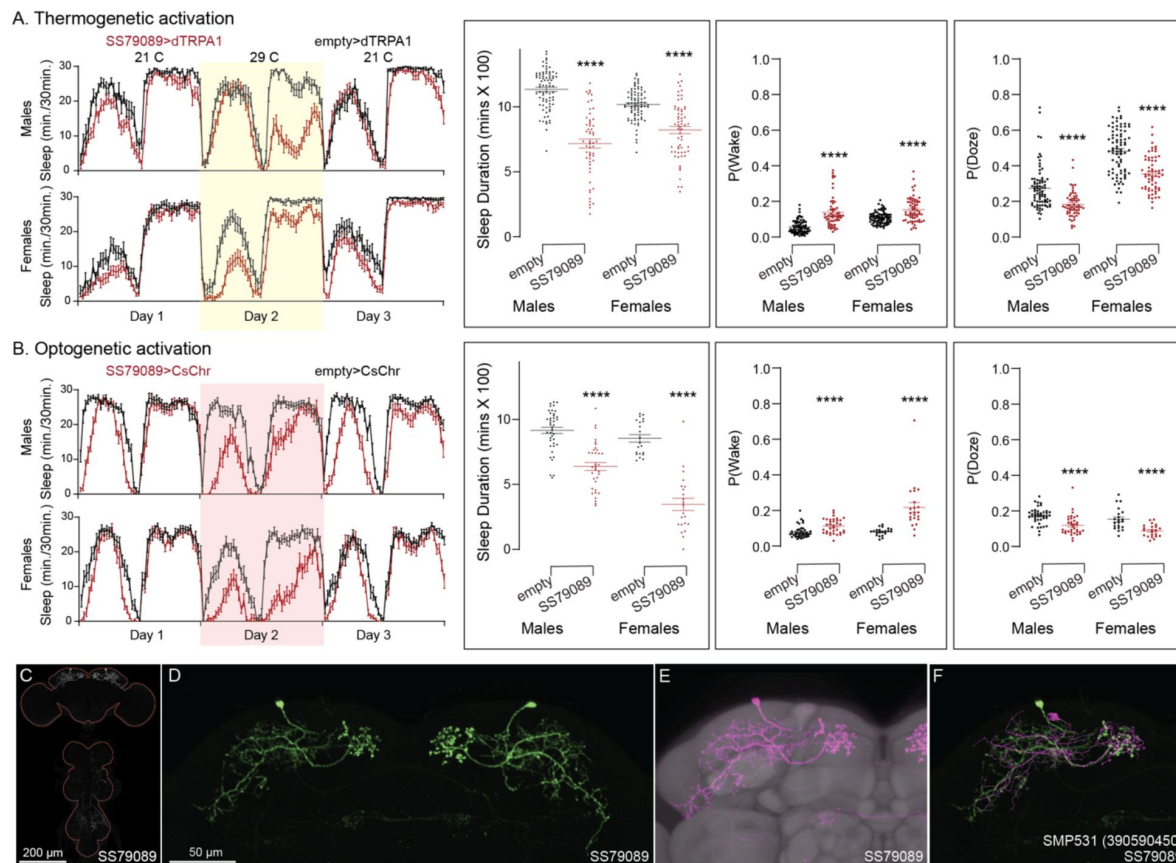


Figure 11—figure supplement 1.

Activation phenotype of SMP531. SMP531 is part of a potential pathway from clock outputs to the CX (see [Figure 12](#) — figure supplement 1). The SMP531 split-GAL4 line SS79089 suppresses sleep when activated thermogenetically (A) and optogenetically (B) in both males and females. Further, activation of this line increases P(Wake) and decreased P(Doze). (C) MIP image of GFP-driven expression in the brain and VNC of SS79089. The brain and VNC are outlined in red. (D) Higher resolution images of the relevant brain area of SS79089. (E) Morphology shown with neuropil reference. (F) Comparison of LM and EM cell morphologies. Original confocal stacks for panels C-E can be downloaded from www.janelia.org/split-gal4. The full genotypes of the driver lines are given there and in Supplementary File 1. Statistical comparisons were made by Kruskal-Wallis and Dunn's post hoc test. Asterisk indicates significance from 0: ****p<0.0001.

Connections between the CX and the circadian clock

Not surprisingly, the connectome reveals that many of the intrinsic CX cell types with sleep phenotypes are connected by wired pathways. The strongest of these connections are diagrammed in [Figure 12](#), with [Figure 12](#)—figure supplement 1 also showing additional weaker connections. The connectome also suggested pathways from the circadian clock to the CX. Some of these have been previously noted. Links between clock output DN1 neurons to the ExR1 have been described in [Lamaze et al. \(2018\)](#) and [Guo et al. \(2018\)](#), and [Liang et al. \(2019\)](#) described a connection from the clock to ExR2 (PPM3) dopaminergic neurons. We found two SMP cell types, SMP368 and SMP531, that were very strongly wake-promoting when activated suggesting they might be components of previously undescribed wired pathways from the clock to the CX.

In addition to these wired pathways, our work supports the possibility of signaling from the clock over considerable distances to the CX using neuropeptides. Our RNA profiling of ER5 cells ([Figure 9—figure supplement 1](#)), which are known to be regulators of sleep and sleep homeostasis ([Liu et al., 2016](#)), revealed expression of receptors for both PDF and Dh44. The presence of the PDF receptor in ER5 cells was suggested by prior work ([Im & Taghert, 2010](#); [Parisky et al., 2008](#); [Pirez et al., 2013](#)). We confirmed these observations and showed that ER5 cells make Dh31 ([video 1](#)). Dh44 has been implicated as a clock output that regulates locomotor activity rhythms ([Barber et al., 2021](#); [Cavanaugh et al., 2014](#)) and the DH44R1 receptor has been shown to function in sleep regulation in non-CX cells ([King et al., 2017](#)). However, the presence of the Dh44R2 receptor in the EB was unexpected. Dh31 is expressed by many cells in the fly brain (see [Figure 6F](#)) including DN1s ([Kunst et al., 2014](#)) and has been shown to play a role in sleep regulation; the cellular targets of Dh31 released from ER5 are unknown, however previous work ([Goda et al., 2017](#); [Mertens et al., 2005](#); [Shafer et al., 2008](#)) has shown that Dh31 can activate the PDF receptor raising the possibility of autocrine signaling. [Andreani et al. \(2022\)](#) also showed a functional link between the clock and ER5 cells, but the circuit mechanism was not elucidated.

Concluding Remarks

We provide a greatly enhanced set of genetic reagents for manipulating the intrinsic cell types of the CX that will be instrumental in fully elucidating the many functions of the CX. We illustrate their use in discovering cell types involved in activity regulation, and uncovered new potential wired and peptidergic connections between the circadian clock and the CX. We surveyed neuropeptide and neuropeptide receptor gene expression in the adult central brain. Neuropeptides fell into two broad categories, those that are expressed in only a few cells and those that are expressed in dozens to hundreds of cells. We observed that neuropeptide receptor genes were much more broadly expressed than those of their cognate neuropeptides. Finally, we generated the largest available dataset of co-expression of neuropeptides and neurotransmitters in identified cell types. Unexpectedly, we found that all neuropeptide-expressing cell types also expressed a small neurotransmitter. Our data reveal the pervasive potential for peptidergic communication within the CX—more than half of the cell types we examined expressed a neuropeptide and one-third of those expressed multiple neuropeptides.

Materials and Methods

Generation of split-GAL4 lines

Split-GAL4 lines were generated as previously described ([Dionne et al. 2018](#)). Databases of expression patterns generated in the adult brain by single genomic fragments cloned upstream of GAL4 ([Jennet et al., 2012](#); [Tirian et al., 2012](#)) were manually or computationally ([Meissner et al.,](#)

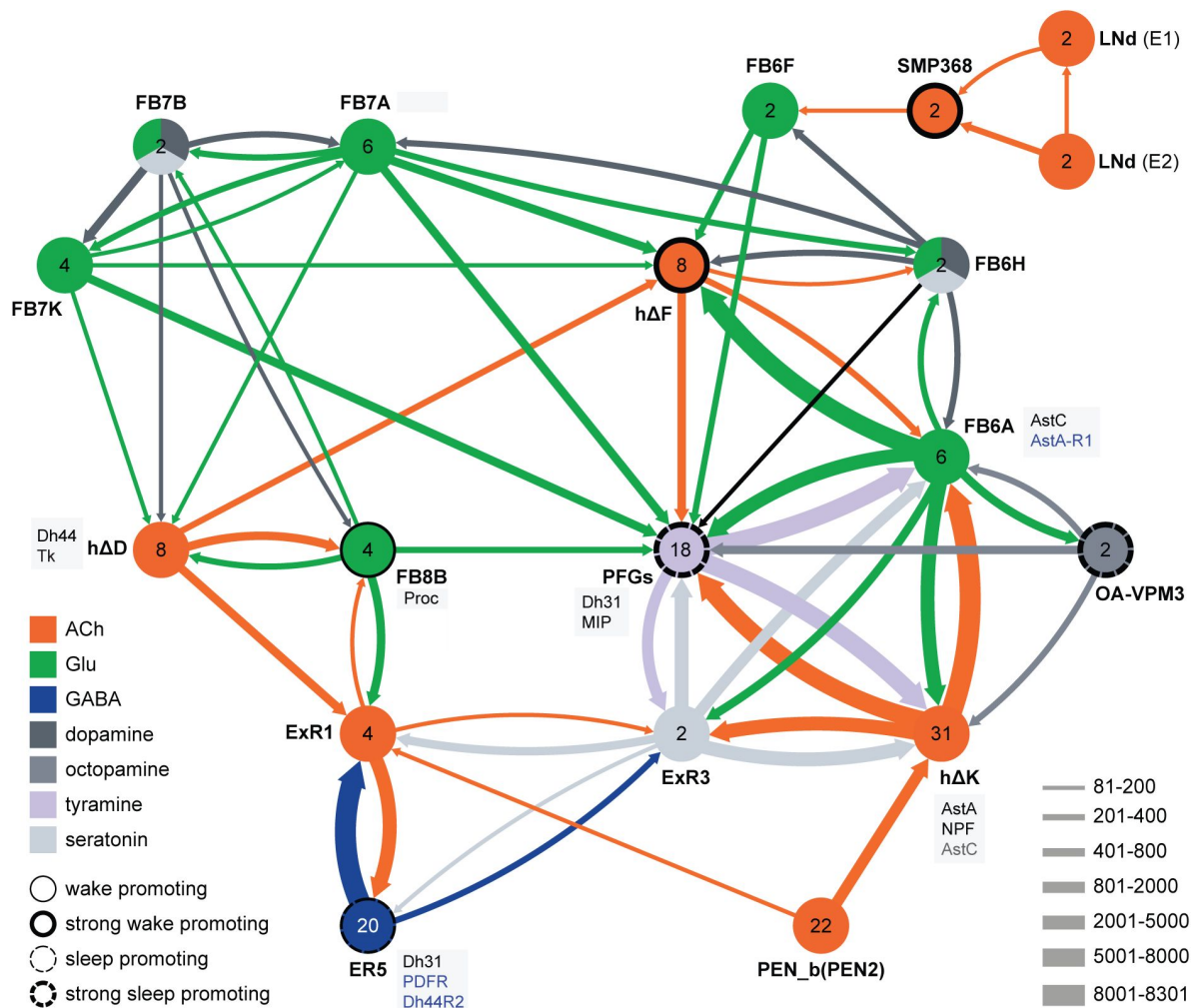


Figure 12.

Circuit diagram of CX cells implicated in regulating sleep. Selected CX cell types, plus non-CX cell types SMP368, LNd and OA-VPM3, are shown. The number within each circle denotes the number of cells in that cell type. The neurotransmitters used by each cell type are indicated by color coding, and the number of synapses between cell types is represented by arrow width. Cell types that have been shown to promote sleep or wake when activated are indicated. Experimental evidence for the wake promoting effects of OA-VPM3 is from Reyes, M and Sitaraman D (in preparation). In cases where we have experimentally determined expression of neuropeptides or neuropeptide receptor genes by either EASI-FISH or RNA profiling, this information is indicated in the boxes next to the relevant cell type. Cell type names are from the hemibrain release 1.2.1 except for the LNd neurons whose names have been modified based on morphology and connectivity; they have been grouped into two types: LNd (E1) corresponds to hemibrain body IDs 5813056917 + 5813021192 and LNd (E2) corresponds to hemibrain body IDs 511051477 (5th LNd) + 5813069648 (LNd6) (Shafer et al., 2022). Because the CX is a central body and the inputs from CX cells that have their soma in right or left hemisphere appear to be comingled on their downstream targets, the synaptic strengths shown represent the combined number of cells of each type, regardless of soma position. For example, the arrow thickness between FB6F and hDeltaF reflects the total number of synapses (368) from FB6F_R and FB6F_L to all hDeltaF cells; the individual synapse number between each of the two FB6F cells to each of the eight hDeltaF cells, which ranges from 7 to 39, can be found in neuPrint. The sole exception is the LNd cells where the synaptic strength represents only the output of LNds in the right hemisphere. See Figure 53 of Hulse et al. (2021) for additional connected cells.

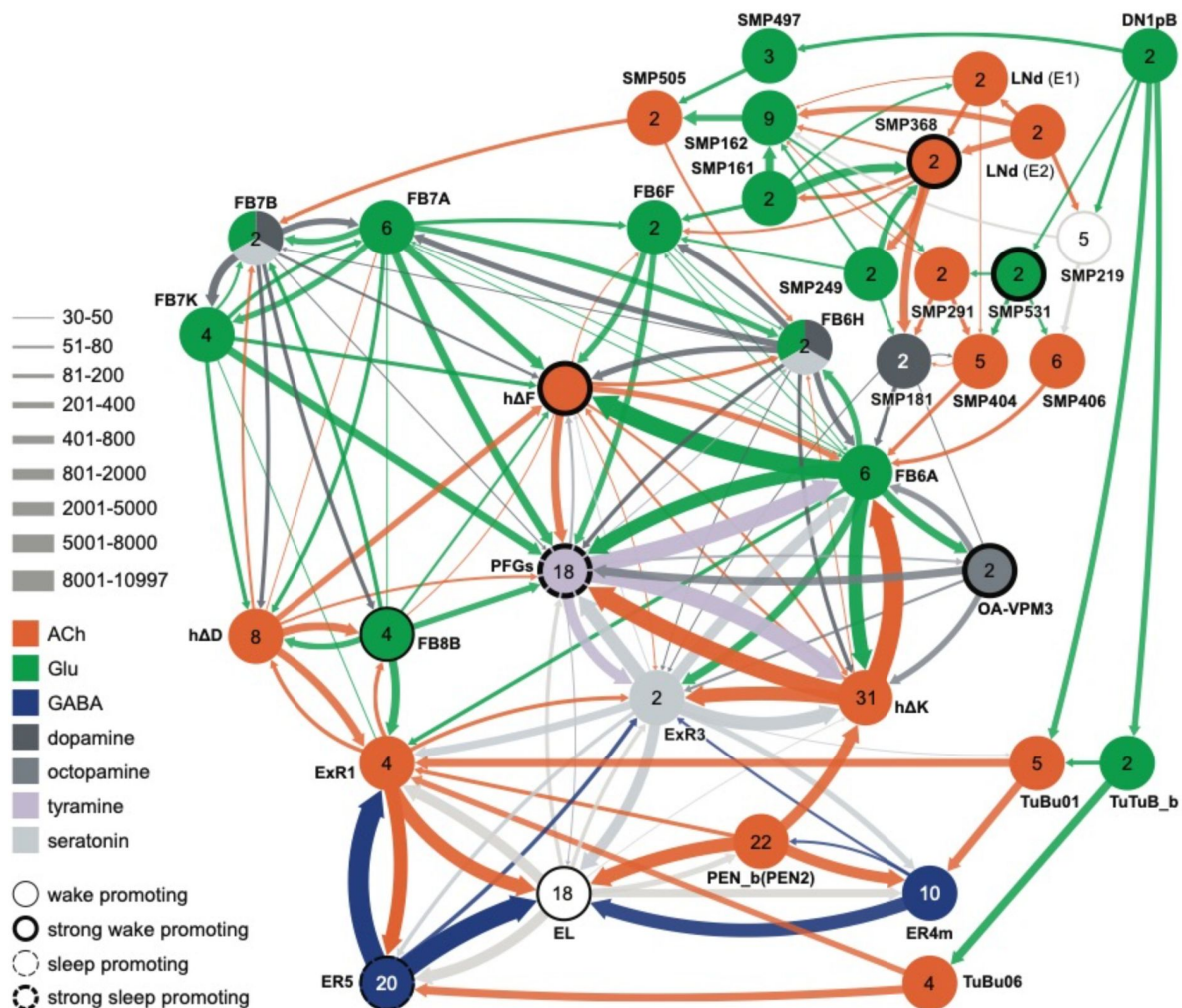


Figure 12—figure supplement 1.

Circuit diagram showing some additional cell types potentially involved in regulating sleep. Other studies have identified roles for some of these cell types as well as additional cells not shown in this diagram. Links between clock output DN1 neurons to the ExR1 have been described in Lamaze et al. (2018) [DOI](#) and Guo et al. (2018) [DOI](#). Liang et al. (2019) [DOI](#) described a connection from the clock to ExR2 (PPM3) dopaminergic neurons.

2023 [DOI](#)) screened. Individual enhancers that showed expression in the desired cell type were then cloned into vectors that contained either the DNA-binding or activation domain of GAL4 (Luan et al., 2008; Pfeiffer et al., 2012). These constructs were combined in the same individual and screened for expression in the desired cell type by confocal imaging. Over 15,000 such screening crosses were performed to generate the new split-GAL4 lines reported here. Successful constructs were made into stable lines.

The lines listed in Supplementary File 1 are currently being maintained at Janelia and the majority of these have also been deposited in the Bloomington Drosophila Stock Center.

Characterization of split-GAL4 lines

Lines were characterized by confocal imaging of the entire expression pattern in the brain and VNC at 20X. Most lines were also imaged at higher magnification (63x) and/or subjected to stochastic labelling (MCFO; Nern et al., 2015 [DOI](#)) to reveal the morphology of individual cells. Split-GAL4 images are shown as MIPs after alignment to JRC2018 (Bogovic et al., 2020 [DOI](#)). Over 1,800 confocal stacks derived from over 450 lines generated during this work are presented in, and can be downloaded from, an on-line database (janelia.org/split-GAL4 [DOI](#)). Images for the additional lines used in the sleep screen are available from flylight-raw.janelia.org [DOI](#).

Determining the correspondence between the cell-types present in each split-GAL4 line and those described in the connectome (Hulse et al., 2021 [DOI](#)) was based solely on morphology. Even when assigning correspondence between cells in two different connectomes, where information on connectivity can also be employed, the process is not always straightforward (see Schlegel et al., 2024 [DOI](#)). Because of the similarity in morphology of many of the CX cell types it was often challenging to assign correspondence to the cell types defined by connectome analysis. For this reason, we rated our confidence in our assignments as Confident, Probable or Candidate and include this information for each line at janelia.org/split-GAL4 [DOI](#). To be considered confident, we judged our opinion had a >95% chance of being correct. Such assignments were generally only possible for cell types which had morphological features clearly distinct from those in other cell types. Most assignments were rated as Probable indicating 70-95% confidence. Lines whose cell type assignments are listed as Probable have been rigorously examined and the assignments are the most accurate that the available data allow. In the absence of single-cell data available in MCFO brains (the case for many lines) or additional data (for example, physiological data on connectivity), cell types that are morphologically very similar cannot be distinguished with complete confidence. Lines whose cell type assignments are listed as Candidate are even less certain (30-70% confidence).

RNA *in situ* hybridization

Adult females (5 to 7 days post eclosion) were expanded, probed, and imaged using the EASI-FISH method as described in (Eddison and Ihrke, 2022 [DOI](#); Close et al., under review). The oligo probes and HCR hairpins were designed by, and purchased from, Molecular Instruments, Inc. Imaging was performed on a Zeiss Z7 microscope equipped with a 20X objective. Laser power and exposure time was optimized to maximize the signal-to-noise ratio. For neurotransmitters, the specific probes used for each cell type are indicated in **Figure 9** [DOI](#) and in **Supplemental File 1** [DOI](#). For neuropeptides, each of the 17 selected NP probes shown in **Figure 5—figure supplement 1** [DOI](#) was used on all cell types in Figure 9 except those marked by “—” in the neuropeptide column.

RNA profiling

The data shown in **Figure 9—figure supplement 1** [DOI](#) were generated as described in Aso et al., 2019 [DOI](#). See NCBI Gene Expression Omnibus (accession number GSE271123) for the raw data and additional details.

Sleep measurement and analysis: Thermogenetic activation screen

Split-GAL4 flies were crossed to 10X UAS-dTrpA1 (attP16) (Hamada et al., 2008) and maintained at 21–22°C in vials containing standard dextrose-based media (7.9g Agar, 27.5g Yeast, 52g cornmeal, 110g dextrose, 8.75 ml 20% Tegosept and 2 ml propionic acid/liter).

Virgin female progeny or male progeny (as specified in the figures), 3–7 days post-eclosion, (n = 16–32/trial) were placed in 65 mm × 5 mm transparent plastic tubes with standard cornmeal dextrose agar media and placed in a Drosophila Activity Monitoring system (Trikinetics). Food composition was kept consistent between rearing and experimentation. Locomotor activity data were collected in 1-min bins for 5–7 days. Activity monitors were maintained with a 12 hr:12 hr light–dark cycle at 40–65% relative humidity. Total 24-hr sleep amounts (daytime plus nighttime sleep duration), Pwake, and Pdoze were extracted from the locomotor data as described in (Donelson et al., 2012; Wiggin et al., 2020) using MATLAB-based SCAMP program. (Sitaraman et al., 2024; Vecsey et al., 2024)

Sleep duration was defined as 5 min or more of inactivity (Hendricks et al., 2000; Shaw et al., 2000). Representative sleep profiles were generated representing average (n = 24–32) sleep (min/30 min) for day 1 (baseline), day 2 (activation), and day 3 (recovery/post activation). In addition to permissive temperature controls, split-pBDPGAL4U/dTrpA1 were used as genotypic controls for hit detection. pBDPGAL4U (attP40, attP2), has enhancerless GAL4-AD construct and GAL4-DBD constructs inserted on chromosome II and III (Dionne et al., 2018), as is the case for split-GAL4 driver lines in behavioral assays. Each split-GAL4 line was tested at least twice in independent trials. For all screen hits, wake activity was calculated as the number of beam crossings/min when the fly was awake. Statistical comparisons between experimental and control genotypes were performed using Prism (Graphpad Inc) by Kruskal Wallis One way ANOVA followed by Dunn's post-test. Pairwise comparisons between the empty (split-pBDPGAL4U) control and experimental lines were made using Mann-Whitney U test.

Sleep measurement and analysis: Optogenetic activation screen

Split-GAL4 flies were crossed to 20XUAS-CsChrimson-mVenus-trafficked (attP18) (BDSC:55134) and maintained at 21–25°C in vials containing standard cornmeal food supplemented with 0.2% retinal. Male and virgin female progeny were collected into separate vials containing standard cornmeal food with 0.4% added retinal and kept in a 25°C incubator on a 12:12 light:dark schedule for 3–5 days before loading. Typical sleep experiments lasted 6–7 days. Flies were loaded into 96 well plates or individual tubes using CO₂ anesthesia. Flies were allowed to recover from anesthesia and acclimatize to the experimental chambers for 16–18 hours prior to starting the experiment.

The behavioral setup for video recording system was adapted from (Guo et al., 2016). Flies were briefly anesthetized and loaded into 96 well plates (Falcon™ 96-Well, Non-Treated, Fisher Scientific Inc) containing 150 µl per well of 5% sucrose, 1% agarose and 0.4% retinal. The plates were covered with breathable sealing films. Small holes (one per well) were poked with fine forceps into the film to further ensure air exchange and prevent condensation. The entire setup was housed in an incubator to control light/dark conditions and temperature. The 96-well plates with flies were placed in holders, constantly illuminated from below using an 850 nm LED board (Smart Vision Lights Inc) and imaged from above using a FLIR Flea 3 camera (Edmund Optics Inc). 635 nm red light (for optogenetic activation) was provided using an additional backlight, low levels of white light (to provide a light-dark cycle) were supplied from above. Optogenetic activation was for a 24 h period (starting in the morning at the same time as the white light was turned on for the day) and was delivered in pulses of 25 ms duration at 2 Hz frequency. Each experiment also included at least one full day without the red light preceding and following the activation day (matching the general design of the thermogenetic experiments).

Fly movement was tracked using single fly position tracker (GitHub - [cgoina/pysolo-tools](#)) and processed for sleep duration and other sleep parameters using SCAMP. In addition to the 5 min criteria, used to define total duration of sleep, P(Doze), the probability that an active fly will stop moving, and P(Wake), the probability that a stationary fly will start moving provide key additional measures of inactivity and activity and were included in our analyses (Wiggin et al., 2020). These sleep measures are presented in **Figure 10** and **11** (and supplements) and supplementary files 3,4,5, and 6.

Supplemental Files for sleep phenotypes

The supplemental files present data on day1 (baseline) and day 2 (activation). Given the large effects of environmental conditions on activity, we calculated p values between experimental and control group within the same environmental conditions. However, we also present mean differences between the days as a complementary way to identify lines that modified sleep when activated.

Acknowledgements

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Additional information

Author Contributions

TW, ME, DS and GMR conceived the study. TW, AN and GMR created split-GAL4 lines. TW analyzed lines for CX cell types, assigned them to cell types, and scored EASI-FISH experiments. ME designed, conducted, and scored EASI-FISH experiments. NC, PS and DS designed and conducted behavioral assays. GMR wrote the paper with input from all authors.

Additional files

Supplemental File 1. Table of split-GAL4 lines, organized by CX structure and cell type. [↗](#)

Supplemental File 2. Table of additional split-GAL4 lines, organized by CX structure and cell type [↗](#)

Supplemental File 3. Thermogenetic screen of selected stable-split lines in males [↗](#)

Supplemental File 4. Thermogenetic screen of selected stable-split lines in females [↗](#)

Supplemental File 5. Optogenetic screen of selected stable-split lines in males [↗](#)

Supplemental File 6. Optogenetic screen of selected stable-split lines in females [↗](#)

Video 1. Expression of genes encoding the neuropeptide receptors PDFR and Dh44R2 as well as the neuropeptide Dh31 in ER5 cells of the EB. [↗](#)

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Reviewer #1 (Public review):

Summary:

This work is meant to help create a foundation for future studies of the Central Complex, which is a critical integrative center in the fly brain. The authors present a systematic description of cellular elements, cell type classifications, behavioral evaluations and genetic resources available to the *Drosophila* neuroscience community.

Strengths:

The work contributes new, useful and systematic technical information in compelling fashion to support future studies of the fly brain. It also continues to set a high and transparent standard by which large-scale resources can be defined and shared.

Weaknesses:

Manuscript revisions by the authors addressed all proposed weaknesses from the original version.

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Reviewer #2 (Public review):**Summary:**

In this paper, Wolff et al. describe an impressive collection of newly created split-GAL4 lines targeting specific cell types within the central complex (CX) of *Drosophila*. The CX is an important area in the brain that has been involved in the regulation of many behaviors including navigation and sleep/wake. The authors advocate that to fully understand how the CX functions, cell-specific driver lines need to be created. In that respect, this manuscript will be of very important value to all neuroscientists trying to elucidate complex behaviors using the fly model. In addition, and providing a further very important finding, the authors went on to assess neurotransmitter/neuropeptides and their receptors expression in different cells of the CX. These findings will also be of great interest to many and will help further studies aimed at understanding the CX circuitries. The authors then investigated how different CX cell types influence sleep and wake. While the description of the new lines and their neurochemical identity is excellent, the behavioral screen seems to be unfinished and could have been more matured.

Strengths:

- (1) The description of dozens of cell-specific split-GAL4 lines is extremely valuable to the fly community. The strength of the fly system relies on the ability to manipulate specific neurons to investigate their involvement in a specific behavior. Recently, the need to use extremely specific tools has been highlighted by the identification of sleep-promoting neurons located in the VNC of the fly as part of the expression pattern of the most widely used dorsal-Fan Shaped Body (dFB) GAL4 driver. These findings should serve as a warning to every neurobiologist, make sure that your tool is clean. In that respect, the novel lines described in this manuscript are fantastic tools that will help the fly community.
- (2) The description of neurotransmitter/neuropeptides expression pattern in the CX is of remarkable importance and will help design experiments aimed at understanding how the CX functions.

Weaknesses:

- (1) I find the behavioral (sleep) screen of this manuscript to be incomplete. It appears to me that this part of the paper is not as developed as it could be. The authors have performed neuronal activation using thermogenetic and/or optogenetic approaches. For some cell types, only thermogenetic activation is shown. There is no silencing data and/or assessment of sleep homeostasis or arousal threshold. The authors find that many CX cell types modulate sleep and wake but it's difficult to understand how these findings fit one with the other. It seems that each CX cell type is worthy of its own independent study and paper. I am fully aware that a thorough investigation of every CX neuronal type in sleep and wake regulation is a herculean task. So, altogether I think that this manuscript will pave the way for further studies on the role of CX neurons in sleep regulation.
- (2) Linked to point 1, it is possible that the activation protocols used in this study are insufficient for some neuronal types. The authors have used 29{degree sign} for thermogenetic activation (instead of the most widely used 31{degree sign}) and a 2Hz optogenetic activation protocol. The authors should comment on the fact that they may have missed some phenotypes by using these mild activation protocols.
- (3) There are multiple spelling errors in the manuscript that need to be addressed.

Comments on revisions:

I am satisfied with the authors response. This paper provides excellent starting points for additional studies into the role of different CX cell types in sleep and wake.

Author response:

The following is the authors' response to the original reviews.

Public Reviews:

Reviewer #1 (Public review):

Summary:

This work is meant to help create a foundation for future studies of the Central Complex, which is a critical integrative center in the fly brain. The authors present a systematic description of cellular elements, cell type classifications, behavioral evaluations and genetic resources available to the Drosophila neuroscience community.

Strengths:

The work contributes new, useful and systematic technical information in compelling fashion to support future studies of the fly brain. It also continues to set a high and transparent standard by which large-scale resources can be defined and shared.

Weaknesses:

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"The central complex (CX) of the adult Drosophila melanogaster brain consists of approximately 2,800 cells that have been divided into 257 cell types based on morphology and connectivity (Scheer et al., 2020; Hulse et al. 2021; Wolff et al., 2015)."

The 257 accumulated cell types have informational names (e.g., PBG2-9.s-FBI2.b-NO3A.b) in addition to their associations with specific Gal4 lines and specific EM Body IDs. All this is very useful. I have one suggestion to help a reader trying to get a "bird's eye view" of such a large amount of detailed and multi-layered information. Give each of the 257 CX cell types an arbitrary number: 1 to 257. In fact, Supplemental File 2 lists ~277 cell types each with a number in sequence, so perhaps in principle, it is there. This could expedite the search function when a reader is trying to cross-reference CX cell type information from the text, to the Figures and/or to the Supplemental Figures. Also, the use of (arbitrary) cell type numbers could expedite the explanation of which cell types are included in any compilation of information (e.g., which ones were tested for specific NT expression).

In this report we adhered to the nomenclature introduced in Hulse et al. 2021. We agree that the nomenclature of cell types in the CX is imperfect. There are inherent limitations to what can be done with present data. Even between the hemibrain and FAFB/Flywire EM datasets, it was not possible to derive a one-to-one correspondence in many cases, largely because we do not yet have enough information to distinguish between natural variation within a cell type and distinct cell types (see Schlegel et al. 2024). Moreover, many cell type distinctions depend on connectivity differences that are observable only in EM datasets but not in LM images. Several research groups are currently engaged in a comprehensive and collaborative effort to update the CX nomenclature that will extend over the next few months as additional connectomes become available. This work will require hundreds of hours of effort from anatomical and computational experts in multiple laboratories who have a strong interest in the CX. Since the correspondence between the established Hulse et al nomenclature we use and this new nomenclature will be made clear, it will be easy to transfer our data to that new

nomenclature. For all these reasons, we believe we should not unilaterally introduce any new naming systems at this time.

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"Figure 2 and Figure 2-figure supplements 1-4 show the expression of 52 new split-GAL4 lines with strong GAL4 expression that is largely limited to the cell type of interest. We also generated lines of lesser quality for other cell types that in total bring overall coverage to more than three quarters of CX cell types."

This section describes the generation and identification of specific split Gal4 lines, and the presentation is generally excellent. It represents an outstanding compendium of information. My reading of the text suggests ~200 cell types have Gal4 lines that are of immediate use (having high specificity or v close-to-high). Use of an arbitrary number system (mentioned above) could augment that description for the reasons stated. For example, which of the 257 cell types are represented by split Gal4 lines that constitute the ~1/3 representing "high-quality lines"? A second comment relates to this study's functional analysis of the contributions of CX cell types to sleep physiology. The recent literature contains renewed interest in the specific expression patterns of Gal4 lines that can promote sleep-like behaviors. In particular Gal4 line expression outside the brain (in the VNC and outside the CNS) have been raised as important elements that need be included for interpretation interpretation of sleep regulation. This present study offers useful information about a large number of expression patterns, as well as a basis with which to seek additional information., including mention of VNC expression in many cases However, perhaps I missed it, but I could not find a short description of the over-all strategy used to describe the expression patterns and feel that could be helpful. Were all Gal4 lines studied for expression in the VNC? and in the peripheral NS? It is probably published elsewhere, but even a short reprise would still be useful.

We added a couple of sentences to clarify that the lines were imaged in the adult female brain and VNC and many were also imaged in males. These data, including the ability to download the original confocal stacks, are contained in an on-line web source cited in the text. We also make clear that we did not assay expression outside of the brain, optic lobes and VNC. Therefore, we cannot rule out expression in the peripheral nervous system (other than detected in the axons of sensory neurons in the CNS) or in muscle or other non-neuronal cell types.

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Neurotransmitter expression in CX cell types

"To determine what neurotransmitters are used by the CX cell types, we carried out fluorescent in situ hybridization using EASI-FISH (Eddison and Irkhe, 2022; Close et al., 2024) on brains that also expressed GFP driven from a cell-type-specific split GAL4 line. In this way, we could determine what neurotransmitters were expressed in over 100 different CX cell types based on"

Reading this description, I was uncertain whether the >100 cell types mentioned were tested with all the NT markers by EASI-FISH? Also, assigning arbitrary numbers to the cell types (same suggestion as above) could help the reader more readily ascertain which were the ~100 cell types classified in this context.

The specific probes used for each cell type are indicated in Figure 9 and in Supplemental File 1.

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"Our full results are summarized below, together with our analysis of neuropeptide expression in the same cell types."

I recommend specifying which Figures and Tables contain the "full results" indicated.

We changed the wording to read:

"Our full results are summarized, together with our analysis of neuropeptide expression in the same cell types, in Figures 5 -9 and in Supplemental File 1."

NP expression in CX cell types

Similar to the comments regarding studies of NT expression: were all ~100 cell types tested with each of the 17 selected NPs? Arbitrary numerical identifies could be useful for the reader to determine which cell types/ lines were tested and which were not yet tested.

We expanded the description in Methods to now read:

"For neurotransmitters, the specific probes used for each cell type are indicated in Figure 9 and in Supplemental File 1. For neuropeptides, each of the 17 selected NP probes shown in Figure 5—figure supplement 1 was used on all cell types in Figure 9 except those marked by "—" in the neuropeptide column."

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"The neuropeptide expression patterns we observed fell into two broad categories."

This section presents information that is extensive and extremely useful. It supports consideration of peptidergic cell signaling at a circuits level and in a systematic fashion that will promote future progress in this field. I have two comments. First, regarding the categorization of two NP expression patterns, discernible by differences in cell number: this idea mirrors one present in prior literature. Recently the classification of the transcription factor DIMM summarizes this same two-way categorization (e.g., doi: 10.1371/journal.pone.0001896). That included the fact that a single NP can be utilized by cell of either category.

We inserted a sentence to acknowledge this earlier work:

"Such large neurosecretory cells often express the transcription factor DIMM (Park et al. 2008)."

Second, regarding this comment:

"In contrast, neuropeptides like those shown in Figure 6 appear to be expressed in dozens to hundreds of cells and appear poised to function by local volume transmission in multiple distinct circuits."

Signaling by NPs in this second category (many small cells) suggests more local diffusion, a smaller geographic expanse compared to "volume" signaling by the sparser larger peptidergic cells. Given this, I suggest re-consideration in using the term "volume" in this instance, perhaps in favor of "local" or "paracrine". This is only a suggestion and in fact rests almost entirely on speculation/ interpretation, as the field lacks a strong empirical basis to say how far NPs diffuse and act. A recent study in the fly brain of peptide co-transmitters (doi: 10.1016/j.cub.2020.04.025) provides an instructive example in which differences between the spatial extents of long-range (peptide 1) versus short-range (peptide 2) NP signaling may be inferred in vivo.

We have modified the text to now read:

“those shown in Figure 6 are expressed in dozens to hundreds of cells and appear poised to function by transmission to nearby cells in multiple distinct circuits.”

Spab was mentioned (Figure 6 legend) but discarded as a candidate NP to include based on a personal communication, as was Nplp1. The manuscript did not include reasons to do so, nor include a reference to spab peptide. I suggest including explicit reasons to discard candidate NPs.

While there is strong supportive evidence for many NPs in *Drosophila*, the fact that other transcripts express NPs is more circumstantial often relying simply on sequence analysis and without convincing evidence for a specific cognate receptor. We note that Spab is not listed as a neuropeptide in the current release of FlyBase. In these cases, we relied on the opinion of individuals with extensive experience in studying *Drosophila* NPs. The results obtained with the probes for Spab and Nplp1 are still available in Supplemental File 1.

*In Fig 9-supplement 1, neurotransmitter biosynthetic enzymes were measured by RNA-seq for given CX cell types to augment the cell type classification. The same methods could be used to support cell type classification regarding putative peptidergic character (in Figure 9 supplement 2) by measuring expression levels of critical, canonical neuropeptide biosynthetic enzymes. These include the proprotein convertase dPC2 (amon); the carboxypeptidase dCPD/E (silver); and the amidating enzymes dPHM; dPal1; dPal2. PHM is most related to DBM (dopamine beta monooxygenase), the rate limiting enzyme for DA production, and greater than 90% of *Drosophila* neuropeptides are amidated. If the authors are correct in surmising widespread use of NPs by CX cell types (and I expect they are), there could be diagnostic value to report expression levels of this enzyme set across many/most CX cell types.*

In our admittedly limited experience, most cells express these enzymes and the level we observed in confirmed NP expressing cell types was not reproducibly higher. (The complete data for all genes for the cell types we assayed are available from our deposition in the NCBI Gene Expression Omnibus with accession number GSE271123.) Given our small sample size we chose not to comment on this in the paper.

Comment #6

Screen of effects on Sleep behavior

This work is large in scope and as suggested likely presents excellent starting points for many follow-up studies. I again suggest assigning stable number identities to the elements described. In this case, not cell types, but split Gal4 lines. This would expedite the cross-referencing of results across the four Supplemental Files 3-6. For example, line SS00273 is entry line #27 in S Files 3 and 4, but line entry #18 in S Files 5 and 6.

We believe the interested reader can make this correspondence by searching the supplemental files which are excel spreadsheets. We note that both driver lines and cell types have stable identifiers that are used across Figures and Tables: the line numbers (for example, SS00273) for driver lines and the Hulse et al cell type names for cell types.

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Clock to CX

"Not surprisingly, the connectome reveals that many of the intrinsic CX cell types with sleep phenotypes are connected by wired pathways (Figure 12 and Figure 12-figure supplement 1)."

Do intrinsic CX cells with sleep phenotypes also connect by wired pathways to CX cells that do not have sleep phenotypes?

Yes, but we do not have high confidence that negative sleep phenotypes in our assays indicate no role in sleep.

"The connectome also suggested pathways from the circadian clock to the CX. Links between clock output DN1 neurons to the ExR1 have been described in Lamaze et al. (2018) and Guo et al. (2018), and Liang et al. (2019) described a connection from the clock to ExR2 (PPM3) dopaminergic neurons."

The introduction to this section indicates a focus on connectome-defined synaptic contacts. Whereas the first two studies cited featured both physiological and anatomic evidence to support connectivity from clock cells to CX, the third did not describe any anatomical connections, and that connection may in fact be due to diffuse not synaptic signaling

I could not easily discern the difference between Figs 12 and 12-S1? These appear to be highly-related circuit models, wherein the second features more elements. Perhaps spell out the basis for the differences between the two models to avoid ambiguity.

We clarify the supplemental diagram differs from the one in the main text by the inclusion of additional connections:

"The strongest of these connections are diagrammed in Figure 12, with Figure 12—figure supplement 1 also showing additional weaker connections."

"...the cellular targets of Dh31 released from ER5 are unknown, however previous work (Goda et al., 2017; Mertens et al., 2005; Shafer et al., 2008) has shown that Dh31 can activate the PDF receptor raising the possibility of autocrine signaling."

Regarding pharmacological evidence for Dh31 activation of Pdfr: strong in vivo evidence was developed in doi: 10.1016/j.neuron.2008.02.018: a strong pdfr mutation greatly reduces response to synthetic dh31 in neurons that normally express Pdfr

We added the Shafer et al., 2008 reference.

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"Unexpectedly, we found that all neuropeptide-expressing cell types also expressed a small neurotransmitter."

Did this conclusion apply only to CX cell types? - or was it also true for large peptidergic neurons? Prior evidence suggests the latter may not express small transmitters (doi: 10.1016/j.cub.2009.11.065). The question pertains to the broader biology of peptidergic neurons, and is therefore outside the strict scope of the main focus area - the CX. However, the text did initially consider peptidergic neurons outside the CX, so the information may be pertinent to many readers.

We did not look at other cell types in the current study and so cannot provide an answer.

Reviewer #2 (Public review):

Summary:

*In this paper, Wolff et al. describe an impressive collection of newly created split-GAL4 lines targeting specific cell types within the central complex (CX) of *Drosophila*. The CX is an important area in the brain that has been involved in the regulation of many behaviors including navigation and sleep/wake. The authors advocate that to fully understand how the CX functions, cell-specific driver lines need to be created. In that respect, this manuscript will be of very important value to all neuroscientists trying to elucidate complex behaviors using the fly model. In addition, and providing a further very important finding, the authors went on to assess neurotransmitter/neuropeptides and their receptors expression in different cells of the CX. These findings will also be of great interest to many and will help further studies aimed at understanding the CX circuitries. The authors then investigated how different CX cell types influence sleep and wake. While the description of the new lines and their neurochemical identity is excellent, the behavioral screen seems to be limited.*

Strengths:

(1) The description of dozens of cell-specific split-GAL4 lines is extremely valuable to the fly community. The strength of the fly system relies on the ability to manipulate specific neurons to investigate their involvement in a specific behavior. Recently, the need to use extremely specific tools has been highlighted by the identification of sleep-promoting neurons located in the VNC of the fly as part of the expression pattern of the most widely used dorsal-Fan Shaped Body (dFB) GAL4 driver. These findings should serve as a warning to every neurobiologist, make sure that your tool is clean. In that respect, the novel lines described in this manuscript are fantastic tools that will help the fly community.

(2) The description of neurotransmitter/neuropeptides expression pattern in the CX is of remarkable importance and will help design experiments aimed at understanding how the CX functions.

Weaknesses:

(1) I find the behavioral (sleep) screen of this manuscript to be limited. It appears to me that this part of the paper is not as developed as it could be. The authors have performed neuronal activation using thermogenetic and/or optogenetic approaches. For some cell types, only thermogenetic activation is shown. There is no silencing data and/or assessment of sleep homeostasis or arousal threshold. The authors find that many CX cell types modulate sleep and wake but it's difficult to understand how these findings fit one with the other. It seems that each CX cell type is worthy of its own independent study and paper. I am fully aware that a thorough investigation of every CX neuronal type in sleep and wake regulation is a herculean task. So, altogether I think that this manuscript will pave the way for further studies on the role of CX neurons in sleep regulation.

(2) Linked to point 1, it is possible that the activation protocols used in this study are insufficient for some neuronal types. The authors have used 29°C for thermogenetic activation (instead of the most widely used 31°C) and a 2Hz optogenetic activation protocol. The authors should comment on the fact that they may have missed some phenotypes by using these mild activation protocols.

Our primary goal was to test the feasibility of using these tools in assessing sleep and wake function of neurons within the CX. In the process we uncovered several new neurons within the DFB-EB network that control sleep and make connections with previously identified sleep

regulating neurons. For all single cell type lines and lines with sparse patterns and no VNC expression we present both optogenetics and thermogenetic data. The lines for which we only have thermogenetic but no optogenetic data are those which have multiple cell types or VNC expression. We felt that optogenetic data for these non-specific or contaminated lines would not reliably indicate a role for individual cell types in sleep regulation.

Many previous studies that have used 31 degrees have done so for shorter durations and often using different times of the day for manipulations. The lack of consistency between studies using this temperature may be due in part to the fact that 31 degrees alters behaviors of flies (including controls) and, for this reason, is usually not used for 24-hour activation durations.

To keep the screen consistent and ensure we capture changes in both daytime and nighttime sleep we used 29 degrees. The behavior of control flies is not as disrupted or altered at this temperature, and 29 degrees for activation is routinely used in behavioral experiments.

We similarly selected an optogenetic stimulation protocol that minimizes the response of flies to the red-light pulses. We chose this protocol because we found, in earlier experiments in a different project, that this level of stimulation was able to elicit activation phenotypes across a range of cell types (including several known clock neurons). However, we cannot rule out false negatives in both the TrpA and optogenetic experiments and agree that we might have missed some phenotypes.

Finally, as the reviewer rightfully points out, a thorough, detailed investigation of each cell type is a herculean task. We screened in both genders with very sparse, and often cell-type-specific, driver lines while using two distinct modes of activation and different methods for assessing sleep. For these reasons, we believe the GAL4 lines we identified provide excellent starting points for the additional investigations that will be required to better understand the roles of specific cell types.

(3) *There are multiple spelling errors in the manuscript that need to be addressed.*

Reviewer #3 (Public review):

Summary:

The authors created and characterized genetic tools that allow for precise manipulation of individual or small subsets of central complex (CX) cell types in the Drosophila brain. They developed split-GAL4 driver lines and integrated this with a detailed survey of neurotransmitter and neuropeptide expression and receptor localization in the central brain. The manuscript also explores the functional relevance of CX cell types by evaluating their roles in sleep regulation and linking circadian clock signals to the CX. This work represents an ambitious and comprehensive effort to provide both molecular and functional insights into the CX, offering tools and data that will serve as a critical resource for researchers.

Strengths:

(1) The extensive collection of split-GAL4 lines targeting specific CX cell types fills a critical gap in the genetic toolkit for the Drosophila neuroscience community.

(2) By combining anatomical, molecular, and functional analyses, the authors provide a holistic view of CX cell types that is both informative and immediately useful for researchers across diverse disciplines.

(3) The identification of CX cell types involved in sleep regulation and their connection to circadian clock mechanisms highlights the functional importance of the CX and its

integrative role in regulating behavior and physiological states.

(4) The authors' decision to present this work as a single, comprehensive manuscript rather than fragmenting it into smaller publications each focusing on separate central complex components is commendable. This decision prioritizes accessibility and utility for the broader neuroscience community, which will enable researchers to approach CX-related questions with a ready-made toolkit.

Weaknesses:

While the manuscript is an outstanding resource, it leaves room for more detailed mechanistic exploration in some areas. Nonetheless, this does not diminish the immediate value of the tools and data provided.

Appraisal:

The authors have succeeded in achieving their aims of creating well-characterized genetic tools and providing a detailed survey of neurochemical and functional properties in the CX. The results strongly support their conclusions and open numerous avenues for future research. The work effectively bridges the gap between genetic manipulation, molecular characterization, and functional assessment, enabling a deeper understanding of the CX's diverse roles.

Impact and Utility

This manuscript will have a significant and lasting impact on the field, providing tools and data that facilitate new discoveries in the study of the CX, sleep regulation, circadian biology, and beyond. The genetic tools developed here are likely to become a standard resource for Drosophila researchers, and the comprehensive dataset on neurotransmitter and neuropeptide expression will inspire investigations into the interplay between neuromodulation and classical neurotransmission.

Additional Context

The breadth and depth of the resources presented in this manuscript justify its publication without further modification. By delivering an integrated dataset that spans anatomy, molecular properties, and functional relevance, the authors have created a resource that will serve the neuroscience community for years to come.

Recommendations for the authors:

Reviewing Editor:

The reviewers suggest that a nomenclature, perhaps a numbering system, be adopted for different cell types and Gal4 drivers in order to facilitate reading of the manuscript and cross-referencing.

We agree that a comprehensive reanalysis of the CX nomenclature is in order, but it is premature for us to attempt that as part of this study. This is best done after additional connectomes are generated to help resolve the degree of variation in morphology and connectivity between the same cell in multiple animals.

Reviewer #3 (Recommendations for the authors):

The authors have characterized a large number of split-GAL4 drivers targeting individual or small subsets of CX cell types. This manuscript delivers a detailed anatomical, molecular, and functional mapping of the CX.

By integrating data on neurotransmitters, neuropeptides, and their receptors, the authors provide a holistic view of CX cell types that will undoubtedly serve as a foundation for future studies.

The use of these genetic tools to identify CX cell types affecting sleep, as well as those linking the circadian clock to the CX, represents a significant advance. These findings hint at the diverse and integrative roles of the CX in regulating both behavior and physiological states.

The authors' decision to present this work as a single, comprehensive manuscript rather than fragmenting it into smaller publications each focusing on separate central complex components is commendable. This decision prioritizes accessibility and utility for the broader neuroscience community, which will enable researchers to approach CX-related questions with a ready-made toolkit.

While the manuscript leaves room for further exploration and mechanistic studies, the breadth and depth of the resources presented are more than sufficient to justify publication in their current form.

The data on neuropeptide and receptor expression patterns, especially the observation that all examined CX cell types co-express a small neurotransmitter, opens intriguing new avenues of inquiry into the interplay between classical neurotransmission and neuromodulation in this region.

This manuscript has provided a much-needed resource for the Drosophila neuroscience community and beyond. This work will facilitate important discoveries in CX function, sleep regulation, circadian biology, and more.

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