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Publication Date

2026-09-08

Peer reviewed|Thesis/dissertation

UNIVERSITY OF CALIFORNIA

Los Angeles

Four neurons coordinate rhythmic activity brain-wide to support circuit development

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy in Neuroscience

by

Jun Reichl

2026

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ABSTRACT OF THE DISSERTATION

Four neurons coordinate rhythmic activity brain-wide to support circuit development

by

Jun Reichl

Doctor of Philosophy in Neuroscience

University of California, Los Angeles, 2026

Professor Orkun Akin Chair

During development, the brain becomes electrically active even before it is ready to receive sensory stimuli. Such intrinsic developmental activity has been studied for 3 decades in vertebrate model systems and is known to be critical for the proper wiring of neural circuits. However, the complexity of the vertebrate brain and the technical limitations of studying its development have challenged progress towards identifying the precise mechanisms that initiate activity, whether and how it occurs throughout the brain, and its relevance to adult behavior and health. The recent discovery of patterned, stimulus-independent, neural activity (PSINA “see-nah”) in *Drosophila* has made it feasible to begin addressing these open questions. PSINA begins at the onset of synapse formation and engages the entire brain in highly coordinated and structured cycles of activity throughout the last half of metamorphosis. The global activity patterns are further textured with cell-type specific dynamics that are more correlated between future neuron-pairs compared

to neurons that do not synapse together. A small neuronal population (<2% of the brain) expressing the cation channel *Trpγ* serves as an activity template for the rest of the brain. Attenuated PSINA, either in *trpγ* mutants or from pan-neuronal silencing approaches, leads to altered synaptic structure at the cell-type level in the visual system. Here, I describe how neuropeptide signaling functions to regulate the wildtype activity patterns of PSINA. Specifically, I detail how the *Trpγ*⁺ neuron activity template is patterned by four neurons that produce the neuropeptide SIFamide (SIFa). Signaling through the SIFa Receptor (SIFaR), SIFa modulates the activity of both SIFa and *Trpγ*⁺ neurons to establish the brainwide activity cycles of PSINA. In turn, *Trpγ*⁺ neurons regulate SIFa neuron activity through a recurrent interaction. This work highlights a novel role for neuropeptide signaling in the modulation of developmental activity. Lastly, I conclude with a chapter on how disrupted PSINA may lead to changes to adult behavior.

The dissertation of Jun Reichl is approved.

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Jeffrey Donlea

Mark Frye

Orkun Akin, Committee Chair

University of California, Los Angeles

2026

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ACKNOWLEDGEMENTS

My journey to this Ph.D. in neuroscience started back in my undergraduate studies at UCLA. I can trace it back to a specific moment actually, when I took an honors physics course taught by Dr. Arisaka (who is still at UCLA as of this writing) where he had us research and present on a topic that relates biology with physics. I remember fervently taking notes through the chapters on learning and memory in *Principles of Neural Science* by Eric Kandel in the Biomedical Library at the Center for Health Sciences and feeling a sense of awe and inspiration at the process of how we came to better understand the molecular and cellular mechanisms of plasticity. David Glanzman, a former postdoc of Kandel, graciously let me volunteer in his lab to study those very same mechanisms in the zebrafish. Although I spent only a brief time in the Glanzman Lab, it left such an impact that I could never shake the feeling of wanting to dive deeper into the mysteries of the brain even after I had started to build a successful career as a high school science teacher and coach. I am still in disbelief that when I reached out to David after 7 years, he still remembered who I was and even wrote a letter of recommendation for the several graduate programs I applied to throughout the country. I would not have the privilege of writing this today if it wasn't for the incredible teachers, mentors, and colleagues I got to learn from as an undergraduate at UCLA and beyond, but I especially want to acknowledge David's unconditional support throughout my life.

I want to thank the UCLA NSIDP for admitting me into the graduate program. After having served on the admissions committee in my 4th year, I know that just a few professors and students would've had to not only hand-pick me out of a large and highly competitive applicant pool, but continue to choose me through the multiple rounds of elimination. Whoever you are for fighting for my chance to be here, I am forever grateful. From the moment I was admitted, the continued support from the program leadership, especially Jenny Lee, in helping me navigate through the

challenges of graduate school has been indispensable to my success. The institutional support from UCLA as a whole, which ranges from grant and fellowship funding that directly enabled the work in this dissertation to affordable gym access, transportation, and tax-free meals for students at the Reagan hospital, has been a major reason why I have spent nearly a decade with this community.

My first rotation was with the Akin Lab and it was the positive relationships I developed with Orkun Akin and the members of the lab at the time – Bryce Bajar, Nguyen Phi, and Harpreet Randhawa, that shaped my decision to join in the following summer. These positive relationships kept me coming to lab and grew over the years to include fellow graduate students Sassan Suarez and Julia Miller, the undergraduates Pranav Kadiyala, Anagha Srikanth, Joshua Ann, and Nico Momjian, as well as the members of the Donlea Lab; Jeff Donlea, Ceazar Nave, Jackie Weiss, Abby Aleman, Prabhjit Singh, and Natalie Moncada. My good rapport with Mark Frye and Laura DeNardo and their respective expertise made it an easy decision to ask them to join my thesis committee. I am thankful that they agreed because, along with Orkun and Jeff, I could not have had a better team to guide my research training.

The list of friends and family outside of my direct research and their support throughout my doctoral training deserves its own chapter. At the least, I want to directly express gratitude to my immediate family and my partner Connie for holding me up during the most challenging feat of my life. Thank you for believing in me even when I didn't believe in myself.

My development as a scientist and my contributions to the work described in this dissertation is completely owed to all of you. I cherish the times you taught me new skills and ways of thinking, or when we got to work together to create novel solutions to complex, but sometimes silly, problems, or even just to go to the gym or grab coffee or go karaoke. Thank you all for making the good times great and the bad times manageable.

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Bajar, B. T., Phi, N. T., Isaacman-Beck, J., **Reichl, J.**, Randhawa, H., & Akin, O. (2022). A discrete neuronal population coordinates brain-wide developmental activity. *Nature*, 602(7898), 639–646.

Roberts, A. C., **Reichl, J.**, Song, M. Y., Dearing, A. D., Moridzadeh, N., Lu, E. D., Pearce, K., Esdin, J., & Glanzman, D. L. (2011). Habituation of the C-Start Response in Larval Zebrafish Exhibits Several Distinct Phases and Sensitivity to NMDA Receptor Blockade. *PLOS ONE*, 6(12), e29132.

Awards and Fellowships

2025 UCLA Jessamine Hilliard Neurobiology Graduate Student Grant

2023 UCLA BRI Flexible Travel Award

2020 Rose and Sam Gilbert Fellowship

Selected Talks and Poster Presentations

2025 Four neurons pattern brain-wide developmental activity through neuropeptide signaling. *Neurobiology of Drosophila*, Cold Spring Harbor Laboratory; NY

2025 Neuropeptide signaling regulates neural activity during development. *Synapse to Circuit Seminar*, Brain Research Institute, University of California Los Angeles, CA

2024 Neuropeptides regulate activity in the developing brain. *Invertebrate NeuroAffinity Seminar*, University of California Los Angeles, CA

2023 Flies with altered developmental neural activity have sleep deficits as adults. *Drosophila Research Conference*, Chicago, IL

Chapter 1

Patterned activity in the developing brain

1.1 Introduction

Who we are and the way we interact with the world is shaped, quite literally, by how our brains are wired. Our thoughts and emotions, our personalities, our “natural-born” talents, as well as our acquired knowledge and skills, are all rooted in the connectivity of our neural circuits. On the other hand, major disruptions to neural circuit wiring underlie a vast number of neurological disorders, from developmental to psychiatric to degenerative (Di Martino et al. 2014; Xiong et al. 2023). This is because the diverse functions of the brain are borne out of the ability of neurons to communicate, which occurs along the connections of neurons with one another and with other cell types. Neuroscience researchers have made significant progress in understanding how the environment shapes neuronal connectivity, yet how the first connections are assembled during early stages of development, which lays the foundation for experience-dependent adaptations, has seen less advancements. Progress in understanding the early “connectome” has been hindered by the technical challenges of studying the developing brain, while at the same time, the importance of early brain architecture and its influence on long-term health trajectories is supported by a continuously growing body of evidence (Exposito-Alonso and Rico 2022; G. Li et al. 2025). Thus, elucidating the mechanisms that give rise to the early connectome remains a central goal of developmental neurobiology.

Across the animal kingdom, brain development progresses similarly in a series of overlapping stages – From neurogenesis to proliferation, differentiation to migration, neurite growth to synaptogenesis, local circuit assembly to larger network integration, and ultimately to the continued refinement and maintenance of these functional networks through experience (**Figure 1.1**). Prior to synaptogenesis, brain development is primarily driven by the tightly timed regulation of genetic programs and molecular guidance cues, with sparse bouts of electrical

activity occurring on a cell-by-cell basis (Spitzer 2006). As synapses first begin to emerge, electrical activity becomes patterned and correlated across neighboring cells and eventually to distant brain regions (Blankenship and Feller 2010a). These intrinsically driven activity patterns, which I will frequently refer to as “developmental activity,” occurs before sensory stimuli can be received and continues for days to weeks. The current consensus is that developmental activity refines immature circuits into functional networks through correlated neuronal firing – disrupting developmental activity leads to mis-wired topographic maps and altered circuit function (Wu et al. 2024a). However, where developmental activity is generated, how it is coordinated across separate regions, and the mechanisms that refine connectivity at the level of individual cell-types and synapses is not fully understood. Furthermore, while changes to wiring that results from disruptions to developmental activity leading to the emergence of pathologies (e.g. neurodevelopmental disorders) is predicted (Iyer et al. 2015), a direct link has not been established.

Developmental activity was first discovered in vertebrates nearly 40 years ago (Fitzgerald 1987) and occurs in many regions throughout the vertebrate brain, including the retina, cochlea, hippocampus, neocortex, cerebellum, brainstem, and spinal cord (Blankenship and Feller 2010a). Activity is recorded through calcium imaging and/or electrophysiology and characterized by cycles of large, slow depolarizations with periods of quiescence in-between. In the neural pathways that process vision, hearing, and touch, developmental activity in the respective projection neurons shapes the regionalization of upstream, higher-order brain regions: Silencing activity in these sensory epithelia or in the corresponding cortical regions themselves alters the organization of topographic maps and the segregation of sensory inputs (Leighton and Lohmann 2016). Furthermore, in the auditory system, disrupting cochlear developmental activity changes the neural responses to stimuli and reduces the size of auditory processing centers (Kersbergen et al. 2022a).

Comparable activity has also been observed in deeper, sub-cortical regions such as the hippocampus, where the “giant depolarizing potentials” of pyramidal cells may activate silent (i.e. pure-NMDA) synapses through Hebbian mechanisms (Kasyanov et al. 2004) to refine local circuitry.

Developmental activity may also be integrated with spontaneous muscle movements in motor-related regions to facilitate circuit maturation. Rhythmic activity patterns in the developing spinal cord are linked to the spontaneous movements of embryonic limbs (Inácio et al. 2016) and facilitates motor neuron axon pathfinding (Hanson et al. 2008), the balance of excitatory and inhibitory synapses (Gonzalez-Islas and Wenner 2006), and the assembly of local pattern-generating circuits (Marder and Rehm 2005; Myers et al. 2005). Rhythmic developmental activity in the cerebellum (Watt et al. 2009), paired with muscle twitches (Sokoloff et al. 2015), may facilitate the wiring of cerebellar outputs with downstream nuclei to support subsequent cerebellar-dependent learning in early postnatal rats.

Remarkably, although the underlying circuit configurations and individual activity dynamics vary from region to region (Blankenship and Feller 2010a), there are general parallels in the overall structure and progression of activity patterns throughout development – activity is initially sparse and occurs in small clusters of neurons, followed by larger and longer bouts that are highly synchronous, and lastly, activity transitions into desynchronized patterns until the brain is ready to process environmental input (Wu et al. 2024a). Despite these gross parallels, whether and how activity is coordinated at a global level throughout the brain is a critical question that remains unanswered – an interest that is further underscored by the fact that most behaviors require cross-functional integration of multiple brain regions.

Figure 1.1

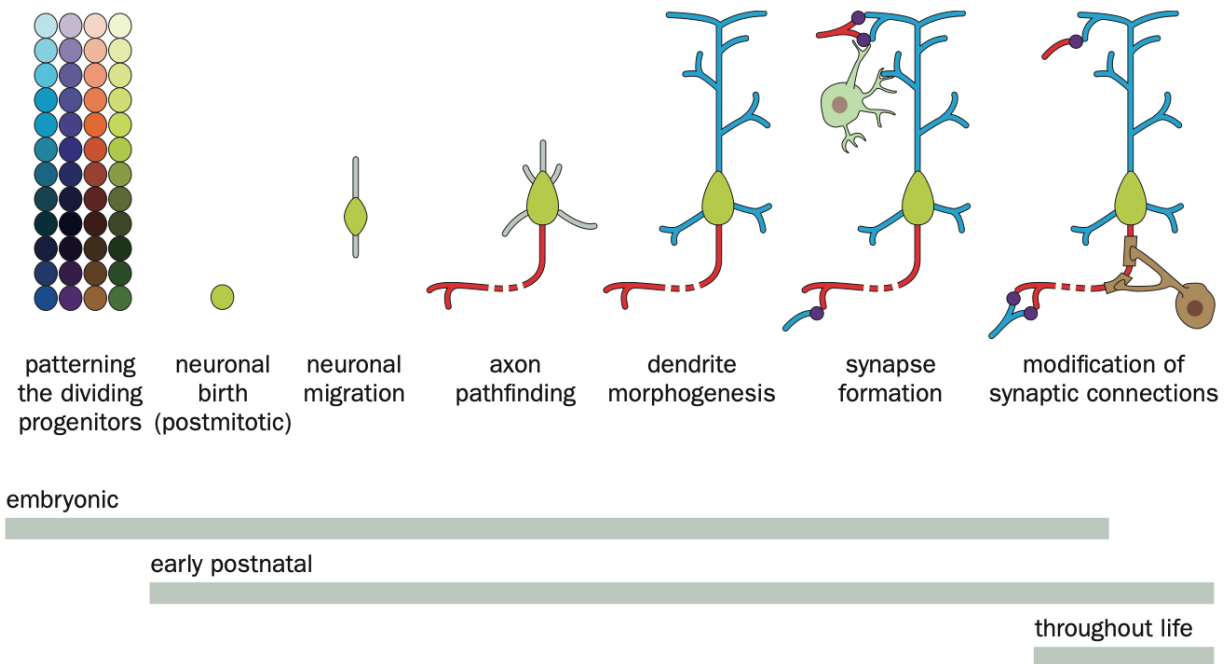


Figure 1.1 Progression of nervous system development

Early neural progenitor cells are patterned by developmental events based on different positions to produce distinct neuronal cell types. Post mitotic neurons then migrate to their destinations, extend axons towards their post-synaptic partners, establish dendrite branching patterns, form synaptic connections, and modify those synaptic connections. Patterned, intrinsic developmental activity occurs in these last two stages, before the onset of receiving sensory input. Adapted from Figure 7-1 (Luo 2020)

1.2 Patterned, Stimulus-Independent Neural Activity (PSINA) in the developing fruit fly, *Drosophila Melanogaster*

Until recently, invertebrate brain development was thought to occur exclusively through genetically hard-wired programs, independent of any influence from neural activity. The discovery of comparable developmental activity in the fruit fly (Akin et al. 2019), *Drosophila melanogaster*, has revealed that patterned activity is a fundamental and conserved process for nervous system development across evolution (Akin and Zipursky 2020). Notably, the fly has greatly expanded the experimental access to investigate the underlying mechanisms that drive developmental activity to a level that was not possible in more complex model systems, through 1. A vast library of driver lines that provide cell-type-specific access (Jenett et al. 2012); 2. A rich selection of reagents to manipulate and measure neuronal activity (Simpson and Looger 2018); 3. An electron microscopy (EM)-level connectome for the whole brain (Scheffer et al. 2020); 4. Single cell sequencing methods to assess gene expression changes throughout development (Kurmangaliyev et al. 2020; Özel et al. 2021); and 5. A wealth of behavioral paradigms, mapped to defined circuits (Griffith 2012).

Drosophila are holometabolous, undergoing two distinct periods of nervous system development – the embryonic stage builds the larval brain, which is then largely re-organized into the adult brain during the 100 hours of metamorphosis, or pupal development (**Figure 1.2**). In the first 50 hours after puparium formation (hAPF), neurogenesis, morphogenesis, and axon guidance are mediated by the expression of cell-surface molecules to orient the vast array of cell-types into their appropriate regions (Hadjieconomou et al. 2011) – primed to assemble into functional neural circuits and larger integrated networks. Coincident with the appearance of the first structural synapses around 50hAPF (Y. Chen et al. 2014), flies expressing a genetically-encoded calcium

indicator (GECI), GCaMP6s (T.-W. Chen et al. 2013), in all neurons, begin to exhibit waves of rhythmic fluorescent activity that continues for the remainder of metamorphosis (**Figure 1.3**). Interestingly, activity shuts off roughly 1 hour before the juvenile-adult fly emerges from its pupal case (i.e. eclosion). This activity courses throughout the whole brain in highly structured and stereotyped cycles comprising globally coordinated active and silent phases. Embedded within each active phase are oscillatory peaks known as sweeps. Earlier, during the “periodic phase,” the defining feature of PSINA is its steady rhythmicity, until it transitions ~75 hAPF to the “turbulent phase”, where active phases become longer and silent phases become shorter.

At both the level of the whole brain and that of individual cell-types in the visual system, GCaMP6s dynamics correlate with glutamate release and membrane voltage changes and is severely attenuated with the application of tetrodotoxin. These results establish that the GCaMP6s reporter reflects *bona fide* electrical activity. Furthermore, activity patterns in the optic lobes were unchanged in blind animals compared to controls, indicating that visual system activity occurred independent of sensory stimuli. Taken together, these characteristics lead to the naming of this activity as patterned, stimulus-independent, neural activity (PSINA, “see-nah”). Notably, simultaneous and orthogonal recordings of GECI changes in astrocytes and neurons revealed that astrocytes oscillate in anti-phase with PSINA (**Figure 1.4**) (Akin et al. 2019; Bajar, Phi, Randhawa, et al. 2022). Ablating astrocytes, which are known to contribute to synapse maturation (Muthukumar et al. 2014) leads to a ~50% reduction in PSINA, while silencing all neurons nearly abolishes calcium changes in astrocytes, indicating that neuron-astrocyte interactions are critical for PSINA. The specific mechanisms by which neurons and astrocytes work together to drive PSINA and contribute to synaptic development and circuit wiring remains unexplored.

An additional defining characteristic of PSINA is the stereotyped specificity of patterns at the level of individual cell-types (**Figure 1.5**). PSINA in 15 different visual system neuron-types was recorded with 2-photon microscopy and compared with both brain wide dynamics and with one another. While the broader temporal structure of each individual neuron-type overlaps with that of the whole brain, i.e. individual neuron-types are on or off in parallel with the rest of the brain and dynamics progress similarly throughout development, the finer spatiotemporal properties of activity are distinct from cell-type to cell-type in a stereotyped fashion across animals. Furthermore, when comparing the type-specific activity patterns between visual system neurons, they correlate more in neuron-pairs that are known to be future synaptic partners than with those that do not wire together in the adult brain. The higher correlation between wired neuron-pairs is lost when blocking synaptic vesicle release via expression of tetanus toxin in the pre-synaptic component, while the correlations do not change with the same approach between neurons that do not partner together. In other words, the synchrony in neuronal partners depends on synaptic coupling, suggesting that PSINA may be playing an instructive role in assembling the adult connectome (Akin et al. 2019).

Figure 1.2

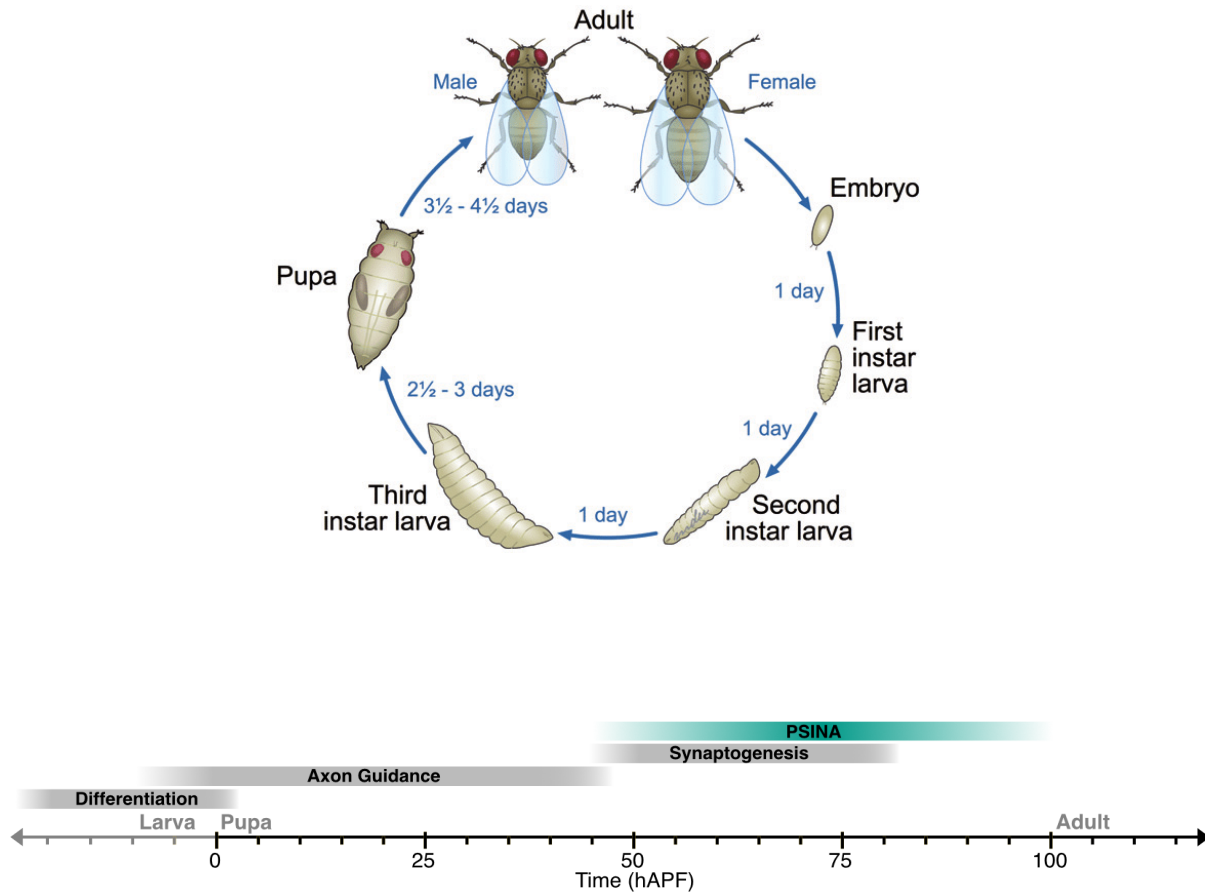


Figure 1.2. The fly life cycle and PSINA

(Top) The fly life cycle from fertilization to adulthood takes ~10 days at 25°C. Adapted from (Ong et al. 2015). (Bottom) Pupal development lasts ~100 hours at 25°C and coincident with the appearance of the first structural synapses around 50 hours after puparium formation (hAPF), Patterned Stimulus-Independent Neural Activity (PSINA) courses throughout the brain in highly structured cycles of active and silent phases until ~1 hour before eclosion.

Figure 1.3

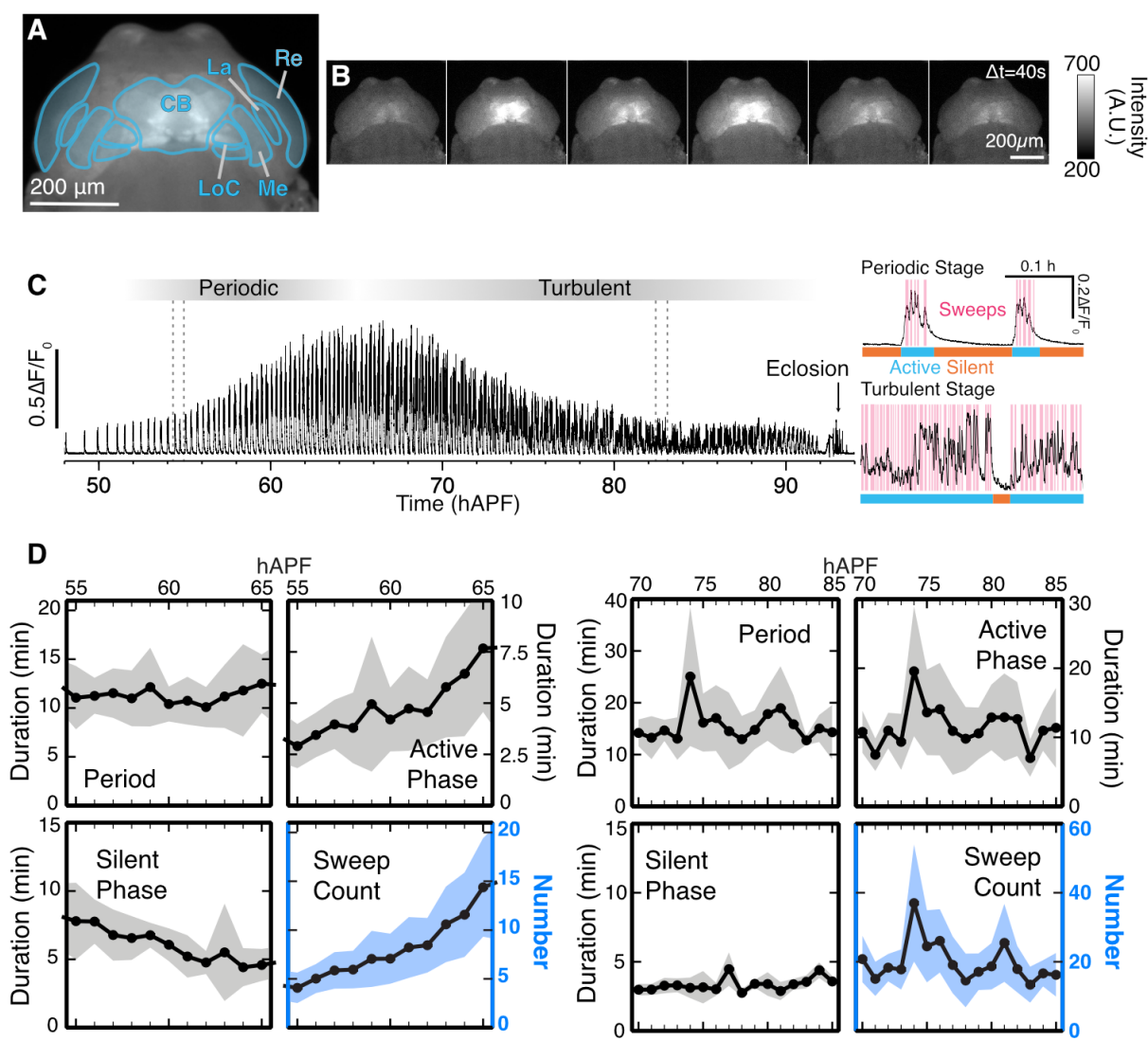


Figure 1.3 Key Features of PSINA

A. (Left) Average intensity projection (AIP) from wide-field fluorescence series of a pupa expressing pan-neuronal GCaMP6s. CB, central brain. La, lamina. Re, retina. LoC, lobula complex. Me, medulla. Scale bar, 200 μ m. **B.** Panels span one active phase at 60 hAPF in control pupae. Frames ~40 seconds apart. Scale bar, 200 μ m. **C.** Representative trace of PSINA, recorded with wide-field fluorescence imaging from pupa expressing pan-neuronal GCaMP6s. This animal eclosed at ~93 hAPF. Dotted lines mark limits of inset traces (right) from periodic (top) and turbulent (bottom) stages. Highlights (magenta) mark individual sweeps; bars mark active (cyan) and silent (orange) phases. **Figure 1A-C** Adapted from Figure 1 of (Bajar, Phi, Isaacman-Beck, et al. 2022) **D.** Average traces of cycle metrics in the periodic stage (n = 54 columns from 6 flies). Shaded area represents SD. Adapted from Figure 1 of (Akin et al. 2019).

Figure 1.4

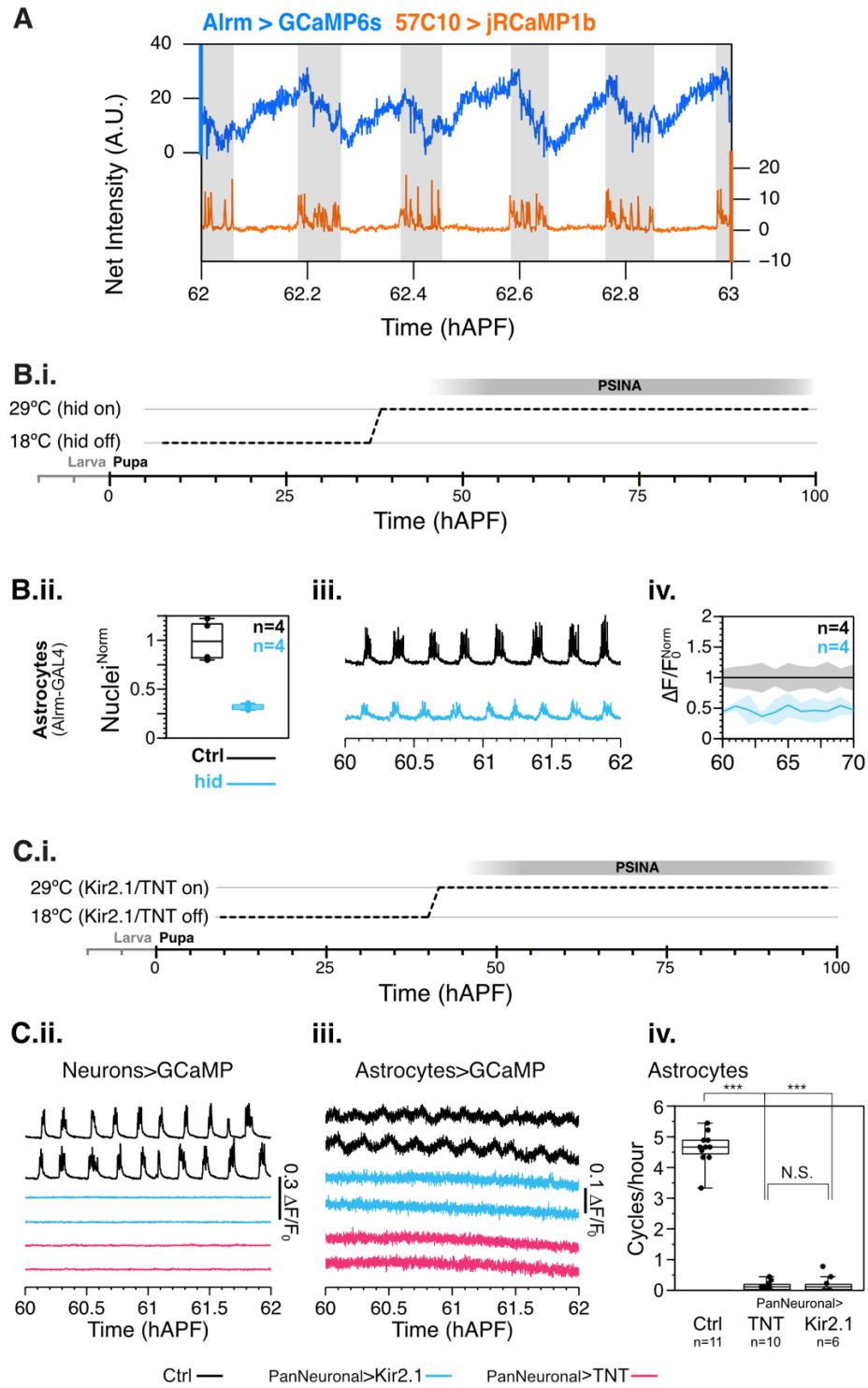


Figure 1.4 PSINA requires neuron-astrocyte interactions

A. Representative traces comparing activity in astrocytes expressing GCaMP6s (blue) and activity in all neurons expressing jRCaMP1b (orange) between 62 and 63 hAPF. Active phases of the neuronal cycles are shaded in gray. Adapted from Figure 3 of (Akin et al. 2019). **B. (i.)** Expression control of Alrm-GAL4 driven UAS-hid (programmed apoptosis) with TARGET (McGuire et al. 2003); animals shifted from 18 to 29°C at 38 hAPF. **(ii.)** Quantification of astrocyte ablation in control (black) and hid-expressing (teal) animals. Sample counts indicated in top right corner. **(iii.)** Representative traces from neurons expressing jRCaMP1b in control (black) and in genetic background of astrocyte ablation (teal). **(iv.)** Active phase average amplitude binned by hour and normalized to wildtype activity between 60 and 70 hAPF. Shaded areas, standard deviation. Colors match genotypes in **(ii.)** Sample sizes in top right corner. **C. (i.)** Expression control of UAS-Kir2.1 or TNT with TARGET; animals shifted from 18 to 29°C at 38 hAPF. **(ii. – iii.)** Representative traces of pupae expressing GCaMP6s in neurons **(ii.)** or astrocytes **(iii.)**. Wildtype activity in the empty-GAL4>Kir2.1 condition (black, top), and reduced activity in the pan-neuronal-GAL4>Kir2.1 (blue, middle) and pan-neuronal-Gal4>TNT condition (magenta, bottom). **(iv.)** Astrocyte calcium cycles per hour from 60 to 70 hAPF. Box-and-whiskers mark 5th, 25th, 50th, 75th, and 95th percentiles. ***, $p < .0001$ by Tukey's post hoc test following ANOVA. **Figure 1.4B and C** adapted from (Bajar, Phi, Randhawa, et al. 2022).

Figure 1.5

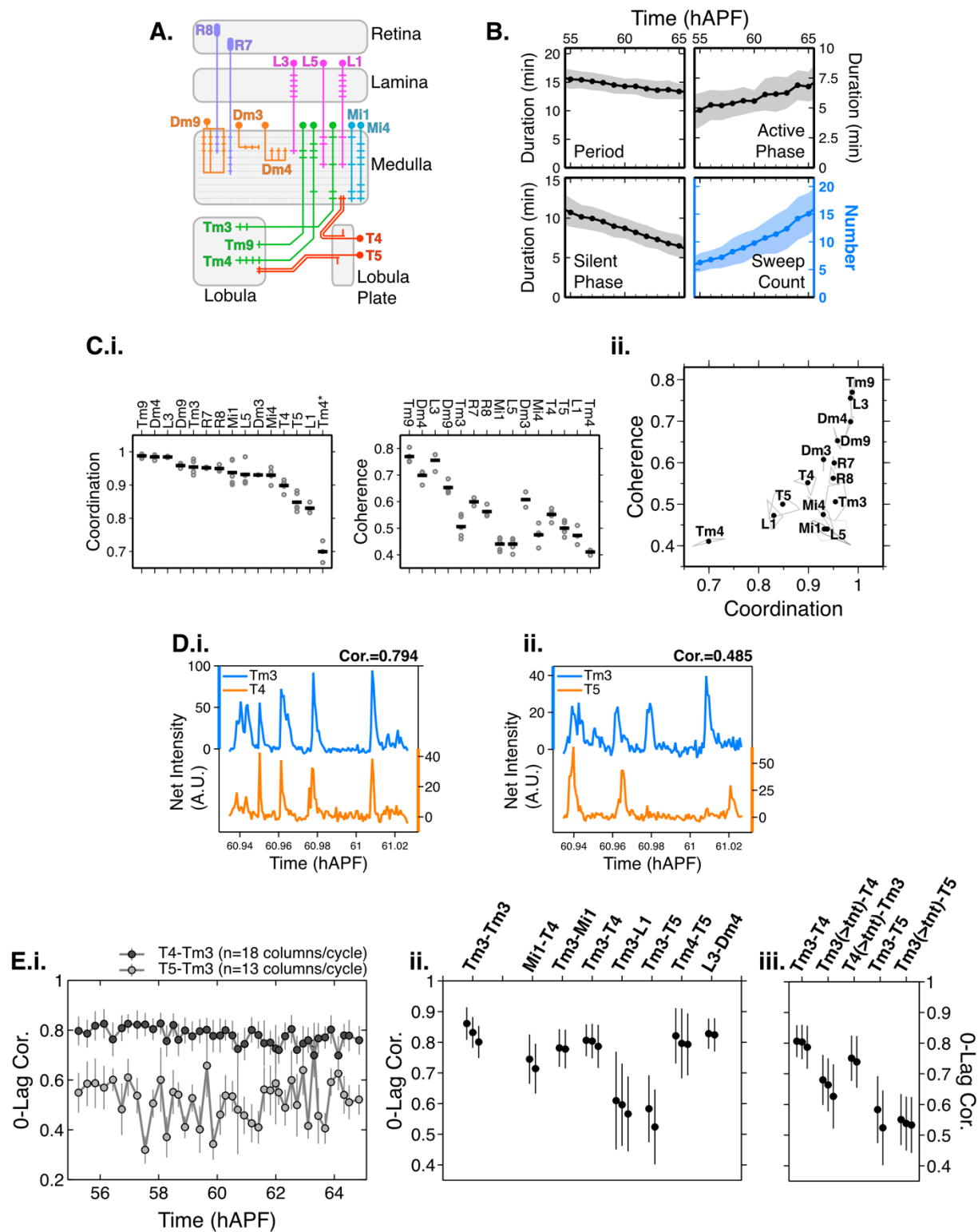


Figure 1.5 Cell-type specific activity dynamics in PSINA

A. Schematic of visual system cell types described in Figures 1.5B-E. **B.** Cycle metrics in the periodic stage, averaged over 15 cell types and 55 time series (i.e., flies). Shaded areas represent SD. **C. (i.)** Coordination (left) and coherence (right) values calculated for different cell types. Coordination is the average of the fraction of columns that participate in each sweep. Coherence is the largest fraction of columns that peak within the same time point, averaged over all sweeps. Round gray markers are individual time series, black bars are the average for each cell type. Data from two to six flies shown for each cell type. Metrics for each time series calculated over 55–65 hAPF, using an average of 41 ± 9 cycles and 10–20 columns per cycle. Asterisk indicates the outlier coordination value of Tm4 is due to sparse labeling of this cell type with the driver used. **(ii.)** Scatterplot of coordination versus coherence. Vertices of light gray polygons represent individual time series, and black dots represent the average for each cell type. **D. (i.-ii.)** Tm3-T4 **(i.)** and Tm3-T5 **(ii)** net fluorescence intensity. **E. (i.)** 0-lag cross correlation values between 55 and 65 hAPF for Tm3-T4 (dark gray) and Tm3-T5 (light gray) for the time series used **D.** Markers show the average correlation value for 10–20 columns per cycle, and gray vertical lines represent SD. **(ii.)** 0-lag correlation values for pairs of cell types, averaged over 55–65 hAPF. Black markers and vertical lines are the average and SD for each time series. Data from two or three flies shown per pair. 43 ± 10 cycles with 15 ± 3 columns per cycle used for each fly. The Tm3-Tm3 pair represents the highest correlation we expect to observe for a perfect match given the signal-to-noise statistics of the data. **(iii.)** TNT expression in Tm3 reduces Tm3-T4 correlation but has no effect on Tm3-T5 correlation (data statistics as in **(ii.)**). Unperturbed pairs are reproduced from **(ii.)** for ease of comparison. **Figure 1.5 A-E** adapted from Figures 3 and 4 in (Akin et al. 2019)

1.3 Trp γ ⁺ neurons serve as the activity template for brain wide PSINA

The global coordination of PSINA points to a dedicated network that can organize activity throughout the brain. We searched for the genetic basis of such a potential network and identified the critical role of the Transient Receptor Potential gamma (Trp γ) channel in PSINA (Bajar, Phi, Isaacman-Beck, et al. 2022). In *trp γ* mutants, brain wide PSINA amplitude is attenuated by ~50% and the sweep count per active phase is increased, while cycle frequency and duration are unaltered compared to heterozygous controls (**Figure 1.6**). These mutant phenotypes can be rescued by the expression of the isoform, Trp γ -D, only when spatiotemporally targeted to the last half of pupal development in Trp γ ⁺ neurons. Together, these results indicate *Trp γ* is both necessary and sufficient for wildtype PSINA during the last half of pupal development. In addition, the cell-type specific activity patterns in the visual system are disrupted in *trp γ* mutants – generally, the trends converge across the 10 cell-types examined where fewer cells of a given type were active, but those that were showed greater synchrony compared to controls (**Figure 1.7**). Furthermore, whole-animal loss of *trp γ* also alters synaptic development in visual system neurons, which can be rescued with the heterologous expression of Trp γ -D in Trp γ ⁺ neurons. Notably, the synaptic defects in the visual system are due to a non-cell autonomous requirement for Trp γ – in other words, it is the activity of the Trp γ ⁺ neurons that influences synaptic structure rather than the expression of Trp γ in the visual system cell-types themselves. Furthermore, fully silencing PSINA with pan-neuronal expression of the inward rectifying potassium channel, Kir2.1, or tetanus toxin, showed visual system synaptic defects that were indistinguishable from *trp γ* mutants, indicating that the disruptions to cell-type specific activity dynamics from loss of Trp γ is as severe a perturbation to synapse development as the complete loss of developmental activity.

Consistent with the importance of Trpγ⁺ neuron electrical activity on synapse maturation, spatiotemporally targeted expression of Kir2.1 in Trpγ⁺ neurons during the last half of pupal development nearly abolishes brain wide PSINA (**Figure 1.9**). Further restricting the silencing to just the Central Brain sub-population of Trpγ⁺ neurons also severely attenuates brain wide PSINA. The converse approach, activation of Trpγ⁺ neurons, produces a continuous train of sweeps, suggesting that Trpγ⁺ neurons are sufficient to generate brain wide oscillations during PSINA. Lastly, comparing the activity dynamics of the ~2000 Trpγ⁺ neurons with the rest of the brain through orthogonal GECI reporter expression in each respective population in the same animals reveals that brain wide PSINA correlates strongly with Trpγ⁺ neuron patterns. Importantly, this high correlation persists when comparing the two neuronal populations in *trpγ* mutants, establishing that Trpγ⁺ neurons serve as the template of activity for brain wide PSINA. More simply put, what happens to the Trpγ⁺ population is what happens to the rest of the brain, and they minimally function as a PSINA relay network.

Trpγ is a cation channel with a preference for Ca²⁺ and has a wide range of reported functions, from sensory processing including phototransduction (Xu et al. 2000), mechanosensation (Akitake et al. 2015), and lipid metabolism (Nath et al. 2025), to complex behaviors such as fine motor control (Akitake et al. 2015), food selection (Dhakal et al. 2022), sleep, courtship, and learning and memory (Palacios-Muñoz et al. 2022). Notably, prior to our study (Bajar, Phi, Isaacman-Beck, et al. 2022), there were no known developmental roles for Trpγ. Trpγ is expressed in ~2000 neurons (<2% of the brain), which includes all neurotransmitter types, some neuromodulatory (dopaminergic and serotonergic), and a few neuropeptidergic (SIFamide, DH44, and PDF). The expression of mCherry-nLS (nuclear localization signal) with the gene-trap driver, Trpγ-GAL4 (Akitake et al. 2015), which captures the endogenous expression domain of

the gene, along with immunohistochemical staining with a Trpγ antibody, shows that Trpγ production in the brain begins at ~24hAPF, peaks around 72hAPF, and is undetectable by 3 days after eclosion (Figure 4 in (Bajar, Phi, Isaacman-Beck, et al. 2022) and **Figure 1.9**). The relatively sharp rise and fall of Trpγ⁺ nuclei counts in the brain suggests this population may exist for the sole purpose of coordinating PSINA during pupal development. The presence of transient populations has been observed in mammalian models of developmental activity (Luhmann et al. 2016; Tritsch et al. 2007; Wang et al. 2015), raising the prospect that temporary, dedicated circuits may be driving developmental activity across evolution.

The behavioral phenotypes reported in the various studies mentioned above did not include temporal control of Trpγ expression. Taken with how Trpγ expression is not present in the brain in 5-day old adults, there is a reasonable possibility that the reported behavioral phenotypes originate in circuit disruptions because of altered PSINA from Trpγ loss of function during pupal development. I extensively explored whether altered PSINA directly resulted in adult behavioral deficits in the context of sleep, in collaboration with Dr. Jeff Donlea, in Chapter 4.

Figure 1.6

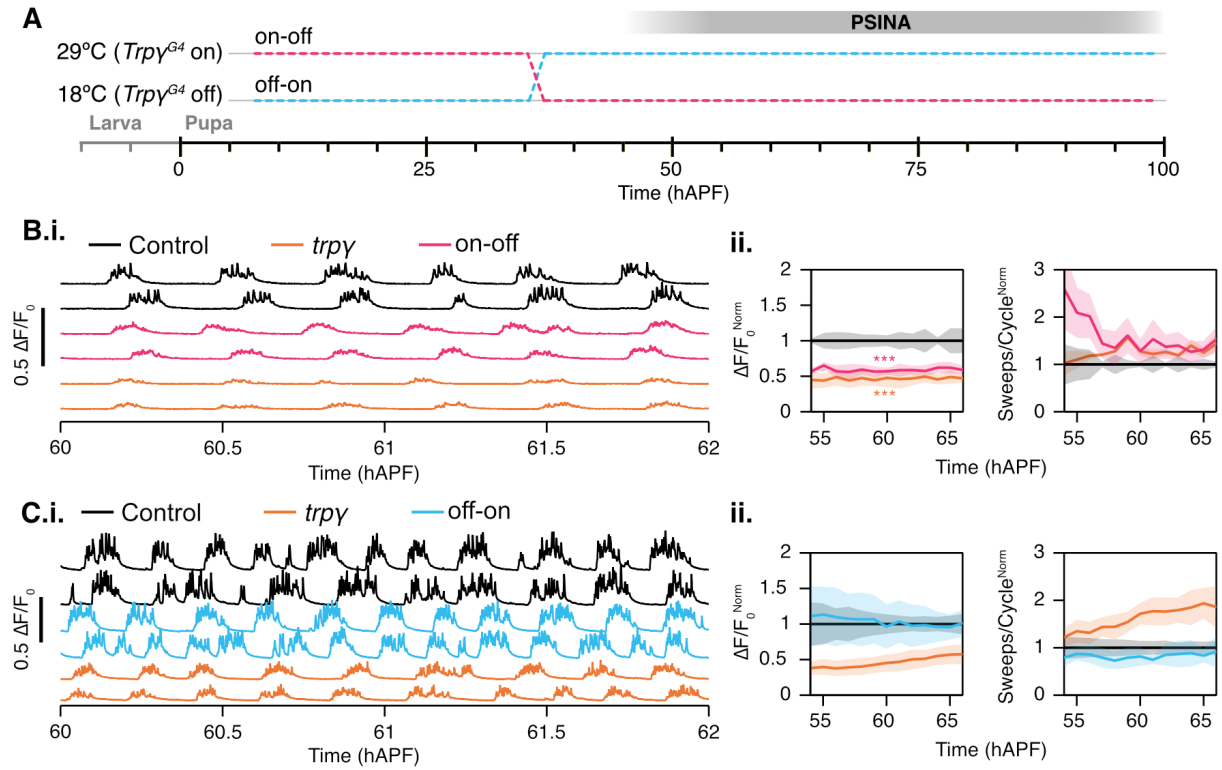


Figure 1.6 Trpy is necessary and sufficient for wildtype PSINA

A. Expression control of UAS-Trpy-D with TARGET (GAL80ts); temperature shifts at 36 hAPF.

B. (i.) PSINA traces and **(ii.)** active phase average amplitude (left) and sweeps per cycle (right) in control (black, $n = 8$ flies), *trpy* mutants (orange, 8 flies) and ‘on–off’ pupae (magenta, $n = 5$ flies) binned by hour and normalized to control.

C. (i.) PSINA traces and **(ii.)** active phase average amplitude (left) and sweeps per cycle (right) in control (black, $n = 3$ flies), *trpy* (orange, $n = 3$ flies) and ‘off–on’ pupae (cyan, $n = 3$ flies) binned by hour and normalized to control.

* $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Welch’s t-test following Shapiro–Wilk test, tested against control at 60 hAPF. Figure adapted from Figure 1 of (Bajar, Phi, Isaacman-Beck, et al. 2022)

Figure 1.7

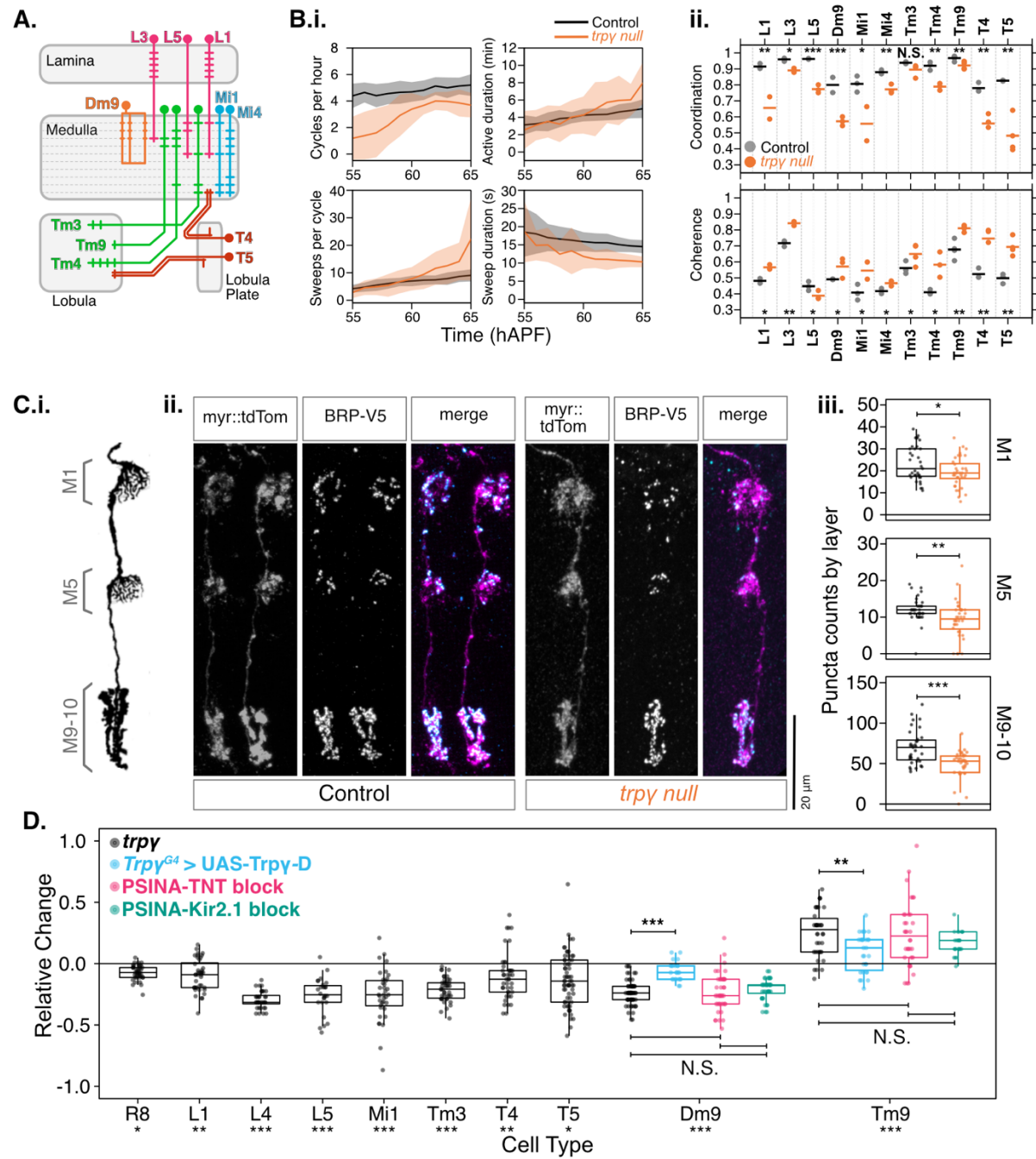


Figure 1.7 Cell-type specific activity dynamics and synapse structure depend on Trpy

A. Schematic of visual system cell types studied. **B. (i.)** Average metrics pooled from two-photon microscopy imaging of all cell types in control (black, $n = 31$ flies) and *trpy* (orange, $n = 33$ flies) pupae. Shaded areas indicate s.d. **(ii.)** Coordination (top) and coherence (bottom) values over 55–65 hAPF calculated for different cell types in control (black) or *trpy* (orange) pupae. Round markers are values from individual time series, bars are averages for each cell type. $n = 2–3$ time series for each cell type. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Welch's t-test following Shapiro–Wilk test. **C. (i.)** Schematic of Mi1, with processes in medulla layers M1, M5 and M9–10 (adapted from ref. 41). **(ii.)** Micrographs of Mi1 neurons in control (left set) and *trpy* (right set) flies with cell membranes (*myr::tdTomato*, magenta in merged) and presynaptic sites (BRP-V5, cyan in merged) labelled. Scale bar, 20 μm . **(iii.)** Mi1 Brp puncta counts by layer in heterozygous control (black, $n = 35$ cells) and *trpy* (orange, $n = 36$ cells) flies. Each data point shows the number of Brp puncta counted in the arborization of one Mi1 neuron in one of three medulla layers. Box-and-whiskers mark 5th, 25th, 50th, 75th and 95th percentiles. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Welch's t-test following Shapiro–Wilk test. **D.** Cell-type-specific Brp puncta counts in *trpy* mutants (black, $n = 26–65$ cells per cell type), in the *trpy*, *TrpyG4>UAS-Trpy-D* rescue condition (cyan, $n = 24$ cells for Dm9, $n = 30$ cells for Tm9), in flies expressing TNT (magenta, $n = 43$ cells for Dm9, $n = 35$ cells for Tm9) or Kir2.1 (green, $n = 40$ for Dm9, $n = 25$ for Tm9) pan-neuronally displayed as change relative to control averages ($n = 18–61$ cells per cell type), calculated by dividing raw Brp counts in the mutant set by the average of the corresponding control set and subtracting 1 from each new ratio value. Data displayed as in c. Asterisks below cell type names report tests of *trpy* mutants versus control. * $P < 0.05$; ** $P < 0.01$; *** $P < 0.001$ by Welch's t-test

following Shapiro–Wilk test or Tukey’s post hoc test following ANOVA for multiple groups.

Figure 1.7 A-D adapted from Figures 2 and 3 in (Bajar, Phi, Isaacman-Beck, et al. 2022).

Figure 1.8

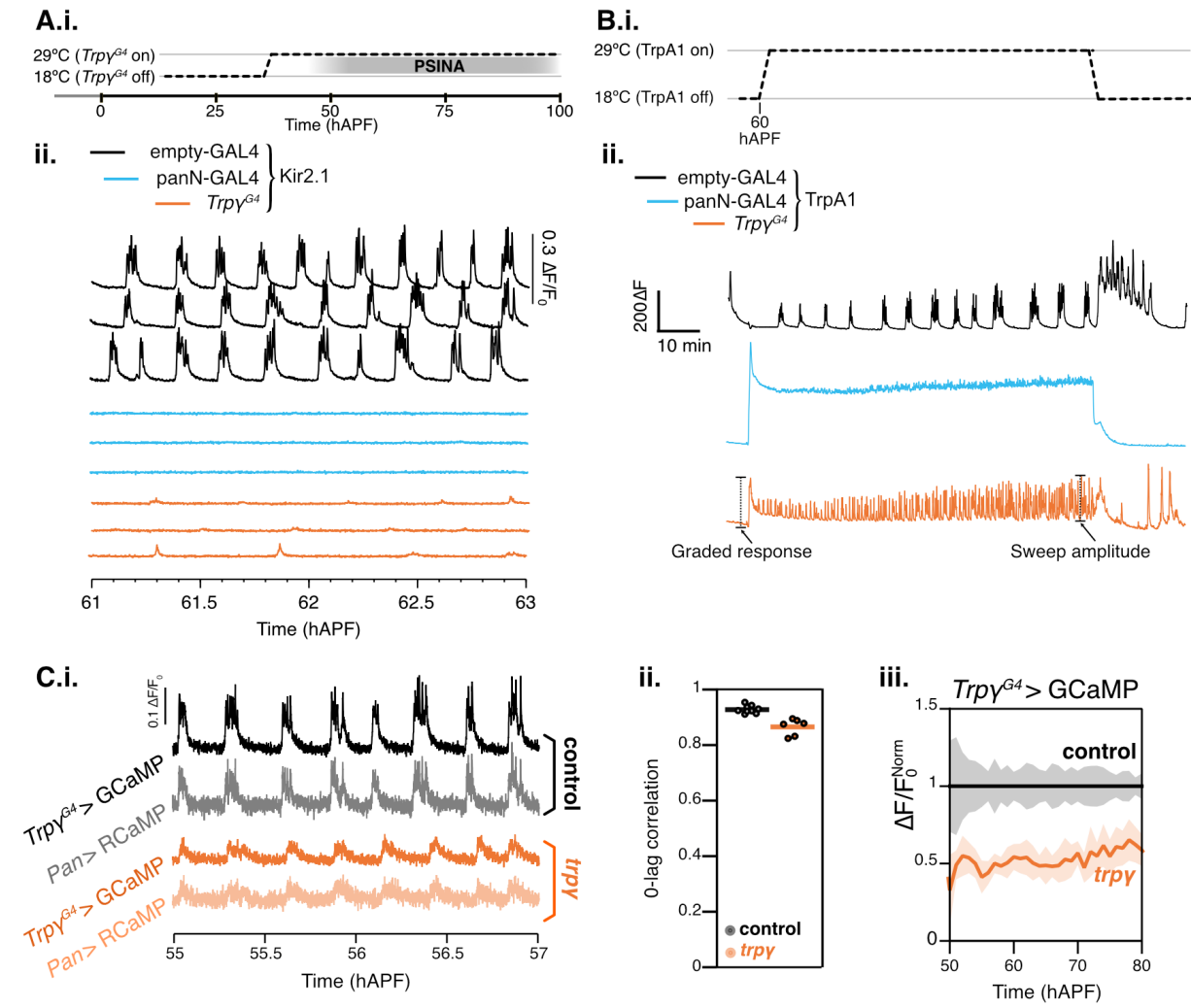
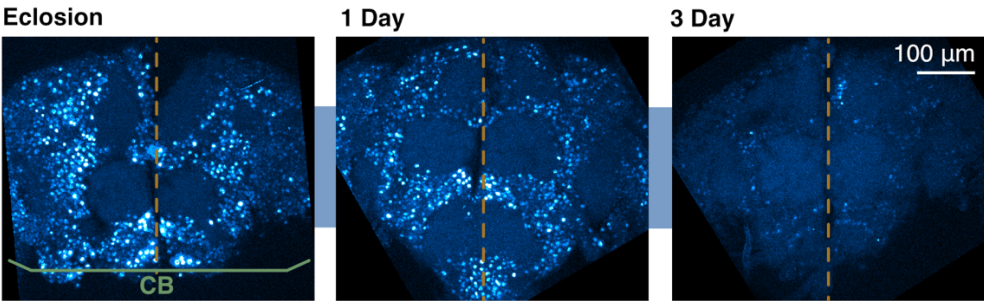


Figure 1.8 $Trp\gamma^+$ neurons act as the activity template for brain wide PSINA

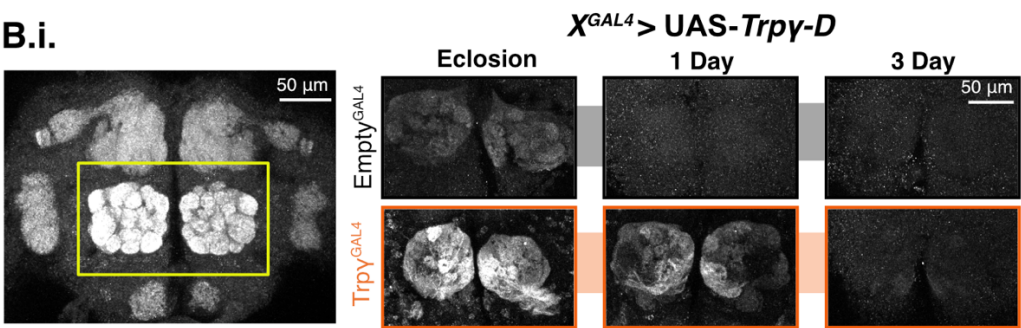
A. (i.) Expression control of UAS-KiR2.1 with TARGET; flies shifted from 18 °C to 29 °C at 40 hAPF. **(ii.)** PSINA traces from pan-neuronal GCaMP6s in control (empty-GAL4, black), panN-GAL4>KiR2.1 (blue), and $Trp\gamma^{G4}$ >KiR2.1 (orange) pupae. **B. (i.)** Expression control of thermogenic channel TRPA1. Pupae were reared at 18 °C and shifted to 29 °C at 60hAPF. **(ii.)** Activity from pan-neuronal GCaMP6s in control (empty-GAL4, black), panN-GAL4>TrpA1 (blue) and $Trp\gamma^{G4}$ >TrpA1 (orange) pupae. Initial graded response to temperature shift and maximum sweep amplitude are marked. **C. (i.)** PSINA traces in control (top two, grayscale) and $trp\gamma$ (bottom two, orange) pupae, recorded from co-expressed GCaMP6s in $Trp\gamma^+$ neurons (darker hues) and pan-neuronal RCaMP1b (lighter hues). Traces acquired with wide-field imaging. **(ii.)** Zero-lag correlation of PSINA in $Trp\gamma^+$ and pan-neuronal expression domains. Round markers show a single time series; bars show genotype average. $n = 9$ control flies; $n = 6$ $trp\gamma$ flies. **(iii.)** Active phase average amplitude recorded in $Trp\gamma^+$ neurons binned by hour and normalized to control. Shaded areas indicate s.d. $n = 9$ control flies, $n = 6$ $trp\gamma$ flies. **Figure 1.8A-C** adapted from Figures 4 and 5 in (Bajar, Phi, Isaacman-Beck, et al. 2022).

Figure 1.9

A. *Trpy^{G4}* > UAS-mCherry-NLS



B.i.



ii.

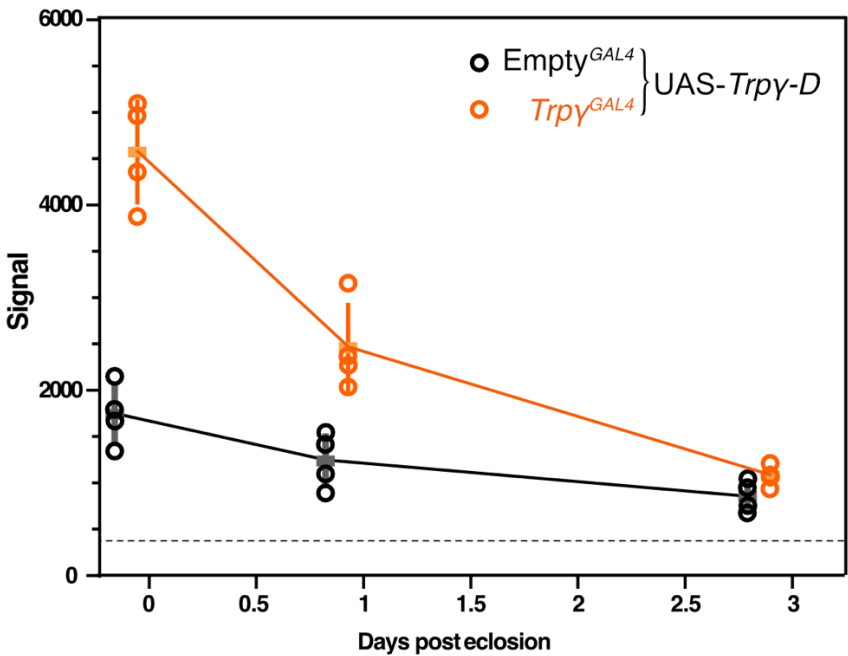


Figure 1.9 Trp γ expression in the brain severely diminishes post-eclosion

A. Maximum Intensity Projection (MIP) comparisons of native fluorescence of nuclei with Trp γ -GAL4 driven UAS-mCherryNLS in the Central Brain (CB) of adult flies. Note: This result stands in contrast to the published measurements of Trp γ ⁺ nuclei in (Bajar, Phi, Isaacman-Beck, et al. 2022), which indicates that Trp γ ⁺ decreases after 72hAPF but maintains ~50% of peak nuclei counts up to 5 days post-eclosion. The key distinction is that the nuclei counts in the report were done with immunohistochemistry to maximize the visualization of cell bodies, whereas this figure depicts native fluorescence. **B. (i.)** Representative MIPs of adult Antennal Lobes labelled with a Trp γ antibody in heterozygous control (Empty-GAL4, black) and homozygous *trp γ* mutant with heterologous (Trp γ -GAL4, orange) expression of UAS- Trp γ -D. Dashed line represents background levels.

1.4 Summary

Developmental activity shapes the early formation of neural circuits throughout the animal kingdom. A commonly shared feature is the emergence of intrinsically driven cycles of active and silent phases coincident with the formation and refinement of synapses. While predicted, a direct link between mis-wired circuits due to altered developmental activity and the advent of neuropathologies has not been made. The discovery of developmental activity in the fly model system opens a parallel track to tackle the most pressing questions about this process – what are the fundamental mechanisms that drive and coordinate activity throughout the brain? In turn, how exactly is the activity itself, let alone its unique patterns, facilitating the maturation of neural circuits?

Patterned, Stimulus-Independent Neural Activity (PSINA) occurs as the earliest structural synapses appear during pupal development. PSINA courses throughout the brain in highly stereotyped cycles of active and silent phases and lasts until 1 hour before eclosion. Within each global active phase, which ranges from 10-15 minutes, are cell-type specific activity patterns that are more synchronous between synaptic neuron-pairs. In anti-phase with neuronal cycles are oscillations of astrocytes, which themselves rely on neural electrical activity but when ablated, reduces brain wide PSINA by ~50%. Furthermore, PSINA is coordinated by a small population of neurons (<2% of the brain) marked by their expression of *Trpγ*. Loss of *Trpγ* leads to ~50% decrease in brainwide activity and altered active phase patterns, while silencing the *Trpγ*⁺ nearly abolishes brain wide PSINA. Mutants of *Trpγ* show disrupted cell-type specific activity dynamics and display synaptic defects that are equivalent to flies with near total loss of electrical activity during the last half of pupal development. Lastly, *Trpγ*⁺ neurons serve as the template for

brainwide PSINA – the cell-autonomous phenotype of Trpy loss is mirrored in the brain wide activity patterns.

Chapter 2

The role of neuropeptide signaling in developmental activity

2.1 Neuropeptide signaling modulates pacemaker circuits

In current models of vertebrate systems, pacemaker-like neurons and recurrent network interactions are thought to initiate and sustain cycles of developmental activity at a local level (Blankenship and Feller 2010). Until more recently, most of the vertebrate studies of developmental activity have been conducted *in vitro*, indicating that even isolated groups of developing neurons can generate patterned activity. The rhythmic nature of PSINA in the fly suggests that pacemakers or central pattern generators (CPGs), i.e. circuits that generate rhythmic output without rhythmic input, are critical for organizing developmental activity across evolution. Notably, with Trp γ ⁺ neurons silenced, low-amplitude peaks of periodic activity persist, suggesting that a pacemaker-like module sits upstream of the Trp γ ⁺ domain (Bajar, Phi, Isaacman-Beck, et al. 2022). Furthermore, when silencing the Central Brain (CB) subset of Trp γ ⁺ neurons, where the core engine that drives PSINA is thought to reside, periodic activity continues in the optic lobes, demonstrating that local generation of activity may be a shared feature of nascent circuits across the animal kingdom. How these localized patterns of activity are coordinated throughout the developing brain is still unknown.

The best studied CPGs, that of the crustacean stomatogastric nervous system (STNS) (Bartos and Nusbaum 1997), are modulated by neuropeptide signaling (Blitz 2023). The STNS is composed of several modulatory projection neurons that bridge sensory and higher-order inputs with two feeding-related CPG outputs to facilitate context-appropriate behaviors. A hallmark of neuromodulatory signaling is its ability to transform the functions of hard-wired circuits, i.e. “circuit-switching,” by altering their intrinsic properties and synaptic strengths (Marder 2012). For instance, in the crab STNS, one such modulatory projection neuron (MCN5) co-releases the neuropeptide Gly-SIFamide and the neurotransmitter glutamate onto a CPG neuron (LPG) to

transform its rhythmic output from a single network (filtering only) to a dual network (filtering and chewing) role (Gnanabharathi et al. 2024). Furthermore, modulators can synchronize the rhythms of two independent networks through selective strengthening of synapses – release of the neuropeptide Red Pigment Concentrating Hormone (RPCH) in the STNS conjoins the rhythms of cardiac and gastric CPGs, which otherwise operate independently from one another (Dickinson et al. 1990). The ability of neuropeptide signaling to synchronize independent rhythms is of particular interest in the context of PSINA.

Neuropeptides are the largest, most diverse, and possibly oldest class of neuroactive compounds (Nässel and Zandawala 2019). Accordingly, they regulate virtually every developmental, physiological, and behavioral process, are expressed in a vast array of cell-types and tissues, and a given neuropeptide can have multiple functions. Neuropeptides are produced in the soma through enzymatic cleavage of larger precursor proteins, are often further post-translationally processed, and trafficked and stored in dense core vesicles to axonal terminations or dendrites. Although in many cases neuropeptides act as co-transmitters alongside small-molecule fast transmitters, a distinctive method of their delivery is extra-synaptic volume transmission, which affects a much larger area and at longer time scales. Some neuropeptides are even released into the circulation to act as hormones (Nässel and Zandawala 2022). Taken together, neuropeptides represent a dynamic, higher order “chemical connectome” overlaid on the conventional synaptic connectome to guide context-appropriate physiology and behavior (Jékely and Yuste 2024).

2.2 A screen of neuropeptides involved in PSINA

In *Drosophila*, there are ~50 genes that encode neuropeptides and roughly the same number for their receptors, which are predominantly G-Protein Coupled Receptors (GPCRs) (Nässel and Zandawala 2019). We conducted a KiR2.1-mediated, neuronal silencing-based screen of *Drosophila* neuropeptide expression domains and recorded their effects on PSINA through our “widefield assay” (**Figure 2.1** and Chapter 3 “Methods” for details). Briefly, using a standard epifluorescence microscope, we simultaneously recorded up to 100 pupae expressing the genetically encoded calcium indicator, GCaMP6s, in all neurons to visualize brain-wide activity. We targeted effector expression (KiR2.1 and RNAi for follow up, see below) to different classes of cells with the GAL4-UAS and LexA-LexAop systems. We restricted the timing of effector expression to the last half of pupal development with GAL80ts (McGuire et al. 2003), which inhibits GAL4 at 18°C, but allows for full expression at 29°C. Below, we refer to this approach as temporally-targeted expression.

Starting with KiR silencing before individual genetic knockdown or knockout provided several advantages – First, KiR silencing is expected to inhibit all forms of chemical and electrical transmission to produce the strongest possible phenotype. Second, the KiR approach was fully modular, giving us the ability to target multiple GAL4 expression domains at once (during early stages this was around 4-5 different groups of 5-6 replicates each but has since expanded to double the groups and replicates) and thus was much faster and efficient. Our expectation, therefore, was that there would be many hits, some of which would be false positives with respect to the gene of interest. As detailed below, the plan was to then follow up on KiR hits with targeting the neuropeptide gene. Lastly, we also hoped that some smaller expression domains identified as KiR hits could reveal new PSINA-active neurons, even if we confirmed that they were not participating

through peptidergic signaling (i.e., a negative result from the genetic manipulations), which would have served our broader research objective of mapping the circuitry that drives PSINA.

For many neuropeptide genes, individual research groups have generated gene-derived, synthetic promoter driven GAL4s to label the expression domains. More recently, a number of driver collections which incorporate GAL4 into native genetic loci have become available (Deng et al. 2019b; Diao et al. 2015). We were partial to utilizing these so-called gene-trap GAL4 drivers, which are expected to better capture the native expression domain of the gene of interest. We followed up on all cell-silencing hits with pan-neuronal RNAi-mediated knockdown of the given neuropeptide. When available, we tested multiple independent RNAi constructs in genetic backgrounds heterozygous for the targeted gene to improve the specificity and efficacy of the knockdown. If expression knockdown qualified as a hit, we assessed how mutants (i.e. whole-animal loss of function) of the given neuropeptide gene, if viable, would influence PSINA.

Across the screening pipeline, we classified a “hit” as a >20% change in global signal amplitude or any alterations to the stereotyped spatiotemporal structure of PSINA relative to controls. While we utilize several metrics to quantify the spatiotemporal properties of PSINA, such as active phase duration, cycle counts, and sweeps per cycle, the most powerful approach to visualize the overall temporal organization of PSINA is calculating the power spectral density (PSD). The PSD provides the power distribution of the different frequencies that make up the total original signal (i.e. a PSINA trace). In wildtype PSINA, most of the signal power is carried by a band around 10^{-3}Hz , which corresponds to a cycle period of 10-15 minutes. By focusing on this stereotyped feature of wildtype PSINA, we can easily detect relative changes to the temporal structure that may result from our manipulations.

After about ~35 neuropeptide and neuropeptide-receptor domains screened, we identified a select few hits to ask if neuropeptide signaling is required for PSINA. First, temporally targeted silencing with the pan-neuropeptide driver, c929- aka dimm-GAL4, leads to a complete loss of PSINA cycles (**Figure 2.2**). This early result was one of the motivators of the neuropeptide screen. Notably, the qualitative changes to PSINA from silencing dimm-GAL4 cells are similar to the effects of removing neuromodulatory input in the crustacean STNS – a temporary loss of activity which later recovers with altered dynamics (Khorkova and Golowasch 2007). Our screen ultimately identified 3 specific neuropeptides that are critical for wildtype PSINA – ion transport peptide, isoform D (ITP.D), SIFamide (SIFa), and myoinhibitory peptide (MIP, aka Allatostatin B).

Temporally targeted silencing of the ITP.D-GAL4 domain severely disrupts PSINA – the periodic cycles are abolished and what remains are unstructured, high frequency oscillations with regular, high-amplitude single peaks (**Figure 2.3**). While homozygous mutants of ITP are embryonic-lethal (Gáliková et al. 2018), temporally-targeted knockdown of all 5 ITP isoforms (C-E) with ITP.D-GAL4 in a heterozygous background led to comparable changes to PSINA as with silencing, albeit with fewer oscillations and even higher amplitude single peaks. ITP.D-GAL4 captures sparse populations of neurons; it also targets 4 of the 5 classes of fly glia (Deng et al. 2019) (astrocytes, cortex glia, ensheathing glia, and perineural glia). Notably, ITP.C-GAL4, which appears to label the same small neuronal population as ITP.D-GAL4 but has no expression in glia, does not alter PSINA in the context of KiR silencing. We have shown that astrocytes are required for wildtype PSINA (Bajar, Phi, Randhawa, et al. 2022), but where and how ITP.D functions during pupal development to regulate PSINA has yet to be explored.

SIFa is produced in four neurons in the Pars Intercerebralis, a neuroendocrine region analogous to the mammalian hypothalamus, while its only known receptor, SIFa-R, is broadly expressed throughout the brain. Silencing the four SIFa neurons abolishes the characteristic periodicity that defines wildtype PSINA. Furthermore, whole-animal loss (i.e., mutants), as well as temporally targeted knockdown of SIFa and SIFa-R reproduce the cell-silencing phenotype, indicating that SIFa and SIFa-R operate as an exclusive ligand-receptor module during the last half of pupal development to regulate PSINA cycles brain-wide. I extensively cover the roles of SIFa and SIFaR in PSINA in Chapter 3.

Two other neuropeptides, Hugin and MIP, directly influence SIFa neuron activity in the adult brain to regulate feeding behavior (Martelli et al. 2017). Although silencing with Hugin-GAL4 did not affect PSINA, silencing with MIP-GAL4 led to a semi-penetrant phenotype – about half of the pupae showed altered cycle structure (**Figure 2.4**). When we followed up with temporally targeted pan-neuronal knockdown of MIP and its receptor, sex peptide receptor (SPR), we found that the duration of late-stage PSINA active phases was reduced, which contributed to a ~20% decrease in average global signal. We also observed the same brain-wide phenotype with temporally targeted knockdown of SPR in just the four SIFa neurons, suggesting that MIP-SPR signaling influences SIFa neuron activity to regulate the cycle structure of late-stage PSINA. As of this writing, we are actively investigating the genetic and circuit-level mechanisms by which MIP and SPR participate in PSINA.

As anticipated, we identified several hits in our primary Kir2.1 screen. However, in most cases, our initial cell-silencing hits did not hold up when testing for a genetic requirement (see *Dh44*, *Dh44-R*, *Gyc76C*, *Nplp1*, *sNPF*, and *TkR* in **Tables 2.1-2.3**), confirming that the silencing phenotype we observed resulted from targeting PSINA-active neurons captured by the driver

domain that do not require the expression of the expected gene of interest. For instance, the Dh44-R1-derived driver line 21A07-GAL4 produces a strong loss of cycles phenotype when silenced, similar to the effect of silencing SIFamide neurons (see below, and Chapter 3) – yet we found that PSINA was unaltered in Dh44-R1 mutants. We noted that the 21A07-GAL4 also labels the four SIFamide neurons when we looked at the expression of the built-in fluorescent cell-marker (TdTomato) of the KiR2.1 construct in dissected pupal brains, suggesting that the observed silencing phenotype was due to the 21A07- driver capturing the SIFa neurons. For the case of sNPF-R, which did demonstrate phenotypes across the entire screening pipeline (from silencing to genetic loss of function), we did not further investigate its involvement in PSINA due to its very broad expression domain (FlyBase) and the fact that its only known ligand, sNPF (Mertens et al. 2002), showed no genetic requirement for PSINA. In the similar situation with TkR99D, where silencing TkR99D-GAL4+ neurons lead to a severe disruption to PSINA but targeting the TkR99D gene did not. Here, my colleagues in the Akin Lab founds that the expression domain comprised ~4,000 neurons, twice the size of the Trpy+ population, and efforts to fractionate this set were not successful.

2.3 Summary

The discovery of a central role for neuropeptide signaling in coordinating developmental activity creates a new path for investigating the underlying mechanisms of neural circuit maturation. Due to volume transmission, i.e., paracrine signaling, neuropeptides are not bound by the spatiotemporal constraints of conventional synaptic signaling and offer a fitting mode of organizing brain-wide developmental activity in circuits that are undergoing continuous changes to synaptic structure. As our knowledge of brain architecture deepens with continued advances in mapping connectomes across model organisms, there may also be a growing appreciation of the non-synaptic, “chemo-connectome” and its ability to steer the organization and function of neural circuits. In addition, these findings that indicate a novel role for neuropeptide signaling were only possible because of the expansive tool kit that the fly model system offers. We hope that these insights can inform future investigations to enhance the decades of research on developmental activity in mammalian model systems.

Figure 2.1

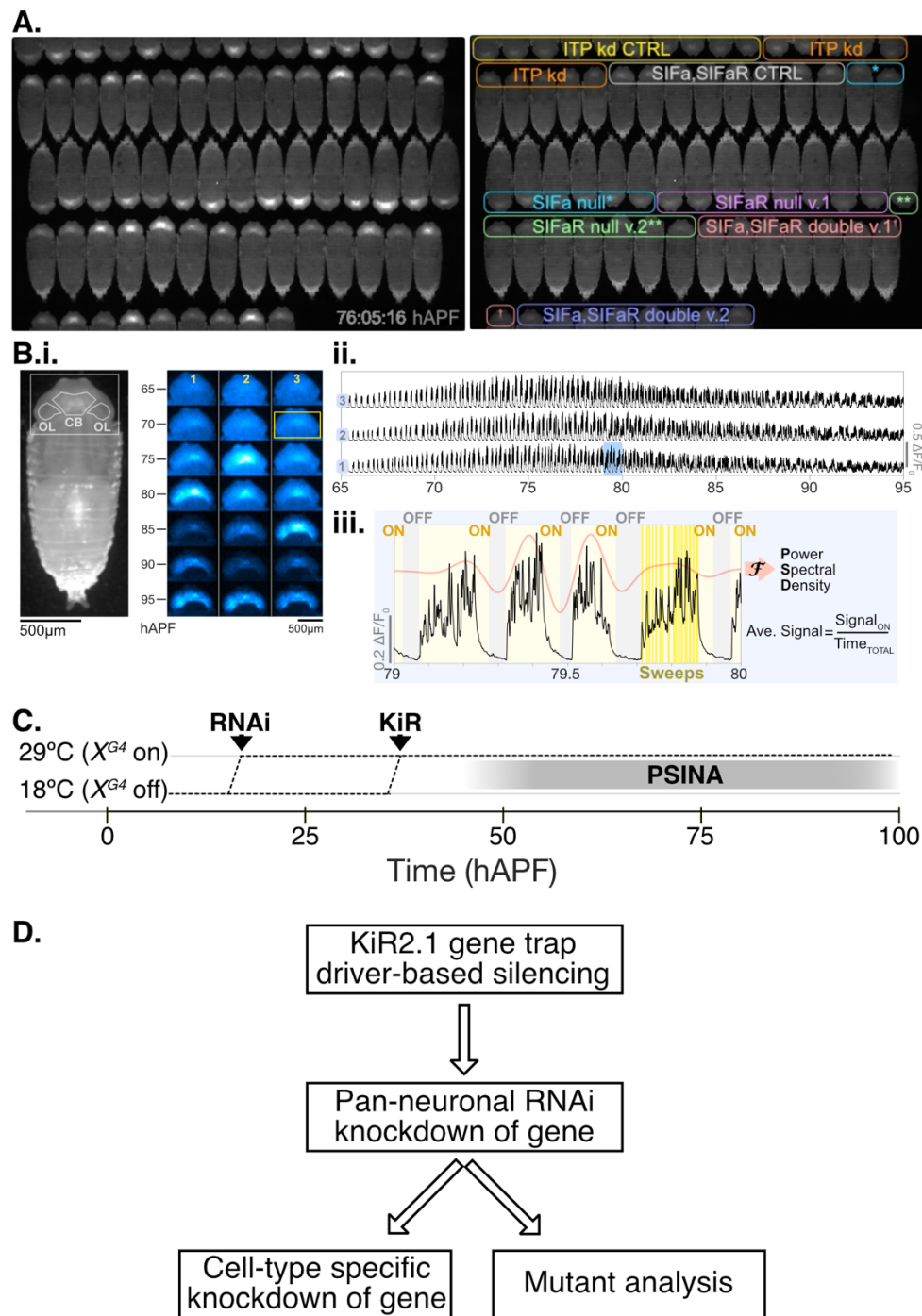


Figure 2.1 Widefield assay and neuropeptide screen pipeline

A. (Left) Example widefield imaging preparation for recording brain wide PSINA from 68 pupae. Snapshot at 76hAPF. (Right) Labels associated with example run of 8 genotypes with 7-9 replicates each. **B. (i.)** (Left) 65 hAPF *D. melanogaster* pupa. Cuticle around the head (boxed) has been removed for widefield imaging. Brain is outlined: CB, central brain; OL, optic lobe. Scale bar, 500µm. (Right) Montage of pupal heads from one widefield imaging session. Signal is fluorescence from pan-neuronally expressed GCaMP6s. Yellow rectangle (top-right) is typical region-of-interest (ROI) used to extract PSINA traces. All time axes in this and following figures are in units of hAPF. Scale bar, 500µm. **(ii.)** Wildtype PSINA recorded from enumerated pupae in **(i.)**; sampling rate is 0.5Hz. **(iii.)** Expanded view of PSINA from the blue highlighted 1h span above depicting the active (ON) and silent (OFF) phases of activity cycles, sweeps, and average signal metric used in analyses. PSD, power spectral density: PSINA traces were filtered to remove sweeps (coral overlaid profile), and PSD was calculated using the Discrete Fourier Transform ('F'). Adapted from Figure 1 of (Reichl et al. 2025). **C.** Temperature regime used to temporally target Kir2.1 and RNAi expression to pupal development. **D.** Neuropeptide screen pipeline.

Figure 2.2

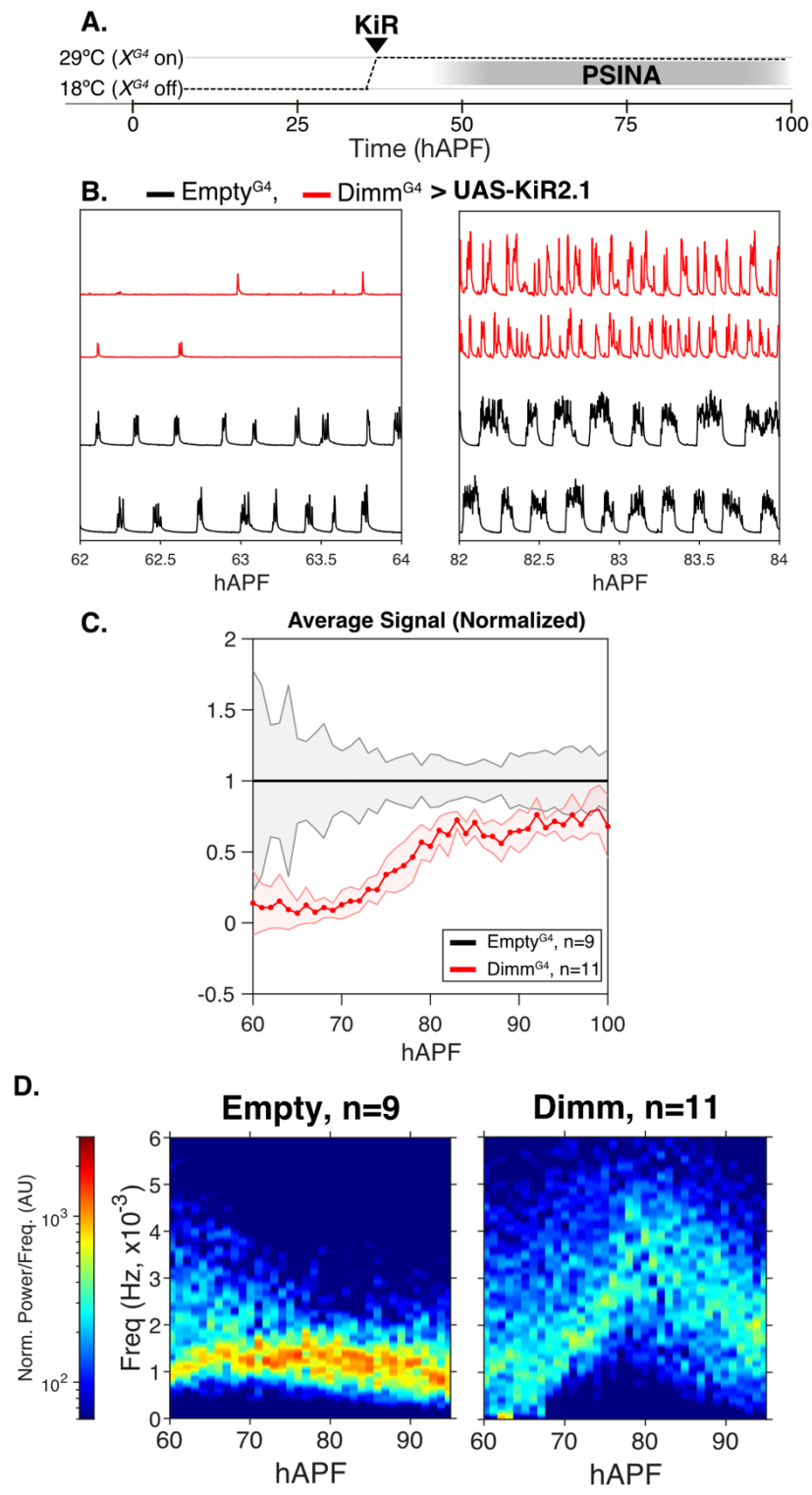


Figure 2.2 KiR silencing with Dimm-GAL4 leads to loss of PSINA cycles

A. Temperature regime used to temporally target KiR2.1 expression to pupal development. **B.** Representative PSINA traces during two, two-hour segments, of control (Empty^{G4}, black), and experimental (Dimm^{G4}, red). **C.** Average signal normalized to control (Empty^{G4}, black). Shaded areas are standard deviation. Sample sizes indicated. Closed circles mark where experimental distribution differs from control ($p > 0.05$) by two-sample KS test. **D.** Average Power Spectral Density (PSD) from control (Empty^{G4}, left), and experimental (Dimm^{G4}, right). Sample sizes indicated.

Figure 2.3

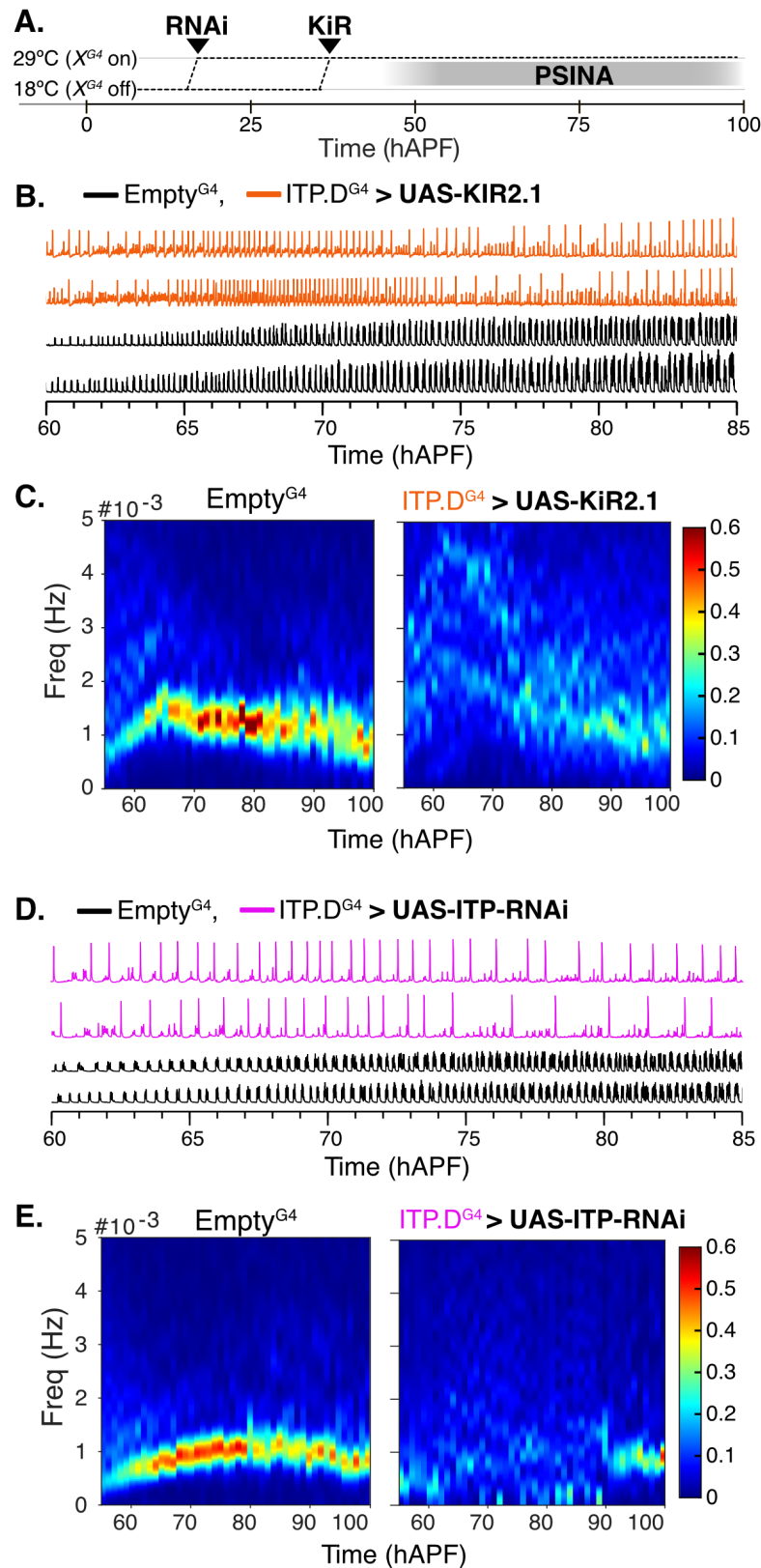


Figure 2.3 ITP.D is necessary for wildtype PSINA

A. Temperature regime used to temporally target KiR2.1 and RNAi expression to pupal development. **B.** Representative PSINA traces from KiR silencing of control (Empty^{G4}, black) and experimental (ITP.D^{G4}, orange), color matched to genotypes shown above. **C.** Hourly normalized PSDs of PSINA from KiR silencing. n=8 per genotype. **D.** Representative PSINA traces from ITP(isoforms C-E) knockdown of control (Empty^{G4}, black) and experimental (ITP.D^{G4}, purple), color matched to genotypes shown above. **E.** Hourly normalized PSDs of PSINA from ITP knockdown.

Figure 2.4

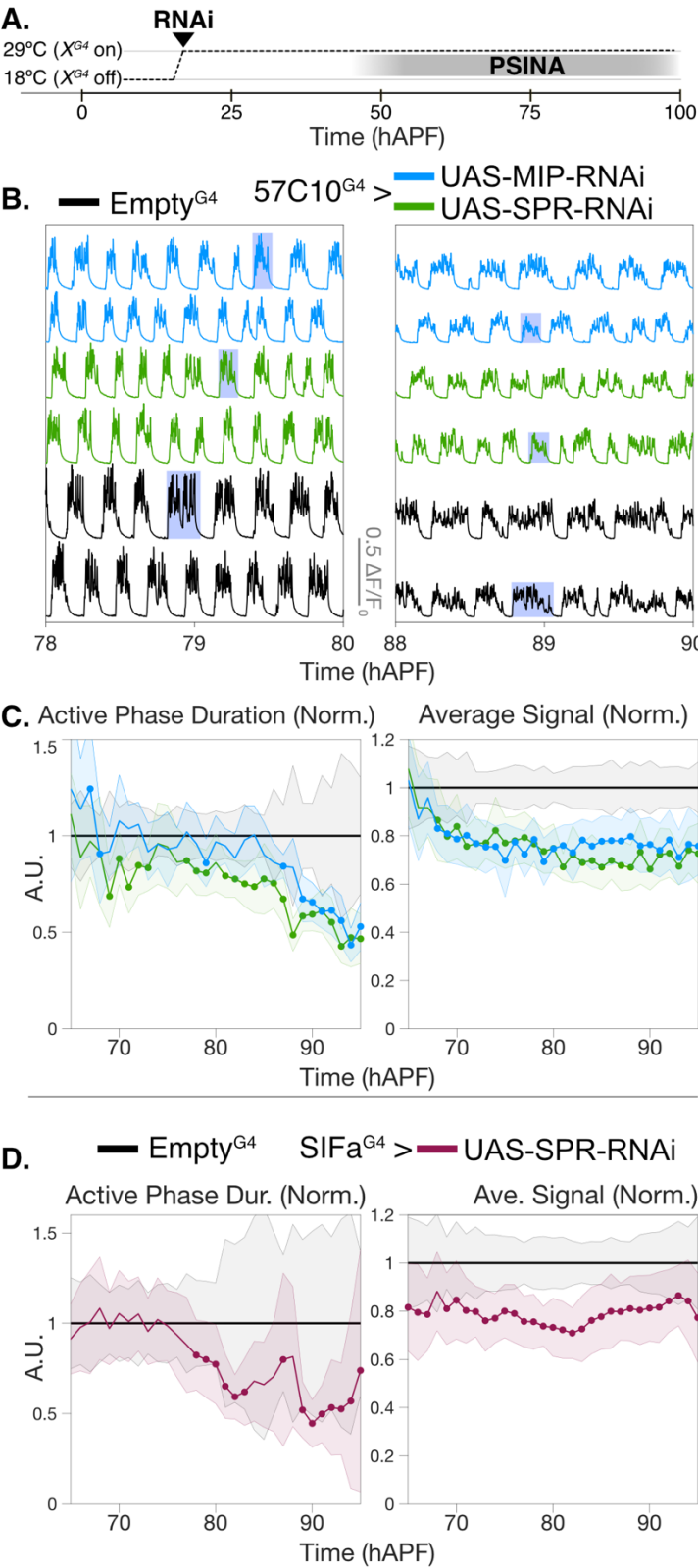


Figure 2.4 MIP and SPR are required for late-stage PSINA cycle structure

A. Temperature regime used to temporally target RNAi expression to pupal development. **B.** Representative PSINA traces during two, two-hour segments, of control (Empty^{G4}, black), and experimental (57C10^{G4}, pan-neuronal) knockdown with UAS-MIP-RNAi (blue) and UAS-SPR-RNAi (green). Blue highlights mark single active phases. **C.** Active phase duration (left) and average signal (right), normalized to control (Empty^{G4}, black, N=18), of pan-neuronal knockdown with UAS-MIP-RNAi (blue, N=11) and UAS-SPR-RNAi (green, N=15). **D.** Same as (C.) normalized to control (Empty^{G4}, black, N=22), of SIFa^{G4} driven knockdown with UAS-SPR-RNAi (green, N=24). For **C-D.**, shaded areas are standard deviation and closed circles indicate where experimental distributions differ from control ($p < 0.05$) by two-sample KS test.

Table 2.1 KiR2.1 silencing of neuropeptide-driver expression domains

Symbol	Gene Name	BDSC #	driver	Result	Notes
AstA	Allatostatin A	84593	AstA[2A-GAL4]	Marginal effects	
AstC	Allatostatin C	84595	AstC[2A-GAL4]	Marginal effects	
Capa	Capability	84597	Capa[2A-GAL4]	No phenotype	
CG30340	N/A	84611	CG30340[2A-GAL4]	No phenotype	putative ITP receptor
Crz	Corazonin	51977	Crz-GAL4.391	No phenotype	
CCAP	Crustacean cardioactive peptide	25686	CCAP-GAL4.P	No phenotype	
Dh31	Diuretic hormone 31	84623	Dh31[2A-AC.GAL4]	Marginal effects	
		84624	Dh31[2A-D.GAL4]	Marginal effects	
Dh44	Diuretic hormone 44	80985	Kurs58-GAL4 (Dh44 + SIFa)	No phenotype	
		93500	Dh44[CR60171-TG4.0]	No phenotype	
		84627	Dh44[2A-GAL4]	Altered cycles	
		39387	GMR65C11-GAL4	No phenotype	

Dh44-R1	Dh44-Receptor-1	83237	Dh44-R1[CR00985-TG4.2]	Brief flat line early, then low activity with high amp. single spikes	
		49855	GMR21A07-GAL4	Loss of cycles. Sparse bouts early and unstructured oscillations ~65- 70hAPF and on	Similar effects to SIFa silencing. Dh44R1 gene not required and this driver labels the SIFa neurons, suggesting phenotype is due the SIFa neurons
			57E06-GAL4	No phenotype	
Dh44-R2	Dh44-Receptor-2	66865	Dh44-R2[MI09411-TG4.2]	Similar to Dh44- R1[CR00985-	

				TG4.2], but higher oscillatory activity	
dim	dimmed	25373	c929-GAL4	Loss of cycles. Sparse activity early to increasingly higher frequency, unstructured oscillations by 65hAPF and on	
Dsk	Drosulfakinin	84630	Dsk[2A-GAL4]	No phenotype	
EH	Eclosion Hormone	6301	Eh-GAL4	No phenotype	
Fs	Follistatin	66838	Fs[MI04308-TG4.1]	Marginal effects	Stock no longer available
Gyc76C	Guanylyl Cyclase at 76C		Gyc76C[2A-GAL4]	Loss of cycles. Single sweeps to	(Kondo et al. 2020)

				unstructured oscillations	
Hug	Hugin	84646	Hug[2A-GAL4]	No phenotype	
		58769	Hug-GAL4	No phenotype	
Ilp1	Insulin-like peptide 1	66006	Ilp1-GAL4.L	No phenotype	
ITP.D	Ion Transport Peptide Isoform D	84702	ITP[2A-D.GAL4]	Sweep amplitude greatly increased and loss of cycles	
ITP.C	ITP Isoform C		ITP.C-GAL4 (DK 0165)	Marginal effects	
Lk	Leucokinin	51993	Lk-GAL4.TH	Marginal effects	
Mip	Myoinhibiting peptide precursor	84651	Mip[2A-GAL4]	No phenotype	
		51984	MiP-GAL4 (2nd Chrom)	No phenotype	
		51983	Mip-GAL4 (3rd Chrom)	Semi-penetrant; ~half with altered cycles	
Ms	Myosuppressin	84652	Ms[2A-GAL4]	No phenotype	

Natalisin	Natalisin	84668	natalisin[2A-GAL4]	Marginal effects	Ligand for Tk-R86C
NPF	neuropeptide F	25681	NPF-GAL4.1	No phenotype	
Nplp1	Neuropeptide-like precursor 1	86349	Nplp1[CR01375-TG4.0]	Major decrease in activity	
Nplp4	Neuropeptide-like precursor 4	84674	Nplp4[2A-GAL4]	Marginal effects	
Proc	Proctolin	51972	Proc-GAL4.TH	Marginal effects	
SIFa	SIFamide	84690	SIFa[2A-GAL4]	Loss of cycles. Sparse bouts early and unstructured oscillations ~65-70hAPF and on	Gene trap kills SIFa neurons and has dominant negative effect
			SIFa-GAL4 (2nd Chrom)	Similar to 2A, but less severe	Vecsey (601)

			SIFa-GAL4 (3rd Chrom)	Similar to 2A, but less severe than SIFa-GAL4(601)	Vecsey (516)
		49257	14F05-GAL4	Loss of cycles. Sparse bouts early and unstructured oscillations ~65-70hAPF and on	Sine Oculus derived
SIFa-R	SIFa Receptor	76670	SIFaR[MI05376-TG4.1]	Abolished activity	
		84689	SIFaR[2A-AC.GAL4]	Decreased cycle frequency late PSINA	
		49087	GMR24F06-GAL4	No phenotype	
		49061	GMR24A12-GAL4	No phenotype	
			GMR23G06-GAL4	No phenotype	

sNPF	short Neuropeptide F precursor	84706	sNPF[2A-GAL4]	~50% decrease in amplitude and fewer cycles	
sNPF-R	sNPF-Receptor	84691	sNPF[2A-GAL4]	~50% decrease in amplitude and fewer sweeps/cycle	
		46547	GMR64H09-GAL4	No phenotype	
		39384	GMR66A02-GAL4	No phenotype	
Tk	Tachykinin	51973	Tk-GAL4.TH	Marginal effects	
		84693	Tk[2A-GAL4]	Marginal effects	
TkR86C	Tachykinin-like receptor at 86C		TkR86C[2A-GAL4]	Loss of cycles	
TkR99D	Tachykinin-like receptor at 99D		TkR99D-GAL4	Loss of cycles	
Misc					
StacL	Stac-Like	66831	StacL[MI02279-TG4.1]	Abolished activity	<u>Near pan-neuronal expression domain</u>

Tdc2	Tyrosine decarboxylase 2	9313	Tdc2-GAL4	No phenotype	Octopamine precursor
Tim	Timeless		Tim-GAL4	No phenotype	

Table 2.2 RNAi screen of neuropeptide KiR hits

Gene	BDSC #	driver	Result
Dh44	25804	TRiP.JF01822	No phenotype
Dh44-R	28780	TRiP.JF03208	No phenotype
	29610	TRiP.JF03289	No phenotype
Gyc76C		TRiP.HMS04516	No phenotype
ITP	25799	TRiP.JF0187	Sweep amplitude greatly increased and loss of cycles
MIP		UAS-MIP-RNAi	Decreased late stage active phase duration
Proc	29570	TRiP.JF03249	No phenotype
SIFa		TRiP.JF03364	Loss of cycles
		TRiP.HMS22876	Loss of cycles
SIFa-R		TRiP.HMS00299	Loss of cycles
		TRiP.JF01899	Loss of cycles
sNPF		UAS-sNPF-RNAi	No phenotype
sNPF-R		UAS-sNPFR-RNAi	No phenotype
SPR		UAS-SPR-RNAi	Decreased late stage active phase duration

Tk-R86C	13392.GD	UAS-TkR86C-RNAi	No phenotype
Tk-R99D	27513	TRiP.JR02663	No phenotype
	55732	TRiP.HMC03749	No phenotype
	43329.GD	UAS-TkR99D	No phenotype
	1372.GD	UAS-TkR99D	No phenotype

Table 2.3 Neuropeptide mutant analysis

Gene	Genotype	Result
Dh44	Dh44[attp]	No phenotype
	Df(3R)BSC468/ Dh44[attp]	
	Dh44[2A-GAL4]/ Dh44[attp]	
	Dh44[CRIMIC-TG4]/ Dh44[attp]	
Dh44-R1	Dh44-R1[attp]	No phenotype
	Dh44-R1[DsRed]/ Dh44-R1[attp]	
	Df(2R)ED2354/ Dh44-R1[attp]	

	Df(2R)BSC700/ Dh44-R1[attp]	
	Dh44-R1[CRIMIC-TG4]/ Dh44-R1[attp]	
Dh44-R2	Dh44-R2[174]	No phenotype
	Df(2R)BSC305/ Dh44-R2[174]	
	Df(2R)BSC829/ Dh44-R2[174]	
	Dh44-R2[Trojan-TG4]/ Dh44-R2[174]	
ITP	Df(2R)ED4061/ ITP[attp](7)	Homozygous mutants embryonic lethal
	Df(2R)BSC602/ ITP[attp](7)	
	ITP[attp](7)	
	ITP[attp](2)	
Nplp1	Df(2R)BSC608/ nplp1[attp]	No phenotype
	nplp1[attp]	
SIFa	SIFa[1]	Loss of cycles
	SIFa[2]/ SIFa[1]	

	Df(2R)BSC780/ SIFa[1]	
	SIFa[2A-GAL4]/ SIFa[1]	
	SIFa[attp]/ SIFa[1]	
SIFa-R	Df(3R)ED10845/ SIFaR[attP]	Loss of cycles
	Df(3R)BSC677/ SIFaR[attP]	
	SIFaR[MI05376-TG4.1]/ SIFaR[attP]	
	SIFaR[GFSTF]/ SIFaR[attp]	
sNPF	sNPF[attp]	No phenotype
	sNPF[MI01807]/ sNPF[attp]	
	sNPF[2A-GAL4]; sNPF[attp]	
	Df(2L)Exel7077/ sNPF[attp]	
	sNPF[attp]/ sNPF[MI01807]	
	sNPF[MI01807](MIMIC)	
	sNPF[2A-GAL4]/ sNPF[MI01807]	
	Df(2L)Exel7077/ sNPF[MI01807]	

sNPF-R	Df(3L)BSC731/ sNPF-R[M108722]	Amplitude climbs from -60% to +60% relative to hets from 50-80hAPF
	Df(3L)BSC830/ sNPF-R[M108722]	
	sNPF-R-GAL4/ sNPF-R[M108722]	
	sNPF-R[attp]/ sNPF-R[M108722]	
	sNPF-R[M108722]	
	Df(3L)BSC731/ sNPF-R[attp]	Amplitude climbs from +20% to +50% from 70-80hAPF
	Df(3L)BSC830/ sNPF-R[attp]	
	sNPF-R-GAL4/ sNPF-R[attp]	
	sNPF-R[attp]	
	sNPF-R[M108722]/ sNPF-R[attp]	
Tk	Df(3R)Excl7312/ Tk[attp]	No phenotype
	Df(3R)Excl8155/ Tk[attp]	
	ΔTk1, UAS-Tk [attp2]/ Tk[attp]	
	ΔTk2, UAS-Tk [attp2]/ Tk[attp]	
	Tk[attp]	
TkR99D	Df(3R) BSCG20/ TkR99D[attp]	No phenotype

	Df(3R) Excl6213/ TkR99D[attp]	
	TkR99D[Mi10336-TG4-2]/ TkR99D[attp]	
	TkR99D[attp]	
	Df(3R) BSCG20/ TkR99D (S222)	No phenotype
	Df(3R) Excl(6?)213/ TkR99D (S222)	
	TkR99D[Mi10336]/ TkR99D (S222)	
TkR86C	Df(3R)Excl6159(Δ 86)/ TkR86C[attp]	No phenotype
	Df(3R)Excl529(Δ 86)/ TkR86C[attp]	
	TkR86C[attp]	

Chapter 3

Four neurons pattern brain-wide developmental activity through neuropeptide signaling

ABSTRACT

The developing brain becomes electrically active before it is ready to process sensory input. During neural circuit maturation, developmental activity is thought to refine synaptic connections by driving neuronal co-activation in rhythmic patterns. The source of this brainwide activity and the mechanisms which regulate its patterns are not well understood. Here we describe cellular interactions that shape developmental activity and their molecular basis. In *Drosophila*, patterned stimulus independent neural activity (PSINA) engages the entire brain in highly stereotyped, globally coordinated cycles of activity. A molecularly-defined population of ~2,000 neurons (Transient Receptor Potential Gamma, *Trpγ*⁺ neurons) act as an activity template for PSINA. We show that this activity template is patterned by four neurons expressing the neuropeptide SIFamide (SIFa). Signaling through the SIFa Receptor, SIFa modulates the activity of both SIFa and *Trpγ*⁺ neurons to establish the brainwide activity cycles of PSINA. In turn, *Trpγ*⁺ neurons regulate SIFa neuron activity through a recurrent interaction. Neuropeptides act through synapse-free, or wireless, signaling; a fitting mode of communication for a process tasked with refining on-going synapse formation. By placing neuropeptide signaling at the core of developmental activity, this work highlights the rich neurophysiological potential of the chemical connectome in shaping the developing brain.

3.1 Introduction

At the onset of synaptogenesis, before the nervous system is ready to process sensory input, the developing brain exhibits waves of patterned rhythmic activity. Such developmental activity occurs in many parts of the mammalian brain, including the visual, auditory, and olfactory systems, the spinal cord, thalamus, and the cortex (Blankenship and Feller 2010b; Wu et al. 2024b; Martini et al. 2021). Altering the activity in sensory systems, where it has been studied in greatest depth, leads to disruptions in topographic mapping (Thompson et al. 2017; Kersbergen et al. 2022b; Antón-Bolaños et al. 2019) and circuit function (Ge et al. 2021; Tiriach et al. 2022). Changes to the activity in the developing cortex are thought to be linked to neurodevelopmental disorders (Wu et al. 2024), highlighting the significance of understanding how this process contributes to the assembly of the brain. While specifics are outstanding, and likely to be case-specific, the consensus view is that the function of the activity is to mediate neuronal co-activation (Willshaw et al. 1976): Selective strengthening or weakening of synapses through Hebbian plasticity (Matsumoto et al. 2024). This framework elevates the importance of the spatiotemporal patterns of activity (Crair 1999) and, by extension, the mechanisms which generate them.

For the few cases where molecular and cellular factors necessary for wildtype activity patterns have been identified (Sernagor and Hennig 2013; Nakashima et al. 2019; Kersbergen and Bergles 2024), they have proved to be valuable handles on the biology of developmental activity. The best studied example is the so called ‘retinal waves’ (Galli and Maffei 1988; Meister et al. 1991) which initiate in the developing retina and spread to other visual areas, including the superior colliculus and the visual cortex (Ackman et al. 2012). In rodents, retinal waves appear two days before birth and persist for two weeks, until eye opening. For much of this time, the waves depend on cholinergic signaling from the network of starburst amacrine cells (SACs), both to each other

and to retinal ganglion cells (RGCs) (Sernagor and Hennig 2013). Loss of the $\beta 2$ subunit of the nicotinic acetylcholine receptor, which mediates this cholinergic communication, severely alters retinal waves (Bansal et al. 2000) and leads to RGC projection defects in higher visual areas (McLaughlin et al. 2003; Rossi et al. 2001). While network interactions among SACs are thought to be sufficient to initiate retinal waves (Sernagor and Hennig 2013), coordination of the activity between the two eyes (Ackman et al. 2012) indicates that there are additional regulatory mechanisms which shape the patterns. Notably, how the core mechanisms that initiate developmental activity are regulated to give rise to the final patterns is not well understood in any part of the developing brain. A promising candidate for bi-lateral retinal wave coordination is a subtype of RGCs which project to the contralateral retinas (Murcia-Belmonte et al. 2019). In addition, brain-to-retina projecting histaminergic neurons from the hypothalamus have been shown to modulate visual responses in mice (Warwick et al. 2024), suggesting that regulation need not arise autonomously in the eyes. The potential for such long-range activity modulation was highlighted by a recent report showing that hypothalamic peptidergic neurons project into nearly all areas of the mouse brain (Jiao et al. 2025).

The discovery of developmental activity in the fruit fly *Drosophila melanogaster* (Carreira-Rosario et al. 2021; Akin et al. 2019) indicates that this phenomenon fundamental to neurodevelopment. The adult fly brain is built during the 100 hours of metamorphosis, or pupal development. Patterned, stimulus-independent neural activity (PSINA, 'see-nah') starts ~50 hours after puparium formation (hAPF) (Akin et al. 2019), co-incident with the appearance of the first structural synapses (Y. Chen et al. 2014a; Muthukumar et al. 2014). For the following two days until an hour before the adult fly emerges, PSINA engages the whole brain in cycles of activity comprising globally coordinated active and silent phases (**Figure 3.1a-c**). Embedded within each

global active phase are stereotyped cell-type-specific activity patterns which are more correlated between future synaptic partners (Akin et al. 2019).

PSINA relies on a population of ~2,000 neurons, genetically defined by their expression of the cation channel *Trpγ* (Bajar, Phi, Isaacman-Beck, et al. 2022). Loss of *trpy* gene function alters PSINA patterns both across the brain and at the single cell-type-level and disrupts synaptic development in a non-cell autonomous manner. Brainwide PSINA in *trpy* mutants mirrors the activity recorded from *Trpγ*⁺ neurons (Bajar, Phi, Isaacman-Beck, et al. 2022), indicating that this small population acts as an activity template for the rest of the brain.

Here, we report that the *Trpγ*⁺ activity template is modulated by the neuropeptide SIFa. SIFa is a highly conserved (Verleyen et al. 2004) arthropod neuropeptide produced principally by four neurons in the Pars Intercerebralis (PI) region (Terhzaz et al. 2007a; Wolff et al. 2025) (**Figure 3.1d**), a neuroendocrine command center homologous to the mammalian hypothalamus (Hartenstein 2006). SIFa neurons (aka DNc01/2 (Namiki et al. 2018)) are present in the larva and undergo extensive remodeling during pupal development to extend processes throughout the adult brain and ventral nerve cord (Terhzaz et al. 2007). The SIFa Receptor, SIFaR (Jørgensen et al. 2006), is a G protein-coupled receptor (GPCR) expressed broadly throughout the brain (Fig. 1d). In the adult, SIFa-SIFaR signaling has been implicated in a range of adult behaviors, including feeding (Martelli et al. 2017), activity rhythms (Dreyer et al. 2019), sleep (Bai et al. 2018; Park et al. 2014), and courtship (Terhzaz et al. 2007a; Sellami and Veenstra 2015). We find that, during pupal development, this signaling module acts on *Trpγ*⁺ neurons to pattern the brainwide activity cycles of PSINA. By identifying four defined neurons that shape brainwide activity through a specific ligand-receptor interaction, our work brings into focus a genetically-

programmed neural engine of discrete composition driving this fundamental phase of neurodevelopment.

3.2 SIFa and SIFaR are necessary for PSINA cycles

To observe PSINA, we use a live imaging approach we term the widefield assay (Supplementary Video 1). We use genetically encoded calcium indicators to visualize neural activity: GCaMP6s (T.-W. Chen et al. 2013) for single cell class recording (e.g., pan-neuronal, PanN, for brainwide PSINA), and GCaMP6s and RCaMP1b (Dana et al. 2016) when imaging two different classes of cells. For independent genetic access to different classes of cells, we use the GAL4-UAS (Andrea H. Brand and Perrimon 1993) and LexA-LexAop (Lai and Lee 2006) expression systems. When needed, we control the timing of GAL4-driven expression using GAL80ts (McGuire et al. 2003), which inhibits GAL4 at 18°C, but allows for full expression at 29°C. Below, we refer to this approach as temporally-targeted expression. We stage and genotype flies at the onset of pupal development, and, at ~48hAPF, prepare them for imaging by removing the cuticle around their heads (**Figure 3.1a,b**). Using a standard long-working-distance fluorescence microscope, we can record simultaneously from up to 80 pupae for two days; that is, until the flies complete metamorphosis and emerge as adults. This medium-throughput assay made it possible to carry out the neuropeptide screen which identified *SIFa*.

To first assess how total loss of SIFa affects PSINA, we recorded brain-wide activity in whole-animal *SIFa* mutants. Compared to PSINA in controls, *SIFa* mutants show lower signal intensity and altered duty cycle, with less time active per hour during early stages (65-75hAPF) shifting to more time active after ~75hAPF (**Figure 3.1e,f**). Most prominently, the temporal organization of PSINA is altered in SIFa mutants: In the wildtype, PSINA is organized into cycles of active and silent phases with periods of 10-15 minutes (**Figure 3.1c**). We visualized these cycles in the frequency domain by taking the Fourier transform of the PSINA traces and calculating the power spectral density (**PSD, Extended Data Fig.1**); the PSD displays how the power in the

original signal is distributed across different frequencies. Most of the power in wildtype PSINA is carried by a band around 10^{-3} Hz (**Figure 3.1g, 1st column**), which corresponds to the 10-15 minute cycle period. Using the PSD representation, we compared wildtype and experimental (**Figure 3.1g, 2nd column**) sets of traces across pupal development and found that the 10^{-3} Hz band is absent in *SIFa* mutants (**Figure 3.1g, 3rd column, bottom panels**). This change to the PSD is equivalent to ~50% of the signal power in the wildtype being carried by other frequencies in *SIFa* mutants (**Figure 3.1g, 3rd column, top panels**). In effect, the stereotyped activity cycles are lost in *SIFa* mutants. Complementation analysis with a deficiency and rescue with a chromosomal duplication confirmed that the *SIFa* mutant alleles are true nulls and the phenotype is monogenic in origin, respectively (**Figure 3.1e-g, Extended Data Figure 3.2a**). We conclude that *SIFa* is necessary for the stereotyped cycles of PSINA.

We next asked how whole-animal loss of SIFaR, the only known receptor for SIFa (Jørgensen et al. 2006), affects PSINA. When paired with a deficiency, the *SIFaR^{null}* allele led to the same PSINA phenotypes we observed in *SIFa* mutants (**Figure 3.1h-j**): altered duty cycle, reduced signal, and lost cycles. We tested a protein trap, *SIFaR^{GFSTF}*, and a gene trap GAL4 driver, *SIFaR^{G4}*, in combination with *SIFaR^{null}* and saw similar changes to PSINA, indicating that these are strong loss-of-function (LoF) alleles (**Figure 3.1h-j, Extended Data Figure 3.2b-d**). Hereafter, we refer to *SIFaR^{GFSTF}* as *SIFaR^{LoF}*. As with *SIFa*, rescue with a chromosomal duplication confirmed the monogenic origin of the *SIFaR* phenotypes (**Figure 3.1h-j, Extended Data Figure 3.2b-d**). We conclude that *SIFaR* is also necessary for the stereotyped cycles of PSINA.

To ask how *SIFa* and *SIFaR* interact, we carried out double mutant analysis using *SIFa*[1] homozygotes and heteroallelic combinations of *SIFaR^{null}* with *SIFaR^{LoF}* or *SIFaR^{G4}* (**Extended**

Data Figure 3.2e-g,h-j). The *SIFaR* single mutants, particularly those carrying the *SIFaR^{G4}* allele, differed from the double mutants in how they affected duty cycle and average signal. We attribute these differences to the *SIFaR^{LoF}* and *SIFaR^{G4}* alleles not being complete nulls. However, for the most prominent phenotype—loss of cycles—the single mutants were indistinguishable from the doubles. The absence of an additive effect in the double mutants is consistent with SIFa and SIFaR functioning as an exclusive ligand-receptor module. Taken together, our findings indicate that SIFa-SIFaR signaling is required for the stereotyped cycles of PSINA.

Figure 3.1

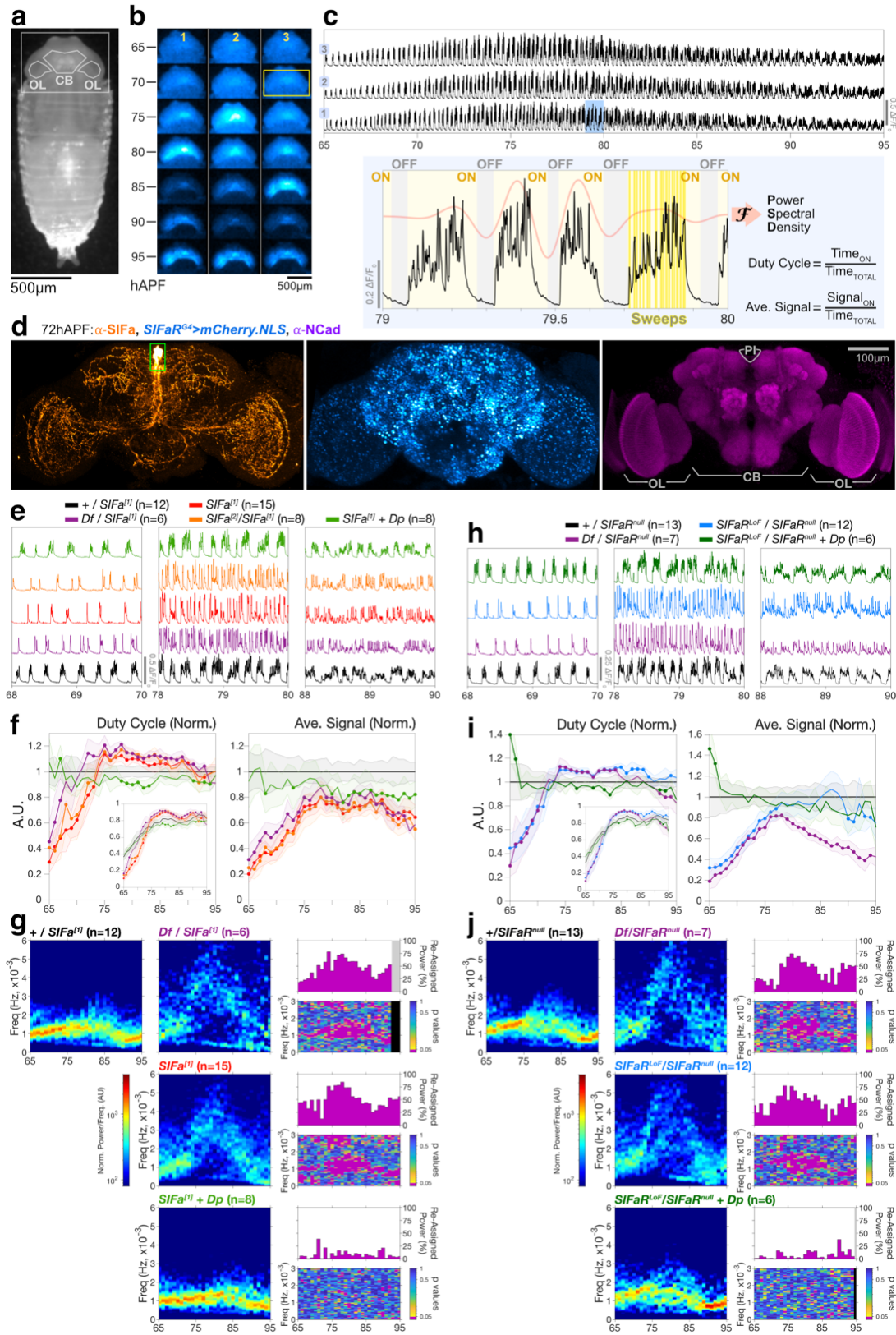


Figure 3.1 SIFa and SIFaR are necessary for PSINA cycles

a. 65 hAPF *D. melanogaster* pupa. Cuticle around the head (boxed) has been removed for widefield imaging. Brain is outlined: CB, central brain; OL, optic lobe. Scale bar, 500 μ m. **b.** Montage of pupal heads from one widefield imaging session. Signal is fluorescence from pan-neuronally expressed GCaMP6s. Yellow rectangle (top-right) is typical region-of-interest (ROI) used to extract PSINA traces. All time axes in this and following figures are in units of hAPF. Scale bar, 500 μ m. **c. Top:** Wildtype PSINA recorded from enumerated pupae in (b); sampling rate is 0.5Hz. **Bottom:** Expanded view of PSINA from the blue highlighted 1h span above depicting the active (ON) and silent (OFF) phases of activity cycles, sweeps, and principal metrics used in analyses. PSD, power spectral density: PSINA traces were filtered to remove sweeps (coral overlaid profile), and PSD was calculated using the Discrete Fourier Transform ('F'). **d.** Maximum intensity projection (MIP) of 72hAPF brain with *SIFaR^{G4}* driving *mCherry.NLS* (native fluorescence, cyan-hot look-up table (LUT)); brain also stained against SIFa (orange-hot LUT) and N-Cadherin (magenta). Green rectangle boxes SIFa soma. CB, central brain; OL, optic lobe; PI, Pars Intercerebralis. Scale bar, 100 μ m. **e-g.** *SIFa* mutant analysis **e.** Representative PSINA traces, color-matched to genotypes shown; numbers are sample sized used in (f) and (g). *Df*, deficiency; *Dp*, genomic duplication. **f.** Duty cycle and average signal, normalized to control genotype (black). Inset in duty cycle plots true values of metric. Shaded areas, standard deviation. Closed circles mark where experimental distribution differs from control ($p < 0.05$) by two-sample KS test. **g.** Average PSDs from control (first column) and experimental (second column) animals. PSD for each PSINA trace was calculated at every hour within a 2-h sliding window and normalized to remove contribution from changes in signal amplitude. Displayed PSDs are averages of replicates for each condition. Labels color-matched to (e,f); sample sizes indicated. PSD

comparison of $SIFa^{[1]}/SIFa^{[2]}$ to $+/SIFa^{[1]}$ omitted for brevity. In third column, bottom panel of each set maps p values from KS tests of control and experimental PSD distributions at each frequency-time coordinate; $p<0.05$ displayed in magenta. Top panel is fraction of control signal power re-assigned to different ($p<0.05$) frequencies in the experimental data. See also Extended Data Fig.1. **h-j.** *SIFaR* mutant analysis with *SIFaR^{LoF}* (i.e., *SIFaR^{GFSHF}*); data presented as in (e-g).

3.3 SIFa gene function and SIFa neuronal activity are necessary during PSINA

Analysis of whole-animal mutants does not address when and where *SIFa* and *SIFaR* are required to influence PSINA cycles. To ask whether *SIFa* is necessary during PSINA, we used a GAL4 driver which specifically labels the four large SIFa neurons in the PI (Wolff et al. 2025) (*SIFa^{G4}* (Terhzaz et al. 2007), **Extended Data Figure 3.3a**) to knockdown *SIFa* expression in a temporally-targeted manner. The resulting PSINA phenotypes were comparable to that seen in the mutants (**Figure 3.2b,c, Extended Data Figure 3.3b**). We conclude that SIFa expression in the four SIFa neurons during pupal development is necessary for wildtype PSINA.

Neuropeptidergic signaling operates alongside co-transmission of other neuroactive compounds (Nässel 2018). We asked if electrical silencing, by exacerbating the *SIFa* mutant PSINA phenotype, would reveal a co-transmitter requirement in SIFa neurons. Contrary to expectation, *SIFa^{G4}* driven, temporally-targeted expression of the hyperpolarizing channel KiR2.1 (Baines et al. 2001) resulted in a weaker PSINA phenotype (**Figure 3.2d,e, Extended Data Figure 3.3d**). This suggested that the strength of the driver was limiting the severity of the perturbation, so we tested another GAL4 which labels the SIFa neurons, *SIFa^{G4-2}* (Aso et al. 2014) (**Extended Data Figure 3.3b**). Temporally-targeted KiR2.1 expression by both drivers altered PSINA in similar fashion, and *SIFa^{G4-2}* led to a more penetrant phenotype, matching and not exceeding the *SIFa* mutant phenotype (**Figure 3.2d,e, Extended Data Figure 3.3d**). These results are consistent with *SIFa* expression being the dominant contribution of SIFa neurons to PSINA.

3.4 SIFaR gene function during pupal development is necessary and sufficient for PSINA

We pursued a similar strategy to establish the spatiotemporal requirements for SIFaR function. Temporally-targeted knockdown of SIFaR expression with the pan-neuronal *PanN^{G4}* driver phenocopied the *SIFaR* mutant phenotype (**Figure 3.2f,g, Extended Data Figure 3.3e**). Knockdown with *SIFaR^{G4}* also disrupted PSINA, but was less effective compared to *PanN^{G4}*, likely due to the dependence of gene trap expression to transcription from the native locus. We conclude that SIFaR gene function is necessary during PSINA.

SIFaR^{G4} captures an expression domain that expands throughout pupal development to include many thousands of neurons, including the SIFa set (**Extended Data Figure 3.4**). This broad expression domain is consistent with a recent fluorescence *in situ* hybridization based characterization of *SIFaR* transcript localization (Wolff et al. 2025). Notably, we found that temporally-targeted knockdown of SIFaR with *SIFa^{G4}* or *Trpy^{G4}* also disrupted PSINA (**Figure 3.2f,g, Extended Data Figure 3.3e**). These results suggest that wildtype PSINA relies on both SIFa autocrine signaling in SIFa neurons and paracrine signaling through *Trpy*⁺ neurons. We present further findings on the relationship between SIFa and *Trpy*⁺ neurons below.

In a complementary set of experiments, we asked whether SIFaR expression during PSINA is sufficient to rescue the loss of cycles. Temporally-targeted expression of *UAS-SIFaR* (Park et al. 2014) with *SIFaR^{G4}* or *PanN^{G4}* in a *SIFaR^{LoF}* background led to an overexpression phenotype (**Extended Data Figure 3.5a-d**): Beyond restoring the cycles, SIFaR expression from either driver extended the duration of active phases, which also increased the duty cycle and average signal. We were able to match wildtype PSINA more closely by rearing the animals below the fully permissive temperature for GAL80ts, (**Extended Data Figure 3.5e-g**) which is expected to reduce the expression levels of the rescue construct. Under both temperature regimes, *SIFaR^{G4}* and *PanN^{G4}*

yielded comparable results, indicating that the *SIFaR^{G4}* gene trap captures the functional expression domain of *SIFaR*. These findings indicate that *SIFaR* expression during PSINA is sufficient for wildtype activity cycles and that active phase duration can be regulated by *SIFaR* levels.

Figure 3.2

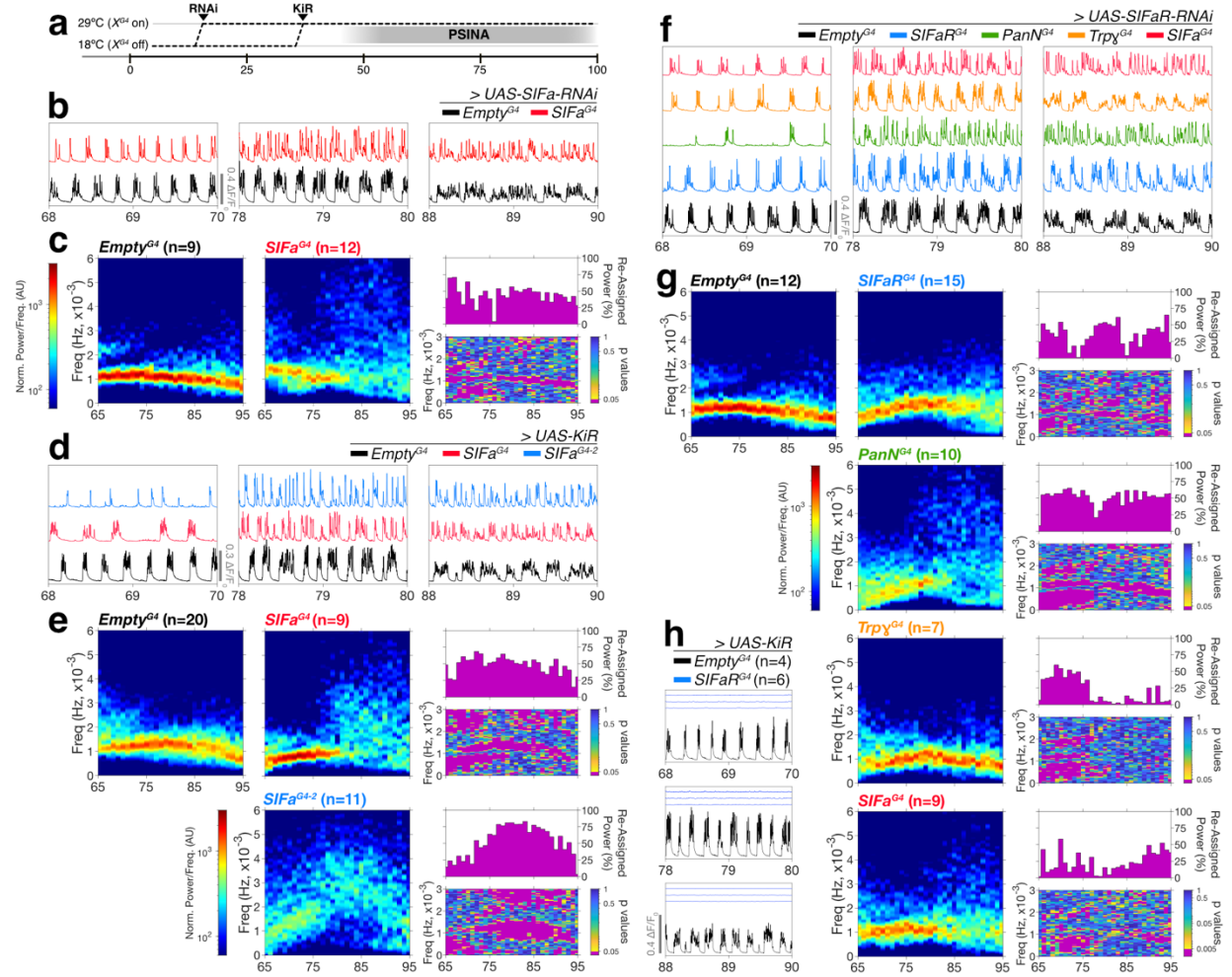


Figure 3.2 SIFa and SIFaR are necessary during PSINA

a. Temperature schedule used to temporally target RNAi knockdown and KiR2.1 expression to pupal development. **b,c.** Temporally-targeted knockdown of *SIFa*. **b.** PSINA traces, color-matched to genotypes shown. **c.** Average PSDs from control and experimental animals; presented as in Fig.1g. **d,e.** Temporally-targeted KiR2.1 expression in *SIFa* neurons; presented as in (b,c). **f,g.** Temporally-targeted knockdown of *SIFaR*; presented as in (b,c). **h.** Temporally-targeted *SIFaR*^{G4}-driven KiR2.1 expression; one control and three experimental traces shown.

3.5 SIFaR in the central brain is necessary for cycles

We had previously reported that electrically silencing only the central brain (CB) subset of *Trpy*⁺ neurons resulted in loss of brainwide PSINA (Bajar, Phi, Isaacman-Beck, et al. 2022). This finding had led to the working model of the PSINA engine: a CB-based network of defined neural circuits that generates the patterned activity and relays it across the brain. To ask whether SIFa-SIFaR signaling is consistent with this model, we used a similar approach (Bajar, Phi, Isaacman-Beck, et al. 2022) to spatially fractionate the broad SIFaR expression domain, which, when silenced in full, leads to complete loss of PSINA (**Figure 3.2h**) This approach takes advantage of how the fly optic lobes (OLs) are positioned as satellite neuropils, whose physical separation from the CB make it possible to assess distinct spatial participation in PSINA (**Figure 3.1a,d**). We restricted GAL4 expression to either the CB or OLs using conditional GAL80 constructs and a mostly OL-specific FLP recombinase (Nern et al. 2015). Within this framework (**Figure 3.3a**), we drove temporally-targeted Kir2.1 expression using *PanN*^{G4}, *Trpy*^{G4}, or *SIFaR*^{G4} (**Figure 3.3b-e**). Silencing the CB fraction of these expression domains virtually abolished PSINA in both the CB and the OLs (**Figure 3.3c,d**). Silencing the OL fraction reduced signal in the OLs by 50-75% and in the CB by ~30% without altering PSINA cycles in either spatial domain (**Figure 3.3c,e**). The non-local effect of OL silencing on signal in the CB may be due to the incompleteness of our spatial fractionation approach or to feedforward/centripetal connections between the OL and the CB (Nern et al. 2025). These results indicate that the CB subset of all three expression domains, including *SIFaR*^{G4}, contain neuronal populations whose activity is necessary for PSINA throughout the brain.

To assess whether there is also a spatial specialization to gene function, we used the same approach to knock down *SIFaR* in either the CB or the OLs (**Figure 3.3f-h**). Consistent with the

silencing results, knockdown in either spatial domain reduced PSINA signal. However, only CB knockdown altered cycles across the brain (**Figure 3.3f,g**). We conclude that while *SIFaR* is used throughout the brain to support wildtype signal amplitude, it is necessary only in the CB for PSINA cycles.

Taken together, these results are consistent with the CB-based PSINA engine model and also suggest a new layer of amplitude modulation through the brainwide influence of neuropeptide signaling.

Figure 3.3

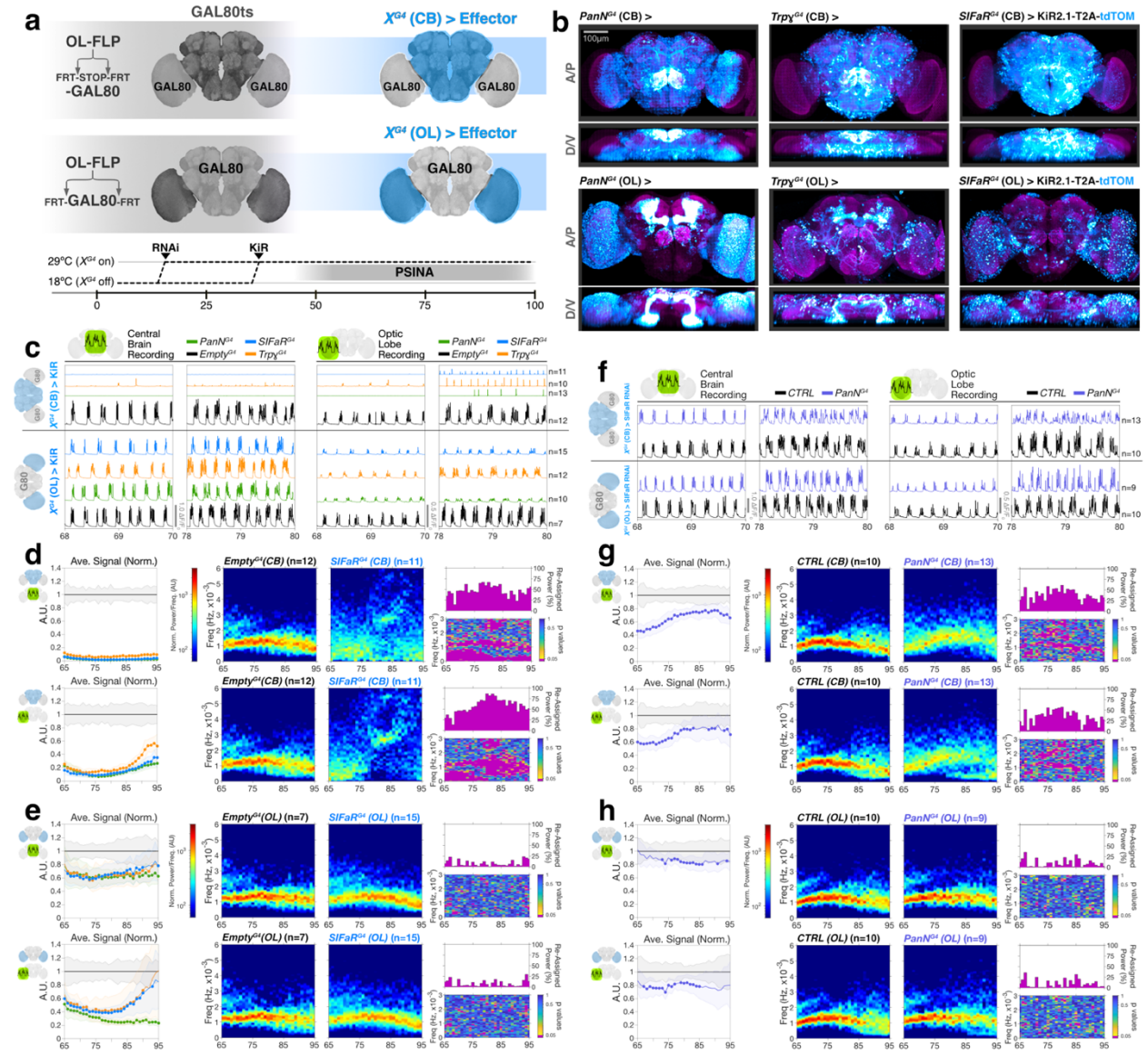


Figure 3.3 SIFaR in the central brain is necessary for wildtype PSINA cycles

a. Spatial fractionation of GAL4 expression. Both schemes carry two variants of *tubP-GAL80*: *GAL80ts* and one of two FLP-responsive conditional alleles. In the optic lobes, OL-FLP (29C07-FLP(Nern et al. 2015)) either turns on *GAL80* expression by removing the interruption cassette ('-STOP-', top) or turns it off by locally excising the FRT-flanked ORF (bottom). Animals are reared at 18°C and shifted 29°C at in the early pupa to unmask these differential *GAL80* expression domains ahead of PSINA onset; *GAL4*-driven effector expression is disinhibited in the complementary domains (blue). CB, central brain. OL, optic lobe. **b.** Anterior-posterior (A/P) and dorso-ventral (D/V) MIPs of 60hAPF brains with *PanN*-, *Trpy*-, or *SIFaR*-GAL4 driving *UAS-KiR2.1-T2A-tdTOM* expression principally in the CB (top row) or the OLs (bottom row). *PanN* labeling reveals some posterior OL neurons in the CB-designated fraction and Kenyon cells in the OL-designated fraction. KiR2.1 expression domain detected through tdTOM native fluorescence (cyan-hot LUT); reference stain is N-Cadherin (magenta). Scale bar, 100µm. **c,f.** Representative PSINA traces from animals in which KiR2.1 (c) or SIFaR-RNAi (f) expression was targeted to the CB (top set) or OL (bottom set) with the indicated *GAL4*s. CB (left columns) or OL (right columns) recordings are from the same animal in each condition. **d,g.** *Left*: Normalized average signal from animals in which KiR2.1 (d) or SIFaR-RNAi (g) expression was spatially targeted to the CB and recorded from the CB (top) or OL (bottom). Genotypes color-matched to (c) and (f); sample sizes indicated in *Right*. Closed circles mark where experimental distributions differ from control ($p < 0.05$) by two-sample KS test. *Right*: Average PSDs from control and experimental conditions described for *Left*; presented as in Fig.1g. **e,h.** Data from animals in which KiR2.1 (e) or SIFaR-RNAi (h) expression was spatially targeted to the OLs; presentation as in (d,g).

3.6 SIFa neuron activity patterns brainwide PSINA cycles

We sought to determine the relationship between the activity of SIFa neurons and brainwide PSINA. The proximity of the oversized SIFa neuronal soma to the dorsal surface of the head made it possible to record from these cells in the widefield assay using GCaMP6s; we followed brainwide PSINA in parallel with RCaMP1b (**Figure 3.4a**). At the level of cycles, SIFa neuron activity is very similar to PSINA (**Figure 3.4b,c**). Closer inspection reveals that SIFa neuron active phases both initiate before and end after PSINA active phases (**Figure 3.4b,d**). We compared this SIFa neuron lead and lag relative to PSINA in the CB to an internal control—the lead and lag spread between PSINA in the CB and the OL (**Figure 3.4d**). Whereas the PSINA active phases across the brain were aligned to within a few seconds, SIFa neurons started out with a lead of tens of seconds and a lag of ~4 minutes relative to PSINA. These spreads decreased steadily until ~80hAPF, and SIFa neuron active phases continued to envelope those of PSINA until the end of pupal development. The consistent temporal lead of SIFa neuron active phases suggests a direct role for these neurons in patterning brainwide PSINA cycles.

Using the same imaging approach, we studied how loss of *SIFa* affects SIFa neuron activity. We found that SIFa neurons also lose their activity cycles in *SIFa* mutant animals (**Figure 3.4b,e**); active and silent phases are replaced by an apparently unstructured train of sweeps (**Figure 3.4b**). Thus, the SIFa neuropeptide regulates the activity patterns of SIFa neurons themselves. The requirement for *SIFaR* expression in SIFa neurons (**Figure 3.2f,g**) indicates that this is achieved, at least in part, through direct autocrine signaling; below, we explore whether SIFa neurons receive feedback from other neurons.

We next asked whether loss of *SIFa* has differential effects on PSINA across different parts of the brain. Comparing PSINA recorded from the CB and the OL in controls and *SIFa* mutants,

we found that the activity remains highly correlated across these different regions in both genotypes (**Figure 3.4f,g**). The persistence of the global coordination of PSINA in the absence of *SIFa* suggests that SIFa-SIFaR signaling operates upstream of an independent brainwide activity template (Bajar, Phi, Isaacman-Beck, et al. 2022).

We studied the patterning roles of SIFa neurons and SIFa-SIFaR signaling in two gain-of-function approaches. First, we overexpressed SIFaR with *SIFaR^{G4}* (**Extended Data Figure 3.6**). This increased the active phase duration of both SIFa neuron activity and brainwide PSINA by >2-fold (**Extended Data Figure 3.6b,c**). SIFa neuron active phases still enveloped those of PSINA with SIFaR overexpression (**Extended Data Figure 3.6a,d**). We did observe some hourly changes in the lead and lag times with overexpression (**Extended Data Figure 3.6d**); however, these changes were not robust enough to establish whether SIFaR expression levels regulate relative timing of active phases. Taken together with the increase in *SIFaR^{G4}* expression during pupal development (**Extended Data Fig.4**), these results suggest that, in the wildtype, SIFaR levels control the increase in the active phase duration over the course of PSINA.

As a second gain-of-function approach, we used the thermogenic cation channel TrpA1 (Pulver et al. 2009) to activate SIFa neurons (**Figure 3.4h-l**). TrpA1-expressing SIFa neurons responded to the activating temperature step with a gradual fluorescence increase; low amplitude activity cycles were visible on top of this graded response (**Figure 3.4h:Inset**). Compared to controls, the cycle frequency of activated SIFa neurons increased by ~80% (**Figure 3.4i**), resulting in a corresponding reduction in the period of ~40%. The lower amplitude of TrpA1-activated SIFa neuron cycles precluded reliable two-channel imaging, so we recorded the response of brainwide PSINA in separate experiments (**Figure 3.4j-l**). We found that PSINA cycles were similarly sped up in response to SIFa neuron activation (**Figure 3.4j,k**). We did not observe any significant

changes to brainwide PSINA when SIFa neurons were activated in *SIFa* mutants (**Figure 3.4j,l**). These results indicate that SIFa neurons can set the cycle period of both their own activity and that of brainwide PSINA in a SIFa-dependent manner.

The only other gene we know to have a specific effect on the cycle period is the phospholipase C β (PLC β) *Norpa* (Akin et al. 2019), an important downstream effector of GPCR signaling. The cycle period in *norpa* nulls is significantly longer, ranging from 200 to 125% of controls between 65-90hAPF (**Extended Data Figure 3.7**). Expression of *UAS-Norpa* in SIFa neurons during pupal development fully restores the cycle period in *norpa* nulls (**Extended Data Figure 3.7**), indicating that this intracellular signaling factor is important for the patterning role of SIFa neurons.

Based on this set of results, we conclude that SIFa neuron activity, which itself is shaped through the interactions of SIFa, SIFaR, and Norpa, patterns PSINA cycles by modulating the brainwide activity template.

Figure 3.4

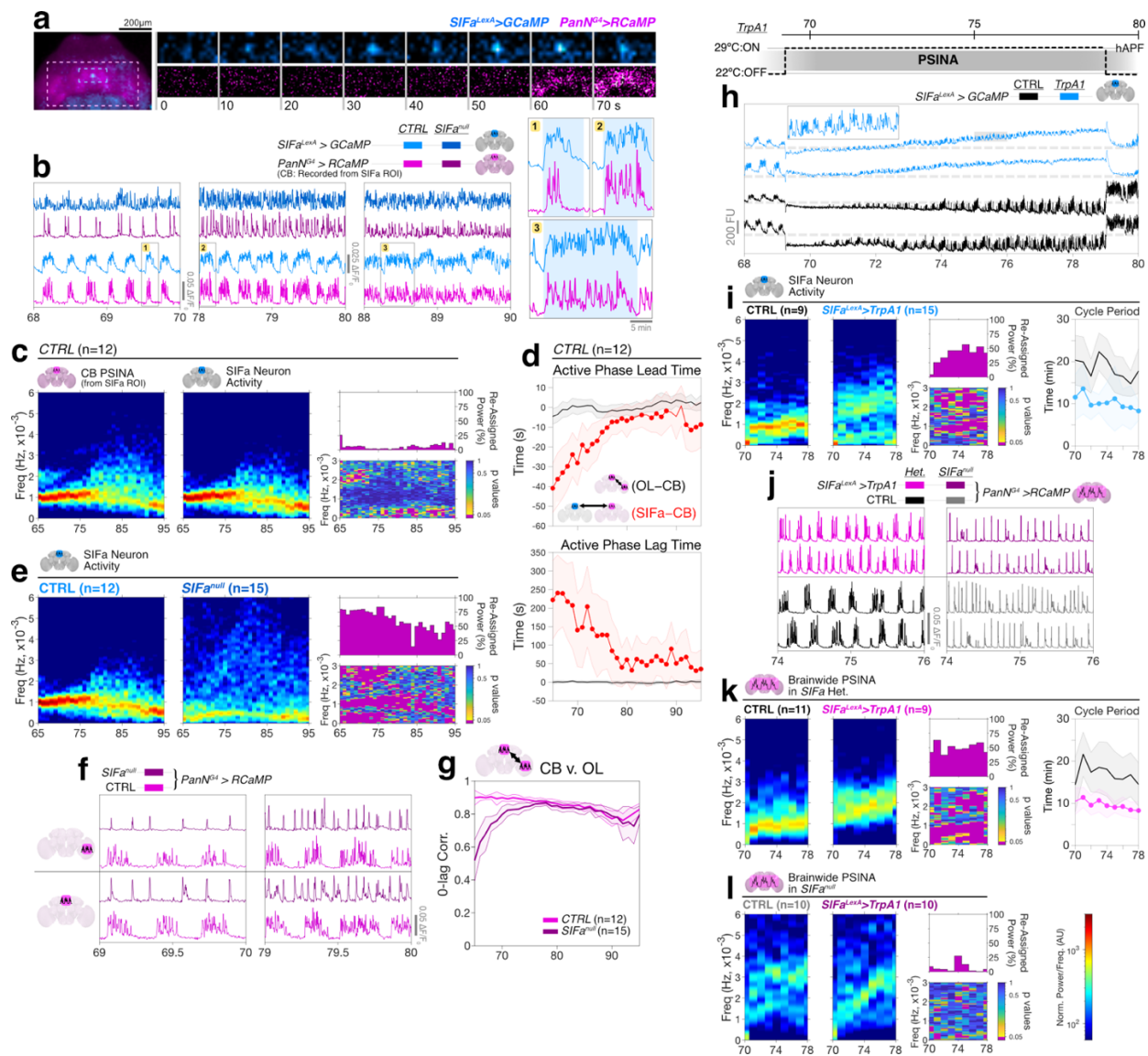


Figure 3.4 SIFa neuron activity patterns brainwide PSINA cycles

a-g. PSINA v. SIFa neuron activity in wildtype and *SIFa* mutant animals. **a.** 2-channel recording of SIFa neuron activity (cyan) and brainwide PSINA (magenta). *Left:* Pan-neuronal>RCaMP and SIFa neuron>GCaMP overlay of pupal head at 69.5 hAPF. *Right:* Progressive frames from boxed span #1 in (b). Color-matched ROIs from *left* shown; intensities adjusted to display fluorescence changes. **b.** *Left:* Representative traces for the indicated conditions. PSINA, as reported by pan-neuronal RCaMP, was recorded from the SIFa neuron ROI in the central brain (CB). *Right:* Expanded views for numbered boxes in *left*; SIFa neuron active phases marked in light blue. **c.** PSD comparison of CB PSINA, as reported by pan-neuronal RCaMP, and SIFa neuron activity in control animals. **d.** Active phase lead (*top*) and lag (*bottom*) time differences between left optic lobe (OL) and CB (black) or SIFa neurons and CB (red) in control animals. ~30 data points per hourly distribution compiled from 12 data sets; shaded areas, s.d. Closed circles mark where the red distributions differ from black ($p < 0.05$) by two-sample KS test. **e.** PSD comparison of SIFa neuron activity in control and *SIFa* mutant animals; sample sizes as indicated. **f.** Representative PSINA traces recorded from the left OL (*top pair*) or the SIFa neuron ROI in the CB (*bottom pair*) in control (magenta) or *SIFa* mutant (purple) animals. **g.** Zero-lag correlation of PSINA recorded from SIFa neuron ROI in the CB and the left OL in control (magenta) and *SIFa* mutant (purple) animals; sample sizes as indicated. **h-i.** TrpA1 activation of SIFa neurons **h.** *Top:* Temperature schedule used. *Bottom:* SIFa neuron activity response to temperature shift, without (black) and with (blue) TrpA1. *Inset:* Expanded view of gray highlighted span. In our hands, both baseline and change in fluorescence recorded from GCaMP6s varies inversely with temperature. When shifted from 22 to 29°C, SIFa neurons in control animals move to a lower baseline (compare to dashed lines marking the baseline at 22°C), and, following a pause of ~1hr, resume periodic activity cycles

at a lower signal amplitude. TrpA1-expressing SIFa neurons respond to the temperature up-shift with a graded fluorescence increase which builds up until the down-shift back to 22°C. **i.** *Left:* PSD comparison of SIFa neuron activity at 29°C without and with TrpA1; sample sizes as indicated. *Right:* SIFa neuron cycle period for the same conditions. Shaded areas, s.d. Closed circles mark where experimental distributions (blue) differ from control (black) ($p < 0.05$) by two-sample KS test. **j.** Representative brainwide PSINA traces at 29°C for the indicated genotypes. **k.** *Left:* PSD comparison of brainwide PSINA activity at 29°C without and with TrpA1; sample sizes as indicated. *Right:* Brainwide PSINA cycle period for the same conditions. Shaded areas, s.d. Closed circles mark where experimental distributions (magenta) differ from control (black) ($p < 0.05$) by two-sample KS test. **l.** PSD comparison of brainwide PSINA activity at 29°C without and with TrpA1 in *SIFa^{null}* animals; sample sizes as indicated.

3.7 SIFa neuron activity is regulated by *Trpγ*⁺ neurons

Next, we studied how SIFa neurons interact with the *Trpγ*⁺ population. During pupal development, *Trpγ* is expressed in ~2,000 neurons, including the SIFa set (Bajar, Phi, Isaacman-Beck, et al. 2022). Electrically silencing *Trpγ*⁺ neurons in the CB eliminates brainwide PSINA (**Figure 3.3** and (Bajar, Phi, Isaacman-Beck, et al. 2022). To ask whether silencing non-SIFa *Trpγ*⁺, or *Trpγ*[±] neurons, alters SIFa neuron activity, we devised a set difference approach (**Figure 3.5a,b**): Using the GAL4-UAS system, we expressed KiR2.1 in *Trpγ*⁺ neurons while excluding SIFa neurons from silencing with LexA-LexAop driven GAL80. The published, cell-type-specific *SIFa^{LexA}* driver (Martelli et al. 2017) (used for SIFa neuron recordings in **Figure 3.4**) uses the transcriptional activation domain of GAL4 and was therefore not compatible with our approach. As an alternative, we identified two other drivers, *SIFa^{LexA.2}* and *SIFa^{LexA.3}*, which make use of the GAL80-insensitive p65 activation domain and are expressed in SIFa neurons during pupal development (**Extended Data Figure 3.8a,b**). Using these drivers in combination to drive GAL80 and GCaMP6s (**Figure 3.5b, Extended Data Fig.8c**), we were able to both suppress KiR2.1 expression in SIFa neurons and record their activity during PSINA (**Figure 3.5c-e**). Silencing *Trpγ*[±] neurons had two major effects on SIFa neuron activity: First, the silent phases are not as clearly defined as they are in controls: Instead of returning to baseline; SIFa neurons continue to engage in sweeps between major active phases (**Figure 3.5c**). This altered pattern, which manifests as broadening of the characteristic frequency band toward higher values in the PSD representation (**Figure 3.5e**), is consistent with the loss of an inhibitory input from *Trpγ*[±] neurons due to KiR2.1 silencing. Secondly, past ~80hAPF, active phase duration does not increase as dramatically as in controls (**Figure 3.5d**), leading to a reduction in both duty cycle and average signal beyond this

time point. This suggests that the SIFaR-dependent active phase extension (**Figure 3.6**) is acting through $Trp\gamma^{\pm}$ neurons. In sum, we conclude that $Trp\gamma^{\pm}$ neurons regulate SIFa neuron activity.

Figure 3.5

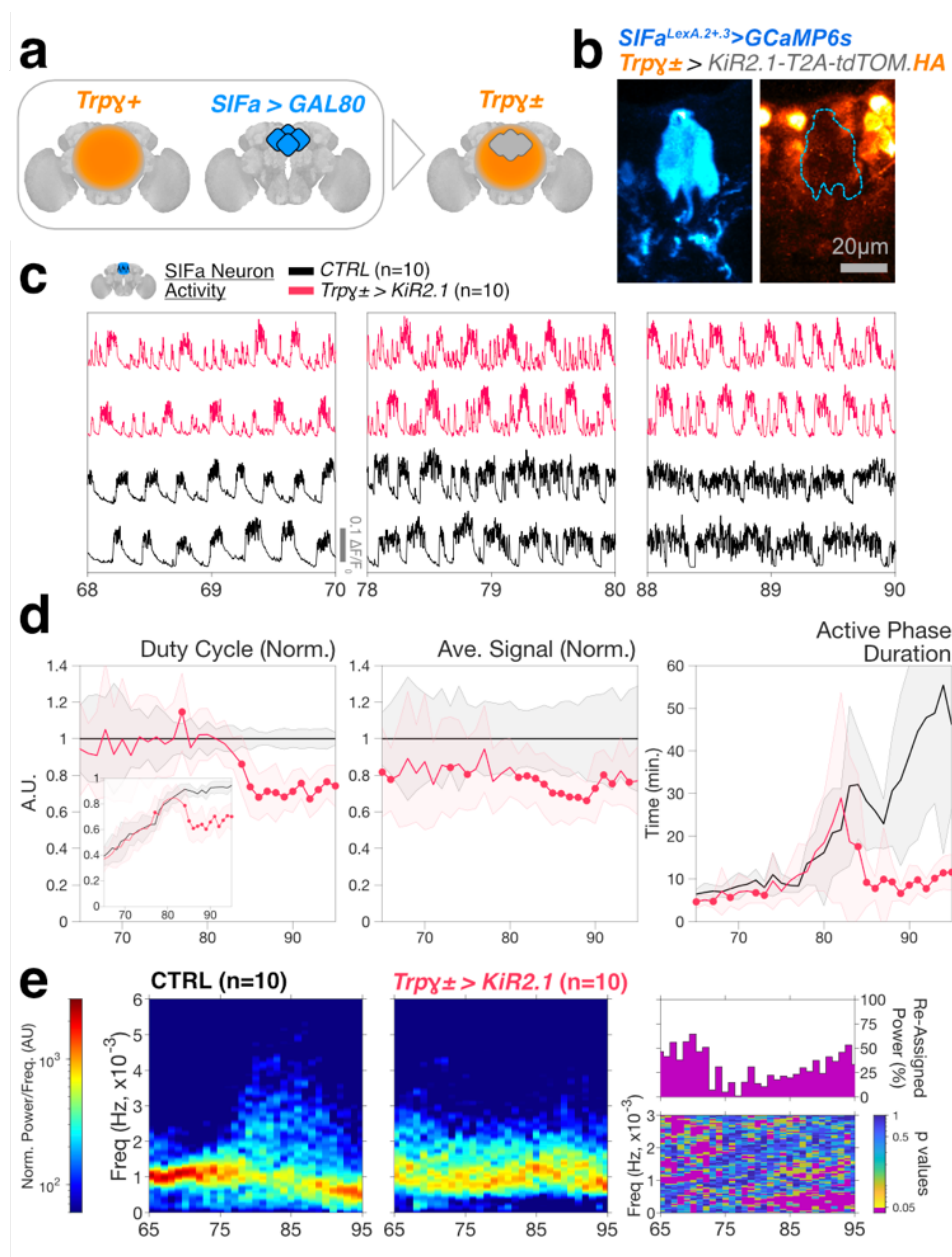


Figure 3.5. SIFa neuron activity is regulated by *Trpγ*⁺ neurons

a. Set Difference: The composite *Trpγ*[±] driver is generated by combining *Trpγ*^{G4} with *SIFa*^{LexA} > *LexAop-GAL80*. **b.** MIP of the PI region from a 72hAPF brain with *SIFa*^{LexA.2+.3} driving *LexAop-GCaMP6s* (anti-GFP, cyan-hot LUT) and *Trpγ*[±] driving *UAS-KiR2.1-T2A-tdTOM.HA* (anti-HA, orange-hot LUT); cyan dashed outline marks SIFa soma location in right panel. See Extended Fig.8c for full brain MIP. **c.** Representative traces of SIFa neuron activity in CTRL animals (black) or with *Trpγ*[±] driving *UAS-KiR2.1* (red). **d.** Normalized and true-valued (inset) duty cycle, normalized average signal, and true-valued active phase duration for the conditions and sample sized indicated in (c). Shaded areas, s.d. Closed circles mark where the experimental distributions differ from CTRL ($p < 0.05$) by two-sample KS test. **e.** Average PSDs from control and experimental animals; presented as in Fig.1g.

3.8 SIFaR expression in *Trpy*⁺ neurons is sufficient to rescue PSINA cycles

The results presented thus far regarding where SIFaR expression is necessary (i.e., *PanN*-, *SIFaR*-, *Trpy*-, and *SIFa*-GAL4 expression domains., **Figure 3.2f,g**) and sufficient (i.e., *PanN*- and *SIFaR*-GAL4 expression domains; **Extended Data Figure 3.5**) are consistent with the possibility that *SIFaR* expression in just the four SIFa neurons is sufficient for wildtype PSINA patterning. We found that this is not the case; *SIFa*^{G4} driven *UAS-SIFaR* did not restore wildtype PSINA in *SIFaR* mutants (**Figure 3.6**). We confirmed the efficacy of this cell-type-specific driver in an overexpression experiment (**Figure 3.6: ‘OE’**): Between 65-80hAPF, the cycle frequency increased by ~37% (Fig.6c), resulting in a corresponding decrease in the period of ~25% (**Figure 3.6b**). Later, from 80-90hAPF, cycle structure was more variable, as seen in the broader frequency spread in the PSD representation (**Figure 3.6c**), and the average active phase duration was longer (**Figure 3.6b**). These effects—increased cycle frequency and extended active phases—are well-aligned with results from SIFa neuron activation (**Figure 3.4h-l**) and *SIFaR*^{G4}-driven overexpression (**Extended Data Figure 3.6**), respectively, and are consistent with autonomous SIFaR expression levels regulating the activity patterns of SIFa neurons.

We next asked if *SIFaR* expression in the *Trpy*⁺ population is sufficient to rescue PSINA in *SIFaR* mutants. We found that *Trpy*^{G4} driven *UAS-SIFaR* restored activity cycles (**Figure 3.6a**). Compared to controls, PSINA in these animals started out with a lower cycle period and maintained higher duty cycle and longer active phases for most of the duration of observation. These differences were similar to what we had observed with *PanN*- and *SIFaR*-GAL4 rescue, which had to be titrated down to match wildtype PSINA (**Extended Data Figure 3.5**). Consistent with this overexpression inference, increasing the dosage of *Trpy*^{G4} driven *UAS-SIFaR* (**Figure 3.6: ‘2x’**) in the context of rescue amplified the differences in duty cycle and active phase duration

(Figure 3.6b). We conclude that *SIFaR* expression in the full $Trp\gamma^+$ domain, i.e., SIFa and $Trp\gamma^\pm$ neurons, is sufficient to pattern the PSINA activity cycles.

Figure 3.6

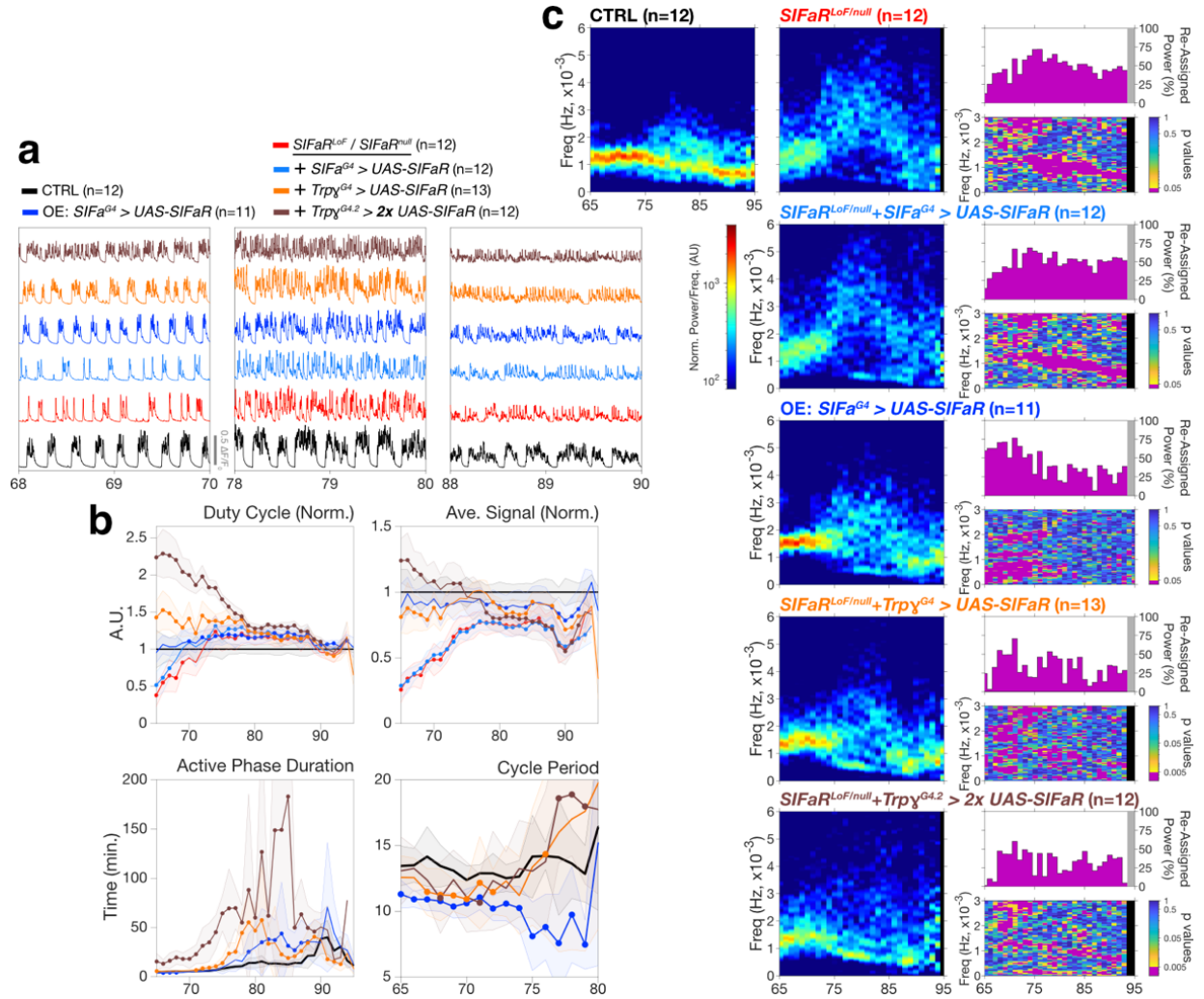


Figure 3.6 *SIFaR* expression in *Trpγ*+ neurons is sufficient to restore brainwide PSINA cycles

a. Representative brainwide PSINA traces for the indicated conditions. ‘OE’; overexpression. **b.** Normalized duty cycle and average signal, true-valued active phase duration and cycle period for the conditions and sample sized indicated in (a). Shaded areas, s.d. Closed circles mark where the experimental distributions differ from CTRL ($p < 0.05$) by two-sample KS test. **c.** Average PSDs from control and experimental animals; presented as in Fig.1g.

3.9 SIFa neurons pattern PSINA by modulating *Trpy*⁺ neurons

Previously, we had shown that brainwide PSINA mirrors the activity recorded from *Trpy*⁺ neurons (Bajar, Phi, Isaacman-Beck, et al. 2022) in *trpy* mutants. As the *Trpy*⁺ neuron activity reflects the cell autonomous phenotype of the *trpy* mutation, this result indicated that the *Trpy*⁺ population acts as an activity template for the rest of the brain. The findings presented here predicted that disruption of *Trpy*⁺ neuron activity underlies the changes to brainwide PSINA seen in *SIFa* mutants. This is what we observed using two-channel imaging: In *SIFa* mutants, *Trpy*⁺ neurons exhibit the same activity phenotypes we described for brainwide PSINA: the two signals are nearly identical (**Figure 3.7a-c**). We conclude that SIFa neurons pattern the cycles of PSINA by modulating the activity of *Trpy*[±] neurons through SIFa-SIFaR signaling (**Figure 3.7d**).

Figure 3.7

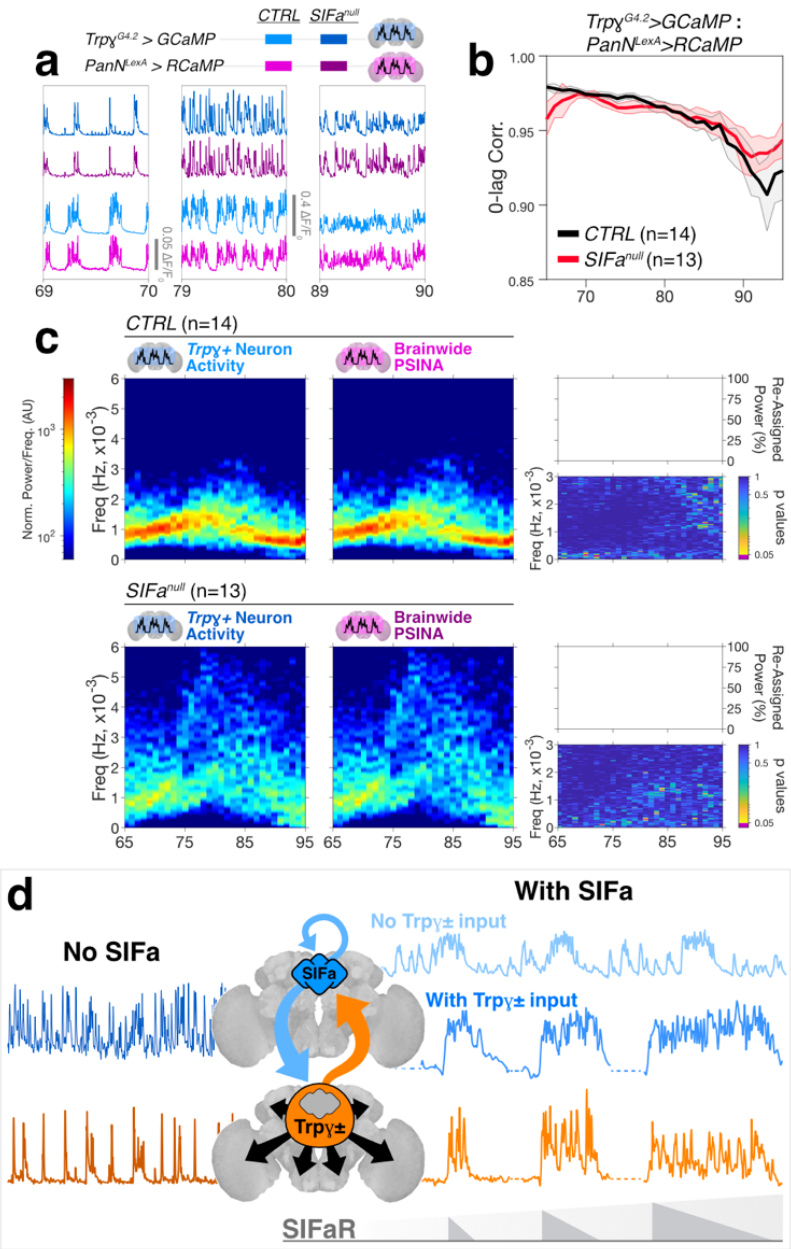


Figure 3.7 SIFa neurons pattern PSINA by modulating *Trpy*⁺ neurons

a-c. PSINA v. *Trpy*⁺ neuron activity in wildtype and *SIFa* mutant animals. **a.** Representative traces from 2-channel recordings of *Trpy*⁺ neuron activity and brainwide PSINA in control and *SIFa* mutant animals. **b.** Zero-lag correlation of *Trpy*⁺ neuron activity to brainwide PSINA in control and *SIFa* mutant animals. **c.** PSD comparison of *Trpy*⁺ neuron activity and PSINA in control (top) and *SIFa* mutant (bottom) animals; sample sizes as indicated. **d.** Model of the core PSINA engine. In the absence SIFa signaling (left, ‘No SIFa’), neither SIFa (blue) nor *Trpy*[±] (magenta) neurons have stereotyped cycles. SIFa-SIFaR signaling patterns both activity profiles into cycles (right, ‘With SIFa’). SIFa neurons regulate their activity through autocrine signaling, and they receive additional input from *Trpy*[±] neurons. Brainwide PSINA follows the *Trpy*[±] neuron activity template. SIFaR expression (light gray gradient ramp) and activity levels (dark gray triangles) control the duration of active phases. See *Discussion* for more details.

3.10 Discussion

We report that the neuropeptide gene *SIFa* and its receptor *SIFaR* are required in the developing central brain to establish the characteristic activity cycles of PSINA (**Figure 3.1-3**). *SIFa* neurons themselves have cycles which initiate before brainwide activity (**Figure 3.4b,d**). Consistent with this implied lead role in patterning PSINA, activating *SIFa* neurons increases the frequency of brainwide cycles (**Figure 3.4**). Additionally, restoring *NorpA* expression in just these four cells rescues the reduction in cycle frequency seen in *norpA* mutants (**Extended Data Figure 3.7**). *SIFaR* is necessary in *SIFa* neurons for wildtype brainwide PSINA (**Figure 3.2f,g** and **Extended Data Fig.3e**) and *SIFa* is autonomously required for *SIFa* neuron activity (**Figure 3.4b,e**), indicating that these neurons are autocrine regulated. Wildtype *SIFa* neuron activity requires additional input from *Trpγ±* neurons (**Figure 3.5**). In turn, *SIFa* neurons pattern the cycles of brainwide PSINA by modulating the activity of *Trpγ+* neurons (**Figure 3.6,7**).

These results build on previous work (Bajar, Phi, Isaacman-Beck, et al. 2022) and suggest a working model of how the core *PSINA engine*—a set of genetically-defined neural circuits in the central brain—patterns brainwide developmental activity (**Figure 3.7d**): *SIFa*-*SIFaR* signaling transforms the oscillations of *SIFa* neurons into cycles. When electrically isolated from *Trpγ±* neurons, the silent phases of *SIFa* neuron cycles are interrupted by sweeps and the active phases do not extend appreciably over the course of development. *SIFa*-*SIFaR* signaling also patterns *Trpγ±* neuron activity into cycles. *Trpγ±* neuron active phases grow progressively longer, in parallel with the developmental increase in *SIFaR* expression (**Extended Data Figure 3.4**), suggesting that receptor availability and activation-dependent desensitization regulate this feature of PSINA. Next, *Trpγ±* neurons feedback onto *SIFa* neurons to establish true silent phases and extend their active phases. Finally, brainwide PSINA follows the *Trpγ±* neuron activity template.

The SIFa-SIFaR dependent patterning of SIFa neuron activity has a precedent in one of the best characterized central pattern generating (CPG) circuits—the crustacean stomatogastric ganglion (STG). In the crab, *Cancer borealis*, SIFa re-organizes the rhythms of the two interdependent CPGs driving food intake and processing (Blitz et al. 2019). Specifically, one defined neuronal type within these circuits responds to SIFa by adding a low frequency bursting pattern on top of an existing high frequency rhythm (Fahoum and Blitz 2021). In the STG, which is subject to significant neuromodulation (Marder 2012), the response to SIFa depends on both the concentration of the neuropeptide as well as the physiology of the responding cells and circuits (Blitz et al. 2019). Our observations are consistent with this expected diversity of responses: In the optic lobes, which appear to follow the central brain generated activity template (**Figure 3.3** and (Bajar, Phi, Isaacman-Beck, et al. 2022)), SIFaR acts as a signal amplifier (**Figure 3.3h**). By contrast, in SIFa and *Trpγ*⁺ neurons, both of which may be intrinsically active populations, SIFaR patterns activity into cycles.

The molecular mechanisms of SIFa-SIFaR neuromodulation are not known. Our results implicate the PLCβ *NorpA* (Akin et al. 2019) (**Extended Data Figure 3.7**) as a candidate effector of SIFaR signaling. Most neuropeptide receptors, including SIFaR, are GPCRs. The primary transducer of GPCR signaling is the heterotrimeric G protein complex comprising a Gα subunit and a Gβγ dimer (Hanlon and Andrew 2015). In response to receptor activation, Gα dissociates from Gβγ to interact with downstream signaling effectors, which, for the Gαq class of Gα proteins, are PLCβs. Recruitment of PLCβ to the plasma membrane ultimately leads to the activation of protein kinase C and mobilization of Ca²⁺ from intracellular stores. *NorpA* may be the principal effector of SIFaR signaling; however, PSINA cycles remain intact in *NorpA* nulls (**Extended Data Figure 3.7**). There is a second PLCβ in the fly, *Plc21C*, which can act redundantly with *NorpA*

(Shieh et al. 2023). It will be possible to resolve this matter and further develop SIFa neurons into an *in vivo* model for neuropeptide signaling using the experimental access established in this work.

Neuropeptides are one of the largest and possibly the oldest class of neuro-active compounds (Beets and Watteyne 2025; Hauser et al. 2022). Bilaterian genomes contain many dozens to over 100 neuropeptide genes; this diversity, along with a similar complement of GPCRs, underscores the neurophysiological potential of the chemical connectome (Ripoll-Sánchez et al. 2023; Deng et al. 2019a; Smith et al. 2019). In the developing brain, neuropeptides have been implicated in neurogenesis, morphogenesis, migration, survival, and circuit assembly (Bakos et al. 2016; Z.-H. Li et al. 2024; Hevesi et al. 2024). While predicted (Hevesi et al. 2025), a direct involvement of neuropeptide signaling in developmental activity had not been demonstrated until the present study.

A defining feature of neuropeptide signaling is extra-synaptic volume transmission. This mode of communication frees neuropeptidergic cells from the constraints of specific post-synaptic contacts, giving them the reach and flexibility to integrate internal and external stimuli to regulate virtually every physiological and behavioral process in the adult (Beets and Watteyne 2025; Jékely and Yuste 2024; Nässel and Zandawala 2019, 2022). During development, the same synapse-free access to neuromodulation would allow neuropeptide signaling to persist through on-going synaptogenesis, resolving the conceptual tension borne by developmental activity as a process tasked with refining synaptic connections it depends on. The possible precedence of neuropeptide signaling in developmental activity also aligns, in ontogeny recapitulates phylogeny fashion, with the chemical brain hypothesis (Jékely 2021). According to this view, neuropeptide signaling, which may predate metazoan origins (Yañez-Guerra et al. 2022), organized the earliest neural

networks prior to the emergence of synaptic transmission, after which neuropeptides took on the more familiar modulatory role.

Activity in the developing mammalian brain has been studied in discrete regions, and, in some cases, circuits or mechanisms which serve as local pattern generators have been identified (Blankenship and Feller 2010). How these distinct, local activity profiles are coordinated to mediate circuit integration (Guillamón-Vivancos et al. 2022) across disparate areas of the brain is not known. In the much smaller fly system, we show that cross brain coordination is driven by a CPG and relay circuit, the PSINA engine, which minimally comprises the SIFa and *Trpy*[±] neurons. Notably, silencing the central PSINA engine reveals local oscillators in the optic lobes (**Figure 3.3c**), suggesting that even at the scale of the fly, global coordination of local foci of activity is a built-in feature. SIFa neurons reside in the PI, the neuropeptide and hormone center of the fly brain, and functional homolog of the mammalian hypothalamus. It was recently reported that hypothalamic peptidergic neurons project into nearly all areas of the mouse brain (Jiao et al. 2025), confirming that the mammalian nervous system has the infrastructure to mediate long-range neuropeptide-mediated coordination; it remains to be seen whether this infrastructure is used during development.

Altering developmental activity is expected to affect behavior, and changes to spontaneous network activity in the developing mammalian cortex are thought to be linked to neurodevelopmental disorders (Wu et al. 2024). In the fly, disruption of SIFa-SIFaR signaling affects a range of behaviors; whether these phenotypes are due to loss of neuropeptide signaling in the adult or during development is a matter of interest (Huang et al. 2021). The advantages of the fly and our improving handle on the PSINA engine will make it possible to address how PSINA contributes to the emergence neurotypical behavior.

3.11 Methods

Experimental model and subject details

Flies were reared at 18°C, 25°C, or 29°C on standard cornmeal/molasses medium; developmental time was matched to the 25°C standard (1x) using relative rates of 0.5x and 1.25x for 18°C and 29°C (Bainbridge and Bownes 1981), respectively. Pupal development was staged relative to white pre-pupa formation (w.p.p., 0hAPF) or head eversion (h.e., 12hAPF). GAL4/UAS (A H Brand and Perrimon 1993) and LexA/LexAop (Lai and Lee 2006) expression systems were used to drive cell-type-specific transgene expression.

The available molecularly-defined null allele of *SIFaR*, *SIFaR*^[attP] (Deng et al. 2019), appeared to be homozygous lethal. However, complementation tests indicated that a second site mutation was the cause of the lethality, and we recovered a viable *SIFaR*^[attP] line through outcrosses. Complete genotypes used in each experiment can be found in Supplementary Table 1.

Widefield imaging

Bottle-scale (~100 virgins, 10-20 males) crosses were setup for each genotype of each experimental set and reared at 18°C-29°C depending on experimental design. Pupae were genotyped and staged at w.p.p. or h.e. All data shown were collected from female pupae; mutant phenotypes of *SIFa* and *SIFaR* (Fig.1) were also confirmed in males (not shown). Imaging sessions typically started ~50hAPF and lasted until eclosion (~100hAPF). To prepare the pupae for imaging, the cuticle around the head was removed with fine forceps and the animals were fixed to a metal plate (McMaster-Carr) with double-sided adhesive tape (3M). The metal plate was placed in a custom environmental chamber to maintain temperature and humidity. This chamber comprised: a PTC1 temperature-controlled breadboard (Thorlabs) to maintain sample temperature

at a designated set-point; a set of four 35-mm dishes filled with deionized water to maintain humidity; and a 150-mm petri-dish lid to provide enclosure. To improve imaging quality, a large format coverslip was fitted into a rectangular opening made in the lid. This coverslip sat in the optical path, between the pupae and the objective lens, and was treated with Barbasol shaving cream (Perio) to prevent condensation during temperature shifts.

Images were acquired using either a Zeiss (1- and 2-channel imaging) Axio Zoom.V16 epifluorescence microscope equipped with a Retiga R1 CCD Camera (QImaging) and X-Cite TURBO 6-Channel LED Light Source (Excelitas Technologies), or a Leica (1-channel imaging) M165FC epifluorescence microscope with a Leica.K5 Camera and CoolLED 3-Channel Light Source (CoolLED). Images were acquired at 0.5 Hz with 100ms exposure time for GCaMP6s and 200ms exposure time for jRCaMP1b (5-20mW under objective power). Acquisition was controlled by Slidebook software (Intelligent Imaging Innovations) on the Zeiss microscope and LAS X (Leica Inc) on the Leica microscope. Time series were processed with Fiji(Schindelin et al. 2012)(ImageJ) and analyzed using MATLAB (Mathworks); see below.

Immunofluorescence and confocal microscopy

Brains were dissected in cold Schneider Medium (Gibco 21720–024) and fixed with 4% v/v PFA (Electron Microscopy Sciences 15710) in Schneider Medium for 20 min at room temperature. Brains were then washed out of fixative into PBS (Quality Biological), solubilized in PBST (0.5% Triton-X100 (Sigma T9284) in PBS) for 1 h, and blocked in PBTN (5% Normal Donkey Serum (Jackson ImmunoResearch no. 017-000-121) in PBST) for 1-2 h, all at room temperature. Brains were sequentially incubated in primary and secondary antibodies diluted in PBTN for 24–48 h at 4 °C, with at least 3 washes through PBST over 2 h at room temperature in

between and afterwards. Brains were post-fixed with 4% v/v PFA in Schneider Medium for 20 min at room temperature, followed by multiple washes into PBST over 10 min. Brains were finally transferred to Everbrite mounting medium (Biotium 23001) and mounted on to slides for imaging.

Primary antibodies and dilutions used in this study were as follows: chicken anti-GFP (Abcam ab13970, 1:1,000), mouse monoclonal anti-V5 (Novus Biologicals NBP2-52703–0.2 mg, 1:150), rat monoclonal anti-Ncad (DSHB DN-Ex 8-c, 1:100), mouse monoclonal anti-Brp (DSHB nc82-c, 1:150), and rabbit polyclonal anti-SIFamide(Terhzaz et al. 2007) (1:1,000).

Secondary antibodies and dilutions used in this study were as follows: Alexa 488 donkey polyclonal anti-chicken (Jackson ImmunoResearch 703-545-155, 1:400), Alexa 488 donkey polyclonal anti-mouse (Jackson ImmunoResearch 715-545-151, 1:400), Alexa 488 donkey polyclonal anti-rabbit (Jackson ImmunoResearch 712-545-153, 1:400), Alexa 568 donkey polyclonal anti-mouse (Jackson ImmunoResearch 715-575-150, 1:400), Alexa 568 donkey polyclonal anti-rabbit (Invitrogen A10042, 1:400), Alexa 647 donkey polyclonal anti-rat (Jackson ImmunoResearch 712-605-153, 1:400), and Alexa 647 donkey polyclonal anti-rabbit (Jackson ImmunoResearch 711-605-152, 1:400)

Immunofluorescence images were acquired using a Zeiss LSM 780 confocal microscope with 20×/0.8 air, 40×/1.4 oil immersion or 40×/1.2 glycerol immersion objectives (Zeiss). Images were prepared for publication using Fiji(Schindelin et al. 2012)(ImageJ).

Analysis of widefield imaging data

User-defined ROIs from time series were selected in Fiji(Schindelin et al. 2012) (ImageJ). Custom scripts written in MATLAB (Mathworks) were used in all subsequent analysis. Raw fluorescence traces from per frame ROI pixel averages (F) were fit to a baseline (F_0) and

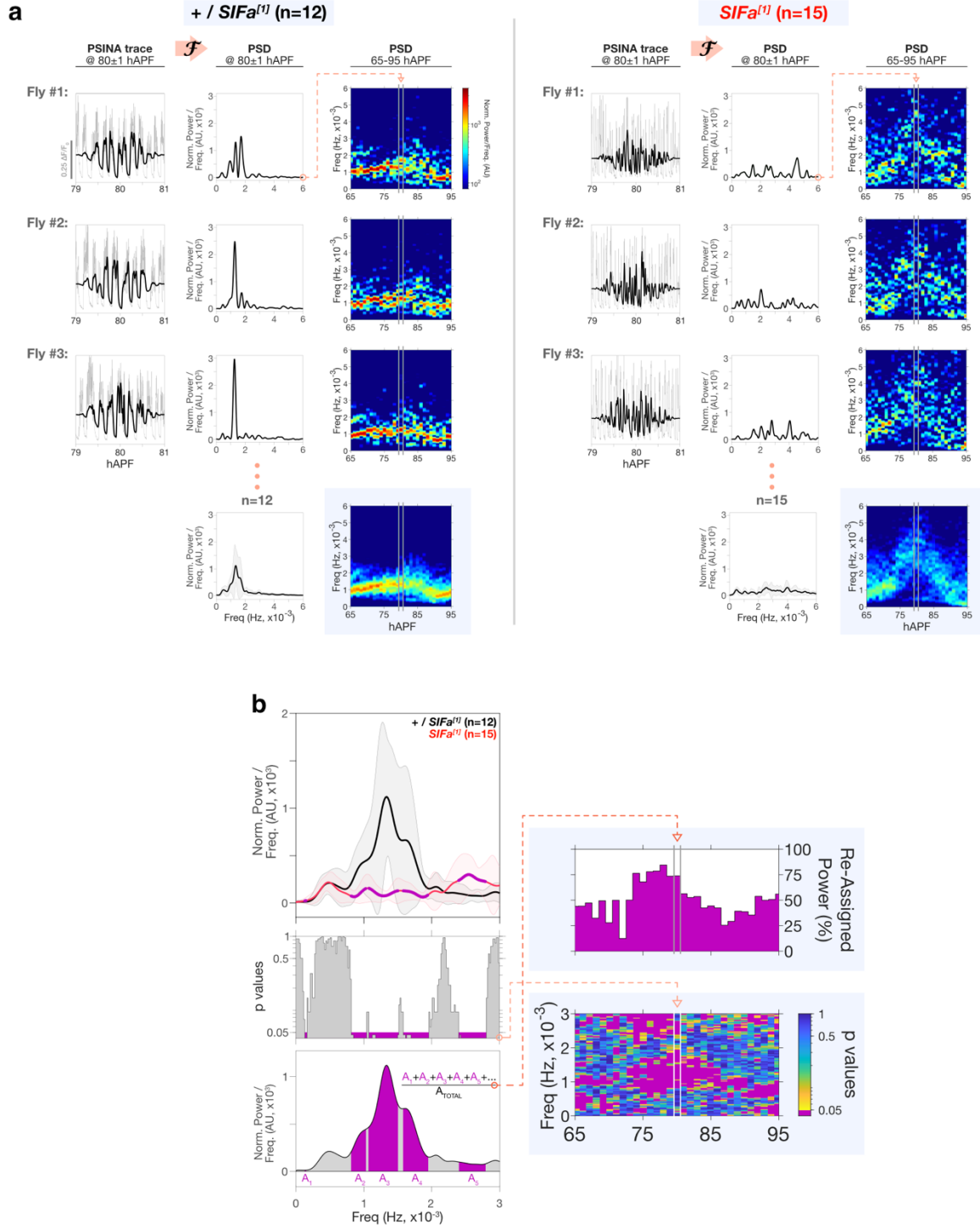
$\Delta F/F_0$ (signal) was calculated by subtracting the baseline from the raw signal and dividing by the baseline. Between 75-85hAPF, the raw fluorescence recorded from intact pupae exhibits a stereotyped sigmoidal decrease in intensity correlated with the maturation of the cuticle around the head. By comparing this trend, captured by the baseline fit (F_0), to staged animals, we found that the inflection point of the curve corresponds to ~ 80 hAPF. In all analyses, this internal, genotype-independent feature was used to synchronize the developmental ages of the animals. The pace of PSINA (e.g., cycle frequency) is temperature sensitive. To compare data collected at different temperatures, we scaled the time axes of the recordings to match the 25°C standard (1x) using the coefficient $T_{\text{Coef}} = 100 / (-0.0465 * T^3 + 4.263 * T^2 - 133.49 * T + 1500)$, where T is the recording temperature in degrees Celsius. The formula is derived from the best fit third order polynomial to the measured temperature-dependent duration of metamorphosis (Bainbridge and Bownes 1981). Active phase limits were defined using a threshold set to 4% of average maximum signal recorded from control genotypes with a minimum floor of 0.5% $\Delta F/F_0$ for low signal-to-noise data (e.g., SIFa neuron recordings).

PSDs were calculated for each hour of recording using a 2-h sliding window (Uhlén 2004). In preparation for Fast Fourier Transform and PSD estimation, each 2-h segment was filtered to isolate target frequencies (standard 120s Gaussian window to remove sweeps or defined low pass cutoffs as noted in Figure legends), centered on the time axis by subtracting its mean, and multiplied with the Hanning window function to reduce spectral leakage. The PSD for each 2-hr segment was normalized relative to its discrete integral over all frequencies; this step effectively removes the contribution of signal amplitude changes to the PSD in order to focus on frequency changes for subsequent comparisons. Each experimental condition thus yielded a distribution of normalized PSD values (as many as replicate number) at each frequency-time coordinate.

Statistical tests between different experimental conditions were carried out between these distributions. PSDs from all replicates of an experimental condition were averaged to generate the average PSD panels shown in the Figures. All statistical analyses were carried out using the non-parametric two-sample Kolmogorov-Smirnov test; p values less than 0.05 were considered significant.

3.12 Extended data figures

Extended data Figure 3.1

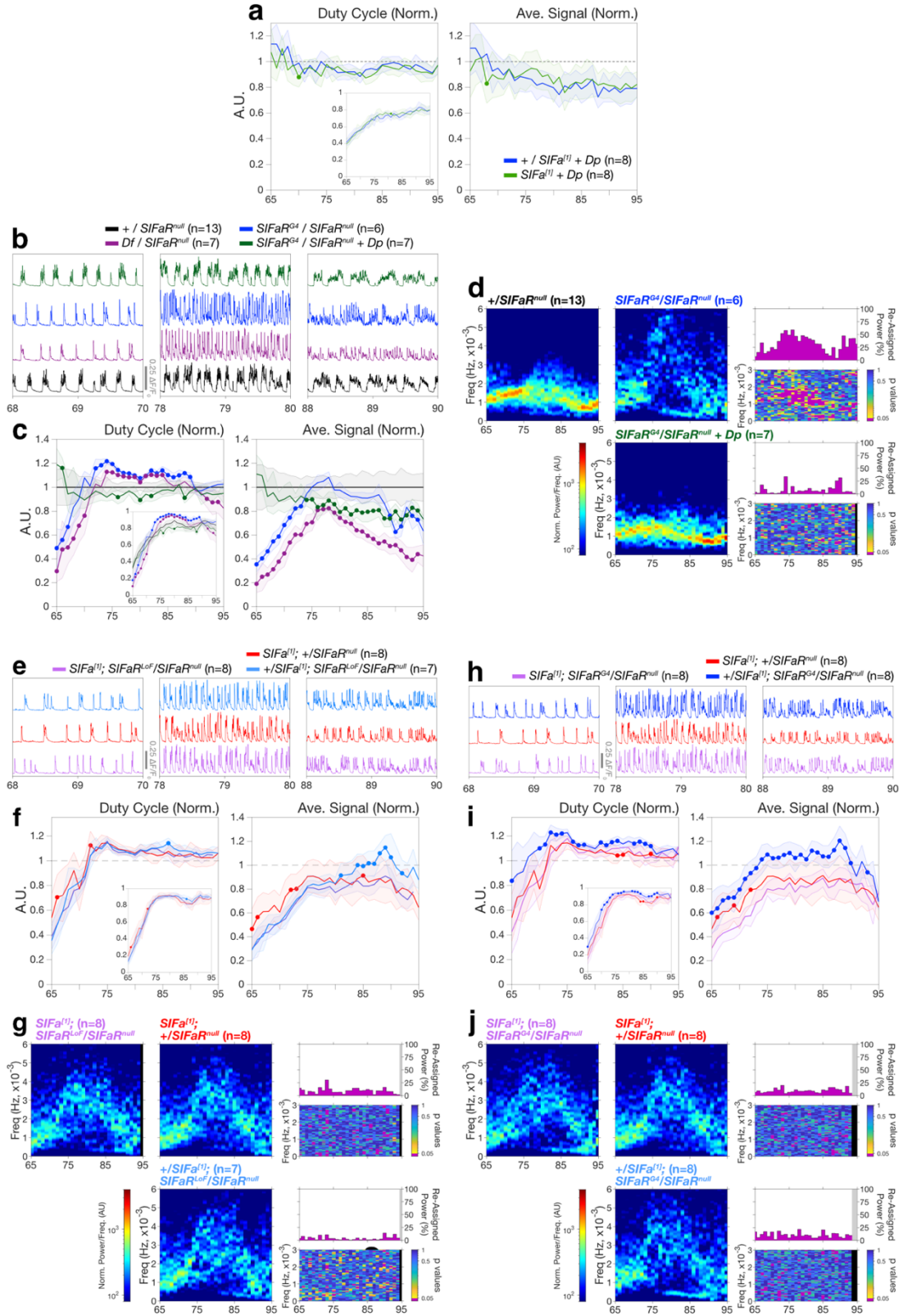


Extended Data Figure 3.1. Power Spectral Density based analysis of PSINA; related to Figure 3.1

Data displayed in Fig.1g ($+/\text{SIFa}^l$ v. SIFa^l ; framed in light blue) is used to illustrate the steps of our PSD-based analysis. **a.** For each fly (e.g., Fly#1) of a genotype ($+/\text{SIFa}^l$ v. SIFa^l), a 2-h long PSINA segment (light gray traces in first column of plots) centered around each hour of recording (e.g., 80hAPF) was processed (black overlaid traces, see Methods) to prepare for Discrete Fourier Transform ('F') and PSD calculation (second column of plots). Concatenating these hourly PSDs (third column of plots) show how the PSINA signal is distributed across different frequencies from 65-95 hAPF. Average hourly PSDs (bottom row of second column; shaded areas, s.d.) were calculated for each genotype and similarly concatenated to generate the PSD representation (bottom row of third column) shown in main figures. This heat map plot only displays the average and not the spread (e.g., s.d.) of the PSD distribution calculated for each genotype. Note that the heat map values for (Normalized Power / Freq.) are plotted in log scale to better display the PSD dynamic range. **b.** *First column, top plot:* The 80hAPF PSD distributions from $+/\text{SIFa}^l$ (black, n=12) and SIFa^l (red, n=15) animals in (a) are overlaid; shaded areas, s.d. Bold line segments (magenta) mark where the SIFa^l distribution differs from $+/\text{SIFa}^l$ ($p < 0.05$) by two-sample KS test. *First column, center plot:* p values calculated at 80hAPF across the displayed frequency range are plotted. Magenta flood line marks where $p < 0.05$; these regions correspond to the bold line segments in the top plot. *Second column, bottom plot:* Hourly p values are concatenated to show when and at what frequency PSINA in the experimental (SIFa^l) genotype differs from control ($+/\text{SIFa}^l$). *First column, bottom plot:* The fraction of normalized signal power in the control PSD at frequencies where the experimental PSD is significantly different is calculated by taking the ratio of the area under the curve at $p < 0.05$ regions to the total area. We defined this fraction, or

100x of this fraction, as percent of control signal power re-assigned to different frequencies. This scalar metric offers a salience measure to the statistical test by describing how much of PSINA power is lost to changes in the PSD. *Second column, top plot:* Hourly re-assigned power values are concatenated across developmental time.

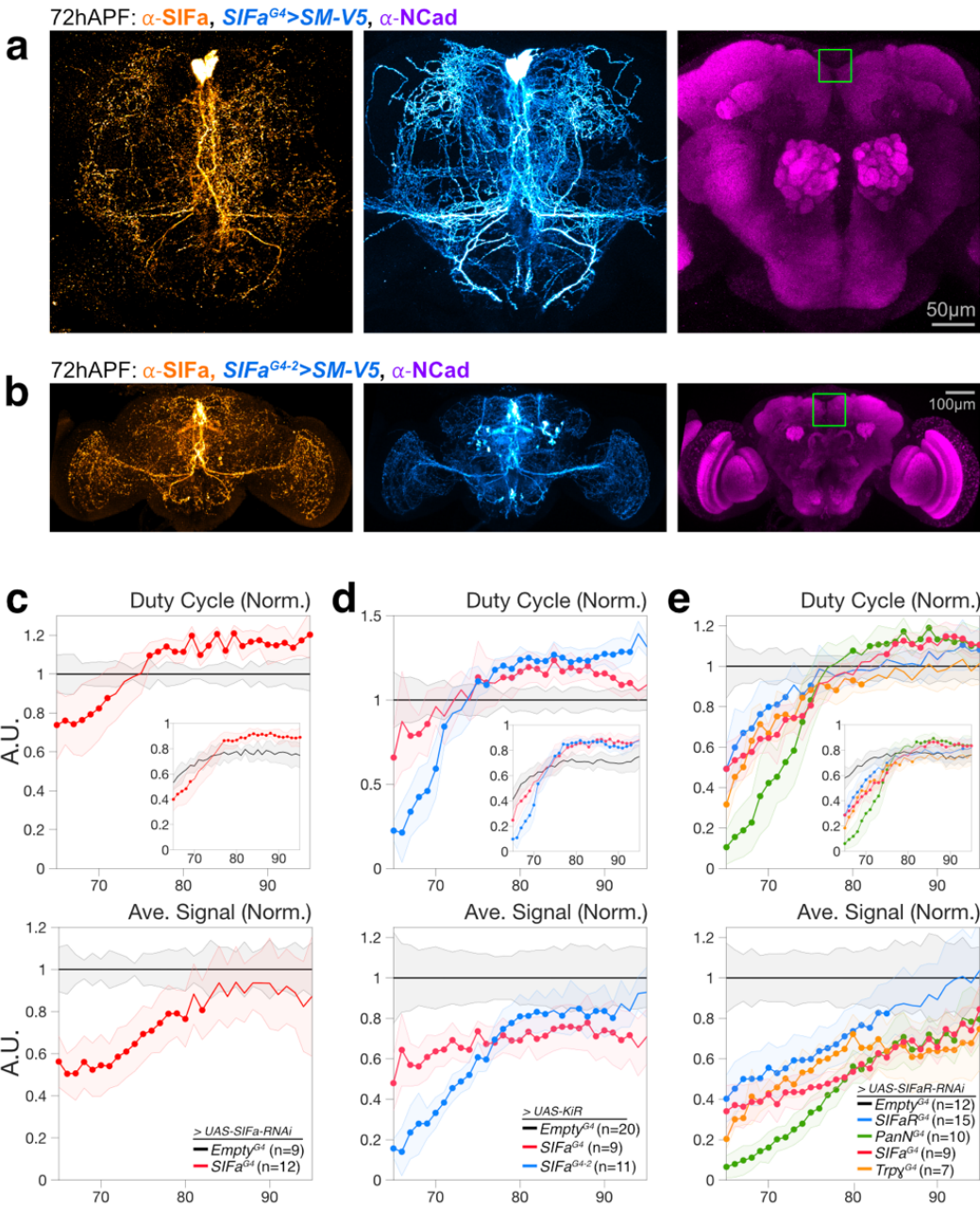
Extended data Figure 3.2



Extended Data Figure 3.2. *SIFa* and *SIFaR* genetic analysis; Related to Figure 3.1

a. Duty cycle and average signal of indicated genotypes, normalized to control in Fig.1e. Inset in duty cycle plots true values of metric. Shaded areas, s.d. Closed circles mark where $+/SIFa[1] + Dp$ (blue) distribution differs from $SIFa[1] + Dp$ (green, reproduced from Fig.1f) ($p < 0.05$) by two-sample KS test. The similarity of these data suggests that the differences between genomic rescue and control (Fig.1f) is due to positional effects from the relocated genomic duplication. **b-d.** *SIFaR* mutant analysis with *SIFaR^{G4}*; data presented as in Fig.1e-g. **e-g.** Double mutant analysis with *SIFaR^{LoF}*; data presented as in Fig.1e-g. Statistical tests carried out against double mutant (magenta) in (f,g). **h-j.** Double mutant analysis with *SIFaR^{TG4}*; data presented as in Fig.1e-g. Statistical tests carried out against double mutant (magenta) in (i,j).

Extended Data Figure 3.3

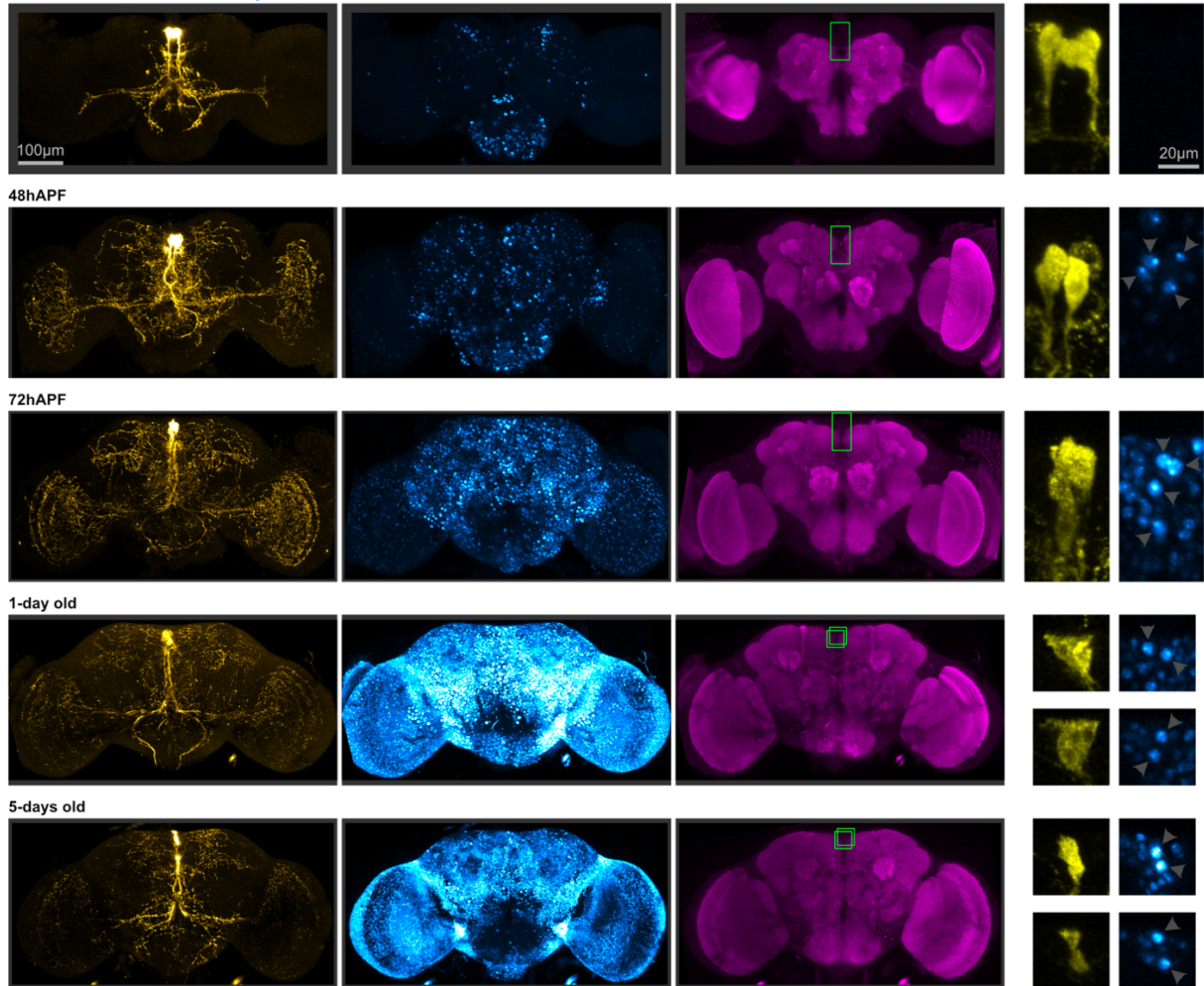


Extended Data Figure 3.3 Related to Figure 3.2.

a. MIP of 72hAPF central brain with *SIFa*^{G4} driving *myr::SM-V5* (cyan-hot LUT); brain also stained against SIFa (orange-hot LUT) and N-Cadherin (magenta). Green square marks location of SIFa soma. Scale bar, 50μm. **b.** MIP of 72hAPF brain with *SIFa*^{G4-2} (i.e., *14F05*^{G4}) driving *myr::SM-V5* (cyan-hot LUT); brain also stained against SIFa (orange-hot LUT) and N-Cadherin (magenta). Green square marks location of SIFa soma. Scale bar, 100μm. **c-e.** Duty cycle and average signal from **c.** *SIFa* knockdown (Fig.2b,c), **d.** Kir2.1 expression in SIFa neurons (Fig.2d,e), **e.** *SIFaR* knockdown (Fig.2f,g) experiments.

Extended Data Figure 3.4

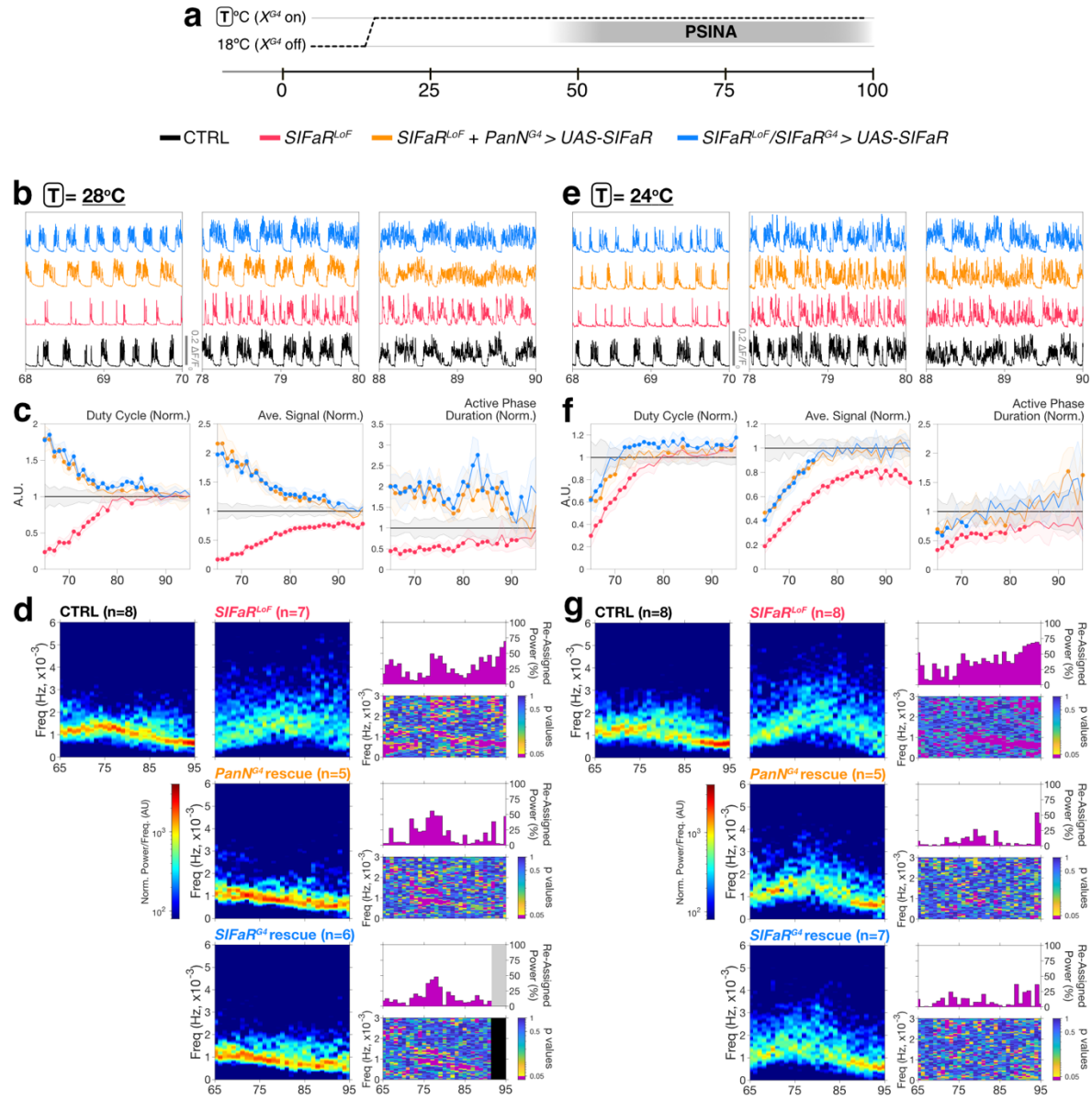
24hAPF α -SIFa, *SIFaR^{G4}*>*mCherry.NLS*, α -NCad



Extended Data Figure 3.4. *SIFaR^{TG4}* developmental expression domain.

Left: MIPs of brains at the indicated ages with *SIFaR^{TG4}* driving *mCherry.NLS* (native fluorescence, cyan-hot LUT); brain also stained against SIFa (yellow-hot LUT) and N-Cadherin (magenta). Green boxes mark location of SIFa soma panels in *Right*. Raw image stacks acquired with 20X objective using consistent image acquisition settings; *mCherry.NLS* and SIFa channel MIPs displayed with same contrast settings. Scale bar, 100µm. *Right:* MIPs of SIFa soma (yellow) and *mCherry.NLS* (native fluorescence, cyan-hot LUT) from brains in *Left*. Raw image stacks acquired with 40X objective using consistent image acquisition settings; SIFa channel MIPs displayed with same contrast settings. Arrowheads mark SIFa neuron nuclei. Scale bar, 20µm.

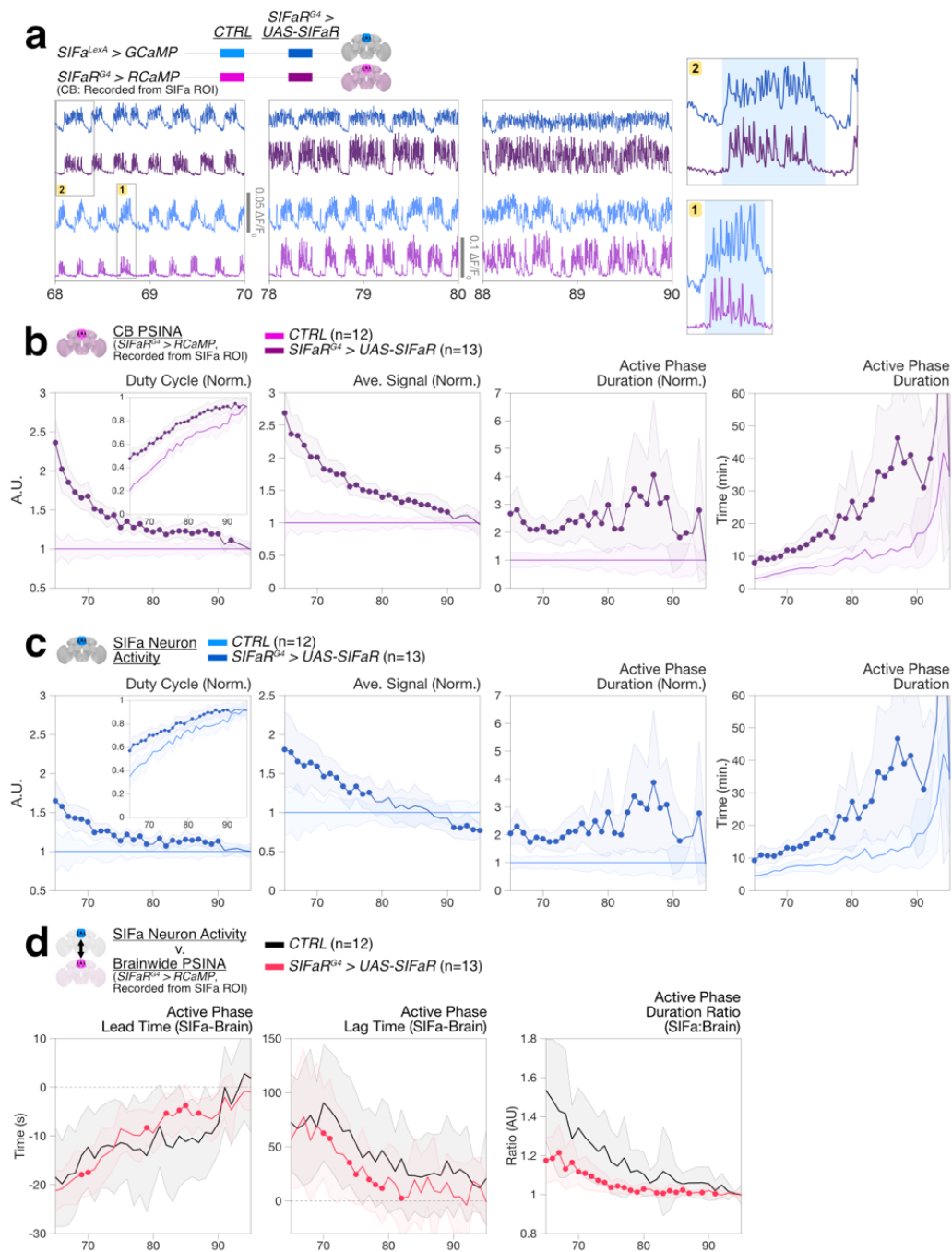
Extended Data Figure 3.5



Extended Data Figure 5. Temporally targeted and tuned rescue of PSINA in *SIFaR* mutants.

a. Temperature schedule and genotypes. **b-d.** *GAL80ts* targeted *GAL4-UAS* rescue of *SIFaR* phenotype at 28°C. **b.** PSINA traces, color-matched to genotypes in (a). **c.** Duty cycle, average signal, and active phase duration, all normalized to control genotype (black). Plots color-matched to genotypes in (a); sample sizes indicated in (d). Shaded areas, s.d. Closed circles mark where experimental distributions differ from control ($p < 0.05$) by two-sample KS test. **d.** Average PSDs from control and experimental animals; presented as in Fig.1g. **e-g.** *GAL80ts* targeted *GAL4-UAS* rescue of *SIFaR* phenotype at 24°C; presented as in (b-d).

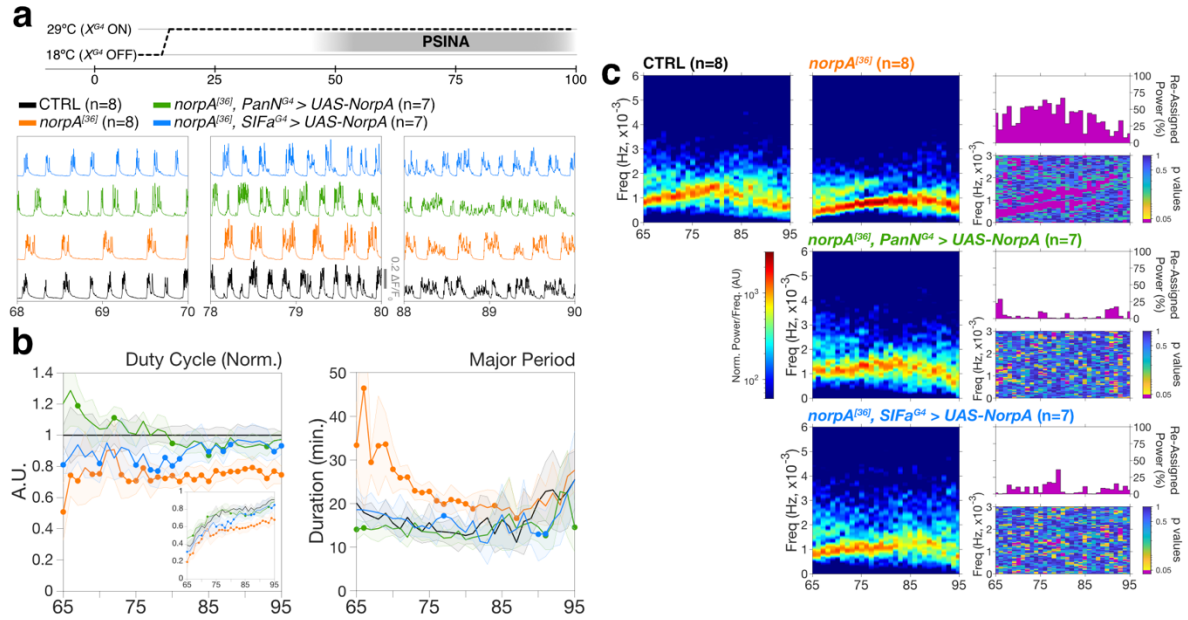
Extended Data Figure 3.6



Extended Data Figure 3.6. SIFaR overexpression increases active phase duration; Related to Figure 3.4.

a. *Left:* Representative traces of SIFa neuron activity (cyan and dark blue) and brainwide PSINA (magenta and purple) in CTRL animals or with SIFaR overexpression. PSINA, as reported by *SIFaR^{G4}>RCaMP*, was recorded from the SIFa neuron ROI. *Right:* Expanded views for numbered boxes in *left*; SIFa neuron active phases demarcated in light blue shaded region. **b,c.** Normalized duty cycle, average signal, and active phase duration, and true-valued duty cycle (inset) and active phase duration plotted for the indicated conditions. Shaded areas, s.d. Closed circles mark where the experimental distributions differ from CTRL ($p<0.05$) by two-sample KS test. **d.** Active phase rise and fall time differences and ratio of active phase durations between SIFa neurons and brainwide PSINA (recorded from SIFa neuron ROI) in CTRL (black) animals or with SIFaR overexpression (red). ~20 data points (range: 8-56) per hourly distribution compiled from each experimental set. Shaded areas, s.d. Closed circles mark where the red distributions differ from black ($p<0.05$) by two-sample KS test. Both genotypes included the same *GAL80ts* allele (see Supplementary Table S1); rearing and data collection were carried out at 29°C to allow for uninhibited expression of the UAS construct.

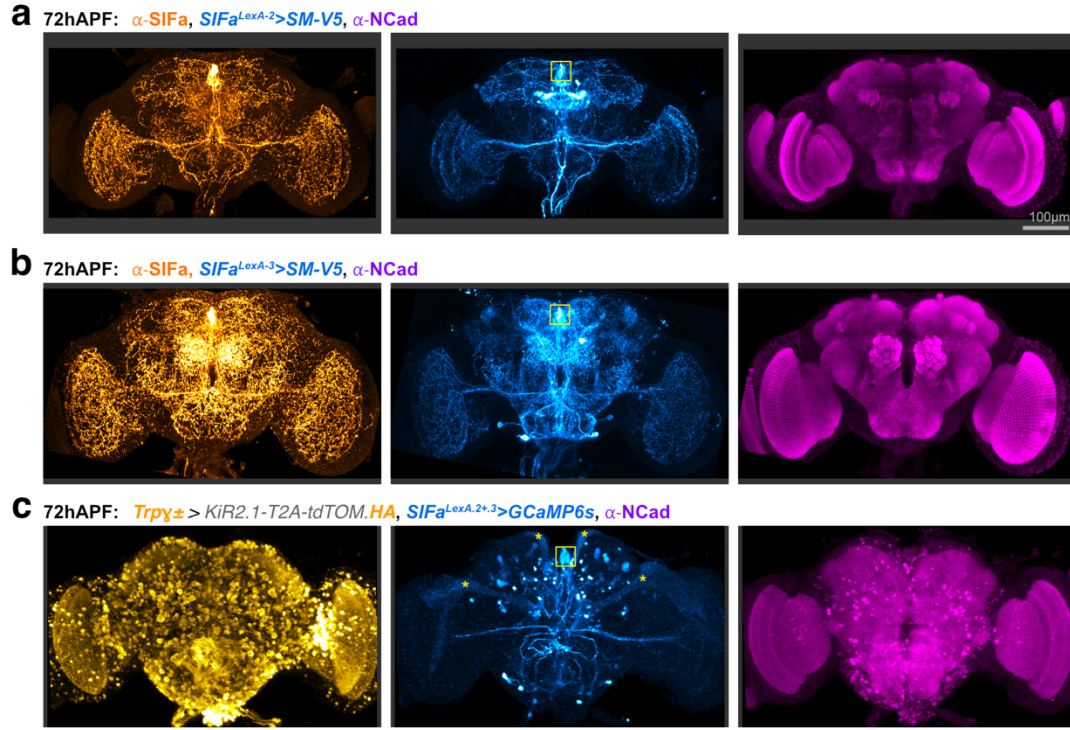
Extended Data Figure 3.7



Extended Data Figure 7. *NorpA* expression in SIFa neurons rescues major *norpA* mutant phenotype.

a. Top: Temperature schedule used. **Bottom:** Representative PSINA traces at 29°C for the indicated genotypes. **b.** Duty cycle, normalized to control, and major period duration for the genotypes and sample sizes in (a). Inset in duty cycle plots true values of metric. Major period corresponds to the dominant frequency in the PSD. Shaded areas, s.d. Closed circles mark where experimental distributions differ from control ($p < 0.05$) by two-sample KS test. **c.** Average PSDs from control and experimental animals; presented as in Fig.1g.

Extended Data Figure 3.8



Extended Data Figure 3.8. *SIFa*^{LexA} drivers; Related to Figure 3.5.

a. MIP of 72hAPF brain with *SIFa*^{LexA.2} (i.e., *14F05*^{LexA}) driving *myr::SM-V5* (cyan-hot LUT); brain also stained against SIFa (orange-hot LUT) and N-Cadherin (magenta). Yellow square marks location of SIFa soma. Scale bar, 100µm. **b.** MIP of 72hAPF brain with *SIFa*^{LexA.3} (i.e., *59A06*^{LexA}) driving *myr::SM-V5* (stained, cyan-hot LUT); brain also stained against SIFa (orange-hot LUT) and N-Cadherin (magenta). Yellow square marks location of SIFa soma. **c.** MIP of a 72hAPF brain with *SIFa*^{LexA.2+.3} driving *LexAop-GCaMP6s* (anti-GFP, cyan-hot LUT) and *Trpy*[±] driving *UAS-Kir2.1-T2A-tdTOM.HA* (anti-HA, orange-hot LUT). Brain also stained against N-Cadherin (magenta); native fluorescence of *tdTOM* is also visible in this channel. Yellow square marks location of SIFa soma. Yellow asterisks mark trachea, visible due to autofluorescence.

Supplementary Table 1 Chapter 3 genotypes

Figure	Label in Figure	Genotype	Notes
3.1b-c		<i>w</i> ; <i>SIFa[1] / +</i> ; <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / +</i>	
3.1d		<i>w</i> ; <i>Sp-CyO / +</i> ; <i>UAS-mCherry.NLS / SIFaR[MI05376-TG4.1]</i>	
3.1e-g	+ / SIFa[1]	<i>w</i> ; <i>SIFa[1] / +</i> ; <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / +</i>	
	Df / SIFa[1]	<i>w</i> ; <i>SIFa[1] / Df(2R)BSC780</i> ; <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / +</i>	
	SIFa[1]	<i>w</i> ;	

		<i>SIFa[1];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / +</i>	
	SIFa[2] / SIFa[1]	<i>w;</i> <i>SIFa[2] / SIFa[1];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / +</i>	
	SIFa[1] + Dp	<i>w;</i> <i>SIFa[1];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) /</i> <i>Dp(2;3)GV-CH321-58A04 (VK31)</i>	
3.1h-j	+ / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-opGCaMP6s (su(Hw)attP5) / +;</i> <i>SIFaR[attP] / +</i>	FLP element not used in experiments
	Df / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-opGCaMP6s (su(Hw)attP5) / +;</i>	FLP element not used in experiments

		<i>SIFaR[attP] / Df(3R)ED10845</i>	
	SIFaR LoF / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-opGCaMP6s (su(Hw)attP5) / +;</i> <i>SIFaR[attP] / SIFaR[MI05376-GFSTF.1]</i>	FLP element not used in experiments
	SIFaR LoF / SIFaR null + Dp	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-opGCaMP6s (su(Hw)attP5) /</i> <i>Dp(3;2)GV-CH321-04D21 (VK37);</i> <i>SIFaR[attP] / SIFaR[MI05376-GFSTF.1]</i>	FLP element not used in experiments
3.2b,c	Empty G4	<i>UAS-Dcr2, w / w;</i> <i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-</i> <i>opGCaMP6s (su(Hw)attP5) / +;</i> <i>TRiP.JF03364 / empty-GAL4 (attP2)</i>	
	SIFa G4	<i>UAS-Dcr2, w / w;</i>	

		<i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / SIFa-GAL4;</i> <i>TRiP.JF03364 / +</i>	
3.2d,e	Empty G4	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / w;</i> <i>tubP-GAL80[ts](10) / +;</i> <i>UAS-His-RFP, 13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / empty-GAL4 (attP2)</i>	
	SIFa G4	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / w;</i> <i>tubP-GAL80[ts](10) / SIFa-GAL4;</i> <i>UAS-His-RFP, 13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / +</i>	
	SIFa G4- 2	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / w;</i> <i>tubP-GAL80[ts](10) / +;</i> <i>UAS-His-RFP, 13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / R14F05-GAL4 (attP2)</i>	
3.2f,g		<i>UAS-Dcr2, w / w;</i>	

Empty G4	<i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / +;</i> <i>TRiP.JF01849 / empty-GAL4 (attP2)</i>
SIFaR G4	<i>UAS-Dcr2, w / w;</i> <i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / Sp-CyO;</i> <i>TRiP.JF01849 / SIFaR[MI05376-TG4.1]</i>
PanN G4	<i>UAS-Dcr2, w / w;</i> <i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / R57C10-GAL4 (JK22C);</i> <i>TRiP.JF01849 / SIFaR[attP]</i>
Trpy G4	<i>UAS-Dcr2, w / w;</i> <i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / TrpGamma[G4];</i> <i>TRiP.JF01849 / SIFaR[attP]</i>
SIFa G4	<i>UAS-Dcr2, w / w;</i>

		<i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / SIFa-GAL4;</i> <i>TRiP.JF01849 / SIFaR[attP]</i>	
3.2h	Empty G4	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / w;</i> <i>tubP-GAL80[ts](10) / +;</i> <i>UAS-His-RFP, 13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / empty-GAL4 (attP2)</i>	
	SIFaR G4	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / w;</i> <i>tubP-GAL80[ts](10) / Sp-CyO;</i> <i>UAS-His-RFP, 13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / SIFaR[MI05376-TG4.1]</i>	
3.3b-e	Empty G4 (CB)	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / R29C07-FLP1</i> <i>(su(Hw)attP8);</i> <i>tubP-GAL80[ts](10), tubP(FRT.stop)GAL80;2 / +;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / empty-GAL4 (attP2)</i>	

	PanN G4 (CB)	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / R29C07-FLP1</i> <i>(su(Hw)attP8);</i> <i>tubP-GAL80[ts](10), tubP(FRT.stop)GAL80}2 / +;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) /</i> <i>R57C10-GAL4 (attP2)</i>
	Trpy G4 (CB)	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / R29C07-FLP1</i> <i>(su(Hw)attP8);</i> <i>tubP-GAL80[ts](10), tubP(FRT.stop)GAL80}2 / TrpGamma[G4];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / +</i>
	SIFaR G4 (CB)	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / R29C07-FLP1</i> <i>(su(Hw)attP8);</i> <i>tubP-GAL80[ts](10), tubP(FRT.stop)GAL80}2 / +;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) /</i> <i>SIFaR[MI05376-TG4.1]</i>
	Empty G4 (OL)	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / R29C07-FLP1</i> <i>(su(Hw)attP8);</i>

	<i>tubP-GAL80[ts](10), alphaTub84B(FRT.GAL80)}2 / +;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) /</i> <i>empty-GAL4 (attP2)</i>
PanN G4 (OL)	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / R29C07-FLP1</i> <i>(su(Hw)attP8);</i> <i>tubP-GAL80[ts](10), alphaTub84B(FRT.GAL80)}2 / +;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) /</i> <i>R57C10-GAL4 (attP2)</i>
Trpy G4 (OL)	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / R29C07-FLP1</i> <i>(su(Hw)attP8);</i> <i>tubP-GAL80[ts](10), alphaTub84B(FRT.GAL80)}2 / TrpGamma[G4];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / +</i>
SIFaR G4 (OL)	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / R29C07-FLP1</i> <i>(su(Hw)attP8);</i> <i>tubP-GAL80[ts](10), alphaTub84B(FRT.GAL80)}2 / +;</i>

		<i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / SIFaR[MI05376-TG4.1]</i>	
3.3f-h	CTRL (CB)	<i>UAS-Dcr2, w / R29C07-FLP1 (su(Hw)attP8); tubP-GAL80[ts](10), tubP(FRT.stop)GAL80}2 / +; 13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / TRiP.JF01849</i>	
	PanN G4 (CB)	<i>UAS-Dcr2, w / R29C07-FLP1 (su(Hw)attP8); tubP-GAL80[ts](10), tubP(FRT.stop)GAL80}2 / R57C10-GAL4 (JK22C); 13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / TRiP.JF01849</i>	
	CTRL (OL)	<i>UAS-Dcr2, w / R29C07-FLP1 (su(Hw)attP8); tubP-GAL80[ts](10), alphaTub84B(FRT.GAL80)}2 / +; 13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / TRiP.JF01849</i>	
		<i>UAS-Dcr2, w / R29C07-FLP1 (su(Hw)attP8);</i>	

	PanN G4 (OL)	<i>tubP-GAL80[ts](10), alphaTub84B(FRT.GAL80)}2 / R57C10-GAL4 (JK22C);</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / TRiP.JF01849</i>	
3.4a-g	CTRL	<i>13XLexAop2-opGCaMP6s (?, su(Hw)attp8) / w;</i> <i>SIFa[1] / +;</i> <i>R57C10-GAL4(attP2), 20XUAS-IVS-NES-jRCaMP1b-p10 (VK5) /</i> <i>SIFa-LexA.GAD</i>	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions
	SIFa null	<i>13XLexAop2-opGCaMP6s (?, su(Hw)attp8) / w;</i> <i>SIFa[1];</i> <i>R57C10-GAL4(attP2), 20XUAS-IVS-NES-jRCaMP1b-p10 (VK5) /</i> <i>SIFa-LexA.GAD</i>	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions

3.4h,i	CTRL	<i>13XLexAop2-opGCaMP6s</i> (? , <i>su(Hw)attp8</i>) /w; <i>SIFa[1]</i> / +; <i>R57C10-GAL4(attP2)</i> , <i>20XUAS-IVS-NES-jRCaMP1b-p10 (VK5)</i> / <i>SIFa-LexA.GAD</i>	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions
	TrpA1	<i>13XLexAop2-opGCaMP6s</i> (? , <i>su(Hw)attp8</i>) /w; <i>SIFa[1]</i> / <i>13XLexAop2-IVS-dTrpA1-WPRE (su(Hw)attP5)</i> ; <i>R57C10-GAL4(attP2)</i> , <i>20XUAS-IVS-NES-jRCaMP1b-p10 (VK5)</i> / <i>SIFa-LexA.GAD</i>	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions
3.4j-l	Het, CTRL	<i>13XLexAop2-opGCaMP6s</i> (? , <i>su(Hw)attp8</i>) / w; <i>SIFa[1]</i> / +;	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions

		<i>R57C10-GAL4(attP2), 20XUAS-IVS-NES-jRCaMP1b-p10 (VK5) / SIFa-LexA.GAD</i>	
Het, TrpA1		<i>13XLexAop2-opGCaMP6s (?, su(Hw)attP8) / w;</i> <i>SIFa[1] / 13XLexAop2-IVS-dTrpA1-WPRE (su(Hw)attP5);</i> <i>R57C10-GAL4(attP2), 20XUAS-IVS-NES-jRCaMP1b-p10 (VK5) / SIFa-LexA.GAD</i>	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions
SIFa null, CTRL		<i>13XLexAop2-opGCaMP6s (?, su(Hw)attP8) / w;</i> <i>SIFa[1];</i> <i>R57C10-GAL4(attP2), 20XUAS-IVS-NES-jRCaMP1b-p10 (VK5) / SIFa-LexA.GAD</i>	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions

	SIFa null, TrpA1	<i>13XLexAop2-opGCaMP6s</i> (? , <i>su(Hw)attp8</i>) / <i>w</i> ; <i>SIFa[1]</i> / <i>13XLexAop2-IVS-dTrpA1-WPRE</i> (<i>su(Hw)attP5</i>), <i>SIFa[1]</i> ; <i>R57C10-GAL4(attP2)</i> , <i>20XUAS-IVS-NES-jRCaMP1b-p10 (VK5)</i> / <i>SIFa-LexA.GAD</i>	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions
3.5b-e	CTRL	<i>13XLexAop2-opGCaMP6s</i> (? , <i>su(Hw)attp8</i>) / <i>w</i> ; <i>R14F05-lexA (attP40)</i> , <i>Trpy[CR70048-TG4.2]</i> / <i>R59A06-LexA</i> (<i>attP40</i>); <i>8XLexAop2-IVS-GAL80-WPRE (attP2)</i> / +	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions
	Trpy $\pm$ > KiR2.1	<i>13XLexAop2-opGCaMP6s</i> (? , <i>su(Hw)attp8</i>) / <i>yw</i> , <i>UAS-Kir2.1-2A-</i> <i>tdTOM-3xHA (attP3)</i> ; <i>R14F05-lexA (attP40)</i> , <i>Trpy[CR70048-TG4.2]</i> / <i>R59A06-LexA</i> (<i>attP40</i>);	

		<i>8XLexAop2-IVS-GAL80-WPRE (attP2) / +</i>	
3.6a-c	CTRL	w; <i>UAS-SIFaR / +;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / empty-GAL4 (attP2)</i>	
	OE: SIFa G4>UAS-SIFaR	w; <i>UAS-SIFaR / SIFa-GAL4;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / +</i>	
	SIFaR LoF/ SIFaR null	w; <i>UAS-SIFaR / +;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / SIFaR[MI05376-GFSTF.1]</i>	
		w; <i>UAS-SIFaR / SIFa-GAL4;</i>	

	+ SIFa G4>UAS- SIFaR	<i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / SIFaR[MI05376-GFSTF.1]</i>	
	+Trpy G4 >UAS- SIFaR	w; <i>UAS-SIFaR / TrpGamma[G4];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / SIFaR[MI05376-GFSTF.1]</i>	
	+Trpy G4.2 >2x UAS- SIFaR	w; <i>UAS-SIFaR / UAS-SIFaR, Trpy[CR70048-TG4.2];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / SIFaR[MI05376-GFSTF.1]</i>	
3.7a-c	CTRL	w, <i>20XUAS-IVS-opGCaMP6s (su(Hw)attP8) / w;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-jRCaMP1b (su(Hw)attP5),</i> <i>SIFa[1] / Trpy[CR70048-TG4.2];</i> <i>20XUAS-IVS-opGCaMP6s (su(Hw)attP1) / +</i>	Fluorescent eye marker was removed from Trpy[CR70048-TG4.2] using Cre

	SIFa null	<i>w</i> , 20 <i>XUAS-IVS-opGCaMP6s (su(Hw)attP8)</i> / <i>w</i> ; <i>R57C10-lexA (attP40)</i> , 13 <i>XLexAop2-jRCaMP1b (su(Hw)attP5)</i> , <i>SIFa[1]</i> / <i>Trpy[CR70048-TG4.2]</i> , <i>SIFa[2]</i> ; 20 <i>XUAS-IVS-opGCaMP6s (su(Hw)attP1)</i> / +	Fluorescent eye marker was removed from <i>Trpy[CR70048-TG4.2]</i> using Cre
ED3.1a,b	+ / SIFa[1]	<i>w</i> ; <i>SIFa[1]</i> / +; 13 <i>XLexAop2-opGCaMP6s (su(Hw)attP1)</i> , <i>R57C10-lexA (VK20)</i> / +	
	SIFa[1]	<i>w</i> ; <i>SIFa[1]</i> ; 13 <i>XLexAop2-opGCaMP6s (su(Hw)attP1)</i> , <i>R57C10-lexA (VK20)</i> / +	
ED3.2a	+ / SIFa[1] + Dp	<i>w</i> ; <i>SIFa[1]</i> / <i>Sp - CyO - +</i> ; 13 <i>XLexAop2-opGCaMP6s (su(Hw)attP1)</i> , <i>R57C10-lexA (VK20)</i> / <i>Dp(2;3)GV-CH321-58A04 (VK31)</i>	
		<i>w</i> ;	Same as in Fig1e-g

	SIFa[1] + Dp	<i>SIFa[1];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) /</i> <i>Dp(2;3)GV-CH321-58A04 (VK31)</i>	
ED3.2b-d	+ / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-opGCaMP6s (su(Hw)attP5) / +;</i> <i>SIFaR[attP] / +</i>	FLP element not used in experiments
	Df / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-opGCaMP6s (su(Hw)attP5) / +;</i> <i>SIFaR[attP] / Df(3R)ED10845</i>	FLP element not used in experiments
	SIFaR G4 / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-opGCaMP6s (su(Hw)attP5) / +;</i> <i>SIFaR[attP] / SIFaR[MI05376-TG4.1]</i>	FLP element not used in experiments

	SIFaR G4 / SIFaR null + Dp	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-opGCaMP6s (su(Hw)attP5) /</i> <i>Dp(3;2)GV-CH321-04D21 (VK37);</i> <i>SIFaR[attP] / SIFaR[MI05376-TG4.1]</i>	FLP element not used in experiments
ED3.2e-g	SIFa[1]; SIFaR LoF / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>SIFa[1];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / SIFaR[MI05376-GFSTF.1]</i>	FLP element not used in experiments
	SIFa[1]; + / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>SIFa[1];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / +</i>	FLP element not used in experiments

	+ / SIFa[1]; SIFaR LoF / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>SIFa[1] / +;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / SIFaR[MI05376-GFSTF.1]</i>	FLP element not used in experiments
ED3.2h-j	SIFa[1]; SIFaR G4 / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>SIFa[1];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / SIFaR[MI05376-TG4.1]</i>	FLP element not used in experiments
	SIFa[1]; + / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>SIFa[1];</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / +</i>	FLP element not used in experiments

	+ / SIFa[1]; SIFaR G4 / SIFaR null	<i>w, hs-FLP.D5 (attP3) / w;</i> <i>SIFa[1] / +;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), SIFaR[attP], R57C10-lexA (VK20) / SIFaR[MI05376-TG4.1]</i>	FLP element not used in experiments
ED3.3a		<i>w, 10XUAS-IVS-myr::smGdP-V5 (attP18) / w;</i> <i>SIFa-GAL4 / ap[Xa];</i> <i>Sb / +</i>	
ED3.3b		<i>w, 10XUAS-IVS-myr::smGdP-V5 (attP18) / w;</i> <i>+ / Sp;</i> <i>R14F05-GAL4 (attP2) / +</i>	
ED3.3c	Empty G4	<i>UAS-Dcr2, w / w;</i> <i>R57C10-lexA (attP40), tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / +;</i> <i>TRiP.JF03364 / empty-GAL4 (attP2)</i>	Same as in Fig2b,c
	SIFa G4	<i>UAS-Dcr2, w / w;</i>	Same as in Fig2b,c

		<i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / SIFa-GAL4;</i> <i>TRiP.JF03364 / +</i>	
ED3.3d	Empty G4	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / w;</i> <i>tubP-GAL80[ts](10) / +;</i> <i>UAS-His-RFP, 13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / empty-GAL4 (attP2)</i>	Same as in Fig2d,e
	SIFa G4	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / w;</i> <i>tubP-GAL80[ts](10) / SIFa-GAL4;</i> <i>UAS-His-RFP, 13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / +</i>	Same as in Fig2d,e
	SIFa G4- 2	<i>yw, UAS-Kir2.1-2A-tdTOM-3xHA (attP3) / w;</i> <i>tubP-GAL80[ts](10) / +;</i> <i>UAS-His-RFP, 13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20) / R14F05-GAL4 (attP2)</i>	Same as in Fig2d,e
ED3.3e		<i>UAS-Dcr2, w / w;</i>	Same as in Fig2f,g

Empty G4	<i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / +;</i> <i>TRiP.JF01849 / empty-GAL4 (attP2)</i>	
SIFaR G4	<i>UAS-Dcr2, w / w;</i> <i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / Sp-CyO;</i> <i>TRiP.JF01849 / SIFaR[MI05376-TG4.1]</i>	Same as in Fig2f,g
PanN G4	<i>UAS-Dcr2, w / w;</i> <i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / R57C10-GAL4 (JK22C);</i> <i>TRiP.JF01849 / SIFaR[attP]</i>	Same as in Fig2f,g
Trpy G4	<i>UAS-Dcr2, w / w;</i> <i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / TrpGamma[G4];</i> <i>TRiP.JF01849 / SIFaR[attP]</i>	Same as in Fig2f,g
SIFa G4	<i>UAS-Dcr2, w / w;</i>	Same as in Fig2f,g

		<i>R57C10-lexA (attP40) , tubP-GAL80[ts](10), 13XLexAop2-opGCaMP6s (su(Hw)attP5) / SIFa-GAL4;</i> <i>TRiP.JF01849 / SIFaR[attP]</i>	
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ED3.4		<i>w;</i> <i>Sp-CyO / + ;</i> <i>UAS-mCherry.NLS / SIFaR[MI05376-TG4.1]</i>	Same as in Fig1d
ED3.5	CTRL	<i>w, 20XUAS-IVS-opGCaMP6s (su(Hw)attP8) / +;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-jRCaMP1b (su(Hw)attP5), UAS-SIFaR / tubP-GAL80[ts](10);</i> <i>SIFaR[MI05376-GFSTF.1] / +</i>	Only RCaMP channel recorded
	SIFaR LoF	<i>w, 20XUAS-IVS-opGCaMP6s (su(Hw)attP8) / +;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-jRCaMP1b (su(Hw)attP5), UAS-SIFaR / +;</i> <i>SIFaR[MI05376-GFSTF.1] / +</i>	Only RCaMP channel recorded

	PanN G4 rescue	<i>w, 20XUAS-IVS-opGCaMP6s (su(Hw)attP8) / +;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-jRCaMP1b (su(Hw)attP5), UAS-</i> <i>SIFaR / tubP-GAL80[ts](10);</i> <i>SIFaR[MI05376-GFSTF.1] / R57C10-GAL4 (attP2), SIFaR[MI05376-</i> <i>GFSTF.1]</i>	Only RCaMP channel recorded
	SIFaR G4 rescue	<i>w, 20XUAS-IVS-opGCaMP6s (su(Hw)attP8) / +;</i> <i>R57C10-lexA (attP40) , 13XLexAop2-jRCaMP1b (su(Hw)attP5), UAS-</i> <i>SIFaR / tubP-GAL80[ts](10);</i> <i>SIFaR[MI05376-GFSTF.1] / SIFaR[MI05376-TG4.1]</i>	Only RCaMP channel recorded
ED3.6	CTRL	<i>13XLexAop2-opGCaMP6s (?, su(Hw)attP8) / w;</i> <i>tubP-GAL80[ts](10) / +;</i> <i>20XUAS-IVS-NES-jRCaMP1b-p10 (VK5), SIFa-LexA.GAD /</i> <i>SIFaR[MI05376-TG4.1]</i>	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions Crosses and progeny reared at 29°C throughout development

	SIFaR G4 > UAS-SIFaR	<i>13XLexAop2-opGCaMP6s</i> (? , <i>su(Hw)attp8</i>) / <i>w</i> ; <i>tubP-GAL80[ts](10)</i> / <i>UAS-SIFaR</i> ; <i>20XUAS-IVS-NES-jRCaMP1b-p10</i> (VK5), <i>SIFa-LexA.GAD</i> / <i>SIFaR[MI05376-TG4.1]</i>	Recombinant of two independent <i>13XLexAop2-opGCaMP6s</i> insertions Crosses and progeny reared at 29oC throughout development
ED3.7	CTRL	<i>w, norpA[36]</i> / <i>w</i> ; <i>SIFa[1]</i> / +; <i>13XLexAop2-opGCaMP6s</i> (<i>su(Hw)attP1</i>), <i>R57C10-lexA</i> (VK20), <i>tubP-GAL80[ts](7)</i> / +	
	<i>norpA[36]</i>]	<i>w, norpA[36]</i> ; <i>SIFa[1]</i> / +; <i>13XLexAop2-opGCaMP6s</i> (<i>su(Hw)attP1</i>), <i>R57C10-lexA</i> (VK20), <i>tubP-GAL80[ts](7)</i> / +	
		<i>w, norpA[36]</i> ;	

	norpA[36], PanN G4	<i>SIFa[1] / R57C10-GAL4 (JK22C);</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20), tubP-</i> <i>GAL80[ts](7)/ UAS-norpA.K</i>	
	norpA[36], SIFa G4	<i>w, norpA[36];</i> <i>SIFa[1] / SIFa-GAL4;</i> <i>13XLexAop2-opGCaMP6s (su(Hw)attP1), R57C10-lexA (VK20), tubP-</i> <i>GAL80[ts](7)/ UAS-norpA.K</i>	
ED3.8a	SIFa LexA.2	<i>w, 10XUAS-IVS-myr::smGdP-HA (attP18), 13XLexAop2-IVS-</i> <i>myr::smGdP-V5 (su(Hw)attP8) / w;</i> <i>R14F05-LexA (attP40) / Sp ;</i> +	UAS element not used in experiment
ED3.8b	SIFa LexA.3	<i>w, 10XUAS-IVS-myr::smGdP-HA (attP18), 13XLexAop2-IVS-</i> <i>myr::smGdP-V5 (su(Hw)attP8) / w;</i> <i>R59A06-LexA (attP40) / Sp ;</i> +	UAS element not used in experiment

ED3.8c	SIFa LexA.2+3	<i>13XLexAop2-opGCaMP6s</i> (? , <i>su(Hw)attP8</i>) / <i>yw</i> , <i>UAS-Kir2.1-2A-tdTOM-3xHA</i> (<i>attP3</i>) ; <i>R14F05-lexA</i> (<i>attP40</i>) , <i>Trpy[CR70048-TG4.2]</i> / <i>R59A06-LexA</i> (<i>attP40</i>); <i>8XLexAop2-IVS-GAL80-WPRE</i> (<i>attP2</i>) / +	Recombinant of two independent 13XLexAop2-opGCaMP6s insertions Same as in Fig5b-e
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Chapter 4

Developmental activity and adult behavior

4.1 Introduction

Sleep is a fundamental process conserved across evolution and necessary for human health: Sleep deprivation and dysfunction of the circadian clock network increase the risk for pathogenesis, including neurodevelopmental, metabolic, immune, and neurodegenerative disease (Beckwith and French 2019). Like us, flies sleep as well. Indeed, the fly has been at the cutting edge of sleep research: groundbreaking work on circadian timekeeping mechanisms carried out in the fly was recognized in 2017 by the Nobel Prize in Physiology.

As in other animals, *Drosophila* sleep is regulated by homeostasis and the circadian clock network (Huang et al. 2021). Although these two processes are tightly linked to control sleep, their underlying pathways are distinct. The sleep homeostat, which increases sleep pressure with more time spent awake, is mediated by several neurochemically-defined processes in *Drosophila*. Working alongside the sleep homeostat, the *Drosophila* circadian network consists of 1. the ~150 clock neurons with endogenous, uncoupled rhythms, 2. the sensory inputs that synchronize them, and 3. the output pathways that control behavior. In addition, growing evidence points to the importance of neuropeptides in modulating and integrating these three major components. Circadian behavior in flies is highly stereotyped: under light-dark (LD) conditions (12hr light and 12hr dark), flies exhibit peaks of activity around the times of lights-on and lights-off. As flies entrain to LD conditions, they increase their activity in anticipation to the light transitions.

To record sleep and circadian behavior in flies, I loaded individual, LD-entrained, 4-6 day old adult male and female flies into Daily Activity Monitoring (DAM) tubes and placed them into Trikinetics monitors at 25°C, or at 18°C and 29°C for TARGET experiments (see below). Periods of inactivity (no beam crossings) lasting at least 5 minutes were categorized as sleep. After a 36-48-hour acclimation period, I binned the number of infrared beam crossings every 1 minute over

the course of 48-72 hours and analyzed the data using SCAMP (Vecsey et al. 2024). These outputs were then compiled across multiple runs with Microsoft Excel and statistically tested (2-way ANOVA) and plotted using GraphPad PRISM.

4.2 *Trpy* is necessary and sufficient for wildtype sleep

I first asked how whole animal loss of function of *Trpy* would affect adult sleep. *Trpy* mutants exhibit sex-specific sleep deficits relative to wildtype controls: *trpy* mutant females sleep significantly more during the day and marginally less at night, while *trpy* mutant males sleep marginally more during the day and significantly less at night (**Figure 4.1**). While the relative trends for total sleep levels in mutants was reciprocal between sexes, the trends for average sleep episode duration, a metric for sleep consolidation, more so overlapped: both sexes of *trpy* mutants have fractured sleep at night, with shorter, more frequent sleep episodes compared to controls, whereas *trpy* mutant females also showed an increase in average sleep bout duration during the day compared to wildtype females.

These initial investigations into sleep with *trpy* mutants would occasionally yield variable results (Not shown). This was especially true for *trpy* mutant females, where the relative daytime sleep increase and nighttime sleep decrease phenotypes were inconsistent across repeated trials. In addition, *trpy* mutant males sometimes showed prominent decreases to daytime sleep as well. Notably, the nighttime sleep loss phenotype in *trpy* mutant males was repeatedly observed across separate trials and became the focus of my subsequent rescue experiments.

Chronic expression of UAS-*Trpy*-D with *Trpy*-GAL4 in males homozygous for the *trpy* mutation restored night-time sleep loss (**Figure 4.1**). Together, these results indicate that *Trpy* is necessary and sufficient for wildtype sleep in male flies. These findings are consistent with a recent

study that reported similar trends, where *trpy* mutant males showed decreased sleep relative to controls that was restored by introducing a wildtype copy of the *Trpy* gene using a genomic rescue construct (Palacios-Muñoz et al. 2022). They also found that *trpy* mutant females showed inconsistent sleep phenotypes relative to controls. Notably, this study was motivated by the homology between *Trpy* and the human gene *TRPC6*, an autism-candidate gene, and they identified several parallels in behavioral anomalies between *trpy* mutant flies with those seen in Autism-Spectrum Disorder patients, including social behavior changes (courtship), hyperactivity, learning and memory deficits, and sex-specific sleep defects.

Figure 4.1

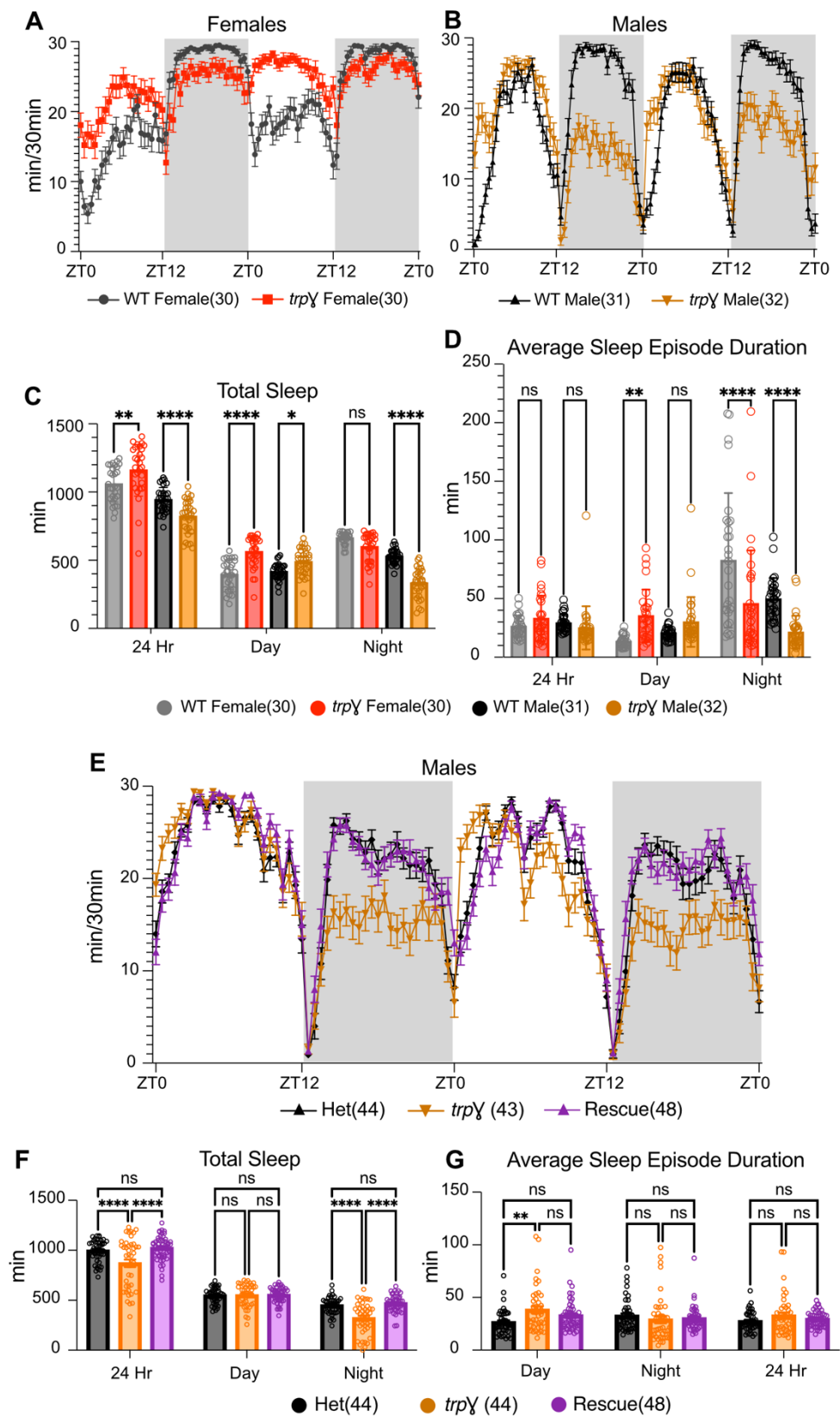


Figure 4.1 *Trpγ* is necessary and sufficient for wildtype sleep in male flies.

A-D. Sleep analysis comparing wildtype females (WT females, charcoal) and males (WT males, black) with *trpγ* mutant females (*trpγ* females, red) and *trpγ* mutant males (*trpγ* males, orange). **A-B.** Group averages of sleep binned every 30min comparing *trpγ* mutant and wildtype females (**A**) and males (**B**) over two days. **C-D.** Total sleep averages (**C**) and Average sleep episode duration (**D**) and SEM of 24hr, Day (ZT0-12), and night (ZT12-24), color matched to genotypes and sexes with corresponding N's. **E-G.** Sleep analysis comparing male flies that are heterozygous (Het, black), homozygous *trpγ* mutants (*trpγ*, orange), and heterozygous flies expressing *Trpγ-GAL4* driven UAS-*Trpγ-D* (Rescue, purple). **E.** Group averages of sleep binned every 30min. **F.** Total sleep averages and SEM. **G.** Average sleep episode duration and SEM. Shaded regions represent night. Two-way ANOVA analysis for all total sleep and average sleep episode duration comparisons. p values: * <0.05, **<0.005, ***<0.0005, ****<0.0001, ns = not significant.

4.3 Temporally targeted rescue of sleep was inconclusive

The analysis of whole-animal *trpy* mutants alone cannot determine when the *Trpy* gene is required for wildtype sleep. We next investigated whether the sleep deficits in male *trpy* mutants was due to disrupted PSINA (**Chapter 1**), and presumably from changes to sleep circuitry development, and/or due to the concurrent requirement for *Trpy* in actively regulating sleep in adults. In a similar fashion to our approaches to establish whether a gene or a cell population was required specifically during PSINA, what we referred to as “temporally targeted expression” (**Chapter 2.2**), we adapted the use of GAL80ts to determine when *Trpy* expression is required for adult sleep. Specifically, we sought to control the timing of *Trpy*-GAL4 driven expression of UAS- *Trpy*-D in males homozygous for the *trpy* mutation by keeping them at the restrictive (18°C) and permissive (29°C) temperatures during development or post-eclosion and monitored sleep and daily activity.

We first monitored sleep in experimental and control male flies at 29°C throughout development and adult life to validate the efficacy of the TARGET rescue construct, with the expectation that sleep would be restored to similar levels to controls as seen in our rescue experiments without GAL80ts (**Figure 4.1**). Across three attempts spanning over six months, there were no significant differences between groups; that is, control, mutant, and experimental genotypes were indistinguishable from each other. Notably, each attempt revealed the same pattern, where experimental and control flies carrying at least one copy of the *trpy* mutation and one allele of a 3rd chromosome insertion of GAL80ts (P{*tubP*-GAL80[ts]}2, BDSCID #7017, henceforth referred to as GAL80ts_2), all showed decreased nighttime sleep, with a characteristic negative slope of decreasing sleep throughout the night (**Figure 4.2**). We subsequently tested flies carrying just one copy of GAL80ts_2 at 25°C and found no sleep deficits relative to controls (not shown), suggesting that the phenotype we observed in our rescue attempts at 29°C could perhaps

be from an unknown genetic interaction, a second site mutation, or a temperature dependent effect. I did not further explore the different possibilities to determine the cause of this peculiar decreasing nighttime sleep effect and instead decided to attempt the same experimental approach with a different allele of GAL80ts, this time on the 2nd chromosome (P{tubP-GAL80[ts]}10, BDSCID #7108, henceforth referred to as GAL80ts_10).

Using this allele restored the expected sleep differences across genotypes at constant 29°C (**Figure 4.2C,D**). However, in the subsequent experiment where we reared these same groups at 18°C during development and shifted them to 29°C post-eclosion, we observed a similar phenomenon as with the GAL80ts_2 runs described above, where all groups showed decreasing nighttime sleep and negative and positive controls were indistinguishable from one another. How this similar pattern emerged across conditions, especially given the successful rescue in chronic 29°C conditions with GAL80ts_10, is unclear.

I also conducted experiments using immunohistochemistry with a Trpγ-specific antiserum to measure *Trpγ* levels in the brain, which revealed that *Trpγ* is transiently expressed only during pupal development (**Figure 1.9**). While this would suggest that the adult sleep loss in *trpγ* mutants is due to *Trpγ*'s role during PSINA, the behavioral experiments to corroborate this possibility produced inconsistent results that were difficult to interpret. We ultimately suspended our attempts at investigating whether altered PSINA can lead to adult sleep deficits until we discovered new genetic handles on PSINA – namely *SIFa* and *SIFaR*, which were also reported to be involved in regulating adult sleep.

Figure 4.2

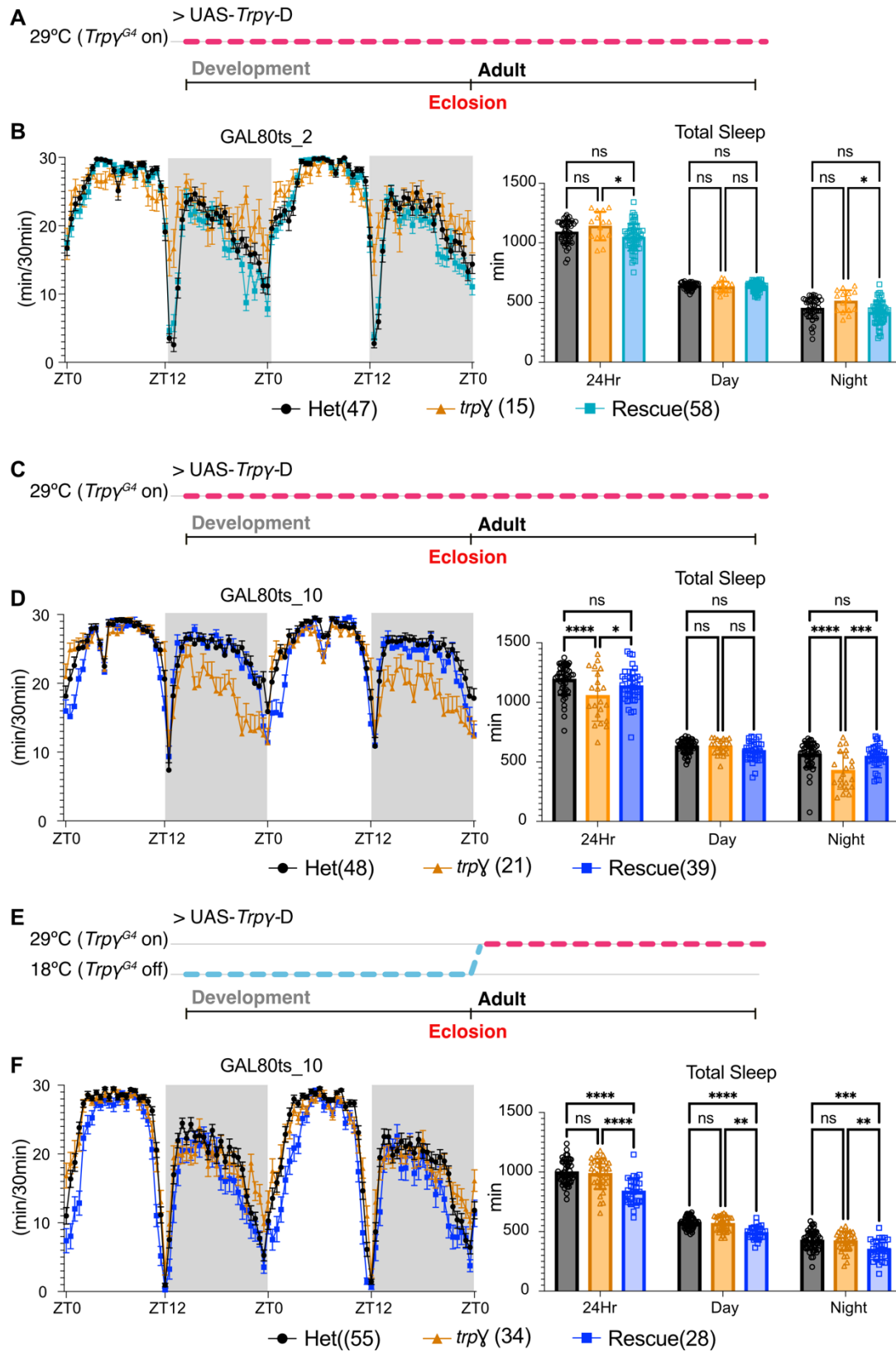


Figure 4.2 Temporally targeted rescue with UAS-*Trpγ*-D was inconclusive

A. Expression control of *Trpγ*-GAL4 driven UAS-*Trpγ*-D with GAL80ts_2 at constant 29°C. **B.** Group averages of sleep binned every 30min (left) and total sleep averages and SEM (right) over two days of male flies that were heterozygous (Het, black), homozygous *trpγ* mutants (*trpγ*, orange), and heterozygous expressing *Trpγ*-GAL4 driven UAS-*Trpγ*-D (Rescue, cyan). **C.** Expression control of *Trpγ*-GAL4 driven UAS-*Trpγ*-D with GAL80ts_10 at constant 29°C. **D.** Group averages of sleep binned every 30min (left) and total sleep averages (right) and SEM over two days of male flies heterozygous (Het, black), homozygous *trpγ* mutants (*trpγ*, orange), and heterozygous expressing *Trpγ*-GAL4 driven UAS-*Trpγ*-D (Rescue, blue). **E.** Expression control of *Trpγ*-GAL4 driven UAS-*Trpγ*-D with GAL80ts_10 reared at 18°C and shifted to 29°C at eclosion. **F.** Group averages of sleep binned every 30min (left) and total sleep averages (right) and SEM over two days of male flies with same genotypes as **D**. Corresponding N's indicated next to genotype labels. Shaded regions represent night. Two-way ANOVA analysis for all total sleep comparisons. p values: * <0.05, **<0.005, ***<0.005, ****<0.0001, ns = not significant.

4.4 Temporally targeted *sifa* knockdown suggests a rearing-temperature effect on adult sleep

Our discovery of the first known developmental role for SIFa-SIFaR signaling provided a new opportunity to establish whether altered PSINA could lead to changes in adult behavior. Previous studies on SIFa and SIFaR indicate their involvement in a wide range of behaviors, including courtship (Terhzaz et al. 2007b; Sellami and Veenstra 2015), feeding (Martelli et al. 2017a; Dreyer et al. 2019), and sleep (Cavanaugh et al. 2014; Park et al. 2014; Deng et al. 2019a; Huang et al. 2021). Importantly, these studies used chronic manipulations without temporal control, raising the question of whether the reported phenotypes were due to altered PSINA and/or a concurrent requirement of SIFa-SIFaR signaling for adult physiology.

To this end, we temporally targeted knockdown of SIFa expression with GAL80ts₁₀ in male flies and monitored sleep (**Figure 4.3**). We controlled the timing of SIFa-GAL4 driven expression of UAS-SIFa-RNAi, restricting the knockdown to either development or adult life and compared them to positive (*SIFa* mutants) and negative (empty-GAL4) controls, as well as to all groups kept at constant 18°C or 29°C. Across all four conditions, homozygous *SIFa* mutants slept less than negative controls, consistent with the findings from the literature. Flies with SIFa knockdown only during development slept at levels comparable to negative controls (empty-GAL4), while flies with SIFa knockdown only during adult life showed decreased sleep levels comparable to *SIFa* mutants. While these findings would be consistent with a concurrent requirement of SIFa for adult sleep, parallel trends occurred in groups kept at constant 18°C or 29°C throughout the experiment. In other words, experimental flies that were kept at constant 18°C were also more similar to *SIFa* mutants, while those kept at constant 29°C were more similar to negative controls. Furthermore, the relative difference between positive and negative controls also followed these same trends according to rearing temperature: *SIFa* mutants showed more sleep

loss when reared at 18°C and less sleep loss when reared at 29°C relative to negative controls. These results suggest that the trends pointing to SIFa expression only being required during adulthood for normal sleep could also be explained by the influence of rearing temperature on flies with disrupted SIFa expression. I discuss the implications and possible mechanisms underlying this phenomenon in the summary section at the end of this chapter.

A $> \text{UAS-SIFa-RNAi}$
 29°C (SIFa^{G4} on)
 18°C (SIFa^{G4} off)
 Development Adult
 Eclosion

B
 min/30min
 ZT0 ZT12 ZT0 ZT12 ZT0 ZT12 ZT0
 ● Empty(28) ■ SIFa[1](27) ▲ SIFaGAL4(37)
 Total Sleep
 ns ns ns ns ns ns ns ns ns ns ns ns
 Day Night 24Hr

C $> \text{UAS-SIFa-RNAi}$
 29°C (SIFa^{G4} on)
 18°C (SIFa^{G4} off)
 Development Adult
 Eclosion

D
 min/30min
 ZT0 ZT12 ZT0 ZT12 ZT0 ZT12 ZT0
 ● Empty(27) ■ SIFa[1](34) ▲ SIFaGAL4(50)
 Total Sleep
 ns ns ns ns ns ns ns ns ns ns ns ns
 Day Night 24Hr

(E-H continued below)

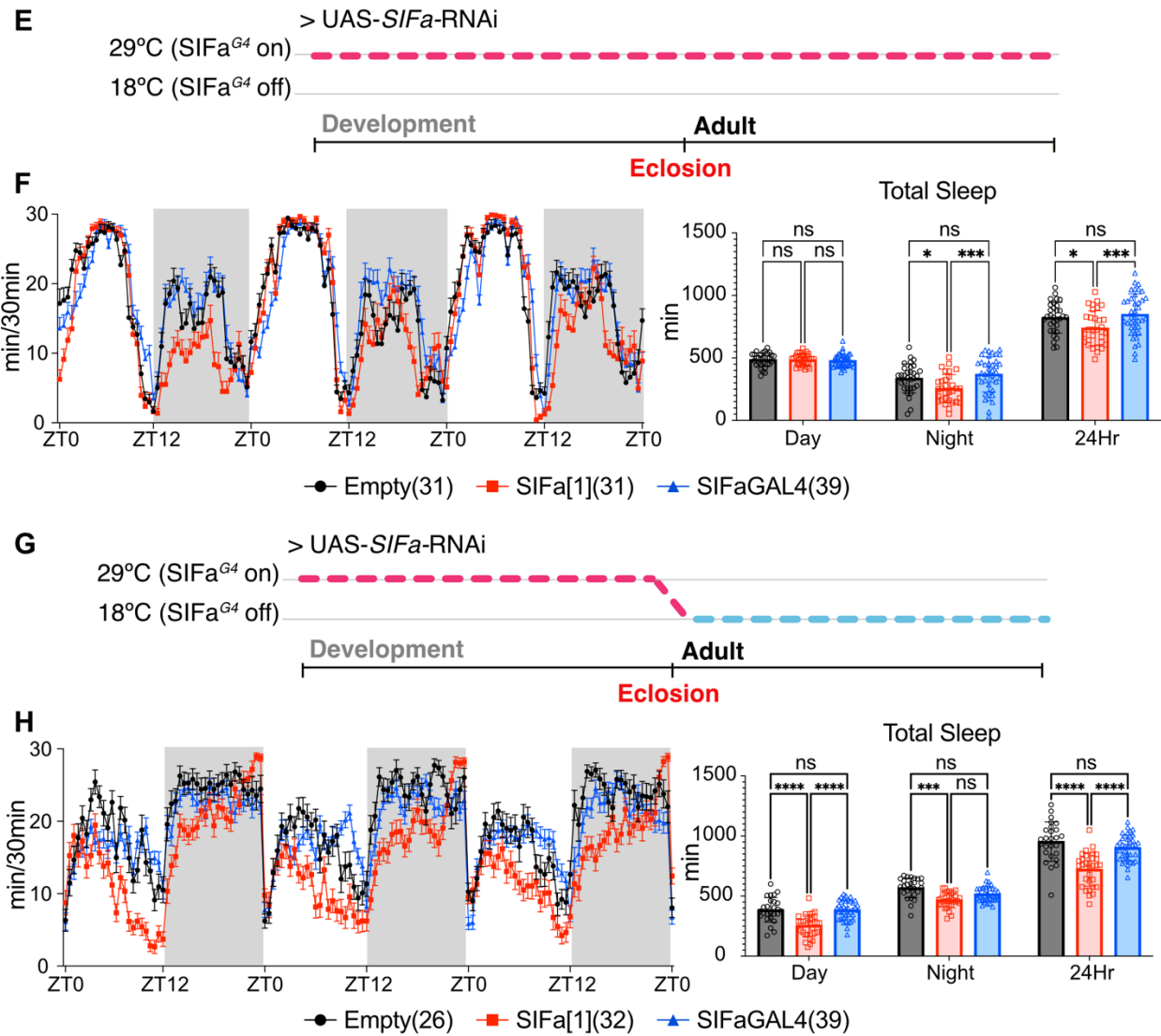


Figure 4.3 Temporally targeted knockdown of SIFa revealed a rearing temperature influence on adult sleep

A-H. Sleep analysis of male flies that are heterozygous (Empty, black), homozygous *SIFa* mutants (SIFa[1], red), and heterozygous with temporally targeted SIFa-GAL4 driven UAS-SIFa-RNAi (SIFa-GAL4, blue). N's indicated next to genotype labels for each condition. **A,C,E,G.** Expression control of SIFa-GAL4 driven UAS-SIFa-RNAi with tubP-GAL80ts₁₀ at 18°C and/or 29°C. Flies were shifted within one day of eclosion for **C** and **G**. **B,D,F,H.** Group averages of sleep binned every 30min (left) and total sleep averages (right) and SEM over three days of genotypes described above. Shaded regions represent night. Two-way ANOVA analysis for all total sleep comparisons. p values: * <0.05, **<0.005, ***<0.005, ****<0.0001, ns = not significant.

4.5 Conditional disruption of SIFaR to assess sleep was inconclusive

A key takeaway from our collective efforts at behavioral analysis was to keep rearing temperatures constant across groups going forward, which essentially voided the traditional application of TARGET. We decided to adapt a recombinase-based, conditional gene disruption and rescue tool known as FlpStop (Fisher et al. 2017). With FlpStop, conditional null or rescue alleles of a modified gene can be irreversibly activated in target cells of interest, along with the red fluorescent marker tdTomato for validation, by controlling the expression of Flp-recombinase. We produced two independent FlpStop alleles for the SIFaR gene, allowing us to induce either heteroallelic mutant cells in heterozygous flies (conditional nulls) or heterozygous cells in heteroallelic mutant flies (conditional rescue).

We first used FlpStop to study the role of SIFaR during PSINA. By bringing the expression of Flp under the precise control of the TARGET system, we spatiotemporally regulated SIFaR expression during pupal development in target cell populations, ranging from all neurons (57C10-GAL4), to the 2000 Trp γ ⁺ neurons (Trp γ -GAL4), to the four SIFa neurons (SIFa-GAL4) and recorded PSINA with our widefield assay. Both contexts of conditional disruption and rescue of SIFaR with FlpStop closely mirrored the results from temporally targeted knockdown and rescue of SIFaR in the respective expression domains (Not shown). For instance, conditional mutagenesis of SIFaR in the four SIFa neurons during pupal development with FlpStop matched the effect of SIFaR knockdown in SIFa neurons during that same period: brainwide PSINA cycles were disrupted.

After confirming that conditional disruption of SIFaR in the four SIFa neurons during pupal development could disrupt brainwide PSINA, we decided to apply the FlpStop tool to assess the requirement for SIFaR in adult sleep. The technical advantage of using FlpStop in this context is

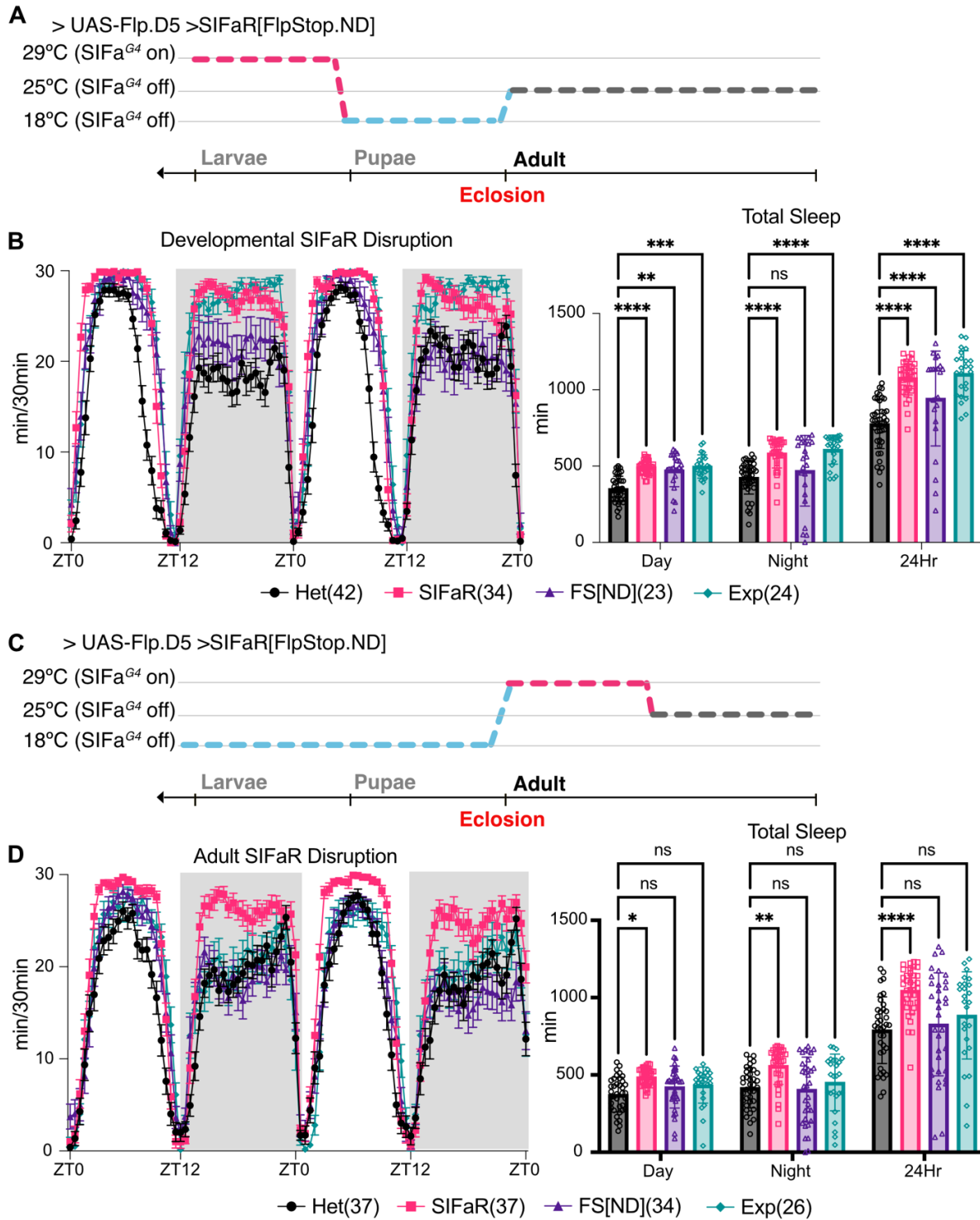
found in the irreversible nature of Flp-recombinase to induce the conditional disruption. Our expectation was that Flp expression was only required for a brief period. We reasoned that we could temporarily disinhibit SIFa-GAL4 driven UAS-Flp expression by exposing flies to the permissive temperature of 29°C for 3-5 days. By strategically timing when this occurred, we could conditionally disrupt SIFaR expression in the four SIFa neurons either before PSINA or after eclosion while keeping the rearing temperature constant during pupal development across all groups.

For our behavioral assays, conditional disruption of SIFaR during development was carried out by rearing flies at 29°C until the 3rd instar larval stage, moving them to 18°C during pupal development, and shifting newly eclosed flies to 25°C for 6 days at 12:12 LD before monitoring sleep at this same temperature. On the other hand, conditional disruption during adult life was carried out by rearing flies at 18°C until eclosion, shifting to 29°C for 3 days at 12:12 LD, and returning them to 25°C for 3 days before monitoring sleep. We independently confirmed that SIFa-GAL4 is expressed during both of the time points that flies would be kept at 29°C. Although the rearing temperature prior to metamorphosis was different between these conditions, this approach ensured that the temperature during pupal development was the same for all groups (18°C) and allowed us to monitor sleep at 25°C. Negative controls were heterozygous flies carrying one copy of the null allele SIFaR[attp] and similarly heterozygous flies that also carried the SIFaR “non-disrupting” FlpStop allele. Positive controls were heteroallelic SIFaR mutants carrying SIFaR[attp] and SIFaR[GFSTF]. The experimental groups carried SIFaR[attp], SIFa-GAL4, and the SIFaR “non-disrupting” FlpStop allele. Lastly, all groups had one copy each of GAL80ts₁₀ and 20X-UAS-Flp.

Notably, SIFaR mutants slept significantly more compared to both negative controls across all conditions (**Figure 4.4**), which contrasts with the sleep loss from SIFa knockdown described above, as well as with the sleep loss from SIFaR knockdown reported in other studies (Park et al. 2014). Notwithstanding, flies that underwent conditional disruption of SIFaR in the four SIFa neurons during development slept at levels close to SIFaR mutants, while flies that underwent conditional disruption after eclosion slept at levels comparable to negative controls. Together, these results suggest that the increase in sleep from SIFaR loss of function is due to a requirement for the SIFaR gene during pupal development only. When validating whether the inversion of the SIFaR FlpStop allele from “non-disrupting” to “disrupting” occurred in the four SIFa neurons by checking for the expression of tdTomato, the native expression of DsRed from the SIFaR[attp] genetic background masked the ability to distinguish between the two because SIFa neurons also fall within the SIFaR expression domain.

To address this critical validation step, we rebuilt our reagents with SIFaR[GFSTF], which does not express DsRed in the SIFaR expression domain. We also added an additional negative control group of heterozygous SIFaR[GFSTF] flies carrying SIFa-GAL4. Running the behavioral assay described above with reagents built with the SIFaR[GFSTF] allele yielded complicated results that were inconsistent with the earlier runs using SIFaR[attp]. First, our negative controls showed significant differences across conditions. Across the two conditions (developmental vs adult SIFaR disruption), homozygous SIFaR[GFSTF] mutants did not consistently sleep more than negative controls. While our experimental group did sleep more with conditional disruption during development and less with conditional disruption post-eclosion, the inconsistent trends in our positive and negative controls hindered the ability to make reliable conclusions from this dataset.

Figure 4.4



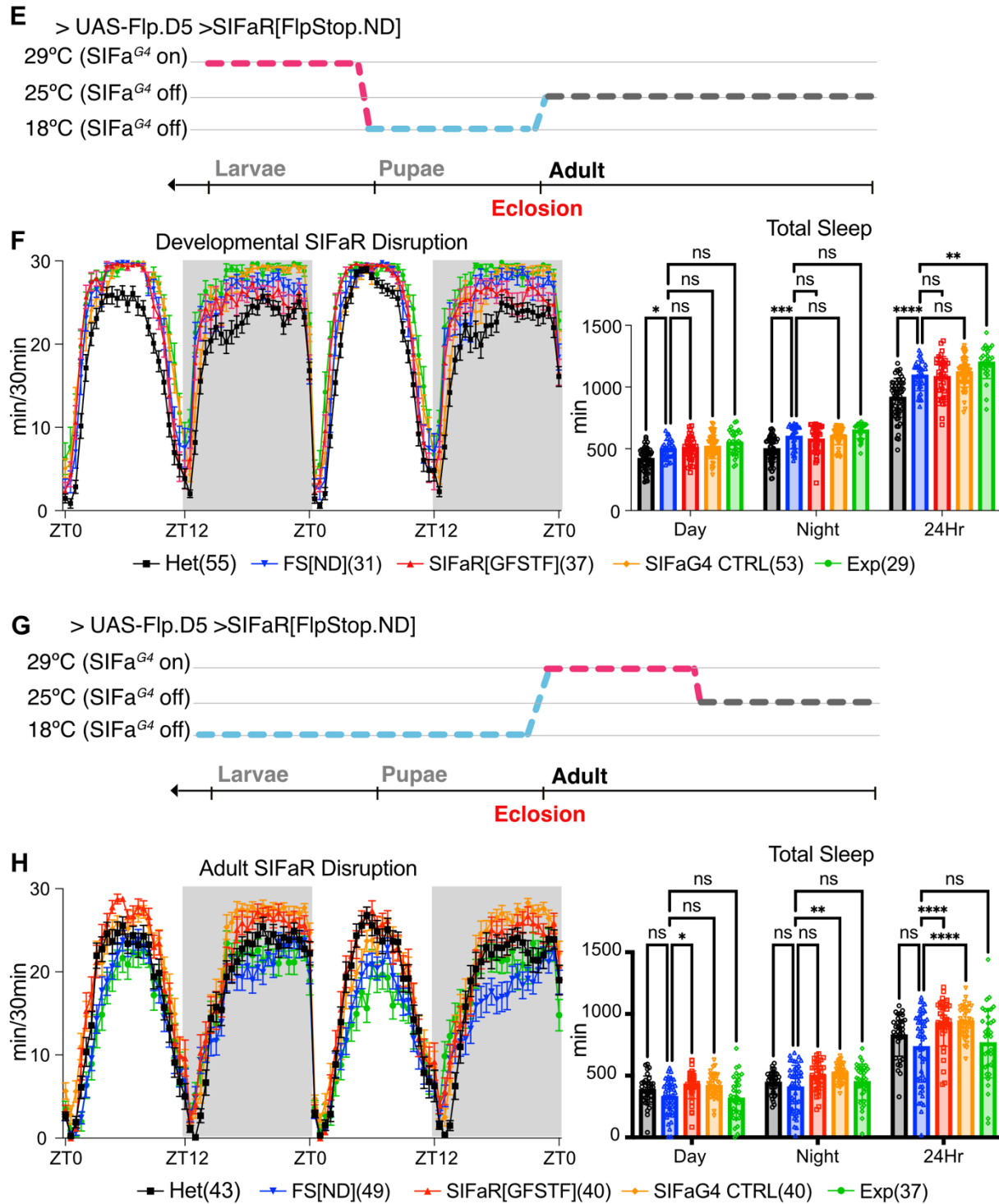


Figure 4.4 Conditional disruption of SIFaR in SIFa neurons using FlpStop to assess sleep was inconclusive.

A-H. Sleep analysis of males with developmental or adult disruption of SIFaR in SIFa neurons compared to controls in the SIFaR[attp] null background (**A-D**) or SIFaR[GFSTF] null background (**E-H**). N's indicated next to genotype labels for each condition. Full genotypes listed in supplementary table 2 at end of chapter. **A,C,E,G.** Expression control of SIFa-GAL4 driven UAS-Flp.D5 with tubP-GAL80ts_10 at 18°C, 25°C, 29°C. For developmental disruption (**A-B, E-F**), flies were reared at 29°C until wandering 3rd instars, kept at 18°C through pupal development, and kept at 25°C for 6 days until loaded into DAM assay. For adult disruption (**C-D, G-H**) flies were reared at 18°C until eclosion, kept at 29°C for 3 days, and moved to 25°C for 3 days until loaded into DAM assay. **B,D,F,H.** Group averages of sleep binned every 30min (left) and total sleep averages (right) and SEM over three days of listed genotypes. Shaded regions represent night. Two-way ANOVA analysis for all total sleep comparisons. p values: * <0.05, **<0.005, ***<0.005, ****<0.0001, ns = not significant.

4.6 Summary

While the link between disruptions to developmental activity and the emergence of neurodevelopmental disorders is predicted, definitive evidence does not exist to support that conclusion across any model system. Our discovery of the genetic handles on developmental activity in the fly created an exciting opportunity to begin empirically addressing this open question through approaches using spatiotemporal regulation of gene expression. My initial findings showing that *Trpγ* is necessary and sufficient for adult sleep in male flies was a promising first step towards this effort. Subsequent sleep experiments aimed at regulating the timing of *Trpγ* expression, and later *SIFa* and *SIFaR*, were not only technically challenging to execute, but produced inconsistent results that made it difficult to reliably draw conclusions. Importantly, they revealed that the approaches we used for regulating gene expression introduced a significant confound when studying adult sleep: temperature, and more specifically, the temperature conditions during development.

A recent study investigated the relationship between developmental temperature conditions and adult sleep patterns. They reported enduring effects on sleep architecture from long-term exposure to non-optimal temperatures during development (Yadav et al. 2025). Notably, flies that were reared at 29°C slept longer and had shorter sleep latencies (the time it takes to enter a sleep state) compared to those reared at 19°C and 25°C. These findings support our observations that rearing temperature can have a lasting influence on sleep/wake behavior during adult life. before monitoring adult sleep patterns and represents an exciting new direction of research.

Supplementary Table 2 Chapter 4 genotypes

Figure	Label in Figure	Genotype
Fig4.1A-D	WT Female, WT Male	<i>CantonS</i> (<i>CantonS</i> / <i>y</i>); +; +
	<i>trpy</i> Female, <i>trpy</i> Male	<i>CantonS</i> (<i>CantonS</i> / <i>y</i>); <i>trpy</i> ; +
Fig4.1E-G	Het	<i>CantonS</i> / <i>y</i> ; <i>trpy</i> / +; <i>UAS-Trpy-D</i> / +
	<i>trpy</i>	<i>CantonS</i> / <i>y</i> ; <i>trpy</i> ; <i>UAS-Trpy-D</i> / +
	Rescue	<i>CantonS</i> / <i>y</i> ; <i>trpy</i> / <i>Trpy-GAL4</i> ; <i>UAS-Trpy-D</i> / +
Fig4.2B	Het	<i>CantonS</i> / <i>y</i> ; <i>trpy</i> / +; <i>UAS-Trpy-D</i> / <i>tubP-GAL80ts(2)</i>
	<i>trpy</i>	<i>CantonS</i> / <i>y</i> ; <i>trpy</i> ; <i>UAS-Trpy-D</i> / <i>tubP-GAL80ts(2)</i>
	Rescue	<i>CantonS</i> / <i>y</i> ; <i>trpy</i> / <i>Trpy-GAL4</i> ; <i>UAS-Trpy-D</i> / <i>tubP-GAL80ts(2)</i>
Fig4.2D,F	Het	<i>CantonS</i> / <i>y</i> ; <i>trpy</i> / +, <i>tubP-GAL80ts(10)</i> ; <i>UAS-Trpy-D</i> / +
	<i>trpy</i>	<i>CantonS</i> / <i>y</i> ; <i>trpy</i> / <i>trpy</i> , <i>tubP-GAL80ts(10)</i> ; <i>UAS-Trpy-D</i> / +
	Rescue	<i>CantonS</i> / <i>y</i> ; <i>trpy</i> / <i>Trpy-GAL4</i> , <i>tubP-GAL80ts(10)</i> ; <i>UAS-Trpy-D</i> / +

Fig4.3	Empty	<i>UAS-DCR2</i> / y; <i>SIFa</i> [1], <i>tubP-GAL80ts(10)</i> / +; <i>Empty-GAL4</i> / <i>UAS-SIFa-RNAi</i> (JF03364)
	SIFa[1]	<i>UAS-DCR2</i> / y; <i>SIFa</i> [1], <i>tubP-GAL80ts(10)</i> / <i>SIFa</i> [1]; <i>UAS-SIFa-RNAi</i> (JF03364) / +
	SIFaGAL4	<i>UAS-DCR2</i> / y; <i>SIFa</i> [1], <i>tubP-GAL80ts(10)</i> / <i>SIFa-GAL4</i> ; <i>UAS-SIFa-RNAi</i> (JF03364) / +
Fig4.4B,D	Het	w / y; <i>tubP-GAL80ts(10)</i> / +; <i>SIFaR</i> [attp], <i>20X-UAS-Flp.D5</i> / +
	SIFaR	y,w / y; <i>tubP-GAL80ts(10)</i> / +; <i>SIFaR</i> [attp], <i>20X-UAS-Flp.D5</i> / <i>SIFaR</i> [GFSTF]
	FS[ND]	y,w / y; <i>tubP-GAL80ts(10)</i> / +; <i>SIFaR</i> [attp], <i>20X-UAS-Flp.D5</i> / <i>SIFaR</i> [FlpStop ND](M13)
	Exp	w / y; <i>tubP-GAL80ts(10)</i> / <i>SIFaGAL4</i> ; <i>SIFaR</i> [attp], <i>20X-UAS-Flp.D5</i> / <i>SIFaR</i> [FlpStop ND](M13)
Fig4.4F,H	Het	w / y; <i>tubP-GAL80ts(10)</i> / +; <i>SIFaR</i> [GFSTF], <i>20X-UAS-Flp.D5</i> / +
	SIFaR[GFSTF]	w / y; <i>tubP-GAL80ts(10)</i> / +; <i>SIFaR</i> [GFSTF], <i>20X-UAS-Flp.D5</i> / <i>SIFaR</i> [GFSTF]
	FS[ND]	w / y; <i>tubP-GAL80ts(10)</i> / +; <i>SIFaR</i> [GFSTF], <i>20X-UAS-Flp.D5</i> / <i>SIFaR</i> [FlpStop ND](M13)
	SIFaG4 CTRL	w / y; <i>tubP-GAL80ts(10)</i> / <i>SIFaGAL4</i> ; <i>SIFaR</i> [GFSTF], <i>20X-UAS-Flp.D5</i> / +
	Exp	w / y; <i>tubP-GAL80ts(10)</i> / <i>SIFaGAL4</i> ; <i>SIFaR</i> [GFSTF], <i>20X-UAS-Flp.D5</i> / <i>SIFaR</i> [FlpStop ND](M13)

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