Anatomical distribution and functional roles of electrical synapses in *Drosophila*

**Highlights**
- An immunohistochemistry-based map of innexin gap junctions in the *Drosophila* CNS
- VS/HS cells are electrically coupled to large cell networks via shakB gap junctions
- Loss of electrical synapses from VS/HS cells induces voltage and calcium oscillations
- Electrical synapses play functional roles in both ON and OFF vision pathways

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**In brief**
Ammer et al. first map the broad distribution of electrical synapses in the fly nervous system. Next, they find that electrical synapses are required for the intrinsic stability of VS/HS cells. Furthermore, electrical synapses play differential roles in ON and OFF visual pathways but are not necessary for the emergence of direction selectivity.
Anatomical distribution and functional roles of electrical synapses in Drosophila

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SUMMARY

Electrical synapses are present in almost all organisms that have a nervous system. However, their brain-wide expression patterns and the full range of contributions to neural function are unknown in most species. Here, we first provide a light-microscopic, immunohistochemistry-based anatomical map of all innexin gap junction proteins—the building blocks of electrical synapses—in the central nervous system of Drosophila melanogaster. Of those innexin types that are expressed in the nervous system, some localize to glial cells, whereas others are predominantly expressed in neurons, with shakB being the most widely expressed neuronal innexin. We then focus on the function of shakB in VS/HS cells—a class of visual projection neurons—thereby uncovering an unexpected role for electrical synapses. Removing shakB from these neurons leads to spontaneous, cell-autonomous voltage and calcium oscillations, demonstrating that electrical synapses are required for these cells’ intrinsic stability. Furthermore, we investigate the role of shakB-type electrical synapses in early visual processing. We find that the loss of shakB from the visual circuits upstream of VS/HS cells differentially impairs ON and OFF visual motion processing pathways but is not required for the computation of direction selectivity per se. Taken together, our study demonstrates that electrical synapses are widespread across the Drosophila nervous system and that they play essential roles in neuronal function and visual information processing.

INTRODUCTION

Neurons communicate via two fundamentally different types of synapses. At chemical synapses, neurotransmitters released from presynaptic sites activate receptors on postsynaptic neurons, leading to direct opening of ion channels or to an initiation of intracellular signaling cascades. In contrast, electrical synapses consist of channel-forming gap junction proteins, which allow for a direct, bidirectional flow of ions between two connected cells. In invertebrates, gap junctions are composed of innexin proteins each, form a functional channel that bridges the cytoplasm of two cells. Apart from forming electrical synapses between neurons, innexins play essential roles in other biological processes such as embryonic development,5 stem cell division,6 the formation of the blood-brain barrier,6 or spermatogenesis.7

In Drosophila, the molecular components, physiology, and function of chemical synapses have been studied in great detail.8 In addition, recent years, large-scale efforts have been undertaken to generate a connectome of the Drosophila nervous system—a complete map of every chemical synaptic connection—based on electron-microscopic9 reconstructions.9,11 Conversely, the nervous-system-wide distribution of electrical synapses is unknown. Importantly, electrical synapses are completely absent from all published Drosophila connectomic studies. This is largely due to their small size of around 10–20 nm,12 which is below the resolution of current electron microscopic imaging techniques used for generating large EM datasets. Thus, most of our knowledge about electrical synapses comes from studies that focused on particular cell types or small neural circuits. The best-studied example is the giant fiber escape circuit of Drosophila. Here, electrical connections formed by shakB-type gap junctions exist at all nodes of the circuit, from sensory neurons to interneurons to motor neurons.13–17 The proposed function of the strong electrical coupling in the giant fiber circuit is to speed up signal transmission for enabling fast escape maneuvers, as electrical synapses essentially introduce no synaptic delay. Additional examples of electrical synaptic connections in the Drosophila brain include coupling between olfactory projection neurons18 and coupling between different types of neurons in the mushroom body.19–21

Extensive electrical connections also exist in the fly visual system. Here, several studies have investigated the vertical system (VS) and horizontal system (HS) cells—subtypes of the lobula plate tangential cells (LPTCs)—in the blowfly Calliphora. LPTCs are wide-field neurons that project their axons from the optic lobe to the central brain or the contralateral optic lobe.22 They receive direction-selective input from small-field T4 and T5 cells, which respond to moving luminance increments and decrements, respectively.23 Spatial integration of these inputs renders LPTCs selective to a particular pattern of optic flow that is dependent on the neuronal subtype.24 In addition to spatial integration, some LPTCs perform nonlinear amplification of
high-frequency inputs in the temporal domain. Different subtypes of LPTCs form electrical synapses both with each other and with descending neurons. Electrical coupling between the axons of VS cells broadens their axonal receptive fields and thereby increases the robustness of optic flow representation under noisy conditions. Similarly, HS cells form electrical synapses with other HS cells and additionally with the H2 cell from the contralateral hemisphere. However, which type of innexin is mediating the electrical coupling between LPTCs is unknown. Furthermore, experimental studies that directly test the effects of removing electrical synapses on the physiology of LPTCs are lacking.

Here, we first describe the distribution of innexins across the entire central nervous system (CNS) in adult Drosophila. Next, to assess the importance of electrical synapses in neuronal function, we investigate the role of shakB, the most widely expressed neuronal gap junction protein, in VS and HS cells. We find that loss of electrical synapses drives these cells into spontaneous membrane potential oscillations and induces large periodic calcium fluctuations. These oscillations arise cell autonomously and involve voltage-gated sodium channels (Nav) and Ih channels. Moreover, we record from VS/HS cells and presynaptic T4/T5 cells and demonstrate that loss of shakB affects visual processing in both ON and OFF pathways.

**RESULTS**

**A map of gap junction expression across the central nervous system**

To determine the distribution of electrical synapses in the central nervous system of Drosophila melanogaster, we performed immunostainings against each of the eight innexin gap junction proteins (Figures 1 and S1–S3). We found that six of the eight innexins show expression in the nervous system (Figure 1). Three of those, ogre (inx1), inx2, and inx3, exclusively localized to glial cells. We performed colabeling of these innexins together with markers for glial subtypes. Consistent with earlier work, ogre localized to subperineural glia and partly to perineural glia, which are crucial components of the blood-brain barrier. Inx3 was detected exclusively and strongly in neuropil ensheathing glia, and inx2 colocalized with all of the three glial subtypes. In stark contrast, shakB (inx8) was broadly expressed in the optic lobes, in many regions of the central brain and in the ventral nerve cord (VNC). We obtained similar results by using a different set of antibodies (Figure S1). Together, these protein expression...
patterns are largely in agreement with RNA sequencing data. According to these studies, the mRNAs of ogre, inx2, inx3, and shakB are the most strongly expressed innexin transcripts in the nervous system, whereas zpg (inx4), inx5, inx6, and inx7 were either not detected at all or only at very low levels (Figure S3B).

**Electrical synapses formed by shakB are widely distributed across the CNS**

As shakB was broadly and strongly expressed in the neuropil, we analyzed the expression pattern of this innexin in more detail (Figures 2 and S1H–S1M). Correlation analysis between relative fluorescence intensities of shakB and nc82, a marker for chemical presynaptic sites, revealed a weak anticorrelation (Figure S3C). This suggests that the number of shakB electrical synapses does not simply scale with the number of chemical synapses but that these two types of synapses have distinct anatomical distributions. shakB localized to all four neuropils of the optic lobe in a layered fashion: the proximal lamina; layers 1, 3, 5, and 10 of the medulla; layer 3 of the lobula; and to the lobula plate (Figure 1H). In the central brain, we detected particularly strong expression of shakB in the anterior mechanosensory and motor center, the anterior ventrolateral protocerebrum, the wedge, the subesophageal ganglion, the giant fiber, the posterior slope, and the cervical connective. Additionally, we observed weaker expression in the antennal lobes, the optic tubercle, the superior medial protocerebrum, and the lateral horn (Figures 2A–2J). In the VNC, shakB localized to the leg neuropils, the tectulum, and the wing and haltere ganglion, and the lateral horn (Figures 1A–I). Importantly, no shakB immunolabeling was detected in shakB2-mutant flies that carry a null allele for six of the eight shakB isoforms (Figure S3D). This suggests that the remaining two isoforms (isoforms A, E) are either not expressed in the adult CNS or only at very low levels. Similarly, shakB staining was absent when we expressed an RNAi construct that targets all eight shakB isoforms pan-neuronally (Figure S3E), thereby confirming the specificity of the antibody. Given the abundant and widespread expression of shakB in the *Drosophila* nervous system, we focused our further investigations on this gap junction type.

**Candidate cell types forming shakB-type electrical synapses**

As a next step, we generated a genetic driver line that is based on the MiMIC-Trojan-Gal4 system (Figure S4A). To do so, we used a published fly line in which a MiMIC transposon is inserted into an intron of the shakB gene that is common to the same six isoforms that are affected by the shakB2 mutation. This MiMIC insertion was then exchanged with a Trojan-Gal4 exon that codes for a T2A-sequence followed by the Gal4 transcription factor. Consequently, every cell that expresses one of those shakB isoforms should express Gal4 as well, thereby allowing us to label most shakB-expressing cells. When using the shakB-Trojan-Gal4 line to drive GFP, we observed many labeled cells across the CNS (Figure 2L). The neuropil regions that showed strong shakB immunolabeling were also strongly innervated by neurons labeled by this line (Figures 2M–2O). A second fly line in which the Trojan-Gal4 cassette was integrated into a different intronic region revealed a highly similar expression pattern (Figures S4B–S4I). This confirms that shakB gap junctions are widely expressed in the central nervous system of the fly by a large number of different cell types.

**LPTCs form large electrically coupled networks via shakB gap junctions**

After describing the anatomical distribution of gap junctions across the *Drosophila* nervous system, we sought to study the functional role of electrical synapses in a restricted number of cells. We chose to investigate the VS and HS cells of the lobula plate tangential cell system for several reasons: First, these cells provide output from the optic lobes to the central brain. Given that shakB is strongly expressed in the optic lobes, we speculated that any effects of removing this protein would likely affect the response properties of LPTCs. Second, VS and HS cells have already been shown to be electrically coupled to each other and to other LPTCs. Third, VS and HS cells are easily accessible by electrophysiological and functional imaging experiments.

VS and HS cells project their axons to the posterior slope in the central brain (Figure 3A). Since we observed shakB expression in this brain area, we reasoned that VS and HS cells might possess shakB gap junctions. When we visualized VS/HS cells with GFP and stained for shakB, we indeed observed colocalization between their axon terminals and shakB (Figure 3B). To identify the neurons that are coupled to VS and HS cells via shakB gap junctions, we performed whole-cell patch clamp recordings from individual cells and filled them with the gap junction-permeable molecule neurobiotin. Staining against neurobiotin revealed large dye-coupled neuronal networks, irrespective of the particular subtype of VS or HS cell (dye-coupling in 10/10 cells; Figures 3C, 3D, 3F, 3G, and S5A). VS and HS cells were dye-coupled to neighboring VS and HS cells, respectively, as described before. Moreover, both cell types were strongly coupled to descending neurons. Interestingly, in contrast to HS cells, VS cells were additionally coupled to dozens of smaller neurons (putatively lobula plate columnar cells) as revealed by the labeling of small cell bodies and thin neurites projecting to the central brain. Importantly, dye-coupling was abolished when injecting VS or HS cells in the shakB2-mutant background (dye-coupling in 0/8 cells), demonstrating that electrical synapses between VS/HS cells and other cells are exclusively formed by shakB-type gap junctions (Figures 3E, 3H, and S5B). VS and HS cells are thus part of large electrically coupled networks connected by shakB gap junctions.

**Spontaneous membrane potential oscillations in VS and HS cells of shakB-deficient flies**

To investigate the functional relevance of electrical synapses in VS/HS cells and upstream visual circuits, we performed electrophysiological recordings from these neurons in control and shakB-deficient flies. Without visual stimulation, VS/HS cells from control flies showed small spontaneous membrane potential fluctuations, corresponding to excitatory postsynaptic potentials (EPSPs) and inhibitory postsynaptic potentials (IPSPs) but generally had stable resting potentials (Figures 4A and 4C). In contrast, a large fraction of recorded cells from shakB2-mutant flies spontaneously displayed fast, high-amplitude membrane potential oscillations. Frequently, sudden, large drops in membrane potential, which again occurred periodically, interrupted these fast oscillations (Figure 4B). Both of these types of oscillations also occurred in flies in which the expression of shakB was knocked down pan-neuronally using RNA interference (hereafter referred to as shakB-RNAi flies) (Figure 4D). This makes it unlikely that secondary
mutations or off-target effects cause the oscillations. Although cells from control flies never displayed any of these two types of oscillations (CS-control: 0/21, RNAi-control: 0/16), both in mutant and knockdown flies, fast oscillations occurred in more than fifty percent of the neurons (shakB-mutant: 17/30, shakB-RNAi: 9/16), of which around half showed slow oscillations (shakB2-mutant: 9/17, shakB-RNAi: 4/9) (Figure 4E). Importantly, neurons only oscillated slowly if they also exhibited fast oscillations, indicative of a functional link between these two oscillation types. We also noted that resting membrane potentials were slightly less negative in both types of flies that lacked shakB when compared with controls (Figure 4F). Why does only a subset of neurons oscillate spontaneously? Interestingly, we found that subtle current injections could switch neurons from a non-oscillatory into an oscillatory state and vice versa (Figures S5C–S5F). This suggests that most (or all) cells that lack shakB are intrinsically unstable,
however, yet unknown variables determine whether a given neuron oscillates spontaneously or not.

To analyze the amplitudes and frequencies of the spontaneous oscillations, we performed Fourier spectrum analysis of the cells’ membrane potential fluctuations. Control flies showed a power spectrum that fell off with increasing frequency, indicative of pure low-pass filtering. In contrast, the power spectra of both shakB2-mutant and shakB-RNAi flies exhibited two prominent peaks (Figures 4G and 4H). We defined two frequency bands around these peaks and named them ultraslow-wave (USW, 0.02–0.2 Hz) and β-oscillations (10–30 Hz), respectively, in accordance with the nomenclature of brain oscillations observed in the mammalian cortex.44,45 The average power in both of these frequency bands was strongly increased in shakB-deficient flies when compared with control flies (Figures 4I and 4J). The median oscillation frequencies were 0.04 and 0.05 Hz for USW oscillations and 17.8 and 15.8 Hz for β-oscillations, for shakB2-mutant and shakB-RNAi flies, respectively (Figures 4K and 4L). Notably, the oscillations in the β frequency band displayed maximum frequencies that were just around or above the corner frequency of the low-pass filter-like power spectrum of control flies (Figures 4G and 4H). Interestingly, this frequency range is highly reminiscent of the reported resonant frequency of HS cells.25 Thus, VS and HS cells from flies that lack shakB often show spontaneous large-amplitude membrane potential oscillations, suggesting that, among others, one possible function of electrical synapses in LPTCs is to prevent their membrane from spontaneously falling into an unstable, oscillatory state.

#### Calcium oscillations in the VS and HS cells of shakB-deficient flies

Our electrophysiological experiments only allowed us to record from one cell at a time. To study the oscillations that arise in VS/HS cells at the network level and to link the membrane potential oscillations we observed after shakB removal to corresponding changes in intracellular calcium levels, we performed two-photon calcium imaging of VS and HS cells. Neurons from control flies did not show any spontaneous calcium activity without visual stimulation (Figure 5A). In contrast, neurons from shakB2-mutant flies displayed slow, large periodic rises and decays in calcium levels (Figure 5B; Video S1). We observed calcium oscillations in the majority of cells (on average 5.2 ±57.8%) of maximally 9 labeled LPTCs per fly in these flies. The calcium oscillations were not restricted to the soma but occurred synchronously throughout dendrites and axons as well (Video S2). Next, we performed pan-neuronal calcium imaging in flies that expressed both GCaMP6f and shakB-RNAi in all neurons. Again, LPTCs displayed large calcium oscillations—in fact, these cells were clearly distinguishable from all other labeled cells mainly because of their large fluorescence changes (Figure 5C; Video S3). We did not observe obvious large calcium transients in the rest of the lobula plate, suggesting that the induction of oscillations after shakB removal is specific to LPTCs (Video S3). In addition to shakB2 mutant and shakB-RNAi flies, we also tested flies in which the pan-neuronal knockdown of shakB was restricted to adulthood by using the temperature-sensitive tubGal80ts transgene (shakB-RNAi-tubGal80ts flies). We confirmed that tubGal80ts allowed for adult-specific RNAi knockdown by performing immunohistochemical and electrophysiological control experiments (Figures S6A–S6I). Importantly, shakB-RNAi-tubGal80ts flies showed large spontaneous calcium oscillations similar to
shakB2-mutant flies and flies in which RNAi expression occurred throughout development (Figure 5D). Thus, the oscillations were not due to a defect caused by depletion of shakB during development. Similar to the electrophysiological experiments, the power in the USW band was significantly higher in shakB2-mutant and both shakB-RNAi fly lines when compared with control flies (Figures 5E and 5F). Power spectrum analysis revealed that the frequency of these slow oscillations (shakB2-mutant: 0.035 Hz, shakB-RNAi: 0.030 Hz, shakB-RNAi-tubGal80ts: 0.042 Hz) was similar to the USW oscillations of the cells’ membrane potential, suggesting a correspondence between these two phenomena (Figure 5G). Importantly, the temporal properties of the calcium indicator excluded the detection of possible fast β-oscillations at the calcium level.
Figure 5. Calcium imaging of VS/HS cells from shakB<sup>2</sup>-mutant, shakB-RNAi, and shakB-N rescue flies

(A–D) Calcium traces of VS and HS cells from control (A), shakB<sup>2</sup>-mutant (B), shakB-RNAi (C), and shakB-RNAi-tubGal80<sup>ts</sup> (D) flies.

(E) Power spectra of calcium traces.

(F) Average power in the ultraslow frequency band.

(G) Maximum oscillation frequency in the USW frequency band.

(H) Cumulative probability of the correlation coefficients for shakB<sup>2</sup>-mutant (left), shakB-RNAi (middle), and shakB-RNAi-tubGal80<sup>ts</sup> flies from cells within flies (colors) and across flies (gray).

(I) Average correlation coefficients within (same) and across (diff) flies.

(J and K) Calcium traces of VS/HS cells from shakB<sup>2</sup>-mutant (J) and shakB-N rescue (K) flies.

(L and M) Power spectra (L) and USW power (M) of shakB-N control (gray), shakB<sup>2</sup>-mutant (orange), and shakB-N rescue (light blue) flies.

Data in (F), (G), and (I): Ctrl, n = 4/18; shakB<sup>2</sup>-mutant, n = 16/83; shakB-RNAi-Ctrl, n = 7/35; shakB-RNAi, n = 11/46; shakB-RNAi-tubGal80<sup>ts</sup>, n = 11/16 flies/cells. Data in (L) and (M): shakB-N control, n = 8/31; shakB<sup>2</sup>, n = 11/18; shakB-N rescue, n = 10/41 flies/cells. Boxplots in (F), (G), and (M) show median (horizontal line), interquartile range Q1–Q3 (boxes), and Q1/Q3–1.5*IQR (whiskers). Data in (I) are mean ± SEM. Dots show data from individual cells (F and G) or pairwise correlations (I). Statistical test in (F), (G), and (I): Mann-Whitney U test (Holm-corrected when comparing >2 experimental groups). *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figure S6 and Videos S1, S2, and S3.
As we could image several cell bodies in the same recording, we analyzed if, and to which extent, the calcium oscillations in different cells were synchronized. On average, we found only a weak, yet significant, positive cross-correlation between calcium signals in all shakB-deficient fly lines (Figures 5H and 5I). Thus, calcium levels in LPTCs do not oscillate in synchrony but still exhibit weak positive correlations.

So far, our experimental approach did not allow us to pinpoint the electrical connections whose loss induces oscillations. As cell-type-specific RNAi knockdown of shakB was unsuccessful (Figure S6L), we performed shakB-rescue experiments in VS and HS cells. To do so, we specifically overexpressed the shakB-N isoform in VS/HS cells in an otherwise shakB mutant background (shakB-N rescue flies). Immunostainings confirmed that shakB was localized to the axon terminals of VS/HS cells but was absent from the rest of the brain (Figures S6J and S6K). Calcium imaging in these flies revealed that rescuing electrical synapses specifically between VS/HS cells prevents calcium oscillations (Figures 5J–5M). Thus, the loss of electrical synapses from VS/HS cells themselves (and not other cell types) is responsible for inducing an oscillatory state.

**Cell-intrinsic mechanisms generate oscillations**

The low correlation between calcium oscillations in different cells points toward a mainly cell-autonomous origin. To directly test if chemical synaptic connections between LPTCs themselves or chemical input from other cells drive or affect these oscillations, we used pharmacology to block chemical synaptic transmission. VS and HS cells receive mainly cholinergic, glutamatergic, and GABAergic inputs. Therefore, we simultaneously applied mecamylamine (MEC) to block excitatory cholinergic input and picrotoxin (PTX) to interfere with inhibitory glutamatergic and GABAergic input. By performing electrophysiological recordings, we confirmed that this pharmacological cocktail indeed blocked all fast synaptic input to these cells (Figures S7A–S7F). After blocking chemical synaptic input, LPTCs continued to display calcium oscillations with similar power and frequency as before (Figures 6A, 6B, 6E, 6I, and 6J; Videos S4 and S5). This suggests that after removal of electrical synapses, VS/HS cells start to oscillate spontaneously and cell autonomously, without any synaptic drive. Interestingly, we found that the average cross-correlation between calcium signals in these cells dropped to zero, suggesting that the weak correlation was caused by weakly synchronizing chemical synaptic input (Figures 6F and 6G). This could be due to common synaptic input from T4 and T5 cells or possible chemical connections between LPTCs themselves.

**Voltage-gated sodium and Ih channels are involved in generating oscillations**

Our finding that the calcium oscillations in VS/HS cells arise cell autonomously prompted us to investigate which cell-specific conductances generate these oscillations. Two prominent channels that were shown to be involved in many neuronal oscillators are hyperpolarization-activated Ih channels and voltage-gated sodium Na_v channels (in *Drosophila* termed “paralytic”). In addition, Na_v channels have been implicated in generating the frequency-dependent amplification of synaptic inputs in *Calliphora* HS cells mentioned earlier. First, to test the involvement of Ih channels, we applied the Ih-antagonist ivabradine together with MEC and PTX to block chemical synaptic input. After application of ivabradine, calcium oscillations did not cease but slowed down (Figures 6C and 6H–6J). Under the premise that ivabradine is similarly effective and specific at blocking *Drosophila* Ih, as it is against rabbit Ih, these results suggest that Ih channels play a role in setting the frequency of these oscillations.

To test the potential role of Na_v channels, we blocked chemical synaptic input pharmacologically and then applied tetrodotoxin (TTX), a specific Na_v-channel blocker. Application of TTX led to a complete cessation of calcium oscillations in all cells, showing that Na_v channels are necessary for generating them (Figures 6D, 6H, and 6I). The dominant component of Na_v currents rapidly inactivates within several milliseconds. This, and our hypothesis that fast voltage oscillations and slow voltage/calcium oscillations are functionally linked, prompted us to test whether silencing Na_v channels also eliminates fast membrane potential oscillations. In line with this prediction, VS and HS cells from shakB flies that displayed slow and/or fast oscillations turned completely silent after application of TTX (Figures 6K–6M). These experiments suggest that Na_v channels are directly involved in generating the fast spontaneous membrane potential oscillations. Notably, a subset of *Drosophila* Na_v channels exhibit persistent sodium currents that could contribute to slow oscillations as well. However, Na_v channels alone cannot account for the large hyperpolarized phases of the slow oscillations. Thus, we propose that fast oscillations secondarily lead to slow voltage and calcium oscillations by a mechanism that might involve Ih channels among others.

**Loss of electrical synapses impairs visual responses of VS and HS cells**

Finally, we investigated whether VS and HS cells of flies that lack shakB-type electrical synapses still respond to visual stimuli, despite showing membrane potential oscillations. We performed electrophysiological recordings and excluded cells with slow membrane potential oscillations from the analysis. VS/HS cells of control flies responded to moving sine wave gratings in a direction-selective manner by depolarizing to their preferred direction and hyperpolarizing to their null direction. VS and HS cells from shakB-deficient flies still exhibited direction-selective responses, albeit at strongly reduced response amplitudes (Figures 7A–7D). Interestingly, direction selectivity, as calculated by the normalized vector sum of the responses (“LDir”), was unaffected (Figure 7E).

To test whether ON and OFF motion pathways are differentially affected by removing shakB, we stimulated VS/HS cells by showing moving ON or OFF edges. Responses to ON and OFF motion stimuli were both reduced, but the OFF pathway was affected more strongly (Figures 7F and 7G). In addition to direction-selective input via T4/T5 cells, VS and HS cells receive signals from a parallel luminance-sensitive pathway. To investigate whether shakB gap junctions are important components of this “flicker pathway,” we presented full field bright and dark flashes to the flies. Although ON flicker responses were left untouched by the loss of shakB, OFF flicker responses were strongly reduced (Figures 7H and 7I).

Are the effects on visual processing at the level of VS/HS cells caused by removing shakB from these cells themselves or rather by removing shakB from neurons in their upstream circuitry? To decide between these alternatives, we measured visual calcium
Figure 6. Pharmacological profile of calcium and voltage oscillations in VS/HS cells from shakB²-mutant flies

(A–D) Calcium traces of VS and HS cells from shakB²-mutant flies before (A) and after application of mecamylamine and picrotoxin (MEC + PTX) (B), ivabradine (IVA) (C), or tetrodotoxin (TTX) (D).

(E) Power spectra of calcium traces for control (gray) and shakB²-mutant flies either untreated (orange) or after application of MEC + PTX (dark blue).

(F) Cumulative probability of the correlation coefficients for shakB²-mutant flies before (left) and after MEC + PTX application calculated within same (colors) or across different flies (gray).

(G) Correlation coefficients within (same) and across (diff) shakB²-mutant flies before and after application of MEC + PTX.

(H) Power spectra of calcium traces for indicated experiments.

(I) Average power in the ultraslow frequency band (USW).

(J) Maximum oscillation frequency in the USW frequency band.

(K) Representative voltage traces of VS/HS cells from shakB²-mutant flies before (orange) and after treatment with TTX (purple).

(L) Power spectra of membrane potentials of shakB²-mutant flies before (orange) and after application of TTX (purple).

(M and N) Average power in the USW frequency band (M) and β frequency band (N) for flies in (K) and (L).

Data in (E)–(J) are from Ctrl, n = 4/17; shakB²-mutant, n = 9/48; shakB² MEC+PTX, n = 9/48; shakB² IVA, n = 6/31; shakB² TTX, n = 4/18 flies/cells. Data in (L)–(N) are from shakB²-mutant, n = 5/5 cells/fly. Boxplots in (I), (J), (M), and (N) show median (horizontal line), interquartile range Q1–3 (boxes), and Q1/Q3–1.5*IQR (whiskers). Data in (G) are mean ± SEM. Dots show data from pairwise correlations (G) or individual cells (I, J, M, and N). Statistical test in (G), (I), (J), (M), and (N): Mann-Whitney U test (Holm-corrected when comparing >2 experimental groups). *p < 0.05, **p < 0.01, ***p < 0.001.

See also Figures S7A–S7F and Videos S4 and S5.
Figure 7. Electrophysiological responses of VS and HS cells from shakB^2-mutant and shakB-RNAi flies to visual stimuli
(A) Average voltage response traces of VS/HS cells from CS-control (gray) and shakB^2-mutant flies (orange) to preferred- (left) and null-direction grating motion.
(B) Similar to (A) but for VS/HS cells from RNAi-control (gray) and shakB-RNAi flies (turquoise).
(C) Directional tuning curves for CS-control and shakB^2-mutant flies (left) and RNAi-control and shakB-RNAi flies.
(D and E) Response strength (PD-ND) (D) and direction selectivity (E) of VS/HS cells to moving gratings.

(legend continued on next page)
responses in flies in which shakB-N was rescued in VS/HS cells. The results of these measurements closely resembled those of the electrophysiological experiments in shakB2-mutant and shakB-RNAi flies: VS/HS cells from shakB-N rescue flies continued to show strongly reduced responses to drifting gratings, although their direction selectivity was unimpaired (Figures 7J–7L). Additionally, responses to moving ON and OFF edges were both reduced in amplitude, however, again with a much more pronounced effect on the OFF pathway (Figure 7M). Thus, the removal of electrical synapses from visual circuits upstream of VS/HS cells is responsible for their impaired responses to visual motion stimuli.

Visual responses of T4/T5 cells mirror VS/HS cell responses in shakB-deficient flies

The results of the previous section predict that similar effects of shakB removal should already be present at the level of the major presynaptic inputs to VS/HS cells—the T4 and T5 cells. To test this prediction, we performed two-photon calcium imaging from the axon terminals of upward-prefering T4/T5c cells while presenting moving ON and OFF edges (Figures S7G–S7J). At the level of calcium, T4 and T5 neurons respond exclusively to either ON or OFF motion31,52 allowing us to separate them by their responses to the stimulus. Calcium signals in the axon terminals of both T4 and T5 cells from control flies were narrowly tuned to upward motion. T4 and T5 cells from shakB2-mutant flies also responded in a direction-selective manner. However, similar to the electrophysiological measurements in postsynaptic VS/HS cells, the response amplitudes were reduced in both T4 and T5, again with a slightly stronger, however more variable, effect on T5 cells (Figures S7H and S7J). When calculating the direction-selectivity index, no difference between controls and shakB2-mutant flies was found (Figure S7J).

Taken together, these results suggest that shakB electrical synapses in the optic lobe—upstream of T4/T5 cells—play crucial roles in processing motion- and direction-related visual information but are not essential for the emergence of direction selectivity.

DISCUSSION

Patterns of gap junction expression in the Drosophila central nervous system

Although large efforts have been made in generating a complete “chemical synaptic connectome” of the adult Drosophila nervous system,8–11 surprisingly, no study has yet investigated the abundance and distribution of electrical synapses throughout that tissue. Here, we took the first steps in filling this gap.

We first described the expression patterns of all eight innexins in the adult central nervous system of Drosophila based on immunohistochemistry. We found that some innexins are not expressed, others exclusively in glial cells, and still others only sparsely in the CNS (Figures 1 and S1–S3). According to our immunostainings, shakB is the only innexin that is widely expressed in many neurons of the brain and VNC. Interestingly, both the immunostainings and the shakB-Trojan-Gal4 reporter lines suggest that shakB gap junctions are more abundant in primary and secondary sensory areas of the brain and much less so in higher brain centers such as the central complex or mushroom body.

Our immunohistochemical results are largely in agreement with published RNA sequencing datasets (Figure S3B).37–40 Here, mRNAs for zpg, inx5, inx6, and inx7 were found to be not or only very weakly expressed. We did not detect zpg and inx7 protein, and inx5 and inx6 only localized sparsely to single brain regions. A study that performed cell-type-specific RNA sequencing of many cells in the optic lobes detected shakB mRNA in ca. 75% of tested cell types.37 Although this is in qualitative agreement with our finding that shakB is widely expressed, our shakB-Trojan-Gal4 reporter lines clearly label less than 75% of cells in the optic lobes or CNS. Further differences to that study37 exist as well: although inx3 mRNA was highly expressed in all sequenced cells, we detected inx3 protein only in neuropil ensheathing glia. Furthermore, we did not detect expression of inx7, despite high RNA levels in all photoreceptor subtypes. Whether such differences can be explained by the fact that high mRNA levels do not necessarily predict high protein levels or by limitations of one or the other method remains to be investigated.

Our description of innexin distribution based on immunostainings has obvious limitations: With the exception of shakB, we did not validate the signal specificity of the antibodies in genetic knockout fly lines. However, most of the antibodies that we obtained from other researchers were validated in publications from these labs.4,7,20,21 Furthermore, we used two different antibodies per innexin and obtained similar results (Figures 1 and S1). One caveat of our immunohistochemical approach is that cells that weakly express innexin proteins might fall below the detection limit. This might explain, for example, why we did not detect clear innexin expression in the mushroom body, whereas
other studies have found several innexin subtypes to be involved in mushroom body function.\(^{19-21}\) As another limitation, the spatial resolution of confocal microscopy does not allow for an unambiguous assignment of innexin expression to specific cell types. Here, a recent study suggests that the use of expansion microscopy can solve this problem.\(^{53}\) Nonetheless, our approach can help to narrow down the list of candidate cells that might form electrical synaptic connections. Similarly, our shakB-Trojan-Gal4 reporter lines do not give definitive evidence about all cell types that express shakB but can serve as a useful guide for identifying cell types for closer investigation.

To circumvent some of the problems mentioned above, promising approaches include the generation of fly lines that allow for the conditional, endogenous tagging of innexin proteins.\(^{43,54,55}\) Once a cell type has been identified to express gap junction proteins, the next step is to test if, and to which cells, it is electrically coupled. In this study, we used whole-cell patch clamp recordings and neurobiotin injections to identify the electrically coupled partners of VS and HS cells. This technique is labor intense and therefore not easily scalable. Alternative techniques that depend on the targeted delivery of gap junction-permeable molecules to a genetically defined cell population circumvent the manual injection step but suffer from low signal-to-noise ratios.\(^{56,57}\) Unfortunately, with both approaches, the identification of coupled cell types is difficult because all connected cells are labeled simultaneously. In the end, improvements in EM technology, such as enhancing the resolution to detect gap junctions in large-scale datasets or genetic tagging of gap junction proteins combined with electron-dense labeling,\(^{58}\) would solve many of the issues discussed above. Such a technique would not only allow for the identification of cells that form electrical synapses but also reveal all of their connected partners.

Intrinsic oscillations and function of electrical synapses in VS and HS cells

Removing shakB gap junctions through a null mutation or pan-neuronal knockdown induced fast and slow membrane potential oscillations as well as large calcium oscillations in VS and HS cells. This is unexpected because in many neural networks, exactly the opposite is the case—electrical coupling is necessary for generating network oscillations by synchronizing neural activity.\(^{39,60}\) Similarly, gap junctions between cardiomyocytes function to stabilize and synchronize electrical activity throughout the myocardium.\(^{51}\) In the present study, VS and HS cells continued to oscillate when isolated from synaptic input and ceased to do so when rescuing shakB cell type specifically. These two findings argue that the oscillations are not due to network effects but arise cell autonomously, owing to a loss of shakB from VS/HS cells themselves. Furthermore, adult-specific RNAi knockdown of shakB suggests that the oscillations are not caused by developmental defects. However, as immediate pharmacological block of gap junctions was unsuccessful (Figure S6M), we cannot rule out that the oscillations are in part shaped by adaptive mechanisms that occur on the timescales of hours or days.

We partially uncovered the biophysical mechanisms that generate oscillations by showing that Na\(_\text{v}\) channels are necessary and \(I_\text{h}\) channels influence their temporal dynamics. Interestingly, application of the Na\(_\text{v}\) antagonist TTX blocked both types of oscillations at the level of the membrane potential and at the level of calcium. Moreover, slow USW oscillations only occurred in cells that show fast β-oscillations. Consequently, we consider it plausible that the fast voltage oscillations directly induce the slow oscillations. The coupling between voltage and calcium oscillations could then potentially occur via calcium-activated potassium channels such as slowpoke. The exact mechanistic links between fast and slow voltage oscillations and calcium oscillations, however, are yet unknown and must be further investigated in the future.

What is the functional role of electrical synapses in the lobula plate network? We speculate that under normal conditions, these connections might form a safety net for VS and HS cells to keep their nonlinear membrane conductances in check by buffering cell-intrinsic noise via dissipating it through the coupled network. Only a synaptic stimulus of the right frequency and strength, acting on one or multiple connected cells synchronously, would engage the nonlinear mechanisms that lead to an amplification of these signals. If electrical synapses are missing, the cell-intrinsic noise itself is sufficient to induce spontaneous oscillations at the resonant frequency of these cells. Interestingly, it has been shown that active conductances in HS cells lead to an amplification of high-frequency inputs that would otherwise be attenuated by the low-pass properties of the passive membrane.\(^{25}\) LPTCs receive such high-frequency inputs when the fly is confronted with fast visual motion. Thereby, this amplifying mechanism increases the dynamic range of these cells. Therefore, we consider it plausible that the spontaneous oscillations we observe in VS/HS cells after removal of shakB and the frequency-dependent amplification of synaptic inputs might be based on the same underlying conductance changes. This hypothesis, however, is difficult to test experimentally and must await further investigations.

Visual processing and electrical synapses

Several studies used the fly Calliphora to investigate how electrical coupling between LPTCs affects their complex receptive field structure.\(^{37,38}\) Subsequent studies then built on these results and performed computational modeling to show that axo-axonal gap junctions between LPTCs can increase robustness and efficiency of coding.\(^{30,33,34}\) The spontaneous oscillations, which arise in VS/HS cells without electrical synapses, complicate the detailed experimental investigation of these models. Thus, we chose to focus on the origin of these oscillations and on the role of electrical synapses in upstream visual circuits in this study.

Loss of shakB electrical synapses led to a reduction of the response magnitudes of T4/T5 and VS/HS cells to both ON and OFF motion stimuli and to an almost complete loss of OFF flicker sensitivity in VS/HS cells. Conversely, the degree of direction selectivity—that is, the sharpness of tuning—was unaffected. Thus, the elementary computation of motion direction does not directly depend on electrical synapses. How and at which level in the visual processing pathway do electrical synapses affect the responses T4/T5 and VS/HS cells? Since shakB mRNA is not or only very weakly expressed in T4/T5 cells,\(^{37}\) the electrical connections responsible for these effects are likely to be found upstream in the medulla or lamina. Future investigations can now pinpoint the neural and synaptic substrates of these effects by identifying candidate cell types and connections. One such promising candidate is the lamina monopolar
cell L4, shakB colocalizes with the dendrites of L4 cells in the proximal lamina and a shakB-Trojan-Gal4 line labels this cell type (Figures S4F–S4I). Additionally, L4 cells are important for OFF motion detection both at the level of VS/HS cells and behavior.62,63 The loss of electrical synapses from L4 cells might impair detection of OFF motion both at the level of VS/HS cells and behavior.62,63 The loss of electrical synapses from L4 cells might impair detection of OFF motion both at the level of VS/HS cells and behavior.62,63 The loss of electrical synapses from L4 cells might impair detection of OFF motion both at the level of VS/HS cells and behavior.62,63

Notably, we only probed a narrow set of visual stimuli. It is likely that electrical synapses play further important roles in other visual regimes, such as under noisy, low-contrast or low-luminance conditions, as is the case for electrical synapses in the mammalian retina.64 Moreover, in the mammalian retina, the strength of electrical coupling between neurons is dynamic and can change depending on ambient luminance or circadian rhythm.65 It will be interesting to see if similar mechanisms are at work in invertebrate visual systems. Furthermore, we only tested the output of a single visual stream. Future research will show which functions electrical synapses exhibit in circuits for phototaxis, color vision, contrast vision, or small object detection but also in other regions of the nervous system such as the central brain or VNC.

Taken together, our study describes the anatomical distribution and demonstrates essential functional roles of electrical synapses in the Drosophila nervous system. Incorporating electrical synaptic connections into future connectomes and brain-wide computational models and using the rich Drosophila tool kit to investigate the functional properties of these connections, will expand our understanding of their contribution to information processing in the fly brain and in nervous systems in general.

STAR METHODS
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SUPPLEMENTAL INFORMATION
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AUTHOR CONTRIBUTIONS
G.A. conceived the study, performed and analyzed all experiments, and wrote the manuscript. R.V. helped with antibody design and validation, R.V. and S.F. generated fly lines. A.B. provided funding. A.B., R.V., and S.F. commented on the manuscript.

DECLARATION OF INTERESTS
The authors declare no competing interests.

INCLUSION AND DIVERSITY
One or more of the authors of this paper self-identifies as an underrepresented ethnic minority in science. One or more of the authors of this paper self-identifies as a member of the LGBTQ+ community.

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### STAR METHODS

#### KEY RESOURCES TABLE

| REAGENT or RESOURCE | SOURCE | IDENTIFIER |
|---------------------|--------|------------|
| **Antibodies**      |        |            |
| Antibodies          |        |            |
| anti-ogre rabbit polyclonal antibody | This paper | N/A |
| anti-inx2 rabbit polyclonal antibody | This paper | N/A |
| anti-inx3 rabbit polyclonal antibody | This paper | N/A |
| anti-zpg rabbit polyclonal antibody | This paper | N/A |
| anti-inx5 rabbit polyclonal antibody | This paper | N/A |
| anti-inx6 rabbit polyclonal antibody | This paper | N/A |
| anti-inx7 rabbit polyclonal antibody | This paper | N/A |
| anti-shakB rabbit polyclonal antibody | This paper | N/A |
| anti-ogre rabbit polyclonal antibody | Reinhard Bauer | N/A |
| anti-inx2 rabbit polyclonal antibody | Reinhard Bauer | N/A |
| anti-inx3 rabbit polyclonal antibody | Reinhard Bauer | N/A |
| anti-zpg guinea pig polyclonal antibody | Guy Tanentzapf | N/A |
| anti-inx5 rabbit polyclonal antibody | Chia-Lin Wu | N/A |
| anti-inx6 rabbit polyclonal antibody | Ann Shyn-Chiang | N/A |
| anti-inx7 rabbit polyclonal antibody | Ann Shyn-Chiang | N/A |
| anti-nc82 mouse monoclonal antibody | DSHB | RRID: AB_2314866 |
| anti-GFP rabbit polyclonal antibody | Invitrogen | Cat# A-11122; RRID: AB_221569 |
| anti-GFP chicken polyclonal antibody | Rockland | Cat# 600-901-215S; RRID: AB_1537403 |
| goat anti-rabbit-Alexa-488 | Invitrogen | Cat# A32731; RRID: AB_2633280 |
| donkey anti-chicken-Alexa-488 | Jackson Immuno Research | Cat# 703-545-155; RRID: AB_2340375 |
| goat anti-rabbit-Alexa-568 | Invitrogen | Cat# A-11011; RRID: AB_2535730 |
| goat anti-mouse-Alexa-647 | Invitrogen | Cat# A32728; RRID: AB_2633282 |
| streptavidin-Alexa-568 | Invitrogen | Cat# S11226; RRID: AB_2315774 |
| streptavidin-Alexa-633 | Invitrogen | Cat# S21375; RRID: AB_2313500 |
| **Chemicals, peptides, and recombinant proteins** | | |
| Tetrototoxin citrate | Abcam | Cat# ab120055 |
| Picrotoxin | TCI | Cat# C0375 |
| Ivalradine hydrochloride | Sigma | Cat# SML0281 |
| Mecamylamine hydrochloride | Sigma | Cat# M9020 |
| Carbexoxolone | Sigma | Cat# C4790 |
| Neurobiotin tracer | VectorLabs | Cat# SP-1120; RRID: AB_2313575 |
| **Deposited data** | | |
| Raw and analyzed data | This paper | https://gin.g-node.org/gammer/Ammer_et_al_2022.git |
| **Experimental models: Organisms/strains** | | |
| Canton S (wildtype) | BDSC | RRID: BDSC_64349 |
| MiMIC02168-shakB; +; + | BDSC | RRID: BDSC_34285 |
| MiMIC15228-shakB; +; + | BDSC | RRID: BDSC_60999 |
| MiMIC02168-shakB-Trojan-Gal4; +; + | This paper | N/A |
| MiMIC15228-shakB-Trojan-Gal4; +; + | This paper | N/A |
| w; +; R57C10-Gal4 (pan-neuronal) | BDSC | RRID: BDSC_39171 |
| w; +; R24E09-Gal4 (VS/HS cells) | BDSC | RRID: BDSC_49083 |
| w; +; R54C07-Gal4 (SPG glia) | BDSC | RRID: BDSC_50472 |

(Continued on next page)
RESOURCE AVAILABILITY

Lead contact
Further information and requests for resources and reagents should be directed and will be fulfilled by the Lead Contact, Georg Ammer (gammer@neuro.mpg.de).

Materials availability
Newly generated fly lines and antibodies are available from the lead contact upon request.

Data and code availability
All data reported in this paper and codes used for analysis are publicly available at: https://gin.g-node.org/gammer/Ammer_et_al_2022.git

EXPERIMENTAL MODEL AND SUBJECT DETAILS

Fly husbandry
All flies were raised on standard cornmeal agar medium at 60% humidity on a 12 h light/12 h dark cycle throughout development. Except when noted otherwise, flies were raised at 25°C. We used female flies for all experiments except for those in which shakB was rescued in VS/HS cells, which were performed with males. For electrophysiology, we used flies that were between 6 hours and 2 days old. For calcium imaging experiments, in order to enhance expression of transgenes, flies were transferred to 29°C after eclosion and then imaged at an age between 4 and 7 days. Flies carrying a tubGal80ts transgene were raised at 18°C. For inducing Gal4 expression, tubGal80ts flies were transferred to 31°C after eclosion, and imaged when 7 days old. For tubGal80ts control experiments, flies were kept at 18°C until the beginning of the experiment. The full genotypes of flies used in this study are listed in Table S1.

METHOD DETAILS

Generation of fly lines
For generating Trojan-Gal4 lines the T2A-TrojanGal4 plasmid with the correct reading frame was injected into embryos of the respective MIMIC insertion lines.
pBS-KS-attB2-SA(2)-T2A-Gal4-Hsp70 (DGRC#1410) was injected into y[1] w[+] M{y[+mDint2]=MIC}shakB[MI15228] (RRID: BDSC_60999)
pBS-KS-attB2-SA(0)-T2A-Gal4-Hsp70 (DGRC#1412) was injected into y[1] w[+] M{y[+mDint2]=MIC}shakB[MI02168] (RRID: BDSC_34285)

Injections were performed by BestGene (https://www.thebestgene.com/).

Antibody generation
We generated polyclonal rabbit antibodies against different innexin proteins by immunizing against the following peptides:

- ogre (inx1): CFACKQVEPSKHDRAK
- inx2: CMSGDHSAHKRPFD
- inx3: CPPVETFLLGGKETET
- zpg (inx4): CAQSLKIPPGADKI
- inx5: CLRTSASGSTLESPV
- inx6: IAEGVGPETRGVTKC
- inx7: CEAPTPAKNYRPEL
- shakB (inx8) purified antibody: CQHHRVPGLKGEIQD
- shakB (inx8) serum antibody: CQHHRVPGLKGEIQD

Both shakB antibodies were raised against a part of the C-terminal sequence that is common to all protein isoforms. The shakB serum antibody was generated in our laboratory using standard procedures. The other eight antibodies were generated by the company GenScript (https://www.genscript.com/).

Immunohistochemistry
Brains (and ventral nerve cords) were dissected in cold phosphate-buffered saline (PBS) and fixed in 4% PFA (in PBS with 0.1% Triton X). Brains were washed three times in PBT (PBS + 0.3% Triton X), blocked in 10% normal goat serum (NGS) in PBT and incubated in the primary antibody solution (antibody in 5% NGS in PBT) for 36-48 hours. Afterwards, brains were washed in PBT overnight and then incubated in the secondary antibody solution for 48-72 hours. Brains were washed in PBT overnight, washed in PBS and then mounted in Vectashield medium (VectorLabs).

Primary antibodies were used at the following dilutions: anti-nc82 (1:25), anti-GFP (1:1000), anti-inx1-genscript (1:1000), anti-inx2-genscript (1:1000), anti-inx3-genscript (1:1000), anti-inx4-Tanentzapf (1:2000), anti-inx5-Wu (1:1000), anti-inx6-Chiang (1:2500), anti-inx7-Chiang (1:200), anti-shakB-serum (1:800), anti-ogre-Bauer (1:50), anti-inx2-Tanentzapf (1:1000), anti-inx3-Bauer (1:50), anti-zpg-genscript (1:1000), anti-inx5-genscript (1:500), anti-inx6-genscript (1:1000), anti-inx7-genscript (1:2000), anti-shakB-genscript (1:2000). All secondary antibodies were used at a dilution of 1:500 or 1:1000.

The specific innexin antibodies that were used in each Figure panel are listed in Table S2.

Confocal microscopy
Images were acquired on a Leica SP5 or SP8 confocal microscope with either a 20× (Leica #115063643) or 63× (Leica #11506353) glycerol immersion objective or a 40× (Leica #11506358) oil immersion objective at a resolution of 1024x1024 pixels. Image processing was performed with Fiji.

Electrophysiology
Electrophysiological whole-cell patch clamp recordings were done as previously described.68 Briefly, flies were waxed to a plexiglass holder with bees wax and the head inserted into an opening in aluminum foil that was mounted in a recording chamber. External saline was added to the preparation, a part of the cuticle on the posterior side of the head removed with a fine needle, and the muscle covering the LPTC cell bodies severed. The glial sheath on the surface of the brain was locally digested by applying Collagenase IV (Gibco) through a pipette with a ~5 μm opening. When the somata of LPTCs were exposed, whole cell recordings were performed with patch electrodes (TW-150F-4, WPI) pulled to a resistance of 5-9 MOhm. Signals were amplified with a BA-1s amplifier (npi electronics), low-pass filtered with a cut-off frequency of 3 kHz and digitized at 10 kHz. Data acquisition was performed with Matlab R2011b (Mathworks) and data analysis was done with Matlab R2011b (Mathworks), Python 2.7.15 and Python 3.8.8. External saline contained the following (in mM): 103 NaCl, 3 KCl, 5 TES, 10 trehalose, 10 glucose, 3 sucrose, 26 NaHCO3, 1 NaH2PO4, 1.5 CaCl2, and 4 MgCl2. The pH of the solution was 7.3 – 7.35 and the osmolarity was around 285 mOsmol. External saline was oxygenated with 95% O2/5% CO2. Internal solution contained the following (in mM): 140 K-aspartate, 10 HEPES, 4 MgATP, 0.5 Na3GTP, 1 EGTA, 1 KCl, and 0.2 Alexa Fluor 568 hydrazide. The pH of the internal solution was adjusted to 7.26 and the osmolarity to ca. 265 mOsmol. Cell types were identified based on the typical response profiles of VS and HS cells to moving gratings and - in most cases - anatomically when cells were properly filled with the Alexa dye. For neurobiotin coupling experiments, we included 2% neurobiotin (VectorLabs) in the patch pipette.
**Calcium imaging**

For functional imaging, we used a custom-built two-photon laser scanning microscope as described previously. Flies were dissected identically to the electrophysiological experiments and imaged in the same extracellular saline. Images were typically recorded at a resolution of 128 × 128 pixels and a frame rate of 3.76 Hz. Some experiments were done at a resolution of 64 × 64 pixels and a frame rate of 12.6 Hz. Data acquisition was performed in Matlab R2013b (MathWorks) using ScanImage 3.8. Data analysis was performed in Matlab R2013b (MathWorks), Python 2.7.15, and Python 3.8.8.

**Visual stimulation**

For electrophysiological recordings, a custom-built LED arena was used for visual stimulation. The arena spanned 170° in azimuth and 90° in elevation, allowed refresh rates up to 600 Hz and had a maximum luminance of 80 cd/m². Moving sine wave gratings were displayed at full contrast, had a spatial wavelength of 30° and moved at a temporal frequency of 0.5 Hz for 3 s. For stimulating ON and OFF pathways independently, we used multiple moving ON or OFF edges. The stimulus started with a standing vertical or horizontal square wave grating that had a wavelength of 42°. Then, either all bright or all dark edges moved for 0.45 s with a velocity of 50°/s. Flicker stimuli consisted of full field flashes at maximum luminance that lasted for 3 s. Stimuli were presented in a randomized manner.

For functional calcium imaging, we used a custom built projector-based arena. Visual stimuli were projected onto the back of an opaque cylindrical screen with two micro-projectors (TI DLP Lightcrafter 3000). The arena covered 180° in azimuth and 105° in elevation. Visual stimuli were displayed with a refresh rate of 180 Hz and a maximum luminance of 276 ± 48 cd/m². For stimulating VS/HS or T4/T5 cells we presented moving ON or OFF edges at full contrast moving with a velocity of 15°/s. Moving gratings had a spatial wavelength of 30° and were shown for 1 s at 1 Hz temporal frequency. Stimuli were presented in a randomized fashion. For imaging spontaneous calcium oscillations in VS and HS cells, the arena was completely dark i.e. no visual stimulus was displayed. Spontaneous activity of VS/HS cells was recorded for 2500 frames at 3.76 Hz which amounts to ca. 663 s.

**Pharmacology**

The following pharmacological substances were used in this study. Tetrodotoxin (TTX, Abcam) 1 mM stock in H₂O, used at a final concentration of 1 μM. Picrotoxin (PTX, TCI) 50 mM stock in DMSO, final concentration 2.5 μM. Mecamylamine (MEC, Sigma) 100 mM stock in H₂O, final concentration: 200 μM. Ibravabide (IVA, Sigma) 20 mM stock in H₂O, final concentration: 200 μM. Carbenoxolone (CBX, Sigma) 5 mM stock in H₂O used at a final concentration of 200 μM. Aliquoted stocks were kept at -20°C, freshly diluted in external saline at the day of the experiment, and added directly to the preparation with a pipette. Recordings were started at least 10 min after addition of the pharmacological substances to allow for diffusion. Carbenoxolone was added to the saline 20 min before the whole cell recording was established.

**QUANTIFICATION AND STATISTICAL ANALYSIS**

**Data analysis**

**Electrophysiology**

For responses to visual motion stimuli, we computed the mean response over the whole stimulation period and averaged that value over sweeps. For responses to full field flicker stimuli, we detected the extrema during the first 500 ms of the response and averaged the value over sweeps. Power spectra were computed using fast Fourier transformations. We classified cells as exhibiting USW- and/or β-oscillations by visually inspecting their membrane potential traces and power spectra.

**Calcium Imaging**

Baseline subtraction and calculation of ΔF/F was performed as previously described. In contrast to electrophysiology, for power spectra we first z-scored the ΔF/F values. This was done because ΔF/F values are relative measures and can vary depending on expression strength, depth of imaging, size of regions of interest (ROIs) and other factors. We then used fast Fourier transformations to calculate power spectra. Cells were classified as active/oscillatory when the variance of their ΔF/F signal was > 0.15 during the whole imaging period and when they exhibited a clear peak in the USW-power spectrum. For T4/T5 imaging we first averaged the response traces over sweeps and then took the maximum ΔF/F value during the stimulation period. The direction selectivity index "LDir" was calculated as the length of the normalized response vector:

$$L_{Dir} = \frac{\sum_{\varphi} r(\varphi)}{\sum_{\varphi} |r(\varphi)|}$$

where $r(\varphi)$ is a vector with the stimulus direction $\varphi$ as its angle and the corresponding neuronal response as its length.

**Statistical analysis**

Gaussianity of data point distributions was assessed by performing Shapiro-Wilk’s test. Based on the outcome we performed either Welch’s t test or Mann-Whitney’s U test and applied Holm’s post-hoc correction when comparing more than two experimental groups. Statistical analysis was performed in Python 3.8.8 using scipy 1.6.2, statsmodels 0.12.2 and scikit_posthocs 0.6.7 packages. More details on the statistical analysis are provided in Table S3.