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Santa Barbara

The *Drosophila* fan-shaped body stores a vector in an allocentric coordinate system

A dissertation submitted in partial satisfaction of the
requirements for the degree Doctor of Philosophy
in Molecular, Cellular, and Developmental Biology

by

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December 2025

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ABSTRACT

The *Drosophila* fan-shaped body stores a vector in an allocentric coordinate system

by

Benjamin Edward Gorko

The nervous systems of animals perform computations that support adaptive behavior. In the case of insect navigation, a region of the insect brain, called the central complex, performs vector computations that facilitate the representation of space in a world-centered (allocentric) reference frame. While there is an in-depth understanding of the anatomy of the central complex in the fruit fly, *Drosophila melanogaster*, the function of most of its cell types remains a mystery. I leveraged the theoretical framework of vector computation in combination with electron microscopy anatomy data to hypothesize a function for a previously uncharacterized cell type called PFL1. Specifically, I propose that it transforms a vector stored in the fly's memory from an allocentric reference frame to an egocentric (fly-centered) reference frame. Through 2-photon functional imaging experiments, I show that PFL1's input output relationship is consistent with this hypothesis. This finding leads me to investigate how the circuitry upstream of PFL1 encodes the stored vector. Via 2-color calcium imaging experiments where I simultaneously record from three cell types at once, I rule out a simple mechanism where the vector is encoded by the calcium activity of the presynaptic neurons, suggesting a more complex mechanism is at play. Through further

imaging experiments, I find that a cell type called FC1 drives local second messenger signaling in the axons of another cell type called FB2B. These results build on our understanding of the fly's navigational system and provide fundamental insights into the nature of neural computation.

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1. Introduction

The navigational behavior of animals reveals that they have an internal representation of their surroundings and their place in them. A remarkable example of such behavior is the waggle dance of bees. Upon returning from a foraging trip, a bee will communicate to other bees the location of a food source by performing a dance¹. The distance to the food source is represented by the duration of a portion of the dance called the waggle run¹. The angle relative to vertical of the waggle run represents the angle relative to the sun of the food source¹. This behavior illustrates not only that the bee retains knowledge of the location of the food source but reveals how this location is stored in its mind: as a distance and a direction. These variables are the components of a polar coordinate system, a mathematical framework for mapping space. Resonating with this behavioral evidence, recent neurobiological discoveries point to a neural substrate for a polar coordinate system referenced to a landmark in the animal's surroundings in a region of the insect brain known as the central complex. Decoding how the central complex enables the kind of mental representations apparent from observations of insect behavior is a remarkable achievement in the pursuit of neuroscience's ultimate goal of describing how the brain relates to the mind.

1.1 Insect central complex overview

The central complex is made up of several neuropils positioned along the midline of the brain: a handlebar-shaped structure named the protocerebral bridge (PB), a donut-shaped structure known as the ellipsoid body (EB), a layered neuropil in the shape of a fan called the fan-shaped body (FB), and a pair of compartmentalized ovoid neuropils known as the noduli (NO)². This brain region is conserved broadly across insects². Some of the most

compelling work on the central complex has been done with the fruit fly, *Drosophila melanogaster*. Due to its experimental accessibility as a genetic model organism, experimenters have been able to record from and manipulate entire populations of identified cell types in the brains of awake behaving flies.

1.2 Head direction system

Johannes Seelig and Vivek Jayaraman used such techniques to identify neurons that track the fly's orientation in its environment, or head direction³. These neurons are called EPG neurons and they have dendrites in the EB, axons in the PB, and mixed processes in a neuropil called the Gall⁴. Each individual EPG neuron occupies a small segment of the EB taking up a sixteenth of its perimeter, called a wedge⁴. Together, all of the EPG neurons tile the entire perimeter of the EB⁴. EPG neurons occupying different wedges have different preferred directions³. Adjacent wedges have preferred directions that are 22.5 degrees apart (1/16 of a full rotation) matching their anatomical separation³. The EPG neurons display a narrow bump of activity that rotates around the EB as the fly's head direction changes³. Both sensory input from landmarks in the environment and self-motion cues contribute to updating the position of the bump to continuously track the fly's orientation³.

As a fly turns, a landmark in its environment will change its azimuth in the fly's visual field, so that if the fly turns left a landmark will move rightward in its visual field. Thus, landmarks are informative of the fly's orientation. Sensory information encoding the azimuth of landmarks is provided to the EPG neurons by a class of neurons called Ring neurons⁵⁻⁸. These neurons have axons that span the entire circle of the ellipsoid body, forming a ring⁹. These neurons are inhibitory^{9,10}. Each ring neuron connects to EPG neurons in every wedge¹¹. Importantly, there is not an anatomical mapping from ring neurons tuned

to specific landmark positions to EPG neurons with matching preferred head directions. Instead, the connections between the ring neurons and the EPG neurons are flexible and plastic. Anti-hebbian plasticity between coactive Ring and EPG neurons gradually sculpts a pattern of synaptic weights creating a functional mapping between Ring neurons and EPG neurons with the same tuning^{12,13}. Specifically, the inhibition between Ring neurons and EPG neurons with the same preferred direction will be weakened. Thus, when a ring neuron is activated, all EPG neurons will be inhibited except the EPG neurons with the same preferred direction.

The EPG neurons also track self-motion information to maintain an accurate estimate of the fly's head direction. The fly's angular velocity is the derivative of its orientation. That means that the fly's orientation can be calculated by integrating its angular velocity. A class of neurons called PEN neurons work in tandem with the EPG neurons to perform this integration^{14,15}. PEN neurons have axons in the EB, dendrites in the PB, and dendrites in the NO⁴. In the EB, the axons of each PEN neuron fill two adjacent wedges, and synapse onto the EPG neurons in those wedges^{4,16}. This two-wedge unit is called a tile. In the PB, each PEN neuron innervates a single glomerulus and is postsynaptic to the EPG neurons^{4,16}. The axons of PEN neurons and dendrites of EPG neurons that innervate the same PB glomerulus are offset in the EB⁴. PEN axons innervate a tile adjacent to the EPG dendrites, where they activate the EPG neurons that innervate a neighboring PB glomerulus⁴. PEN neurons from opposite sides of the PB are offset in different directions⁴. PENs from the right PB are shifted one tile counterclockwise, while PENs from the left PB are shifted one tile clockwise. Thus, right and left PEN neurons are pushing the EPG activity in opposite directions. If they are equally activated, their influence will be balanced, and the bump will

not move. However, the PEN neurons are tuned to angular velocity^{14,15,17}. The right PEN neurons are tuned to right turns, while the left PEN neurons are tuned to left turns. Thus, when the fly turns right, the right PENs will overpower the left PENs and pull the EPG bump counterclockwise, and vice versa for leftward turns^{14,15}. Through this mechanism, the position of the bump encodes the integral of the fly's angular velocity, allowing it to track the fly's orientation even when no landmark is present^{14,15}.

1.3 $\Delta 7$ facilitates vector computations

The PB transforms EPG activity into a vector^{11,18,19}. The PB is segmented into 18 glomeruli⁴. EPG neurons from adjacent wedges project to glomeruli on opposite sides of the PB, so that the left and right PB each contain a full copy of the EPG population activity^{14,16}. In the PB, EPG neurons synapse onto a cell type known as $\Delta 7$ ^{4,16}. EPG excites $\Delta 7$, and $\Delta 7$ is inhibitory^{10,16}. Both the axons and dendrites of $\Delta 7$ neurons are located in the PB⁴. Individual $\Delta 7$ neurons span the entire width of the PB and have axons in one glomerulus on each side of the PB, roughly 7 glomeruli apart⁴. $\Delta 7$ neurons have dendrites in every glomerulus where they don't have axons. Importantly, the input to each of these dendrites from EPG is graded based on how far from the axon it is. Glomeruli more distant from a $\Delta 7$ neuron's axons receive stronger input. The synaptic weight to these dendrites is a sinusoidal function of this distance^{11,19}. As a result, rather than reflecting the narrow bump displayed by the population of EPG neurons in the EB, $\Delta 7$ neurons have a sinusoidal population activity profile^{18,19}.

This feature of $\Delta 7$ function is similar to a theoretical proposal for the neural encoding of vectors known as a sinusoidal array²⁰. In this theory neurons occupy discrete points on an axis, and their activity is a cosine function of their coordinate on the axis²⁰. This sinusoidal activity profile serves as a syntax for the representation of vectors in polar

coordinates, where the amplitude of the sinusoid represents the magnitude of the vector and the phase of the sinusoid represents the direction of the vector²⁰. Any vector computation can be performed by a sinusoidal array. For example, to scale a vector, the activity of all of the neurons in a sinusoidal array should be scaled by the activity of another neuron outside of the array (figure 1.1). To rotate a vector, each neuron in a sinusoidal array should synapse onto a neuron of a subsequent sinusoidal array with a different coordinate (figure 1.2). To calculate the sum of two vectors, a sinusoidal array should sum the input from two presynaptic sinusoidal arrays (figure 1.3). To calculate the inner product between two vectors, each neuron in one sinusoidal array should be scaled by a neuron in another sinusoidal array and then converge onto a single neuron outside of the array that sums the output (figure 1.4). These elementary vector computations can be combined to perform more complex computations.

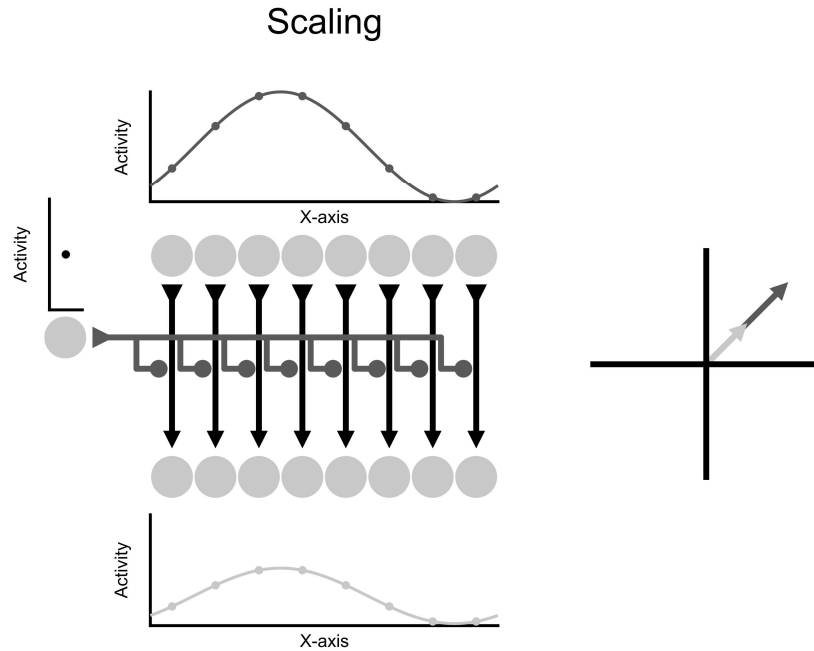


Figure 1.1: Vector Scaling

This schematic illustrates how a sinusoidal array could scale a vector. Each black arrow represents an individual neuron receiving input from a presynaptic sinusoidal array and sending its output to a postsynaptic sinusoidal array. Each neuron in the array also receives input from the same individual neuron outside of the array. The output of each neuron will be the product of the input from the presynaptic sinusoidal array and the input from the neuron on the left. The output of the black arrows, illustrated as the activity of the postsynaptic sinusoidal array, is a sinusoid with the same phase but a different amplitude. The graph on the right shows the vectors represented by the presynaptic (dark gray) and postsynaptic (light gray) sinusoidal arrays. The presynaptic vector is longer than the postsynaptic vector, meaning the function of the array represented by the black arrows is to change the magnitude of the vector.

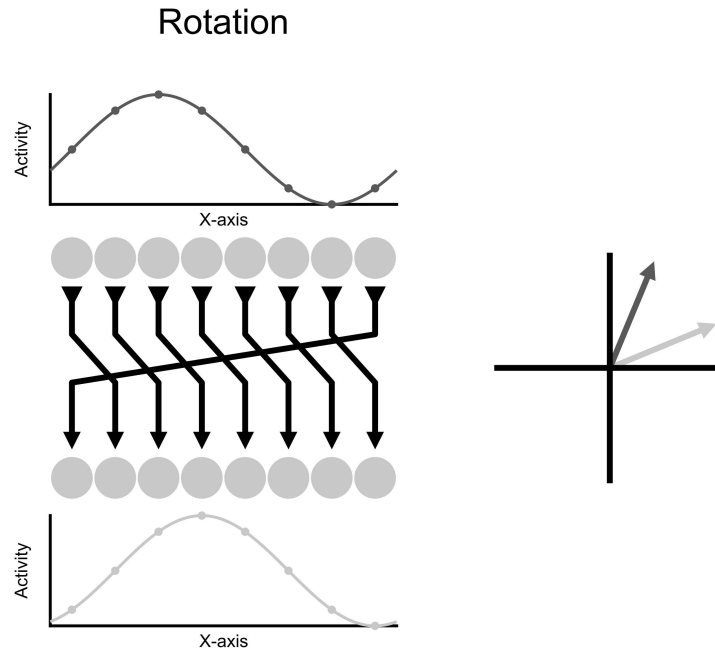


Figure 1.2: Vector Rotation

This schematic illustrates how a sinusoidal array could rotate a vector. Each black arrow represents an individual neuron receiving input from a presynaptic sinusoidal array and sending its output to a postsynaptic sinusoidal array. Each neuron provides output to a neuron with an x coordinate 45 degrees shifted from the neuron it receives input from. This results in the activity profile of the postsynaptic sinusoidal array being 45 degrees out of phase with the presynaptic sinusoidal array. The graph on the right shows the vectors represented by the presynaptic (dark gray) and postsynaptic (light gray) sinusoidal arrays. The postsynaptic vector is rotated 45 degrees clockwise from the presynaptic vector, meaning the function of the array represented by the black arrows is to change the direction of the vector.

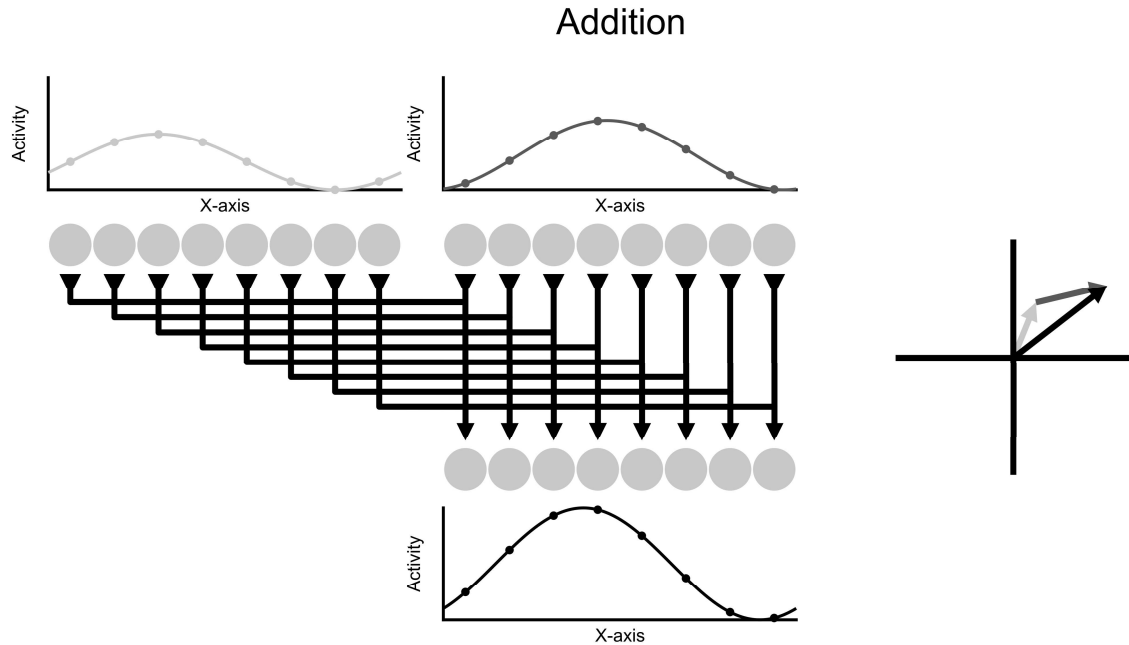


Figure 1.3: Vector Addition

This schematic illustrates how a sinusoidal array could sum two vectors. Each black arrow represents an individual neuron receiving input from two presynaptic sinusoidal arrays and sending its output to a single postsynaptic sinusoidal array. The activity of each neuron is equal to the sum of its two inputs. The output of the black arrows, illustrated as the activity of the postsynaptic sinusoidal array, is a sinusoid with a new phase and amplitude. The graph on the right shows the vectors represented by the two presynaptic (light and dark gray) and postsynaptic (black) sinusoidal arrays. The postsynaptic vector is equal to the sum of the two presynaptic vectors, meaning the function of the array represented by the black arrows is to sum the two input vectors.

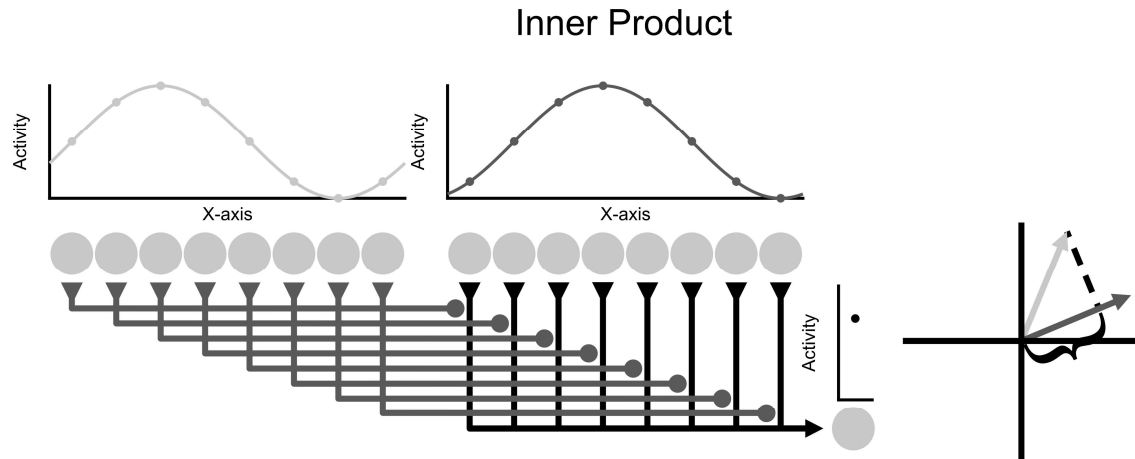


Figure 1.4: Inner Product

This schematic illustrates how sinusoidal arrays could calculate the inner product of two vectors. Each black arrow represents an individual neuron receiving input from two presynaptic sinusoidal arrays and sending its output to a single postsynaptic neuron. The activity of each neuron is equal to the product of its two inputs. The postsynaptic neuron sums the output of the black arrows, meaning the output will be a single scalar value. The graph on the right shows the vectors represented by the two presynaptic arrays (light and dark gray) and a bracket representing the scalar value encoded by the postsynaptic neuron. In this illustration, the two vectors are unit vectors, meaning that their inner product is equal to their projection. The bracket is illustrated as being equal to the projection of one presynaptic vector onto the other one, meaning the function of the array represented by the black arrows is to find the inner product between the two input vectors.

1.4 Fan-shaped body columnar neurons use vector computations to construct an allocentric coordinate system

The first evidence of neurons using sinusoidal arrays to perform vector computations was provided by two parallel studies demonstrating how the PFNv, PFNd, and hDeltaB neurons combine scaling, rotation, and summing to transform information about the fly's translational motion from an egocentric reference frame (defined relative to the fly) into an allocentric reference frame (defined relative to the surrounding world)^{18,19}. PFNv and PFNd are directly postsynaptic to $\Delta 7$ and inherit its sinusoidal activity profile^{11,18,19}. In addition to receiving input in the PB, PFNv and PFNd receive input in the NO¹¹. Left PFNvs, right PFNvs, left PFNd and right PFNd act as 4 separate sinusoidal arrays^{18,19}. The activity of each one is scaled by different neurons tuned to translational motion along one of four perpendicular directions^{18,19}. The neuron that scales left PFNd neurons is tuned to translational motion 45 degrees to the fly's left^{18,19}. Right PFNd neurons are scaled by motion along an axis 45 degrees to the fly's right^{18,19}. The axes of translational motion that scale left and right PFNvs are 135 degrees to the fly's right and left, respectively^{18,19}. These neurons provide output to another sinusoidal array, called hDeltaB, in the FB^{18,19}. In doing so, they induce a rotation of their vector. Left/right PFNd neurons induce a negative/positive 45 degree rotation, respectively^{18,19}. While, Left/right PFNvs induce a positive/negative 135 degree rotation, respectively^{18,19}. hDeltaB then sums the input from all four sinusoidal arrays^{18,19}.

These four sinusoidal arrays act as four basis vectors^{18,19}. Their magnitudes describe the fly's motion in an egocentric reference frame, while their direction tracks the fly's allocentric heading^{18,19}. By summing these four basis vectors hDeltaB calculates the fly's

traveling direction in an allocentric reference frame^{18,19}. This means that a fly that is facing north and moving right will have the same hDeltaB activity as a fly that is facing east and moving forward or a fly that is facing west and moving backward^{18,19}.

Note that the reason this operation requires 4 sinusoidal arrays is because PFNd and PFNv neurons cannot be negatively scaled. In theory, if a PFN type could be negatively scaled, the same operation could be performed with two sinusoidal arrays acting as a pair of basis vectors. A recent study discovered that the cell type PFNa can be negatively scaled via T-type calcium currents²¹. This type of calcium current induces depolarization of the cell following a strong inhibitory input²¹. This mechanism allows a sinusoidal array to invert itself provided strong enough inhibition, meaning it can encode vectors with both positive and negative magnitudes²¹. Therefore, there is no need to have two vectors scaled by axes pointing in opposite directions as there was in the previous example. Demonstrating this concept, the study described how PFNa transforms wind direction information from an egocentric reference frame to an allocentric reference frame with just two sinusoidal arrays, made up of the left and right PFNa neurons²¹.

Another pair of studies showed how cell types can use sinusoidal arrays to read information out of an allocentric reference frame^{22,23}. These studies focused on cell types called PFL2 and PFL3^{22,23}. These cell types receive input in the PB and FB and provide output to a region outside the central complex called the lateral accessory lobes (LAL)¹¹. PFL2 and PFL3 compare a goal direction vector in the FB with the head direction vector in the PB and output egocentric steering commands in the LAL^{22,23}. To do this, they act as 3 different sinusoidal arrays: left PFL3, right PFL3, and PFL2. Each array rotates the head direction vector from the PB and calculates the inner product between it and the goal vector.

The left and right PFL3s rotate their vectors by plus and minus 67.5 degrees, while PFL2 rotates its vector by 180 degrees^{22,23}. The difference between the output of the left and right PFL3 neurons tells the downstream circuitry which direction to turn^{22,23}. However, there is ambiguity whether the fly is facing toward the goal or away from the goal, meaning there will be two equilibrium points a stable equilibrium when the fly faces the goal and an unstable one when the fly is facing 180 degrees away from the goal^{22,23}. PFL2 resolves this ambiguity by signaling whether the fly is facing towards or away from the goal²².

These investigations into PFNs and PFLs demonstrate how the insect brain builds a polar coordinate system referenced to the animal's surroundings and pinpoints the fan-shaped body as the brain region that contains this coordinate system.

1.5 Fan-shaped body circuitry

Hulse, Haberkern, Franconville, Turner-Evans et al. performed a dense reconstruction of the central complex from the hemibrain electron microscopy dataset¹¹. This analysis yielded a comprehensive description of the anatomy of the *Drosophila* central complex including the fan-shaped body. The fan-shaped body contains two broad classes of neurons: tangential neurons and columnar neurons¹¹. Individual tangential neurons span the entire width of the horizontal axis of the structure and occupy a narrow strip of the vertical axis, while columnar neurons occupy a narrow portion of both the width and height of the fan-shaped body¹¹. These broad classes are divided into specific cell types based on both their morphology and their connectivity¹¹. Although each columnar neuron individually takes up a narrow portion of the fan-shaped body, all the neurons of the same columnar cell type span the entire width of the fan-shaped body¹¹. If one thinks of the horizontal axis of the fan-shaped body as an x-axis, then one can think of each type of columnar neuron as a

sinusoidal array, where the position of an individual neuron in the FB defines its x coordinate¹¹.

Thinking of the fan-shaped body as a substrate for an allocentric polar coordinate system provides a framework to generate hypotheses for the function of fan-shaped body cell types that are identified anatomically but are functionally uncharacterized. One such example is a cell type called PFL1. This cell type has broadly similar morphology as PFL2 and PFL3 in that it has dendrites in the PB and FB and axons in the LAL^{4,11}. Thus, one might expect PFL1 to serve a broadly similar function as PFL2 and PFL3 to read information out of an allocentric coordinate system by transforming it into egocentric coordinates.

While they have similarities, there are also key differences between PFL1 and PFL2/PFL3. First, PFL1 neurons synapse onto distinct downstream circuitry to PFL2 and PFL3, suggesting it may have a distinct behavioral role¹¹. Also, PFL1 receives input from different fan-shaped body cell types than PFL2 and PFL3, suggesting that it reads out a different vector¹¹. Furthermore, the circuitry upstream of PFL1 is much simpler than the circuitry upstream of PFL2 and PFL3. PFL2 and PFL3 receive input from a highly interconnected network of columnar cell types¹¹. Whereas PFL1 is downstream of an isolated mostly feedforward network consisting of PFNa connecting to a cell type called FC1 connecting to PFL1¹¹. Such differences in network structure may have different functional implications. PFL1 is ripe for experimental investigation to describe whether and how it supports an allocentric polar coordinate system in the fan-shaped body. Such investigations will further our understanding of how the circuitry of the fan-shaped body facilitates vector computations in order to represent space.

2. PFL1 neurons transform a vector from an allocentric reference frame to an egocentric reference frame

Based on its anatomical position as an output neuron of the fan-shaped body, PFL1 is poised to read information out of an allocentric reference frame. In this chapter, I analyze how PFL1 could perform this function and perform experiments to test whether it does perform this function.

2.1 Anatomically constrained sinusoidal array model of PFL1

I analyzed PFL1 neurons' anatomy using publicly available electron microscopy data. PFL1 dendrites tile two neuropils in the fly brain: the protocerebral bridge (PB) and the fan-shaped body (FB) (Figure 2.1b)²⁴⁻²⁶. Each PFL1 neuron has a dendrite that occupies a narrow portion of the width of each of these neuropils (Figure 2.1b). As a group, PFL1 neurons tile the PB and FB. In the PB, PFL1 neurons are postsynaptic to the EPG neurons, which compute the fly's heading, and $\Delta 7$ neurons, which smooth the fly's heading to two sinusoidal profiles^{16,19}. Finally, PFL1 neurons send axons unilaterally to either the left or right lateral accessory lobe (LAL), a premotor area²⁷.

The tiling pattern in the PB is fairly straightforward, where individual dendrites innervate a single glomerulus (Figure 2.1b). However, in the FB, the pattern is not immediately clear. Hulse, Haberkern, Franconville, Turner-Evans et al. defined a nine column tiling pattern that they sorted the 14 PFL1 neurons into (Figure 2.1c)¹¹. However, I noticed that the anatomy of the PFL1 neurons do not match their columnar assignments. Neurons that are supposed to be in the same column are located as close to each other as they are to neurons of another column (Figure 2.1d). This problem is mirrored by the connectivity of the PFL1 neurons. Plotting neurons based on which columns they receive

input from results in an alternating pattern (Figure 2.1e). Intriguingly, neurons with axons on the left side of the brain are sandwiched between neurons on the right side of the brain.

Looking at the overall morphology of the PFL1 neurons reveals this same pattern, where PFL1 neurons with axons on opposite sides of the brain have interleaving dendrites in the fan-shaped body (Figure 2.1d). All the neurons with axons on a single side of the brain tile the fan-shaped body completely with little overlap (Figure 2.1b). This observation led me to propose a new tiling pattern for the PFL1 neurons. PFL1 neurons tile the fan-shaped body in a 7.5 column pattern, where one neuron per side takes up 1.5 columns and the rest take up a single column (Figure 2.1f).

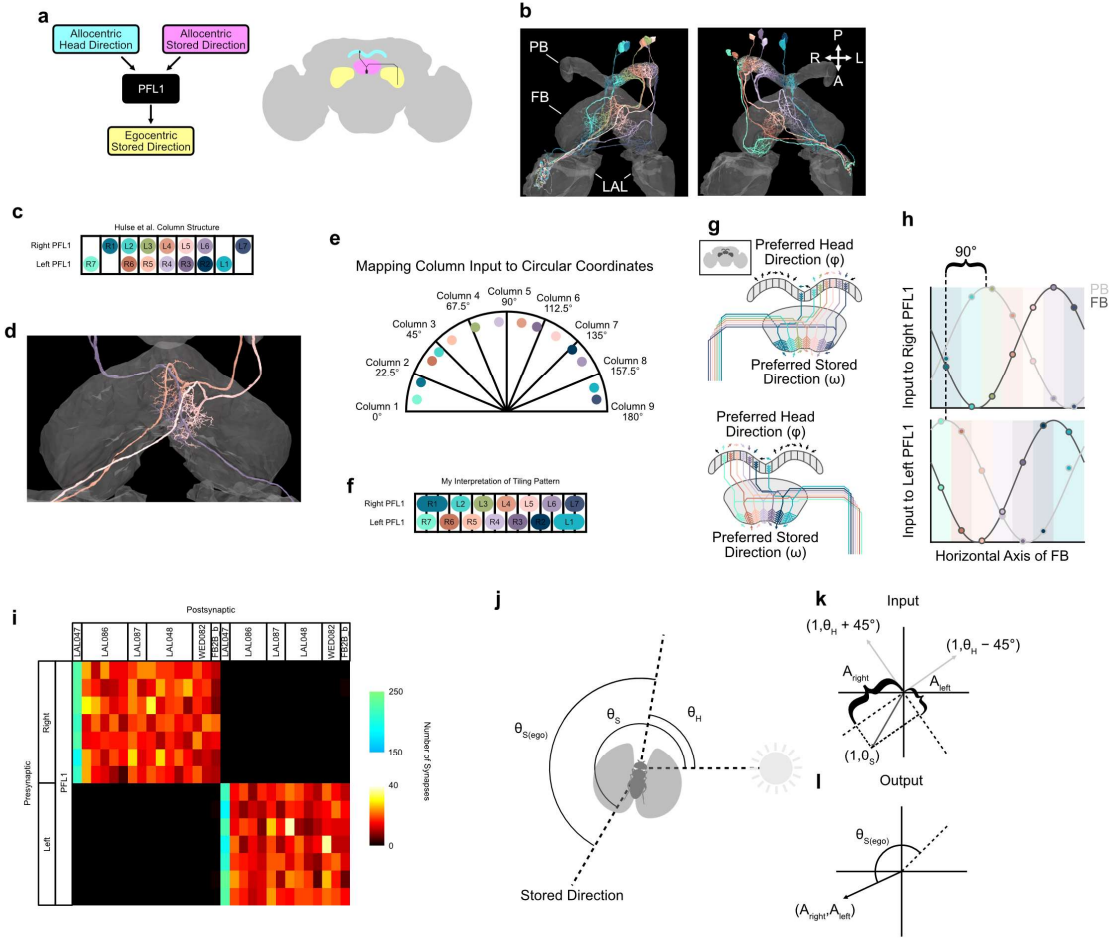


Figure 2.1: PFL1 anatomy supports coordinate transformation model

a, I hypothesize that PFL1 takes as inputs the allocentric head direction and an allocentric stored direction and then outputs the egocentric stored direction. **b**, Rendering of PFL1 neurons from hemibrain dataset. Top: neurons with axons on the right side of the brain. Bottom: Neurons with axons on the left side. P, posterior. L, left. A, anterior. R, right. **c**, Diagram illustrating the proposed nine column tiling pattern. Neurons are identified based on which PB glomerulus they innervate. Right vs Left PFL1 denotes which side of the brain the axons are on. **d**, Close up on FB showing overlap between three neurons. The purple neuron is assigned to the same column as the pink neuron but appears to be sandwiched in between the pink neuron and orange neuron rather than occupying the same position as the

pink neuron. **e**, Plotting PFL1 neurons based on which columns they receive input from. **f**, illustration of tiling pattern based on my observations of PFL1 morphology **g**, Drawing of PFL1 neurons that illustrates how they tile the PB and FB and how these tiling patterns define each neuron's preferred head direction (φ) and preferred stored direction (ω). Inset: drawing of brain with PB, FB, and LAL. **h**, Graph showing the simulated input to PFL1 neurons for the value of head and stored directions shown in **e**. Each dot represents the input to a neuron. The color of the outline of the dot indicates whether the input is from the PB or FB. Light grey indicates the activity in the PB. Dark grey indicates the hypothetical activity in the FB. The x-axis is the horizontal axis of the fan-shaped body. The shaded regions indicate the portion of the fan-shaped body innervated by each neuron. The brace denotes that the PB input to neurons with axons on opposite sides of the brain is 90° out of phase. **i**, Connectivity matrix that shows connections between PFL1 neurons and the top 30 postsynaptic neurons (6 types) by synapse count, sorted by cell type from the Flywire dataset. **j**, Drawing showing the meaning of the variables from the model: head direction (θ_H), stored direction (θ_S), and egocentric stored direction ($\theta_{S(\text{ego})}$). **k**, Graph showing the input to PFL1 as vectors for the values of θ_H and θ_S shown in **j**. The braces denote the output of the model (A_{right} and A_{left}). The coordinates in this graph are polar coordinates. **l**, Graph showing the output of the model (A_{right} and A_{left}) as a vector for the values of θ_H and θ_S shown in **j**. The set of coordinates in this graph are Cartesian coordinates. The dotted line is the direction of the vector when θ_H and θ_S are equal. The difference between this line and the direction of the vector is equal to the egocentric stored direction ($\theta_{S(\text{ego})}$).

To help predict PFL1's function, I built a circuit model constrained by its anatomy. I used the well-characterized head direction tuning of the EPG and $\Delta 7$ neurons, which is based on both the topographical arrangement of these neurons and their physiology, to predict the input to each PFL1 neuron in the PB and define its preferred head direction (Figure 2.1g). Since $\Delta 7$ neurons exhibit a sinusoidal activity profile across the PB^{16,19}, I modelled the input that each PFL1 neuron receives in the PB as a sinusoidal function of the difference between the fly's current head direction and the neuron's preferred head direction (Figure 2.1h). In the FB, on the other hand, the function and the activity profile of the FB neurons presynaptic to PFL1 have not been identified. Extrapolating from known properties of PFL2 and PFL3, which have similar morphologies with PFL1 and receive goal direction information from presynaptic neurons in the FB^{23,28}, I predicted that FB neurons presynaptic to PFL1 encode a vector with an arbitrary direction defined in an allocentric reference frame. I call this the *stored direction*, and I speculate it is encoded by the phase of sinusoidal input to PFL1's FB dendrites. Thus, I modeled the input to PFL1's FB dendrites as a sinusoidal function of the difference between each neuron's preferred stored direction—determined by its position in the FB—and the stored direction (Figure 2.1g,h).

A key feature of PFL1 anatomy is the pattern of dendritic projections to the PB and the FB. PFL1 neurons with axons in the left LAL project their dendrites to the FB in the opposite direction from neurons with axons in the right LAL (Figure 2.1b). This pattern causes left and right PFL1 neurons with dendrites located in a similar part of the FB to have preferred head directions separated by 90° (Figure 2.1g). Thus, this pattern can be thought of as inducing a $\pm 45^\circ$ shift in the phase of the head direction input. As a result of this anatomical feature, two identical sinusoidal population activity profiles of the head direction

signal from each side of the PB become 90° out of phase when they meet information from the FB (Figure 2.1h). Conceptually, two identical copies of a head direction vector are rotated $\pm 45^\circ$ in PFL1 neurons, forming a 90° angle between them when they receive the stored direction vector input from the FB.

In the LAL, downstream neurons pool input from all PFL1 neurons with axons on the same side of the brain (Figure 2.1i). Thus, I modeled the output of PFL1 as two numbers reflecting the sum of the output of all the PFL1 neurons on the right or left side of the brain.

Taking everything together, PFL1 is divided into two populations (left and right), which both receive two vectors as input and each generate a single scalar value as output. This network structure is poised to calculate the inner product between each population's input vectors.

A key assumption for PFL1 to calculate the inner product is that the output of each individual neuron is equal to the product of its two inputs. Through simulations and analysis, I found that two other nonlinear activation functions, unsigned multiplication and square of the sum of the inputs, produce nearly identical results. For an in-depth description of this work see the Mathematical Appendix.

If PFL1 calculates the inner product between its input vectors, mathematically this is identical to calculating projections (braces in Figure 2.1k) of the stored direction vector (dark grey vector in Figure 2.1k) onto two orthogonal vectors that are $\pm 45^\circ$ rotated versions of the head direction vector (light grey vectors in Figure 2.1k). This results in two coordinates (A_{left} and A_{right} in Figure 2.1l) that define an output vector that encodes the stored direction in an egocentric reference frame ($\theta_{\text{s(ego)}}$ in Figure 2.1l). Thus, our modeling suggests a possible function of the PFL1 neurons: They transform an allocentric vector

stored in the FB to an egocentric reference frame by comparing it to the head direction information (Figure 2.1a).

2.2 PFL1 input output relationship supports coordinate transformation model

The coordinate transformation model described above makes three predictions about PFL1's input-output relationship: (i) the output of PFL1 should have a sinusoidal relationship with the head direction, (ii) the phases of the output in the left and the right LALs should be 90° apart, and (iii) the phase of the output relative to the head direction should be determined by the stored direction (Figure 2.2a). I tested whether PFL1's physiology is consistent with these predictions. To simultaneously record the head direction input to PFL1 as well as PFL1's output, I expressed the glutamate sensor iGluSnFR3²⁹ and the red-shifted calcium indicator jRGECO1a³⁰ in PFL1³¹ and performed 2-photon imaging in head-fixed flies flying in a virtual reality environment (Figure 2.2b). The iGluSnFR3 signal in the PB indicates glutamatergic input from $\Delta 7$ neurons representing the head direction. Note, since the glutamatergic input to PFL1 from $\Delta 7$ is inhibitory, the head direction (defined as the phase of the positive PB input) is equal to the population vector of the iGluSnFR3 signal in the PB plus 180°. The jRGECO1a signal in PFL1 axons in the LAL reports the sum of output of the PFL1 neurons on each side.

I divided each experiment into 3-minute trials punctuated by 20 seconds of darkness (Figure 2.2c). As expected, the iGluSnFR3 signal in the PB reliably tracks the fly's heading in the arena (Figure 2.3d). Plotting the axonal activity of PFL1 as a function of the head direction reveals a sinusoidal relationship (Figure 2.2e,h,i). Critically, the right and left outputs in LAL are approximately 90° out of phase from each other (Figure 2.2e,f,g,j,k), as the coordinate transformation model predicts.

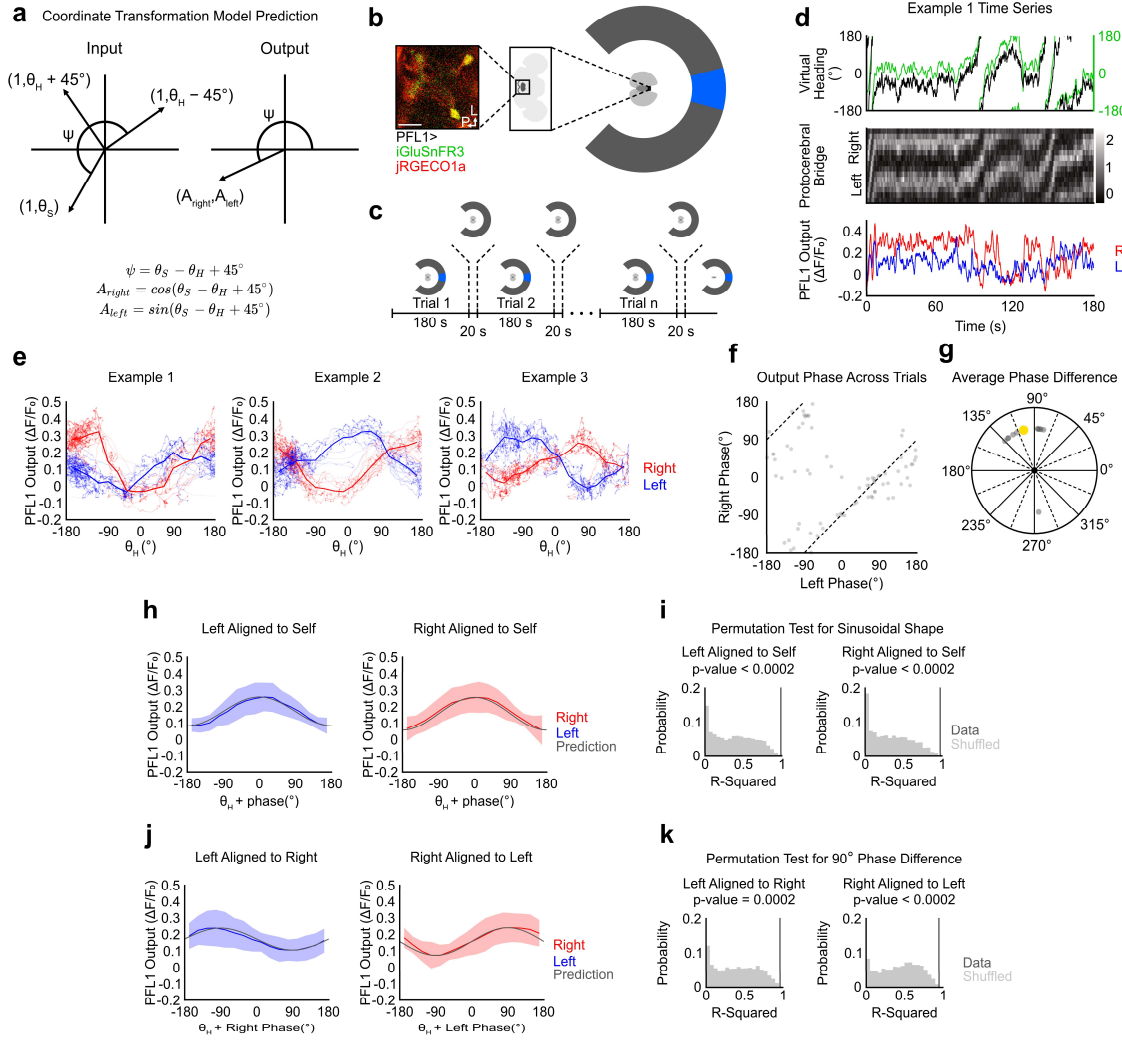


Figure 2.2: Testing predictions about PFL1 input output relationship

a, Prediction about PFL1 input output relationship based on coordinate transformation

model **b**, A schematic illustrating the experiment. Scale Bar 20 micrometers (μm). L, left.

P, posterior. **c**, Trial structure of experiment. **d**, Data time series from an example trial. **e**,

Examples showing the PFL1 output as the function of head direction. Each dot is a time

point. Thick lines are the averages of the dots. Example 1 is the same data from **c**. **f**, A dot is

plotted for each trial based on the phase for the left and right, found by fitting a cosine

function to the entire trial. Dotted line indicates a 90° phase difference between the left and

right. **g**, The average phase difference between the cosines fit to the left and right for each trial (left phase - right phase). Grey dots are single-fly averages. The yellow dot is the across-fly average, 104.9° . **h**, Comparing PFL1 output to a cosine fit. Half the data for each trial was used to fit a cosine function. The other half of the data is aligned and plotted along with a prediction based on the fitting. Red and blue lines are across-fly averages. The shaded region represents a standard deviation. $N=11$ flies. **i**, The R-squared value for the data and the predictions in **a** (dark grey line), compared to a distribution of the same statistic for a shuffled dataset (light grey distribution). **j**, The output of PFL1 aligned to the phase of the opposite side found through fitting a cosine function to the entire trial. Red and blue lines are across-fly averages. The shaded region represents a standard deviation. The grey line is a prediction assuming the two sides are 90° out of phase. $N=11$ flies. **k**, The R-squared value for the data and the predictions in **d** (dark grey line) compared to a distribution of the same statistic for a shuffled dataset (light grey distribution)

The phase of the PFL1 output relative to the head direction varies between flies (Figure 2.2e,f). This cannot be explained if the PFL1 output is only influenced by the head direction information. Furthermore, pooling data across flies does not match a prediction based on a head direction-only model (Figure 2.3e,f,g), strongly suggesting that an additional input, likely from the FB, is influencing the PFL1 output. As we introduced above, I call this information from the FB a stored direction.

I fit the model to infer the stored direction encoded in the FB using the head direction in the PB and the PFL1 output in the LAL for each trial. I did this fitting and simulation using a cross-validation technique (Figure 2.3a). I found that the best-fit stored direction tends to be consistent across trials for the same fly but appears arbitrary across flies (Figure 2.3c), important evidence that the stored direction depends on an individual's distinct experience. My physiology experiments also showed that it can sometimes abruptly change within the same fly (Figure 2.4), suggesting that it is flexible.

Finally, when I aligned the data based on the best-fit stored direction prior to averaging across flies, I found a close match between the data and a prediction based on the coordinate transformation model (Figure 2.3c,d). Together, these results show that the output of PFL1 resembles a set of Cartesian coordinates that encode the stored direction in an egocentric reference frame.

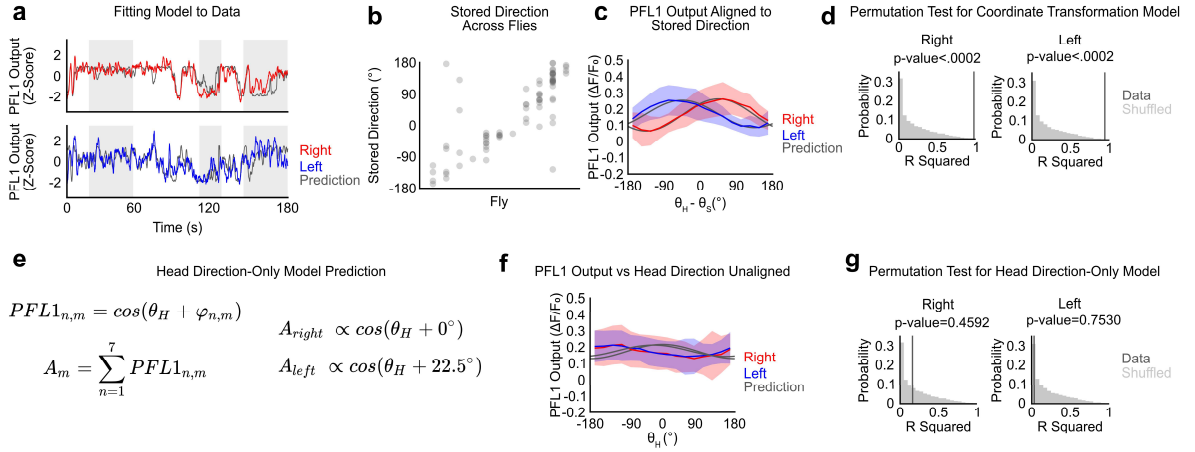


Figure 2.3: Inferring stored direction from PFL1 input output relationship

a, Example showing the model fit to the data with cross-validation method. The grey traces are simulations from the model. Blue and red traces are data. The time intervals with a white background were used for fitting. Time periods with grey backgrounds were held out for testing. These time periods were selected randomly for each trial. **b**, The stored direction for each trial, found through fitting. Each dot is a trial, and each column is a fly. The x-axis is sorted based on the average stored direction. **c**, The output of PFL1 plotted as a function of the difference between the head direction and stored direction. This x-axis aligns the data according to the fitted stored direction. Red and blue lines are across the fly averages. The shaded region represents the standard deviation. N = 11 flies. Grey lines are the prediction from the coordinate transformation model. **d**, R-squared values between the coordinate transformation model and across-fly averages for data (dark grey line) and shuffled data (light grey distribution). **e**, prediction based on head direction only model **f**, Output of PFL1 plotted as a function of the head direction. Blue and red lines are across-fly averages. The shaded region represents the standard deviation. N = 11 flies. Grey lines are predictions based on the head direction-only model. **g**, The same as f for the head direction only model.

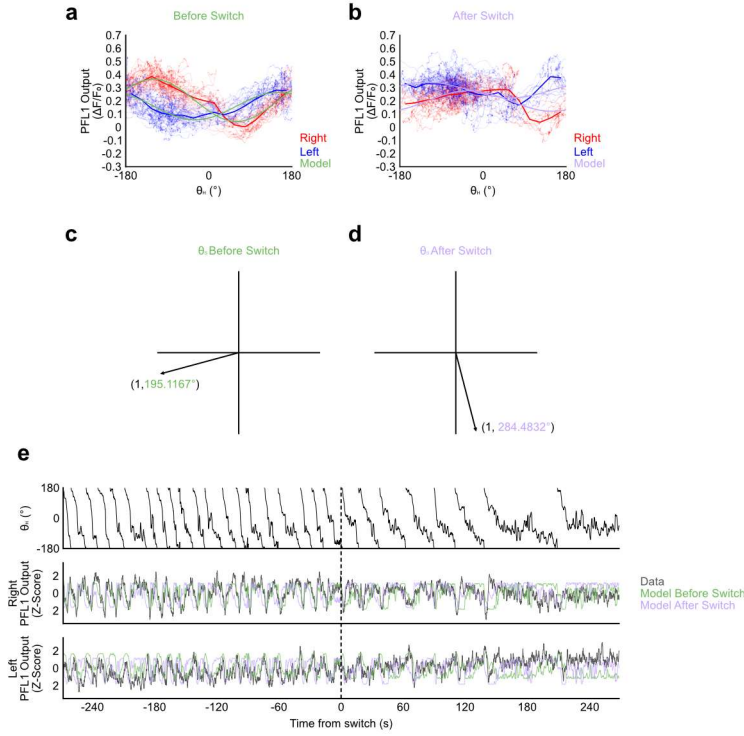


Figure 2.4: Phase of PFL1 output changes abruptly in a single fly

a, PFL1 activity in LALs plotted as a function of the head direction before the change in phase. Green curves are a prediction based on fitting the coordinate transformation model. **b**, PFL1 activity plotted as a function of the head direction after the change in phase. Purple curves are a prediction based on fitting the model. **c**, The stored direction found by fitting the model to the data prior to the switch. **d**, The stored direction found by fitting the model to the data after the switch. **e**, Time series centered on the moment the phase switches. Top: head direction. Middle: Right LAL recording. Bottom: Left LAL recording. The green and purple traces are simulations from the circuit model based on the head direction for each time point and the stored directions from before and after the switch, shown in c and d.

2.3 Dissecting PFL1's contribution to locomotor control

PFL neurons (and their homologs in other insects) are generally thought to control steering, because their axons project to the LAL, a premotor area^{32,33}. I noticed in my calcium imaging experiments that PFL1 activity declined whenever a fly stopped flying (Figure 2.5d,e). This made me wonder if PFL1 might control steering during flight. In my imaging experiments, where I infer a constant stored direction from PFL1's input output relationship, flies are not engaged in goal-directed locomotion. For example, in a fly that is flying in circles, PFL1 still reads out a constant stored direction (Figure 2.5a,b). In fact, most flies I recorded from did not show a preference to fly towards the stored direction (Figure 2.5c). This suggests that the stored direction that PFL1 transforms and conveys to downstream neurons only specifies a goal in a specific behavioral context not present in this experiment. This would be in line with EPG silencing experiments where the CX is not essential for all navigational behaviors, such as object fixation^{34,35}.

To test if PFL1 influences locomotion, I optogenetically activated the neurons. Although a previous study found that activating PFL1 neurons in walking flies does not induce any steering behavior²³, I reasoned based on PFL1 neurons' activity in flying flies that flight may be the appropriate behavioral context. First, I bilaterally activated PFL1 neurons in tethered flying flies by expressing CsChrimson³⁶ in PFL1 using three different split gal4 lines³¹ and pulsing red light on the entire fly. (The flies were rendered blind by a mutation in the *norpA* gene to eliminate any visual response to light³⁷.) Bilateral activation of PFL1 induced a bilateral increase in wing beat amplitude (Figure 2.5f,g).

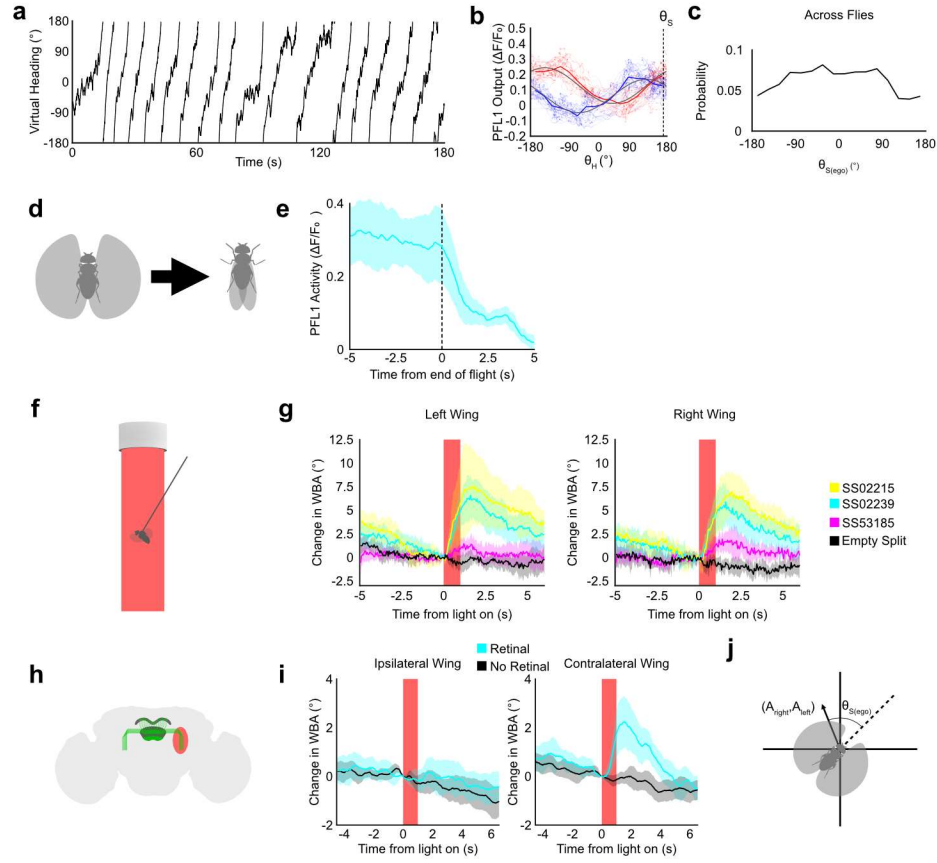


Figure 2.5: Probing the behavioral role of PFL1 activity

a, The behavioral trajectory of an example fly that was flying in circles. **b**, PFL1 output plotted as a function of head direction for the example fly from **a**. Grey lines are the prediction from the coordinate transformation model based on a stored direction found through fitting (dotted line). Each dot is a time point. Red and blue lines are averages of the same color dots. **c**, Histogram showing the egocentric stored direction across flies. If flies preferred to fly in the stored direction, there would be a peak at 0. N=11 flies. **d**, A schematic illustrating the transition from flying to not flying. **e**, Calcium activity in the axons of PFL1 when the fly stops flying. The shaded region represents a 95% confidence interval. N=26 LAL recordings from 13 flies. **f**, A schematic showing illumination of an entire fly with red light. **g**, A change in wing beat amplitude upon bilateral PFL1

stimulation. The shaded region represents the 95% confidence interval. SS02215 N=6 flies. SS02239 N=6 flies. SS53185 N=6 flies. Empty Split N=7 flies. **h**, A schematic showing unilateral red-light stimulation using a digital micromirror device. **i**, A change in wing beat amplitude upon unilateral PFL1 stimulation. The shaded region is the 95% confidence interval. N = 10 flies for retinal. N=10 flies for no retinal. **j**, A schematic showing the relationship between PFL1 output (A_{right} , A_{left}) and the egocentric stored direction ($\theta_{S(\text{ego})}$).

Having established that PFL1 activation induces a behavioral response during flight, I posited that activating PFL1 neurons in only one hemisphere should generate a steering response. To unilaterally activate PFL1 neurons, I used a digital micromirror device to expose the axons of PFL1 on only one side of the brain to red light. I observed an increase in the contralateral wing beat amplitude (Figure 2.5h,i). Such a change in wing beat amplitude would drive an ipsilateral turn in a freely flying fly.

In the context of my model, stimulating the PFL1 neurons in the right LAL would increase one component of the output vector (the X-coordinate in Figure 2.5j), signaling that the egocentric stored direction is to the fly's right, while stimulating the left neurons would signal the egocentric stored direction is to the left (Figure 2.5j). The behavioral response to these stimulations is to turn towards the egocentric stored direction.

Overall, these results suggest that the stored direction may encode a goal direction and that PFL1 neurons provide an egocentric steering signal that the downstream motor circuits can use to turn the animal towards the goal. Yet, animals do not always use it to adjust their locomotion, suggesting that it depends on behavioral context.

3. Characterizing the function of the fan-shaped body circuitry upstream of PFL1

The coordinate transformation model predicts that the phase of sinusoidal input in the FB sets the phase between the output of PFL1 and the head direction. Thus, I sought to use my ability to infer the stored direction from the PFL1 input output relationship to dissect the function of the circuitry upstream of PFL1 in the fan-shaped body.

3.1 Calcium activity of top two presynaptic partners constrains possible mechanisms for encoding the stored direction

I used the electron microscopy connectome data to canvass FB neuron types presynaptic to PFL1. I found that FC1 neurons provide the largest input to PFL1 in the FB by synapse count (Figure 3.1a,b)¹¹.

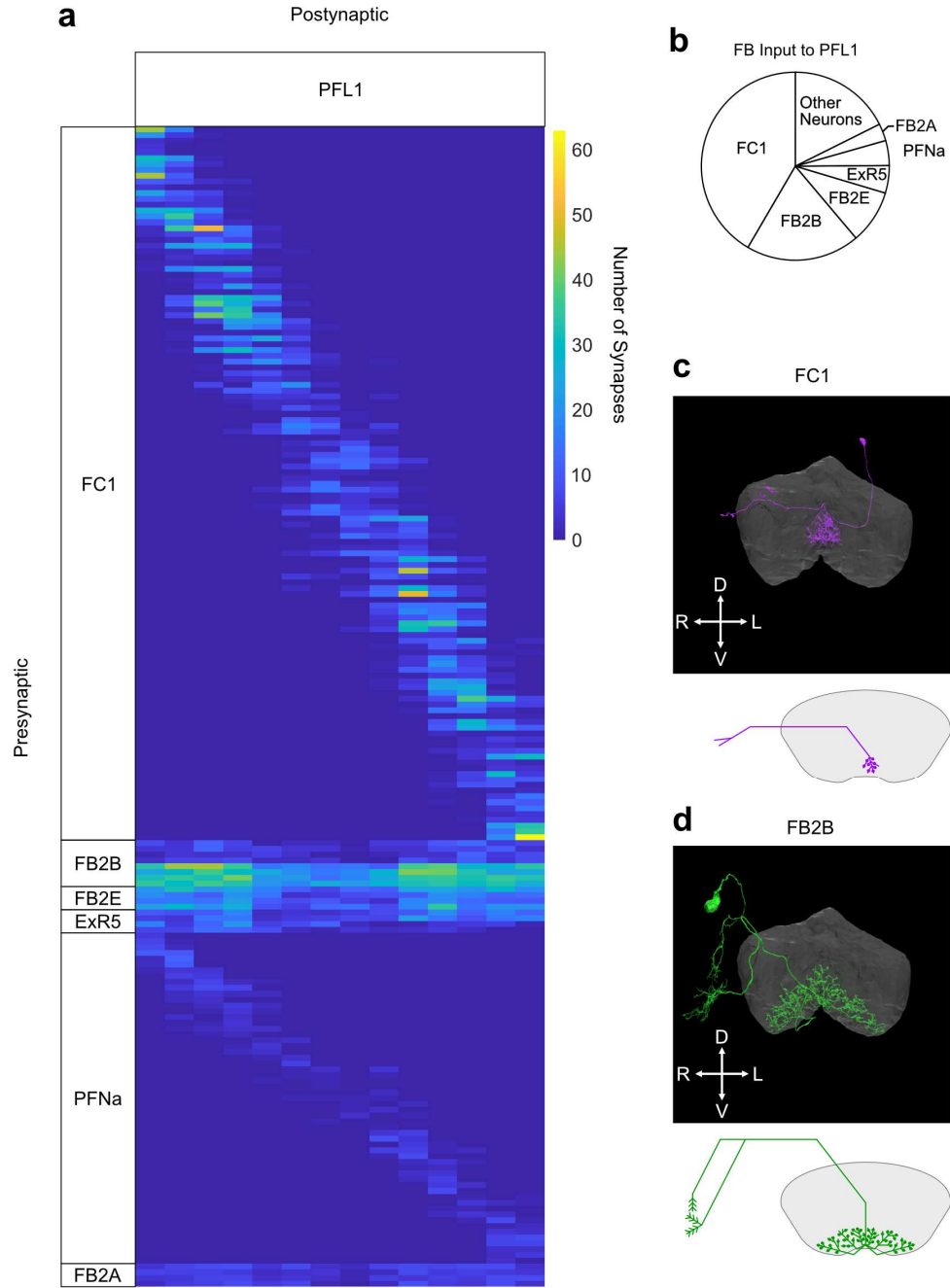


Figure 3.1: FB input to PFL1

a, A connectivity matrix showing the number of synapses in the FB between presynaptic neurons from the top presynaptic cell types and PFL1 in the hemibrain dataset. Note that neurons belonging to these cell types that have no synapses with PFL1 in the FB are excluded from this plot. **b**, A pie chart showing proportion of synapses from each cell type

presynaptic to PFL1 in the Fan-shaped Body, by synapse count. **c**, The image of an individual FC1 neuron from the hemibrain dataset (top). Drawing of an individual FC1 neuron (bottom). Note that FC1 neurons are columnar neurons, so they occupy a narrow portion of the FB. **d**, The same as b, but for an individual FB2B neuron. Note that this neuron is a tangential neuron, so it occupies the full width of the FB. D, dorsal. L, left. V, ventral. R, right.

Because of this strong input to PFL1, I hypothesized that FC1 may encode the stored direction and provide this information to PFL1. On the other hand, FC1 receives input from the PFNa neurons, which convey head direction information to the FB^{11,21}. Thus, another reasonable hypothesis is that FC1 might display head direction tuning. To test these two mutually exclusive hypotheses, I simultaneously recorded the activity of FC1³⁸, $\Delta 7$ ³⁹, and PFL1⁴⁰ by expressing jRGECO1a³⁰ in FC1 and $\Delta 7$ and GCaMP7f⁴¹ in PFL1. Note that the split *gal4* line labels only a subset of FC1, limiting my ability to test the hypotheses for the entire FC1 population. I fit the coordinate transformation model to the $\Delta 7$ and PFL1 data to find the best-fit stored direction for each trial (Figure 3.2a). If FC1 encodes the stored direction, the phase of calcium activity across the width of the FB should be equal to the stored direction I find through fitting the model to the $\Delta 7$ and PFL1 recordings. I found, however, that the phase of the FC1 activity did not match the stored direction (Figure 3.2b,c,e-g). Instead, it tracks the head direction (Figure 3.2b,d-g). Therefore, FC1 calcium activity seems to reflect its input from PFNa rather than the stored direction that I infer PFL1 receives in the FB.

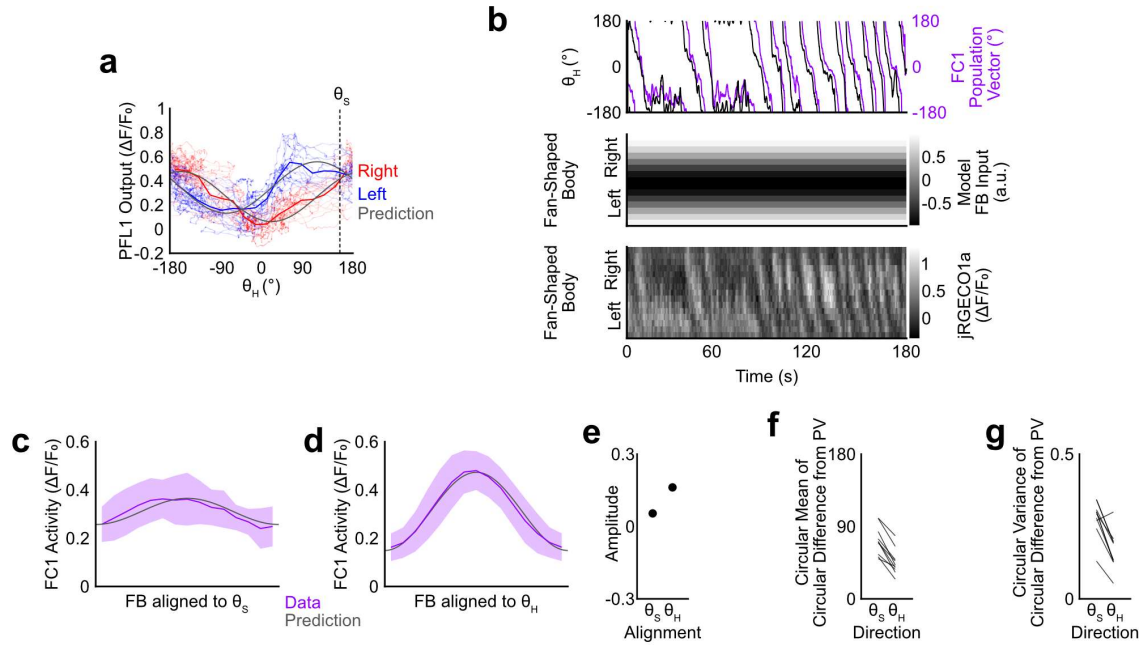


Figure 3.2: Simultaneous recording of $\Delta 7$, FC1, and PFL1

a, Data from example trial with prediction from the coordinate transformation model (grey lines) based on a stored direction found through fitting (dotted line). Each dot is a time point. Red and blue lines are averages of the same color dots. **b**, Data time series from same trial as **a**. Top: Head direction (black) and FC1 population vector (purple). Middle: Predicted FB input from coordinate transformation model. Bottom: Measured FC1 activity. **c**, FC1 activity plotted as a function of the position in the Fan-shaped Body aligned to the stored direction. Purple line is across flies mean. The shaded region represents the standard deviation. The dark grey line shows the predicted result if the phase were to encode the stored direction with an amplitude fit to the data. $N = 10$ flies. **e**, The same as **d** except aligned to the head direction. **d**, The amplitude found by fitting the prediction. **f**, The circular mean of the circular difference between the FC1 population vector and the head direction or the stored direction. **g**, The circular variance of the circular difference between

the FC1 population vector and the head direction or the stored direction. P-value = 0.0039,
Wilcoxon sign rank test

The second largest input to PFL1 in the FB is a cell type called FB2B (Figure 3.1a,b). FB2B is a type of tangential neuron that spans the width of the FB (Figure 3.1d). If the calcium activity reflects the overall activity of the neuron, I would expect calcium to be uniform across the width of the FB. However, a sinusoidal pattern of calcium activity in the axons of FB2B might be a mechanism for providing the stored direction input to PFL1. Prior work identified flight-dependent activity in FB2B but did not describe the pattern of calcium activity across the width of the fan-shaped body⁴². When I simultaneously recorded calcium in FB2B, $\Delta 7$, and PFL1, the calcium signal of FB2B was uniform across the width of the FB (Figure 3.3a,b)^{38,39}. Aligning the FB2B data to either the stored direction or the head direction results in an amplitude close to zero, suggesting its calcium activity does not have a meaningful phase (Figure 3.3c-e).

The results of these experiments constrain possible mechanisms for encoding the stored direction in the fan-shaped body. Whatever the mechanism is, it must be consistent with head direction tuning in FC1 and a uniform calcium concentration across the axons of FB2B.

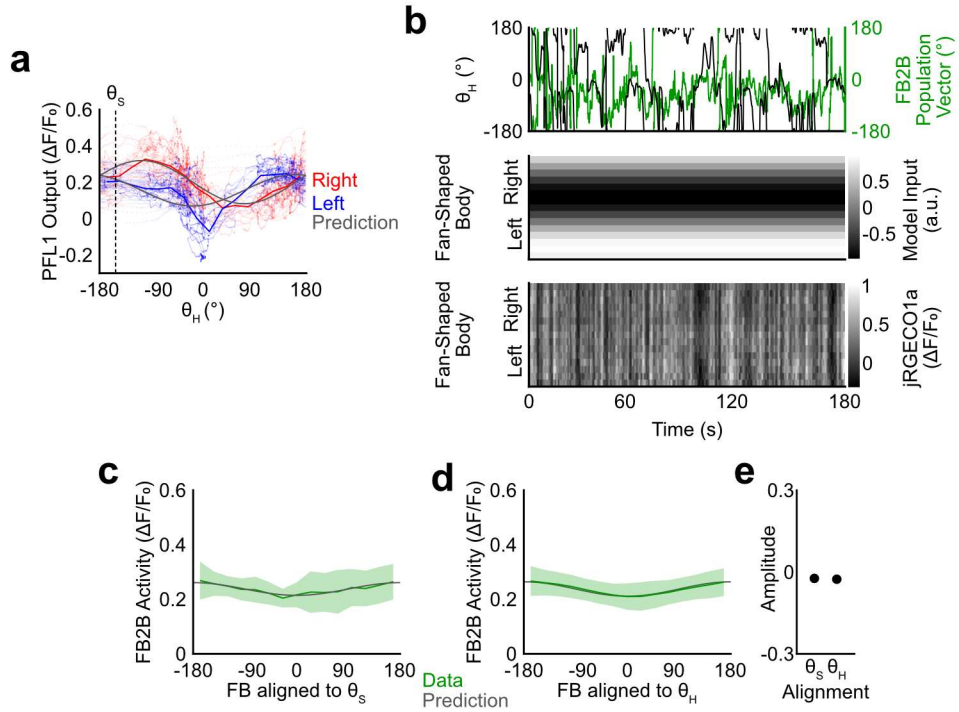


Figure 3.3: FB2B calcium activity is uniform across the FB

a, Data from an example trial, showing the output of PFL1 plotted as a function of the head direction, along with a prediction from fitting the coordinate transformation model to the data. Dots are time points. Red and blue lines are the averages of the dots. The dotted line indicates the stored direction found through fitting. **b**, Data from the example trial in **a**. Top: the head direction and the population vector of FB2B. Middle: The simulated FB input from the coordinate transformation model, predicted based on fitting. Bottom: Calcium recording from FB2B in the FB. **c**, FB2B activity plotted as a function of the position in the fan-shaped body aligned to the stored direction. The green line reflects the across-fly mean. The shaded region represents a standard deviation. The dark grey line shows the predicted result if the phase were to encode the stored direction with an amplitude fit to the data. $N = 10$ flies. **d**, The same as **c** except aligned to the head direction. **e**, The amplitude found by fitting the prediction.

3.2 Recurrent feedback is necessary for the structure of PFL1's input output relationship

FB2B forms a recurrent feedback loop with PFL1 (Fig. 4a-c)¹¹. PFL1 is a major source of input to FB2B's dendrites (Fig. 4b), and FB2B is the second largest input to PFL1's dendrites in the FB (Fig. 3A). The coordinate transformation model does not account for this feature of PFL1 anatomy. I asked what role recurrent feedback might have in shaping PFL1's input output relationship. I blocked all recurrent feedback from PFL1 by expressing tetanus toxin (TNT)⁴³, which blocks synaptic vesicle fusion, while simultaneously expressing iGluSnFR3²⁹ and jRGECO1a⁴¹ to record the head direction input to and output of PFL1³¹.

Qualitatively, PFL1's input output relationship has 3 features: (i) the output of PFL1 has a sinusoidal relationship with the head direction, (ii) the phase of the left and right outputs are 90 degrees apart, and (iii) the phase of the output relative to the head direction is flexible. I asked which of these features, if any, are impacted by blocking the recurrent feedback. I found that the input output relationship remained sinusoidal in flies expressing TNT (Fig. 4d-f). However, the 90-degree phase difference between the left and right outputs was disturbed (Fig. 4d,g,i,j). Blocking recurrent feedback did not reduce the circular variance of the phase of the output, suggesting the phase is still flexible (Fig. 4h). In summary, recurrent feedback between FB2B and PFL1 is necessary for a single feature of PFL1's input output relationship, the 90 degree phase difference between the left and right outputs.

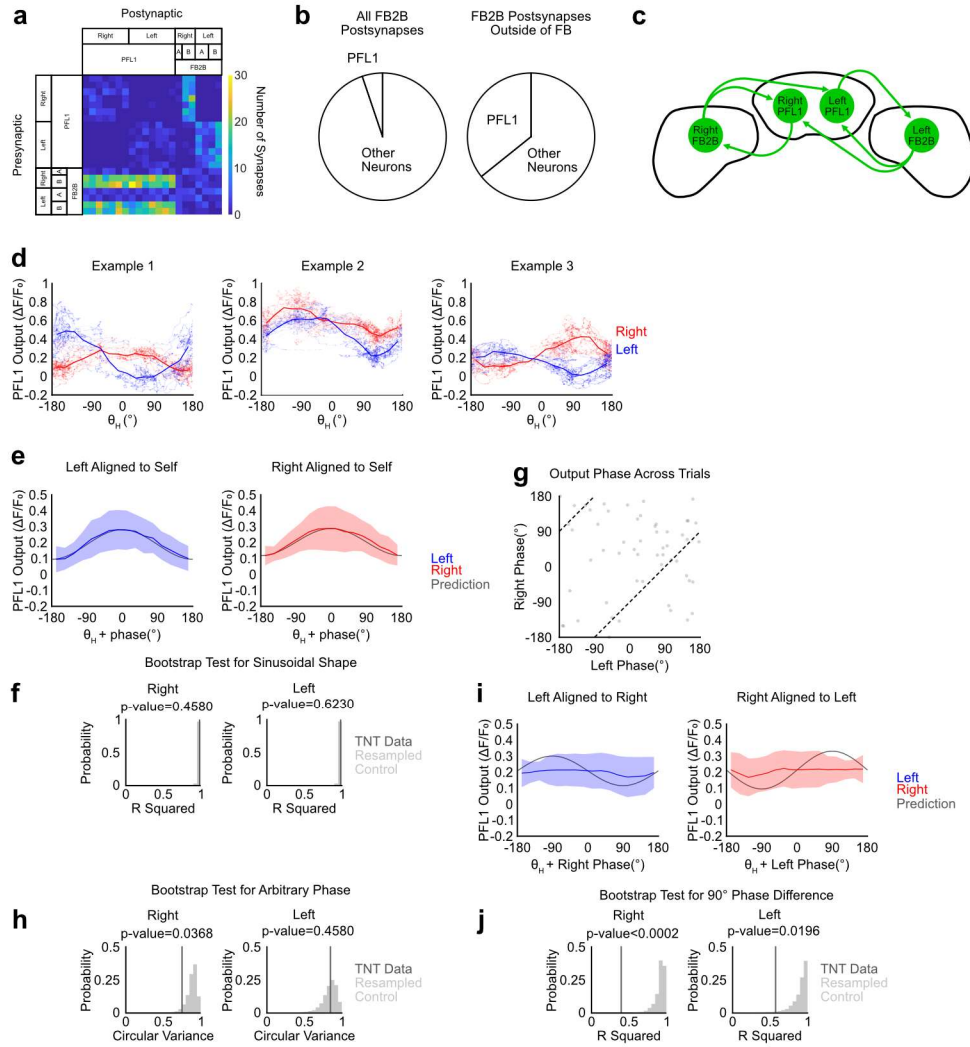


Figure 3.4: Recurrent feedback required for structure of input output relationship

a Connectivity matrix between FB2B and PFL1 based on Flywire data. **b** Pie chart showing proportion of input to FB2B from PFL1 by synapse count across the whole neuron (left) and restricted to FB2B's dendrites outside the FB (right). **c** Schematic illustrating recurrent circuit motif between FB2B and PFL1. PFL1 synapses to either right or left FB2B neurons in the LAL and FB2B synapses onto both right and left PFL1 neurons in the FB. **d** Output of PFL1 plotted as a function of head direction in flies expressing tetanus toxin in PFL1. **e** Phase nulled output of PFL1 neurons as a function of head direction. Grey line is cosine fit. Red and blue lines are across fly averages. Shaded regions are standard deviation. N = 12

flies. **f** R Squared value for across fly average and cosine fit in E (dark grey line) compared to a distribution from resampling the control data (light grey). **g** Individual trials for TNT experiments plotted according to left and right phase of the cosine fit. Dotted lines indicate 90 degree difference between left and right phase. N= _ Trials across 12 flies. **h** The circular variances of the phases of cosine fits for TNT flies (dark grey line) and a distribution of the same statistic found by resampling the control data (light grey). **i** Output of PFL1 neurons aligned to the phase of the opposite side. Grey line is prediction assuming a 90 degree phase difference between left and right. Red and blue lines are across fly means. Shaded region is standard deviation. **j** R Squared value for across fly average and prediction in J (dark grey line) compared to a distribution from resampling the control data (light grey).

This result is surprising, because my model suggests the 90 degree phase difference between the left and right outputs is a consequence of the tiling pattern of PFL1 in the PB and FB. It is possible that recurrent feedback is necessary for the proper wiring of PFL1 during development, and blocking the feedback causes PFL1 to develop differently. One way to test this would be to restrict the expression of TNT to adult flies. However, another possibility is that the acute feedback from FB2B shapes the input output relationship of PFL1. To understand how that works would require further physiological investigations into both how PFL1 input influences FB2B activity and how FB2B influences PFL1 activity as well as further modelling.

3.3 FC1 drives second messenger signaling in the axons of FB2B

My calcium imaging experiments of FC1 and FB2B revealed that the calcium activity in those cell types does not encode the stored direction. How then is the stored direction encoded? One possible clue comes from a 2006 paper about visual learning in flies⁴⁴. The authors found that adenylyl cyclase was required for flies to learn to fly in a certain orientation relative to a visual scene⁴⁴. When they rescued adenylyl cyclase in FB2B, they restored the fly's ability to learn⁴⁴. Based on this paper, I began to wonder whether adenylyl cyclase might have something to do with the stored direction. Adenylyl cyclase signals through the second messenger cyclic adenosine monophosphate (cAMP). To monitor adenylyl cyclase activity in FB2B, I expressed a cAMP sensor called Gflamp2⁴⁵ in the FB2B neurons³⁹. The FB2B neurons display a sinusoidal pattern of cAMP activity across the width of the FB (Figure 3.5a). The phase of this pattern is correlated with the fly's heading in a majority of the fly's that I imaged (Figure 3.5a,b).

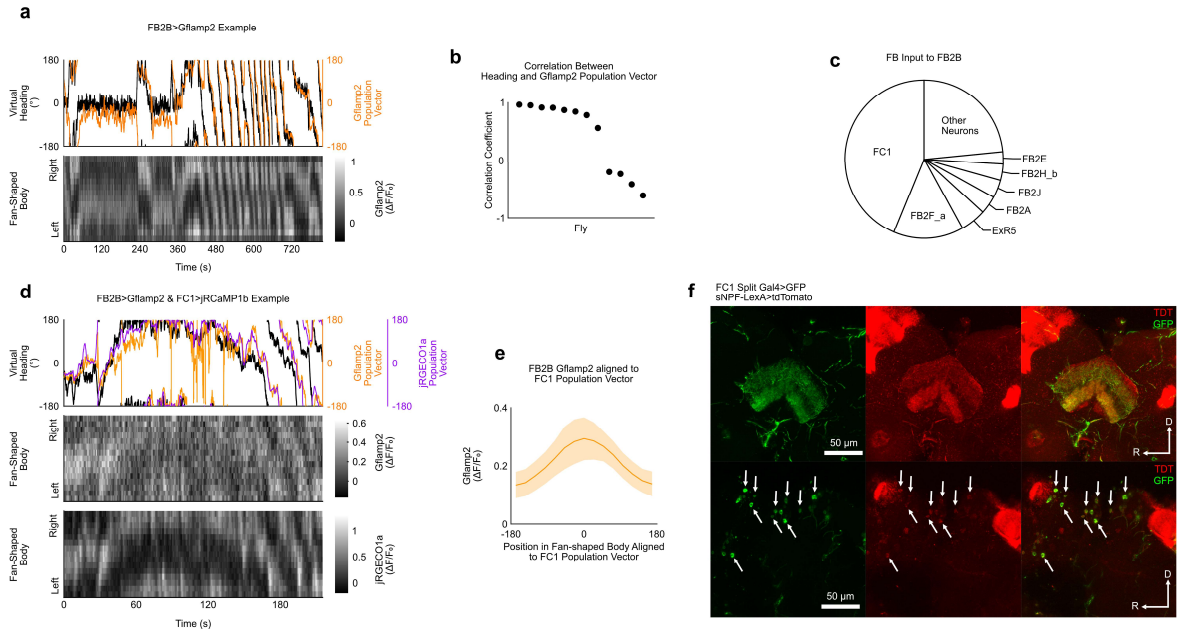


Figure 3.5: Measuring cAMP in FB2B

a, Example of Gflamp2 recording in FB2B. **b**, Pearson correlation coefficients between the unwrapped Gflamp2 population vector and the unwrapped heading across twelve experiments. **c**, Pie chart showing the proportion of the number of FB synapses from each cell type. **d**, Example of simultaneous imaging of calcium in FC1 (Bottom) and cAMP in FB2B (middle). **e**, FB2B Gflamp2 signal aligned to the FC1 population vector. Line is across fly average. Shaded region is standard deviation. N=10 flies. **f**, Double label experiment. Top row z projection showing the FB. Bottom row z projection showing the cell bodies. The two rows come from different z sections of the same confocal image stack.

I asked what is driving the cAMP activity. Adenylyl cyclase is activated by g protein coupled receptor signaling, suggesting it is activated by a presynaptic neuron. Looking at the cell types that are presynaptic to FB2B's axons in the FB revealed that FC1 is the largest presynaptic partner by synapse count (Figure 3.5c). Also, it is the only columnar neuron among the top inputs to FB2B's axons, making it the best candidate to drive localized g protein signaling in FB2B's axons (a tangential neuron would be better positioned to drive g protein signaling across the whole axon).

To characterize the relationship between FC1 activity and cAMP signaling in FB2B, I simultaneously recorded calcium in FC1³⁸ using jRCaMP1b³⁰ and cAMP in FB2B³⁹ with Gflamp2⁴⁵. If FC1 activates adenylyl cyclase, I would expect the cAMP activity to be in phase with the FC1 activity. However, if FC1 inhibits adenylyl cyclase, I would expect FC1 activity to be antiphase with cAMP. I found that the pattern of calcium activity in FC1 is in phase with cAMP in FB2B (figure 3.5d,e).

By comparing electron microscopy reconstructions of FC1 to fluorescence microscopy images, I noticed that some of the enhancer fragments that drive expression in the FC1 neurons are made from sequences of DNA taken from the short neuropeptide F (sNPF) gene⁴⁶. This made me wonder if FC1 neurons express sNPF. To test this, I performed a double label experiment. I drove GFP⁴⁷ in a split gal4 that labels FC1³⁸, and I expressed tdTomato⁴⁸ in a LexA line where LexA has been inserted at the end of the endogenous sNPF gene⁴⁹. In these flies, I found overlap between the GFP signal and the tdTomato signal in both the region of the fan-shaped body containing FC1 processes and in the cell bodies of the FC1 neurons (figure 3.5f). This result suggests that the FC1 neurons do express sNPF. The sNPF receptor is a g protein coupled receptor that could in theory be driving adenylyl cyclase activity in FB2B⁵⁰. However, whether FB2B expresses the sNPF receptor or if it is required for the cAMP signaling is still an open question.

These experiments reveal that FC1 drives local second messenger signaling in the axons of FB2B. Whether and how this second messenger signaling contributes to the stored direction input to PFL1 remain open questions and are exciting future directions.

4 Discussion

Recent research in the *Drosophila* central complex provides a framework for understanding how neurons perform vector computations. My research builds on that framework to provide a mechanistic description of how PFL1 performs vector computations. I leveraged electron microscopy data to demonstrate how PFL1 could perform a coordinate transformation to read a vector out of an allocentric coordinate system and broadcast it to downstream circuitry in an egocentric coordinate system. I performed physiological recordings of PFL1 and found that its input output relationship matches key predictions of the coordinate transformation model. A key feature of this physiological data is that the phase of PFL1's input output relationship is arbitrary across flies. This suggests that the vector read out by PFL1 has a unique direction in each fly.

My model suggests that this vector is encoded in the FB. While the exact mechanism by which the fan-shaped body circuitry encodes this vector remains a mystery, my exploration of this circuitry provides clues that may prove to be important for ultimately understanding the mechanism. The calcium activity of the top two presynaptic partners to PFL1 in the fan-shaped body, FC1 and FB2B, does not encode the vector. The calcium activity of FC1 tracks the head direction, while FB2B displays uniform calcium activity across the width of its axons. In addition to being the top input to PFL1, FC1 is the primary synaptic partner to FB2B's axons. It drives local second messenger signaling in the axons of FB2B, suggesting it might have a modulatory effect. By defining the direction of a vector encoded in the FB as the phase of PFL1's input output relationship, I have established a rigorous way to study vector encoding in the FB.

The function I have described for PFL1 is similar to that of PFL2 and PFL3, however it is not identical. The key functional difference is that I describe the output of PFL1 as a set of cartesian coordinates encoding a vector in an egocentric reference frame. Whereas models describing PFL3 universally describe the output of PFL3 as a one-dimensional steering signal defined as the difference between the output on the left and

right^{11,22,23,51-53}. The reason PFL1 output can be described as a set of Cartesian coordinates is that the left and right head direction input is rotated by $\pm 45^\circ$ to be orthogonal. Each type of PFL neuron rotates the head direction input by a different amount, and in PFL2 and PFL3 the rotations do not result in the head direction vectors being orthogonal. This means that even if one treated the output of these cell types as coordinates, rather than subtracting them to generate a steering signal, there would not be a unique mapping from the FB input vector to the output vector, i.e. there would be multiple inputs that resulted in an identical output. However, in my PFL1 model there is a unique mapping. Practically, this might mean that PFL1 is capable of reading out both the direction and the magnitude of a vector, whereas PFL2 and PFL3 may only read out the direction of a vector.

Apart from the nuanced computational differences between PFL1 and the other PFL neurons, there are obvious anatomical differences. PFL1 receives input from totally distinct circuitry in the FB than PFL2 and PFL3, suggesting that they are reading out different vectors¹¹. Also, PFL1 synapses onto separate downstream circuitry in the LAL than PFL2 and PFL3, suggesting that it is driving different premotor circuitry¹¹. PFL2 and PFL3 receive input from overlapping circuitry in the FB, and synapse onto interconnected downstream circuits as each other¹¹. Their functions appear to complement each other as well. PFL2 breaks a symmetry in the PFL3 steering signal²². Overall, PFL2 and PFL3 act as one output pathway from the FB, and PFL1 acts as a separate pathway. This division might reflect the use of similar computational principles for different behavioral ends.

There are three types of FC neurons in the fan-shaped body, FC1, FC2, and FC3. FC2 encodes the fly's goal direction while it is engaged in goal-directed behavior but otherwise displays head direction tuning²³. FC3 activity reflects its input from PFNa,

encoding the allocentric wind direction when wind is present but otherwise displaying head direction tuning²¹. In my calcium imaging experiments of FC1, I found that FC1 displays head direction tuning. This likely reflects its input from PFNa. FC1 is the largest postsynaptic partner to PFNa^{11,21}. In my experiments, the only stimulus was a visual landmark. Presumably if an additional allocentric stimulus that PFNa is sensitive to, such as wind, was delivered, FC1 would encode the direction of that stimulus like FC3.

If the stored direction is not encoded by the calcium dynamics of the neurons presynaptic to PFL1, how is it encoded? One possibility that has been proposed in computational models but has not been demonstrated experimentally is that the vector is encoded in the synaptic weights of a tangential neuron^{51,54}. This is a very similar mechanism to the mechanism that maps the orientation of a visual scene to a specific value of the head direction representation^{12,13,55}. This mechanism enables a certain orientation of the visual scene, represented by the activation of an individual ring neuron, to be linked with a certain value of the head direction, represented by the activation of EPG neurons in a specific wedge. If the synaptic weights between an FB tangential neuron and the PFL1 neurons were shaped in a sinusoidal pattern, the input to PFL1 from this tangential neuron would have the sinusoidal profile necessary to encode the stored direction. FB2B is a good candidate for this mechanism of encoding the stored direction. It is the type of tangential neuron that provides the most input to PFL1 in the FB. Prior research has implicated it in an orientation memory task^{44,56,57}. I have identified local cAMP signaling in its axons. This signal could serve as an eligibility trace to determine how the synapses of FB2B should be reshaped. An additional cell type could signal when plasticity should occur, triggering the remodeling of the synapses according to the cAMP signal in FB2B's axons. Because FC1 drives the cAMP

signal in FB2B, this mechanism would serve to store a vector encoded by the FC1 neurons at a specific point in time.

In their book *Memory and the Computational Brain*, Gallistel and King point out that in neuroscience memory is often described as a change in a stimulus response relationship⁵⁸. For example, in associative conditioning pairing a stimulus with reward will result in an animal seeking out that stimulus and pairing it with a punishment will result in avoidance. However, Gallistel and King show that in computational systems memory has a different meaning; in these systems the purpose of memory is to store information⁵⁸. They go on to pose the question of how changes in synaptic strength, the mechanism thought to underly associative conditioning, could encode a number, a simple form of informational content⁵⁸.

I have described the fan-shaped body circuitry as a computational system. Computational systems often use a set of simple operations in combination to implement more complex computations. In the introduction, I show how units performing simple arithmetic operations can be arranged in sinusoidal arrays to perform vector computations and how fan-shaped body cell types can combine these vector computations to construct an allocentric coordinate system. In chapter 2, I introduce the stored direction, which is a memory with informational content, i.e. the direction of a vector in an allocentric coordinate system. My physiological measurements of PFL1's input output relationship provide evidence for the existence of this memory. In chapter 3, my investigations into the function of the upstream fan-shaped body circuitry show that one of the molecular signaling pathways canonically thought to underly synaptic plasticity is functional in FB2B⁵⁹. Finally, in this chapter, I proposed a potential mechanism that answers Gallistel and Kings question. Sinusoidal synaptic weights between FB2B and PFL1 could encode a number, i.e. the stored

direction. One key feature of this mechanism is the anatomy of PFL1 forms a computational scaffold that interprets sinusoidal input across its fan-shaped body dendrites as a vector. A second key feature is that the information is encoded in the synaptic strength between individual FB2B neurons and the entire population of PFL1 neurons. Thus, whenever a FB2B neuron is active, the entire PFL1 cell type receives a sinusoidal pattern of input. Thus, activating a FB2B neuron can be thought of as reading the memory stored in its synaptic weights. Further, the activation of the hypothesized additional cell type that induces plasticity, can be thought of as a write signal, overwriting the existing memory with a new value. Thus, investigations into vector computation in the fan-shaped body can not only reveal general principles about neural mechanisms of navigational control but answer fundamental questions about the nature of neural computation.

5 Methods

Model

Coordinate Transformation Circuit Model

I modeled the input to each PFL1 neuron in the PB/FB as a cosine function of the head/stored direction minus the neuron's preferred head/stored direction, which is determined by their horizontal location in each area. In my model, the output of each PFL1 neuron is the product of its two inputs, as in the following equation.

$$PFL1_{n,m} = \cos(\theta_H - \varphi_{n,m}) \times \cos(\theta_S - \omega_{n,m})$$

where $PFL1_{n,m}$ is the output of an individual PFL1 neuron, with n representing an index of a PFL1 neuron and m representing where the axon is (either the left or the right side of the brain). θ_H is the animal's head direction and θ_S is the stored direction in the FB. $\varphi_{n,m}$ and $\omega_{n,m}$ represent the preferred head direction and the preferred stored direction of $PFL1_{n,m}$, respectively. I defined φ to be [-112.5 -67.5 -22.5 22.5 67.5 112.5 180] for $m = right$ and [157.5 -135 -90 -45 0 45 90] for $m = left$. I defined ω to be [-168 -120 -72 -24 24 72 132] for $m = right$ and [-156 -96 -48 0 48 96 144] for $m = left$.

LAL neurons downstream of PFL1 sum the input of all the PFL1 neurons with axons on the same side of the brain. Thus, the final output of the model, A , is the sum of PFL1 neurons on each side.

$$A_m = \sum_{n=1}^7 PFL1_{n,m}$$

Head Direction-Only Model

For the head direction only model, I set the output of each PFL1 neuron to be equal to its protocerebral bridge input, but otherwise the model was the same.

$$PFL1_{n,m} = \cos(\theta_H - \varphi_{n,m})$$

$$A_m = \sum_{n=1}^7 PFL1_{n,m}$$

Fly Rearing

I raised flies on a standard Würzburg recipe, collected them daily, and used them 7 days after collection. I used female flies for all two-photon experiments. For my optogenetic experiments, I used flies with a mutation in the *norpA* gene, which is on the *X* chromosome. Since it is easier to generate males with this mutation because they are hemizygous for *X*, I used male flies for my bilateral optogenetic experiments. Male flies do not fly as well as female flies, however. Therefore, for my unilateral optogenetic experiments, I used female flies. See Table 1 for the genotype of the flies used in each experiment.

Two Photon Imaging

I cold anesthetized flies, removed their front legs to prevent them from pushing the metal holder, waxed their proboscis in place, and tethered flies to a pin using UV curable glue; then, I removed them from cold. I glued the head to the bottom of an inverted stainless-steel pyramid with a small opening at the tip. After filling the pyramid with saline, I removed the cuticle and trachea to expose the brain. Then I placed them in the center of a 270° LED arena (<https://reiserlab.github.io/Modular-LED-Display/G3/>) under the objective of a custom two-photon microscope (<https://www.janelia.org/open-science/mimms-22-2024>)^{60,61}. I covered the surface of the LED arena with gel filters to avoid light leakage to

the PMTs (Roscolux Cerulean Blue R375 and Congo Blue R382), and I added a layer of paper on top of the filters to create a matte surface to prevent reflections in the arena. I tracked the wings in real time using a camera and custom wing tracking software as described previously⁶². I displayed a single bright vertical stripe on the arena, and its azimuthal position was updated based on the flies' left minus right wing beat amplitude.

For two-color imaging, I used a long wavelength of a tunable two-photon laser. I explored and chose the best wavelength for each experiment: 1030nm for the iGluSnFR3 and jRGECO1a experiments; 1000 nm for the $\Delta 7$, PFL1, and FC1 imaging experiments; 1020nm for the $\Delta 7$, PFL1, and FB2B imaging experiments. I used 930nm for imaging of PFL1 during flight offset. I used 930 nm for the FB2B Gflamp2 experiments. I used 1000 nm for the FB2B Gflamp2 and FC1 jRCaMP1b experiments.

Optogenetic Stimulation

Bilateral Stimulation Experiments

I cold anesthetized flies, tethered them to a pin using UV curable glue, and mounted them under a collimator attached to a 625 nm LED via an optic fiber. I tracked wing beat amplitude with a camera, an IR LED for illumination, and custom software. I presented red light in a pulse train of five 1-second pulses interspersed with 10-second interpulse intervals. A fly had to fly for an entire pulse train for the data to be included in the analysis. If flies stopped flying, I applied a short air puff to induce flight.

Unilateral Stimulation

I prepared flies as those for two-photon imaging and mounted them under the two-photon microscope, which I briefly used to locate the PFL1 neurons. I used custom software to draw regions of interest (or masks) around the axons of PFL1 to be optogenetically

stimulated. I aligned a digital micromirror device (DMD) to the light path of the microscope and delivered a 617 nm light (Thor Labs Part Number: M617L3) to the DMD via a liquid light guide. I attached two longpass filters (Semrock Part Number: BLP01-594R) to the output of the DMD to block wavelengths leaking to PMTs. For these experiments, I used a 561 nm single edge dichroic mirror (Semrock Part Number: Di03-R561) to allow the red light from the DMD and the two-photon laser to pass through while reflecting emitted light from the sample to the green PMT. After taking the image to draw the masks, the imaging laser was off for the rest of the experiment. I induced flies to fly and presented 1-second pulses of light every 10 seconds while tracking the flies' wings. I switched the masks between activating the PFL1 axons on the left side and the right side of the brain. I randomized the order of these stimulations between flies. I looped the stimulations until the fly would no longer fly.

Immunohistochemistry

For immunohistochemistry I used a modified version of the flylight antiGFP protocol (<https://www.janelia.org/project-team/flylight/protocols>). I washed flies in ethanol to remove their hydrophobic coating. I dissected the brains in cold Schneider's Insect Medium. I fixed the brains in 2% paraformaldehyde in phosphate buffered saline (PBS) for 55 min. I washed the brains with 4 x 10 min washes in 0.5% Triton X-100 dissolved in PBS (PBT) at room temperature while nutating. I blocked the flies with 5% goat serum. I incubated the brains with primary antibodies consisting of 1 μ L/mL rabbit polyclonal anti-GFP and 4 μ L/mL rat monoclonal anti-RFP for 4 hrs at room temperature and for two overnights at 4°C. I washed the brains in PBT for 3 x 30 min washes. Then, I incubated the brains with the secondary antibodies consisting of 1.25 μ L/mL AF488 Goat anti-rabbit and 2.5 μ L/mL

AF647 Goat anti-rat for 4 hrs at room temperature and 3 overnights at 4°C. After incubating, I performed 3 x 30 min washes in PBT and then 1 20 min wash in PBS. Then I mounted brains were mounted on a poly-L-lysine coated coverslip in Vectashield, placed on a slide, and sealed with Invitrogen Prolong coverslip sealant. Brains were imaged on a Leica SP8 confocal microscope with a 40x water immersion objective.

Analysis

Image Processing

I predetermined the background noise level by measuring the oscillatory noise from the PMT. I then subtracted this level from all imaging data and half-rectified the data before further analysis. I divided the PB into either 14 ROIs for experiments where PFL1 expressed iGluSnFR3 or 18 ROIs for experiments where $\Delta 7$ expressed jRGECO1a to approximate the PB glomeruli. I divided the FB into 15 ROIs horizontally to most closely match the tiling pattern of the PFL1 dendrites. I drew separate polygons around the left and right LAL to define their ROIs. A running average intensity projection of a volume (five planes) at a given time was computed for each pixel. I applied an approximately 1-second rolling average to the data to smooth the data. To calculate the $\Delta F/F_0$, I defined the F_0 for each ROI as the tenth percentile frame for each ROI after smoothing.

Calculating Population Vector

To calculate population vectors for the PB and the FB, I assigned each ROI a vector with a direction based on its position in the neuropil and a length equal to the $\Delta F/F_0$ at that time point. Then, I calculate the average of these vectors to calculate the population vector.

Fitting Model

For fitting the circuit model to the data in Fig. 2.3, I divided each trial into 10 time bins. I randomly selected half of the time bins to use for fitting the model. I left the other half out for testing the model. For every time point, I defined θ_H as the PB population vector, calculated from iGluSnFR3 signal, plus 180 degrees to account for the inhibitory nature of $\Delta 7$ neurons. I used Matlab's `fminbnd` function to find the value of θ_S that maximize Pearson's correlation coefficient between the model prediction and the jRGECO1a signal in PFL1's axons on both sides.

I used the same fitting process in Fig. 2.4. However, I did not break the experiment up into trials, so I manually determined the time periods over which to fit. From visual inspection, it appeared that the difference between the direction of a vector defined by the PFL1 calcium recordings and the head direction changed at a certain point in the experiment. I selected time intervals immediately before and after that time point to fit the model. Unlike Figure 2.3, I left no data from those time periods out of the fitting process, because the model was not quantitatively compared to the data.

For Fig. 3.2 and 3.3, I used a similar fitting process to find an optimal value of θ_S for each trial, given the $\Delta 7$ recordings in the PB and the PFL1 recordings in the LAL. However, I used data from the entirety of each trial to fit the model, because the result of the fitting was being compared with the FB data, which was not used to fit the model.

Shuffling

For the histograms in Fig. 2.2 and 2.3, I generated null distributions for a given test statistic based on chance. I circularly permuted the LAL recordings from each trial by a random interval before performing the analysis. The shuffling and analysis were repeated 5000 times to generate the null distributions.

Bootstrapping

For the histograms in Fig. 3.4, I generated null distributions for a given test statistic based on the control data. I resampled eleven flies with replacement from the control data and calculated each test statistic. The resampling and analysis were repeated 5000 times to generate the null distributions.

Cosine Fitting

In Fig. 2.2 and 3.4, I fit a cosine function to the recordings of PFL1 activity in the LALs. I used Matlab's `fminsearch` function to find the optimal amplitude, phase, and offset for a cosine function of the head direction that minimized the root mean square error between the cosine function and the PFL1 recording for every time point in a trial. To avoid fitting and testing with the same data, I divided trials into 10 time bins and randomly selected half to use for fitting and half for testing (Fig 2.2h,i and Fig 3.4e,f). For analysis where data was not directly compared to a cosine function fit to the same data, I used the entire trial to fit the cosine function (Fig 2.2f,g,j,k and Fig 3.4g,h,i,j).

Aligning FB to Head and Stored directions

To generate the plots in Fig. 3.2 and 3.3, I parameterized the position in the FB by assigning each FB ROI an angle. I assigned the data for each ROI for each time point an x coordinate based on the difference between θ_S or θ_H and the angle assigned to each ROI. I divided the x-axis into 16 bins and calculated the mean of all the time points within each bin for each trial. Then I averaged the bins across all the trials for each fly. I calculated the mean and standard deviation for each bin across flies. I fit a prediction to the across fly mean using Matlab's `fitlm` function. The prediction is equal to the cosine of the x-axis.

I performed a similar alignment in Fig 3.5 to align the Gflamp2 data to the FC1 population vector. In this analysis, the x coordinate for each ROI at each timepoint was based on the difference between the FC1 population vector and the angle assigned to each ROI.

Electron microscopy analysis

For the analysis of PFL1's columnar connectivity in Fig2.1e, I used neuPRINT's (<https://neuprint.janelia.org/>) custom query feature to download the connections between PFL1 and all presynaptic fan-shaped body columnar neurons from the hemibrain data. Neurons in this data are annotated with a columnar assignment. I assigned each number an angle based on its column. I assigned each PFL1 neuron a list of numbers describing its input, where each synapse the PFL1 received was represented by the angle assigned to the presynaptic neuron. For example, if a PFL1 neuron received 24 synapses from a neuron in column 1, I would add 24 zeros to that PFL1 neuron's list of numbers. Then, I found the circular average of these lists to determine the coordinate of each PFL1 neuron.

To find the cell types presynaptic to PFL1 in the FB and plot the connectivity matrix in Fig. 3.1, I used neuPRINT's custom query feature to download a list of PFL1's postsynaptic sites in the FB from the hemibrain data.

Likewise, to find the cell types presynaptic to FB2B in the FB, I used neuPRINT's custom query feature to download a list of FB2B's postsynaptic sites in the FB from the hemibrain data.

To create the connectivity matrices in Fig. 2.1i 3.4a, I used the Connectivity Matrix widget in CATMAID (<https://fafb-flywire.catmaid.org/>).

To find the proportion of FB2B's input that comes from PFL1, I used CAVEclient (<https://github.com/CAVEconnectome/CAVEclient>) to query FB2B's posynapses in the Flywire data with and without a bounding box around the FB.

To create all images of neurons reconstructed from electron microscopy data, meshes of the neurons were downloaded using CloudVolume (<https://github.com/seung-lab/cloud-volume>), and the images were rendered in Blender.

Genotype Table:

Figure	Genotype
2.2,2.3,2.4,2.5a-c	(female) UAS-iGluSnFr3v857-PDGFR (attP18);;UAS-jRGECO1a (VK00005) x (male) SS02239
3.2	(female) 84H05-LexA (attP40), UAS-jRGECO1a (su(Hw)attP5);SS60353 x (male) 55G08-Gal4 (attP2) LexAop-GCaMP7f (VK00005)
2.5g	(female) w ⁺ ,norpA ³⁶ ,UAS-CsChrimson-mVenus (attP18) x (male) SS02215 or SS02239 or SS53185 or empty split
2.5i	(female) w ⁺ ,norpA ³⁶ ,UAS-CsChrimson-mVenus (attP18) x (male) w ⁺ ,norpA ³⁶ ;SS02239
3.3	(female) 84H05-LexA (attP40), UAS-jRGECO1a (su(Hw)attP5);49H05-Gal4 (attP2) x (male) 55G08-Gal4 (attP2) LexAop-GCaMP7f (VK00005)
2.5e	(female) UAS-GCaMP6f (VK00005), UAS-tdTomato (attP2) x (male) SS02239
3.4	(female) UAS-iGluSnFr3v857-PDGFR (attP18);UAS-TNTe;UAS-jRGECO1a x (male) SS02239
3.5a,b	(female) w ⁺ ;UAS-Gflamp2 (vk22) x (male) SS019425
3.5d,e	(female) w ⁺ ;LexAop-Gflamp2 (attP40); UAS-jRCaMP1b (VK00005) x (male) 49H05-LexA (attP40); SS60353
3.5f	(female) LexAop-tdTomato (attP40); UAS-MCD8GFP (attP2) x (male) sNPF[T2A-LexA];SS60353

6 References

1. Frisch, K. von. *The Dance Language and Orientation of Bees*. (Harvard university press, Cambridge (Mass.) London, 1967).
2. Gillet, V., Kluge, J. & Patel, R. N. A historical perspective on the insect central complex: Anatomy, development, and function. *Mol. Psychol. Brain Behav. Soc.* **2**, 19 (2025).
3. Seelig, J. D. & Jayaraman, V. Neural dynamics for landmark orientation and angular path integration. *Nature* **521**, 186–191 (2015).
4. Wolff, T., Iyer, N. A. & Rubin, G. M. Neuroarchitecture and neuroanatomy of the *Drosophila* central complex: A GAL4-based dissection of protocerebral bridge neurons and circuits. *J. Comp. Neurol.* **523**, 997–1037 (2015).
5. Seelig, J. D. & Jayaraman, V. Feature detection and orientation tuning in the *Drosophila* central complex. *Nature* **503**, 262–266 (2013).
6. Sun, Y. *et al.* Neural signatures of dynamic stimulus selection in *Drosophila*. *Nat. Neurosci.* **20**, 1104–1113 (2017).
7. Garner, D. *et al.* Connectomic reconstruction predicts visual features used for navigation. *Nature* **634**, 181–190 (2024).
8. Hardcastle, B. J. *et al.* A visual pathway for skylight polarization processing in *Drosophila*. *eLife* **10**, e63225 (2021).
9. Hanesch, U., Fischbach, K.-F. & Heisenberg, M. Neuronal architecture of the central complex in *Drosophila melanogaster*. *Cell Tissue Res.* **257**, 343–366 (1989).
10. Franconville, R., Beron, C. & Jayaraman, V. Building a functional connectome of the *Drosophila* central complex. *eLife* **7**, e37017 (2018).

11. Hulse, B. K. *et al.* A connectome of the *Drosophila* central complex reveals network motifs suitable for flexible navigation and context-dependent action selection. *eLife* **10**, e66039 (2021).
12. Kim, S. S., Hermundstad, A. M., Romani, S., Abbott, L. F. & Jayaraman, V. Generation of stable heading representations in diverse visual scenes. *Nature* **576**, 126–131 (2019).
13. Fisher, Y. E., Lu, J., D’Alessandro, I. & Wilson, R. I. Sensorimotor experience remaps visual input to a heading-direction network. *Nature* **576**, 121–125 (2019).
14. Green, J. *et al.* A neural circuit architecture for angular integration in *Drosophila*. *Nature* **546**, 101–106 (2017).
15. Turner-Evans, D. *et al.* Angular velocity integration in a fly heading circuit. *eLife* **6**, e23496 (2017).
16. Turner-Evans, D. B. *et al.* The Neuroanatomical Ultrastructure and Function of a Biological Ring Attractor. *Neuron* **108**, 145-163.e10 (2020).
17. Hulse, B. K., Stanoev, A., Turner-Evans, D. B., Seelig, J. D. & Jayaraman, V. A rotational velocity estimate constructed through visuomotor competition updates the fly’s neural compass. Preprint at <https://doi.org/10.1101/2023.09.25.559373> (2023).
18. Lu, J. *et al.* Transforming representations of movement from body- to world-centric space. *Nature* **601**, 98–104 (2022).
19. Lyu, C., Abbott, L. F. & Maimon, G. Building an allocentric travelling direction signal via vector computation. *Nature* **601**, 92–97 (2022).
20. Touretzky, D. S., Redish, A. D. & Wan, H. S. Neural Representation of Space Using Sinusoidal Arrays. *Neural Comput.* **5**, 869–884 (1993).

21. Ishida, I. G., Sethi, S., Mohren, T. L., Abbott, L. F. & Maimon, G. Neuronal calcium spikes enable vector inversion in the *Drosophila* brain. Preprint at <https://doi.org/10.1101/2023.11.24.568537> (2023).
22. Westeinde, E. A. *et al.* Transforming a head direction signal into a goal-oriented steering command. *Nature* **626**, 819–826 (2024).
23. Mussells Pires, P., Zhang, L., Parache, V., Abbott, L. F. & Maimon, G. Converting an allocentric goal into an egocentric steering signal. *Nature* **626**, 808–818 (2024).
24. Scheffer, L. K. *et al.* A connectome and analysis of the adult *Drosophila* central brain. *eLife* **9**, e57443 (2020).
25. Dorkenwald, S. *et al.* Neuronal wiring diagram of an adult brain. *Nature* **634**, 124–138 (2024).
26. Zheng, Z. *et al.* A Complete Electron Microscopy Volume of the Brain of Adult *Drosophila melanogaster*. *Cell* **174**, 730–743.e22 (2018).
27. Namiki, S. & Kanzaki, R. Comparative Neuroanatomy of the Lateral Accessory Lobe in the Insect Brain. *Front. Physiol.* **7**, (2016).
28. Westeinde, E. A. *et al.* Transforming a head direction signal into a goal-oriented steering command. *Nature* **626**, 819–826 (2024).
29. Aggarwal, A. *et al.* Glutamate indicators with improved activation kinetics and localization for imaging synaptic transmission. *Nat. Methods* **20**, 925–934 (2023).
30. Dana, H. *et al.* Sensitive red protein calcium indicators for imaging neural activity. *eLife* **5**, e12727 (2016).

31. Wolff, T. & Rubin, G. M. Neuroarchitecture of the *Drosophila* central complex: A catalog of nodulus and asymmetrical body neurons and a revision of the protocerebral bridge catalog. *J. Comp. Neurol.* **526**, 2585–2611 (2018).
32. Heinze, S. Unraveling the neural basis of insect navigation. *Curr. Opin. Insect Sci.* **24**, 58–67 (2017).
33. Honkanen, A., Adden, A., Da Silva Freitas, J. & Heinze, S. The insect central complex and the neural basis of navigational strategies. *J. Exp. Biol.* **222**, jeb188854 (2019).
34. Dan, C., Hulse, B. K., Kappagantula, R., Jayaraman, V. & Hermundstad, A. M. A neural circuit architecture for rapid learning in goal-directed navigation. *Neuron* **112**, 2581-2599.e23 (2024).
35. Giraldo, Y. M. *et al.* Sun Navigation Requires Compass Neurons in *Drosophila*. *Curr. Biol.* **28**, 2845-2852.e4 (2018).
36. Klapoetke, N. C. *et al.* Independent optical excitation of distinct neural populations. *Nat. Methods* **11**, 338–346 (2014).
37. Pearn, M. T., Randall, L. L., Shortridge, R. D., Burg, M. G. & Pak, W. L. Molecular, Biochemical, and Electrophysiological Characterization of *Drosophila* *norpA* Mutants. *J. Biol. Chem.* **271**, 4937–4945 (1996).
38. Wolff, T. *et al.* Cell type-specific driver lines targeting the *Drosophila* central complex and their use to investigate neuropeptide expression and sleep regulation. Preprint at <https://doi.org/10.7554/eLife.104764.2> (2025).
39. Jenett, A. *et al.* A GAL4-Driver Line Resource for *Drosophila* Neurobiology. *Cell Rep.* **2**, 991–1001 (2012).

40. Pfeiffer, B. D. *et al.* Refinement of Tools for Targeted Gene Expression in *Drosophila*. *Genetics* **186**, 735–755 (2010).
41. Dana, H. *et al.* High-performance calcium sensors for imaging activity in neuronal populations and microcompartments. *Nat. Methods* **16**, 649–657 (2019).
42. Weir, P. T., Schnell, B. & Dickinson, M. H. Central complex neurons exhibit behaviorally gated responses to visual motion in *Drosophila*. *J. Neurophysiol.* **111**, 62–71 (2014).
43. Sweeney, S. T., Broadie, K., Keane, J., Niemann, H. & O’Kane, C. J. Targeted expression of tetanus toxin light chain in *Drosophila* specifically eliminates synaptic transmission and causes behavioral defects. *Neuron* **14**, 341–351 (1995).
44. Liu, G. *et al.* Distinct memory traces for two visual features in the *Drosophila* brain. *Nature* **439**, 551–556 (2006).
45. Liu, W., Liu, C., Ren, P.-G., Chu, J. & Wang, L. An Improved Genetically Encoded Fluorescent cAMP Indicator for Sensitive cAMP Imaging and Fast Drug Screening. *Front. Pharmacol.* **13**, 902290 (2022).
46. Clements, J. *et al.* NeuronBridge: an intuitive web application for neuronal morphology search across large data sets. *BMC Bioinformatics* **25**, 114 (2024).
47. Chalfie, M., Tu, Y., Euskirchen, G., Ward, W. W. & Prasher, D. C. Green Fluorescent Protein as a Marker for Gene Expression. *Science* **263**, 802–805 (1994).
48. Shaner, N. C. *et al.* Improved monomeric red, orange and yellow fluorescent proteins derived from *Discosoma* sp. red fluorescent protein. *Nat. Biotechnol.* **22**, 1567–1572 (2004).

49. Deng, B. *et al.* Chemoconnectomics: Mapping Chemical Transmission in *Drosophila*. *Neuron* **101**, 876-893.e4 (2019).
50. Feng, G. *et al.* Functional characterization of a neuropeptide F-like receptor from *Drosophila melanogaster*. *Eur. J. Neurosci.* **18**, 227–238 (2003).
51. Dan, C., Hulse, B. K., Kappagantula, R., Jayaraman, V. & Hermundstad, A. M. A neural circuit architecture for rapid learning in goal-directed navigation. *Neuron* **112**, 2581-2599.e23 (2024).
52. Rayshubskiy, A. *et al.* Neural circuit mechanisms for steering control in walking *Drosophila*. Preprint at <https://doi.org/10.7554/eLife.102230.2> (2025).
53. Matheson, A. M. M. *et al.* A neural circuit for wind-guided olfactory navigation. *Nat. Commun.* **13**, 4613 (2022).
54. Siliciano, A. F. *et al.* A vector-based strategy for olfactory navigation in *Drosophila*. Preprint at <https://doi.org/10.1101/2025.02.15.638426> (2025).
55. Fisher, Y. E., Marquis, M., D'Alessandro, I. & Wilson, R. I. Dopamine promotes head direction plasticity during orienting movements. *Nature* **612**, 316–322 (2022).
56. Li, W. *et al.* Morphological characterization of single fan-shaped body neurons in *Drosophila melanogaster*. *Cell Tissue Res.* **336**, 509–519 (2009).
57. Pan, Y. *et al.* Differential roles of the fan-shaped body and the ellipsoid body in *Drosophila* visual pattern memory. *Learn. Mem.* **16**, 289–295 (2009).
58. Gallistel, C. R. & King, A. P. *Memory and the Computational Brain: Why Cognitive Science Will Transform Neuroscience*. (Wiley-Blackwell, Chichester, 2009).
59. Kandel, E. R. The Molecular Biology of Memory Storage: A Dialogue Between Genes and Synapses. *Science* **294**, 1030–1038 (2001).

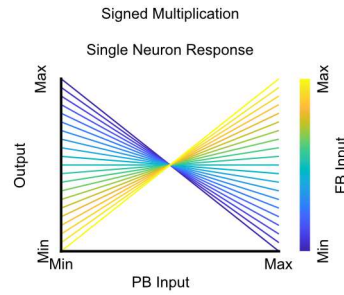
60. Flickinger, D. *et al.* MIMMS: a modular, open design microscopy platform for in vivo imaging of neural tissues. (2010).
61. Pologruto, T. A., Sabatini, B. L. & Svoboda, K. ScanImage: Flexible software for operating laser scanning microscopes. *Biomed. Eng. OnLine* **2**, 13 (2003).
62. Kim, S. S., Rouault, H., Druckmann, S. & Jayaraman, V. Ring attractor dynamics in the *Drosophila* central brain. *Science* **356**, 849–853 (2017).

7 Mathematical Appendix

The main circuit model in this paper used signed multiplication as an activation function for the PFL1 neurons.

$$PFL1_{n,m} = \cos(\theta_H - \varphi_{n,m}) \times \cos(\theta_S - \omega_{n,m})$$

With this activation function, the inputs are allowed to be both positive and negative, and the output of each PFL1 neuron is the product of its inputs. This means that the FB input changes the slope of the relationship between the output of a PFL1 neuron and the PB input, and with a low enough FB input, the slope of this relationship will invert.

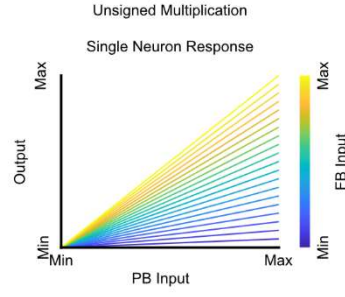


This is a rather unusual activation function that would require PFL1 to have specialized functional properties. However, I found that two other activation functions support a nearly identical result when used in the overall model. This broadens the set of functional properties that PFL1 might have.

The first activation function is unsigned multiplication, where the inputs are modelled as positive, and the output of PFL1 is still the product of its inputs.

$$PFL1_{n,m} = (\cos(\theta_H - \varphi_{n,m}) + 1) \times (\cos(\theta_S - \omega_{n,m}) + 1)$$

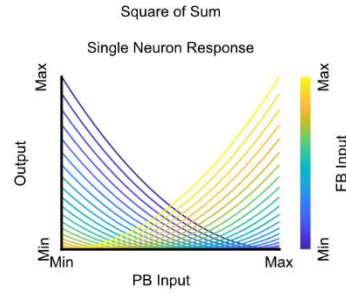
With this activation function, the FB input changes the slope of the relationship between the output of a PFL1 neuron and the PB input, but the slope does not invert. This activation function is essentially a simple gain modulation.



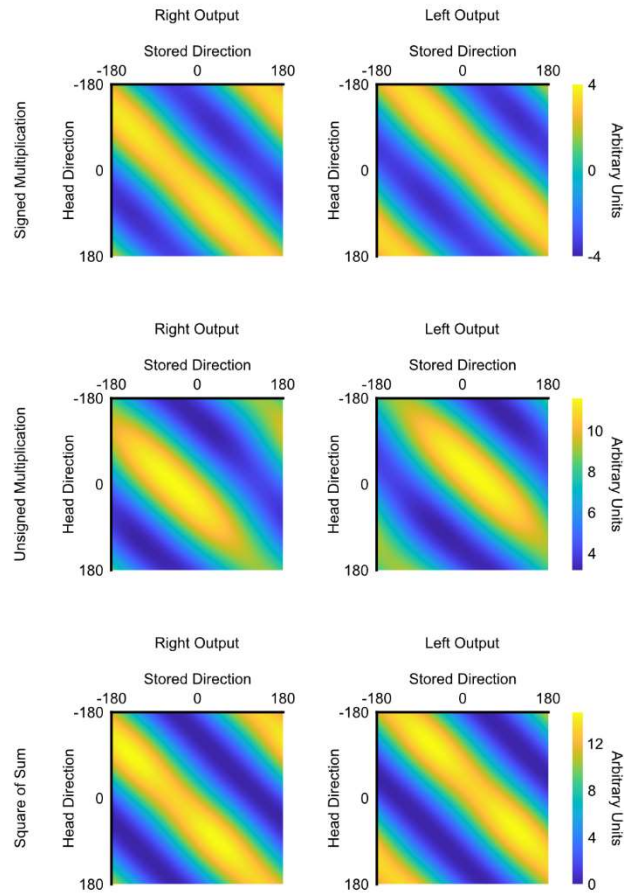
The second activation function is the square of the sum.

$$PFL1_{n,m} = \left(\cos(\theta_H - \varphi_{n,m}) + \cos(\theta_S - \omega_{n,m}) \right)^2$$

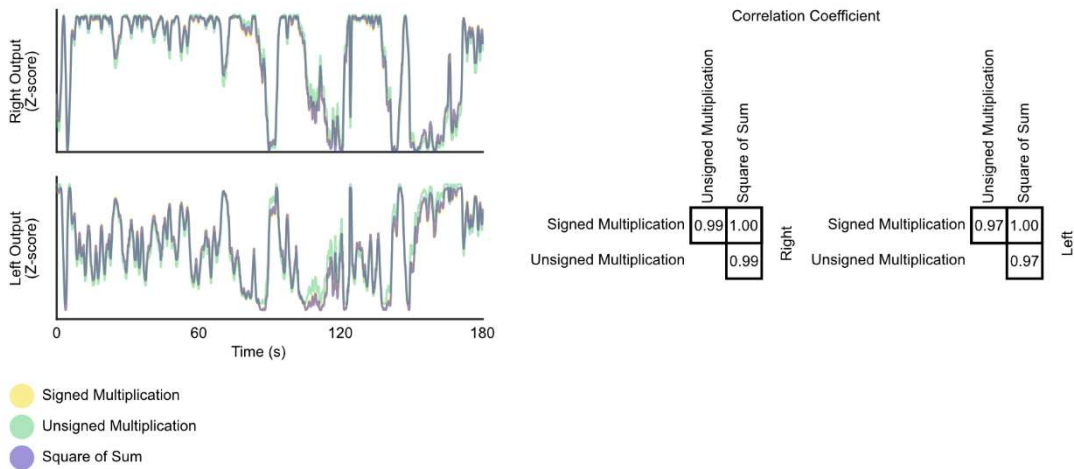
This activation function has an unusual property where as the input decreases past a certain point, the response of the PFL1 neuron starts to increase. The FB input changes the level of PB input at which the output begins to increase.



Simulating the output of the entire model across the full range of head and stored directions using these activation functions yields similar results.



Simulating an example experimental trial yields results that are highly correlated and would be indistinguishable experimentally.



To gain insight into why these simulations yield similar results, I performed algebraic proofs.

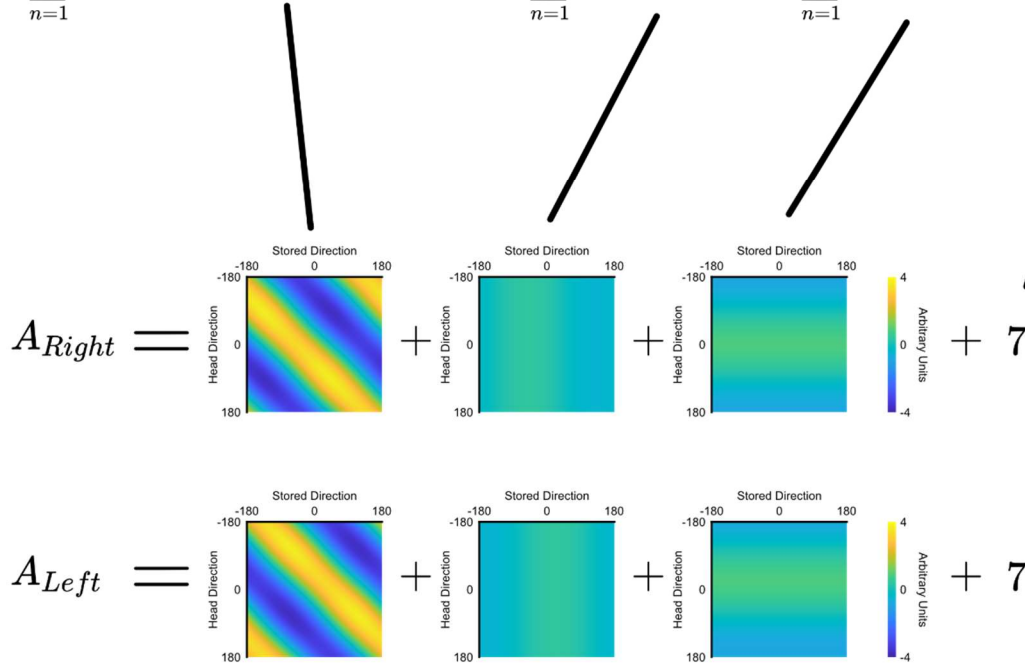
Unsigned Multiplication

$$\begin{aligned} PFL1_{n,m} &= (\cos(\theta_H - \varphi_{n,m}) + 1) \times (\cos(\theta_S - \omega_{n,m}) + 1) \\ &= \cos(\theta_H - \varphi_{n,m}) \times \cos(\theta_S - \omega_{n,m}) + \cos(\theta_S - \omega_{n,m}) + \cos(\theta_H - \varphi_{n,m}) + 1 \end{aligned}$$

The output of the model sums the activity of all the neurons on each side of the brain:

$$A_m = \sum_{n=1}^7 PFL1_{n,m}$$

$$A_m = \sum_{n=1}^7 \cos(\theta_H - \varphi_{n,m}) \times \cos(\theta_S - \omega_{n,m}) + \sum_{n=1}^7 \cos(\theta_S - \omega_{n,m}) + \sum_{n=1}^7 \cos(\theta_H - \varphi_{n,m}) + \sum_{n=1}^7 1$$



The second and third terms are close to zero, and thus the result is nearly identical to the signed multiplication model with a bias.

Square of Sum

$$PFL1_{n,m} = (\cos(\theta_H - \varphi_{n,m}) + \cos(\theta_S - \omega_{n,m}))^2$$

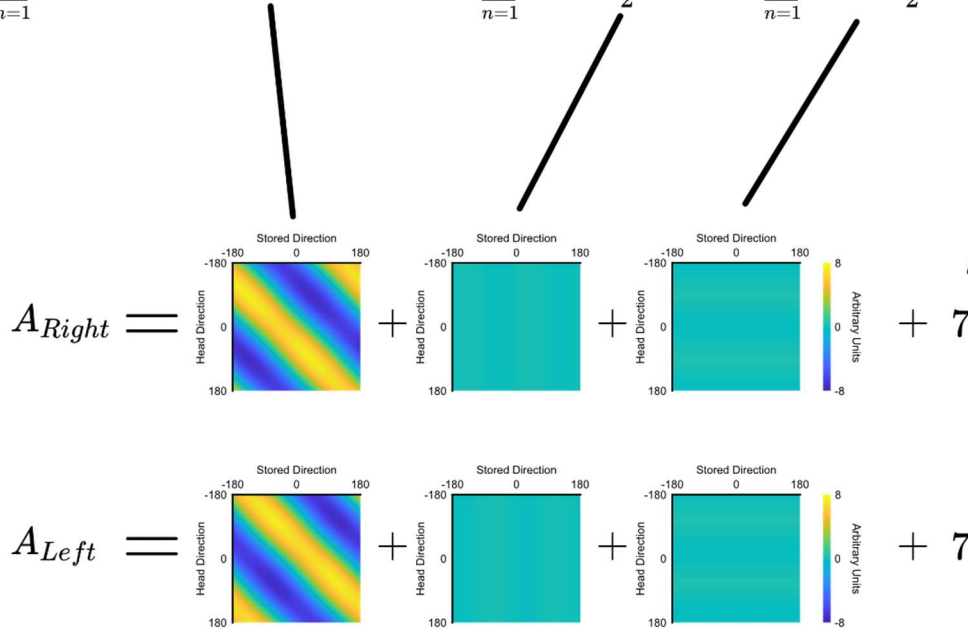
Using trigonometric identities, I can rewrite the equation:

$$\begin{aligned} PFL1_{n,m} &= \left(2 \cos\left(\frac{(\theta_H - \varphi_{n,m}) + (\theta_S - \omega_{n,m})}{2}\right) \times \cos\left(\frac{(\theta_H - \varphi_{n,m}) - (\theta_S - \omega_{n,m})}{2}\right) \right)^2 \\ &= \left(1 + \cos((\theta_H - \varphi_{n,m}) + (\theta_S - \omega_{n,m})) \right) \times \left(1 + \cos((\theta_H - \varphi_{n,m}) - (\theta_S - \omega_{n,m})) \right) \\ &= 1 + 2\cos(\theta_H - \varphi_{n,m}) \times \cos(\theta_S - \omega_{n,m}) \\ &\quad + \left(\cos((\theta_H - \varphi_{n,m}) + (\theta_S - \omega_{n,m})) \right) \\ &\quad \times \left(\cos((\theta_H - \varphi_{n,m}) - (\theta_S - \omega_{n,m})) \right) \\ &= 1 + 2\cos(\theta_H - \varphi_{n,m}) \times \cos(\theta_S - \omega_{n,m}) + \frac{\cos 2(\theta_H - \varphi_{n,m})}{2} + \frac{\cos 2(\theta_S - \omega_{n,m})}{2} \end{aligned}$$

The output of the model sums the activity of all the neurons on each side of the brain:

$$A_m = \sum_{n=1}^7 PFL1_{n,m}$$

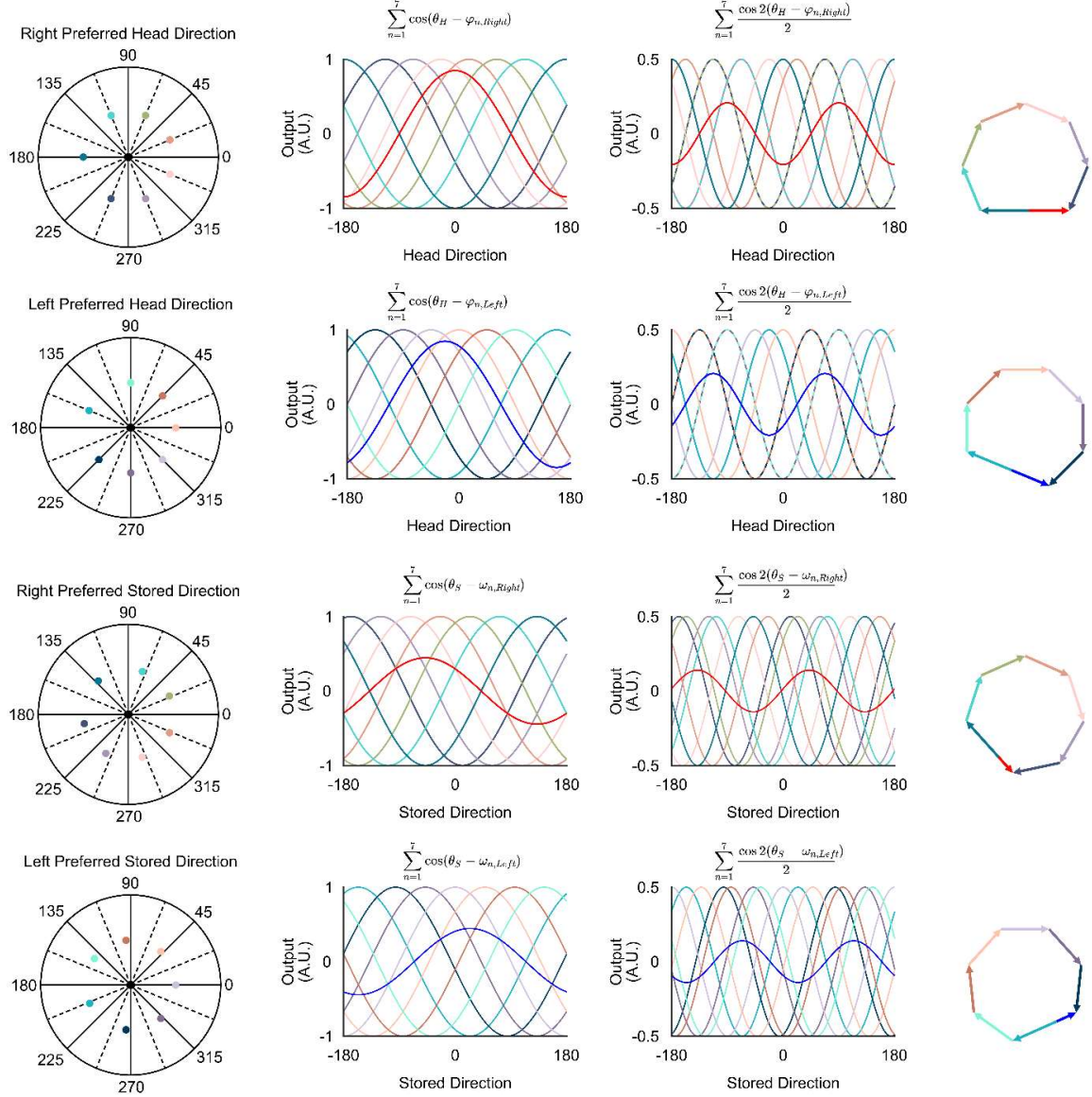
$$A_m = \sum_{n=1}^7 2 \cos(\theta_H - \varphi_{n,m}) \times \cos(\theta_S - \omega_{n,m}) + \sum_{n=1}^7 \frac{\cos 2(\theta_S - \omega_{n,m})}{2} + \sum_{n=1}^7 \frac{\cos 2(\theta_H - \varphi_{n,m})}{2} + \sum_{n=1}^7 1$$



Again, the second and third terms are close to zero. The output is nearly identical to the signed multiplication model except with scaling ($\times 2$) and a bias.

When comparing my model to recordings of neural activity, I use Pearson's correlation coefficient, which is not affected by linear scaling and biasing. Therefore, all three models produce nearly identical results.

The second and third terms are small in both models, because the preferred head and stored directions are spread out across a period, which makes the sum of these terms mostly cancel out. If the preferred directions were perfectly evenly spaced across the period, the sum would equal exactly zero.



Red and blue curves or arrows represent the sum of each term across neurons. Other colored curves and arrows represent the value of the term for individual neurons.