Title
Columnar visual pathways for smooth optomotor steering and saccadic object tracking in Drosophila

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Columnar visual pathways for smooth optomotor steering and saccadic object tracking in

*Drosophila*

A thesis submitted in partial satisfaction of the requirements for the degree Master of Science in

Physiological Science

by

Ivan Lopez

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ABSTRACT OF THE THESIS

Columnar visual pathways for smooth optomotor steering and saccadic object tracking in

*Drosophila*

by

Ivan Lopez

Master of Science in Physiological Science

University of California, Los Angeles, 2019

Professor Mark Frye, Chair

All visual animals stabilize their gaze by moving their eyes, head, or body. Humans, with camera type eyes, use a “fixate and saccade” strategy that combines smooth tracking movements and ballistic eye saccades to stabilize gaze. *Drosophila melanogaster*, having a radically different type of eye, nonetheless move their full body during flight to use these smooth optomotor steering and saccade maneuvers to stabilize visual gaze. Despite the ubiquitous expression of these two behaviors across different species, the neural mechanisms that differentially control these maneuvers remain unknown. In this paper, we utilize the Gal4-UAS system to express a potassium inward rectifier, known as Kir2.1, in T4/T5, T2 or T3 columnar cells to test for differences in a flying fly’s ability to perform smooth optomotor steering and saccade maneuvers. Our data confirm prior work showing that T4/T5 neurons contribute motion
related information to the downstream circuitry responsible for computing the speed of a moving panorama across the fly retina and accurately matching it to smooth fixational optomotor gain. We extend these results to show that T4/T5 are largely dispensable for the control of object tracking saccades. Additionally, our results strongly suggest that T2/T3 neurons contribute to the downstream circuitry responsible for target acquisition mechanisms required for recognizing and initiating saccadic tracking bouts in response to a moving barlike stimulus.
The thesis of Ivan Lopez is approved.

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2019
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INTRODUCTION

All visual animals stabilize their gaze by moving their eyes, head, or body. Humans, with camera type eyes, use a “fixate and saccade” strategy that combines smooth tracking movements and ballistic eye saccades to stabilize gaze (Xivry and Lefèvre, 2007). Gaze stabilization, or keeping the image of the world relatively still on the retina, is especially important to maintain visual acuity to track objects while performing moving activities such as driving, or running to catch a ball. Similarly, flying fruit flies, *Drosophila melanogaster*, despite having a radically different type of eye, nonetheless move their full body during flight to use these same two classes of visual maneuvers (Collett and Land, 1975) – saccades and smooth optomotor steering - to stabilize visual gaze. Collision avoidance saccades, quick movements of the whole fly body to re-orient gaze (and flight direction) in a new direction are instinctively evoked by visual expansion of an approaching object or surface (Censi et al., 2013; Tammero and Dickinson, 2002a; Tammero and Dickinson, 2002b]. Upon triggering a saccade, visual motion plays no role in modulating its dynamics (Bender and Dickinson, 2006; Heisenberg and Wolf, 1988), characterized as brief and hasty, frequently reaching peak angular velocities of 509°/s and lasting approximately 69ms (Mongeau and Frye, 2017). These dynamic properties are necessary for minimizing the time that the retinal image is blurry and visual features are indiscriminable. Additionally, in tethered flight, flies generate saccades without any obvious external stimulus, suggesting that endogenous processes may also evoke saccades (Bender and Dickinson, 2006;
Schnitt et al., 2017). Saccades are contrasted by smooth optomotor steering, smooth rotation of
the whole fly body (and head and eyes), which serves to reduce retinal slip and ‘fixate’ or
stabilize visual gaze against unintended slower course perturbations such as a cross-wind
(Duistermars et al., 2007; Heisenberg and Wolf, 1979; Götz, 1968; Geiger and Nässel, 1981).
Flies employ a combination of both saccades and smooth optomotor steering as strategies to fly
straight and respond to visual stimuli approaching objects or predators or conspecific courtship
targets (Breugel and Dickinson., 2012; Boeddeker and Egelhaaf, 2005); however, despite
ubiquitous expression of these two behaviors across different species, the neural mechanisms
that differentially control these maneuvers remain unknown.

The fly visual system is arranged ‘horizontally’ in a retinotopic manner and projects
‘vertically’ through several neuropils for visual processing. The first layer of visual motion
processing uses signals from photoreceptors R1-R6 in each ommatidium of the compound eye
that provide postsynaptic input to first-order lamina neurons. Lamina neurons L1/L5 and
L2/L3/L4 constitute parallel visual pathways for specific motion computations, and they filter
retinotopic distribution of luminance increments (ON, L1/L5) or decrements (OFF, L2/L3/L4).
ON-pathway lamina neurons send projections through third-order neurons in the medulla that
eventually terminate onto the dendrites of fourth-order T4 neurons. OFF-pathway lamina
neurons supply parallel medulla neurons that terminate onto lobula T5 dendrites (Borst et al.,
2019). Thus, T4 neurons are OFF-selective, whereas T5 are ON-selective. T4/T5 neurons
selective for motion rather than flicker (as all upstream neurons are), show directional selectivity,
have small visual receptive fields, are columnar in structure, retinotopic, and their axons
innervate the dendrites of wide-field tangential cells located in the lobula plate (LPTCs, Figure
1A). LPTCs are widely studied large neurons that map specific wide-field patterns of optic flow.
onto descending pre-motor neurons (Busch et al., 2018; Fisher et al., 2015; Maisak et al., 2013). T4/T5 neurons are the first cells in the visual processing pathway to show strong selectivity for one of the four cardinal directions of motion in a small visual field, and these columnar neurons terminate at the dendrites of LPTCs in the lobula plate (Maisak et al., 2013).

Genetic evidence implicates T4/T5 motion detectors in higher-order motion perception and optomotor behavior. Silencing T4/T5 neurons results in loss of direction-selective responses by LPTCs (Schnell et al., 2012) and behavioral motion blindness leading to the extinction of optomotor responses in walking flies (Bahl et al., 2013). Yet, silencing T4/T5 has little effect on bar tracking behaviors in walking (Bahl et al., 2013), or in flight (Keleş et al., 2019). Thus, these neurons are prime candidates to supply the information needed to separate smooth gaze stabilization from object tracking in Drosophila.

Parallel to the motion vision pathway consisting of a wide-field aggregation of T4/T5 retinotopic neuronal integration by LPTCs, columnar projection neurons from the fourth optic ganglion, the lobula, known as lobula columnar neurons (LCs), have been shown to be non-directional, selective for visual features such as edges or objects, and influence object-dependent visual behaviors such as escape from an approaching threat, or complex features of the landscape that flies navigate through (Ache et al., 2019; von Reyn et al., 2017; Wu et al., 2016). 22 classes of LCs project to the protocerebrum where axon terminals of each class form tight glomerular neuropils (Wu et al., 2016). LC11 is of particular interest due to its uncharacteristic sensitivity to very small contrasting objects moving in the ipsilateral field of view but without any directional preference (Keleş and Frye, 2017). Recently, Keleş et al. (in review) demonstrated that columnar neurons T2 and T3, originating from the medulla and synapsing in the second and third layers of the lobula, provide synaptic input onto LC11 dendrites (Figure 1A). Unlike their T4/T5 cousins
that innervate the lobula plate, T2/T3 neurons are non-selective for direction or contrast polarity (i.e. are ON-OFF detectors), are highly sensitive to small visual objects moving in their receptive field and are suppressed by wide-field background motion (Keleş et al. in review).

By contrast to the physiological properties of T4/T5, which have been broadly implicated for motion vision, T2/T3 neurons and their postsynaptic targets seem to be well suited for feature detection. We therefore wished to compare how T4/T5 and T2/T3 pathways participate in the neural control of feature-tracking saccades and smooth optomotor gaze stabilization. Our results confirm prior work showing that T4/T5 neurons contribute motion related information to the downstream circuitry responsible for computing the speed of a moving panorama across the fly retina and accurately matching it to smooth fixational optomotor gain. We extend these results to show that T4/T5 are largely dispensable for the control of object tracking saccades. Additionally, our results strongly suggest that T2/T3 neurons contribute to the downstream circuitry responsible for target acquisition mechanisms required for recognizing and initiating saccadic tracking bouts in response to a moving barlike stimulus.

MATERIALS AND METHODS

Driver lines and split gal4s
Table 1: Driver Lines and Reagents

<table>
<thead>
<tr>
<th>Driver</th>
<th>Reagents</th>
<th>Source</th>
</tr>
</thead>
<tbody>
<tr>
<td>T4/T5-Split Gal4</td>
<td>$R59E08$-Gal4.AD(attP40); $R42F06$-Gal4.DBD(attP2)</td>
<td>Pfeiffer et al., 2012</td>
</tr>
<tr>
<td>T2-Split Gal4</td>
<td>$VT012791$-Gal4.AD(attP40); $R47E02$-Gal4.DBD(attP2)</td>
<td>Keleş et al., in review</td>
</tr>
<tr>
<td>T3-Split Gal4</td>
<td>$VT002055$-Gal4.AD(attP40); $R65B04$-Gal4.DBD(attP2)</td>
<td>Keleş et al., in review</td>
</tr>
<tr>
<td>10xUAS-Kir2.1-eGFP</td>
<td>$DL^+; JFRC49$-10xUAS-Kir2.1 (attp2)-eGFP</td>
<td>von Reyn et al., 2017</td>
</tr>
<tr>
<td>Enhancerless-Split</td>
<td>$pBPp65ADZpUw; pBPZpGAL4DBDUw$</td>
<td>Hampel et al., 2015</td>
</tr>
</tbody>
</table>

T2- and T3-Split Gal4 candidate lines were checked for expression by crossing them to UAS-GFP before confirming cellular identity using MultiColor FlpOut, a single cell labeling technique (Pfeiffer and Rubin, 2015). This confirmed that R47E02, VT012791 and R65B04 labels T2, whereas VT002055, VT008463 and R65B04 labels T3. Finally, lines that carry different combinations of AD and DBD of aforementioned lines were made. This resulted in T2-Split Gal4 and T3-Split Gal4 (Keleş et al. in review).

Experimental F1 were set up by crossing virgin female 10xUAS-Kir2.1-eGFP flies to either male T4/T5-Split Gal4, T2-Split Gal4, or T3-Split Gal4 flies. Control F1 were set up by crossing virgin female 10xUAS-Kir2.1-eGFP flies to male Enhancerless-Split Gal4 flies. Control
and experimental animals were maintained at 25°C under a 12 h:12h-light: dark cycle with access to food and water ad libitum. All experiments were performed with 5- to 7-day-old adult female flies.

**Cell-specific neural silencing**

To drive expression of transgenes in targeted tissues the Split-Gal4/UAS (Luan et al., 2006) expression system is used. Inward rectifier potassium channel (Kir2.1) establishes a constant outward current of potassium thereby hyperpolarizing the targeted neurons. In neurons, Kir2.1 brings the resting membrane potential closer to the equilibrium potential of potassium, making it harder for the neuron to reach firing threshold and achieve an action potential (Hibino et al., 2010). Complete genotypes are listed in Table 1.

Kir2.1 is attached to eGFP during expression and fluorescence was verified using the ZEISS SteREO Discovery.V12 microscope. Absence or presence of expression was checked sporadically for control and experimental flies, respectively. Confocal imaging was not done for Kir2.1 expression and results showing proof of expression are not provided in this paper.

It is also worth noting that Kir2.1 expression level may differ from cell to cell. This can create disparities in the Kir2.1 adjusted resting membrane voltage, which is expected to be very close to the potassium equilibrium potential when Kir2.1 is expressed. These disparities can affect the strength of stimulus depolarization on Kir2.1 expressing neurons, which may result in variable levels of activity across our targeted cell lines in some animals. However, without the ability to control for Kir2.1 expression across different animals, we instead opted to reduce the intensity of our motion stimulus in order to reduce the effect of variable Kir2.1 expression level in some neurons resulting in variable neuronal silencing.

**Magno Tether visual flight simulator**
Flies were cold-anesthetized by cooling on a stage maintained at approximately 4°C. For the magnetic-tether, a stainless-steel pin (100μm diameter; Fine Science Tools, Foster City, CA) was fixed to the dorsal thorax by applying UV-activated glue. The pins comprised less than 1 percent of the fly’s moment of inertia about the yaw axis. Flies were allowed at least one hour to recover before running experiments. Flies were then placed in the Magno Tether System and after suspension within the magnetic field, they were given several minutes to acclimate. The Magno Tether System has a display consisting of an array of 96 × 16 light emitting diodes (LEDs, each subtending 3.75° on the eye) that wrap around the fly, subtending 360° and vertically 60° (Figure 1B). Flies were suspended between two magnets, allowing frictionless free rotation along the horizontal (yaw) flight axis - they could turn freely in response to displacements of visual stimuli on the LED display (Bender and Dickinson, 2006; Duistermars and Frye, 2008). Flies were illuminated from below with an array of eight 940 nm LEDs. The angular position of the fly body within the arena was recorded at 162 frames per second with an infrared-sensitive camera placed directly below the fly (A602f, Basler, Ahrendburg, Germany).

Each experiment began by eliciting sustained rotation of the fly by revolving a visual panorama on the LED display either clockwise or counterclockwise for 30s at 112.5°/s (Figure 1B). This stimulus elicited a strong rotatory, yaw-based optomotor response. From these data, computing the cumulative sum of all frames and measuring its centroid estimated the fly’s point of rotation. Flies that could not robustly track the rotating panorama were not further used.

Visual stimuli

We sought to test flies’ responses to wide-field panoramic motion and motion of small objects. The wide-field motion stimulus consisted of the rotation of a 96-pixel-by-16-pixel randomly generated pattern of ON and OFF vertical bars. This visual display was rotated at
112.5°/s and the direction of motion (clockwise/counterclockwise) was randomized for each block of trials. To study the flies’ response to rotating bars, a 8-pixel-wide (30°) randomly textured bar was rotated across a similar stationary randomly-generated background pattern. The initial position of the bar was generated from a pseudo-random sequence. The bar’s rotated direction of motion (clockwise/counterclockwise) at 112.5°/s was randomized for each block of trials. Each stimulus was presented for a period of 30s, defining the duration of an individual trial. Between each trial, a fixed visual landscape was presented for 25s for the fly to rest. If flies stopped flying during a trial, the trial was discarded. For analysis, the first second of a trial was ignored in order to exclude startle responses when the stimulus first appears. One experimental block consisted of 4 trials: 2 wide-field motion trials (clockwise and counterclockwise) and 2 bar trials (clockwise and counterclockwise).

**Saccade analysis**

Saccade analysis has been described elsewhere (Mongeau and Frye, 2017). Briefly, to identify saccades, angular position was analyzed on Matlab (Mathworks, Natick, MA, USA). To calculate fly orientation, each frame was converted into a binary image and modeled to the shape of the fly. The head of the fly was manually defined relative to the center of rotation in the first frame to set the initial conditions. Next, a low-pass, Butterworth filter with a cutoff frequency of 25Hz was applied to the position data and the derivative of the filtered data was used to identify saccade events. The saccade-detection threshold was defined as the standard deviation of the background noise of the velocity data for each trial. We defined the noise floor ($\sigma_n$) as:

$$\sigma_n = \text{median}(\frac{|x|}{0.6745}) \quad (\text{Equation 1})$$

where $x$ is the filtered signal, which provides a robust estimate of the noise floor in the presence of spiking events (Quiroga et al. 2004). Saccade threshold was defined as four times the
estimated noise floor, in units of angular velocity. To determine the peak of a velocity spike the local maxima was computed. The period of a saccade was defined as the time when the fly’s angular velocity was greater than one quarter of the peak amplitude (Bender and Dickinson, 2006).

Integrated error, which is how the fly brain integrates the angular position of a moving bar relative to its body axis over time, will reach a threshold value, triggering (with noise) a saccade toward a rotating bar (Mongeau and Frye, 2017). In this paper, integrated error is calculated using custom Matlab scripts and is measured as baseline subtracted integrated error (degree-seconds, °*s). Baseline subtracted integrated error was computed for tracking bouts using trapezoidal numerical integration and is characterized as the change in area between stimulus position and fly heading between two saccades during a saccade bout (Figure 8A, B). Head movement was very small compared to body movement, therefore we did not include head movement in our analysis of integrated retinal position error (Mongeau and Frye, 2017).

Saccade fixation bout classification

To define bouts of bar tracking, correlation between fly position and bar position signals were verified by plotting the fly’s heading with the bar’s position during a trial. Robust tracking was defined as the fly continuously tracking the bar for at least half a revolution around the arena (180°), corresponding to approximately five fixation saccades, and the slope of the fly’s heading during the tracking bout was approximately equal to the speed of the bar (112.5°/s). In some cases, flies performed non-directional saccades relative to the bar’s movement and were classified as spontaneous saccades. During flight, it is expected that flies sparingly perform spontaneous saccades, in addition to tracking saccades (Bender and Dickinson, 2006; Schnell et al., 2017).
Qualification of spontaneous saccades during bar tracking

Saccades are visualized as paired green and red lines perpendicular to the fly’s heading (Figure 2B Inset). A black trace overlapping the fly’s heading represents fly bar tracking segments based on satisfying the criteria listed above (Saccade Fixation Bout Classification); therefore, any saccades appearing perpendicular to a blue trace are likely to be considered spontaneous saccades. In this study, bar-fixation saccades occurred well above the rate of spontaneous saccades for control flies, consistent with previous findings (Mongeau and Frye, 2017).

Bar tracking performance index analysis

Performance Index (PI) is a measurement used to quantify how persistent a fly tracks a moving bar. PI ($\rho$) is defined as:

$$\rho = \frac{\text{fly's total distance tracking}}{\text{bar total distance traveled}} \quad \text{(Equation 2)}$$

and for these experiments, the bar’s total distance traveled is fixed at 2812.5° for a panorama traveling at 112.5°/s for 25 seconds. Calculating a fly’s total distance tracking for PI only takes into account the portions of a trial that have satisfied the criterion (stated in Saccade Fixation Bout Classification) necessary to confidently conclude that a fly is tracking the bar stimulus. Therefore, if a fly tracks the stimulus for 80% of the trial, with a high fidelity, PI would be approximately equal to 0.8 (Figure 3A). If a fly initially starts tracking the stimulus, stops tracking and performs spontaneous saccades, but then performs another series of tracking bouts, the PI only takes into account distance traveled within those two tracking sessions (Figure 3B). Lastly, flies that do not perform any tracking bouts, result in a PI of 0 (Figure 3C). A bar failure refers to a trial with a PI of 0, therefore the success rate is calculated as the proportion of trials with a PI>0 over the entire sample of trials.
Smooth optomotor steering gain analysis

Gain is a measurement used to quantify the quality of smooth optomotor steering response during to reduce retinal slip generated by motion of the visual display. Gain ($\gamma$) is defined as:

$$\gamma = \frac{\text{fly's total distance traveled}}{\text{panorama total distance traveled}} \quad (\text{Equation 3})$$

and for these experiments, the stimulus total distance traveled is fixed at 2812.5° for a panorama traveling at 112.5°/s for 25 seconds.

Statistical analysis

All statistical analysis was performed on MATLAB using custom scripts. Unless otherwise specified, mean ± 1 standard deviation is reported. Significance level ($\alpha$) was set to 0.05 for our hypothesis testing. Additionally, we are assuming data is representative of the entire population, and data is normally distributed according to the Central Limit Theorem. Confidence intervals were calculated at the 95% level.

Data, software availability and resource sharing

Data and software that was used in this study is available upon request to Mark Frye (frye@ucla.edu).

RESULTS

To test whether Kir2.1 expression in any of our candidate cell lines compromises smooth optomotor steering during wide-field motion, gain ($\gamma$) was compared across control and experimental conditions. For saccade bar tracking quantification, performance index, PI ($\rho$), was compared across control and experimental conditions.
Smooth optomotor steering: Kir2.1 expression decreased smooth optomotor steering gain during wide field stimulation in T4/T5 and decreased the proportion of trials exhibiting bar tracking behavior in T2 and T3

During wide-field motion, the $\gamma_{\text{mean}}$ and 95% confidence interval (CI) for control smooth optomotor steering was 0.9842 [0.9281 < $\mu$ < 1.0403] (n=48), which was significantly better than T4/T5 Kir2.1 expressing flies, with a $\gamma_{\text{mean}}$ of 0.7281 [0.6781 < $\mu$ < 0.7780] (n=64, p<0.05). No noticeable difference was seen in the proportion of T4T5 flies that exhibited a bar tracking behavior (51.56%) when compared to control (52.08%, Figure 4A,B). Thus, highly suggesting that T4/T5 serves a primary role in the circuitry responsible for computing the speed of a moving panorama across the fly retina and accurately matching it to smooth fixational optomotor gain.

Flies expressing Kir2.1 in T2 ($\gamma_{\text{mean}} = 0.9015$ [0.8380 < $\mu$ < 0.9650], n=53, p>0.05) or T3 ($\gamma_{\text{mean}} = 1.0379$ [0.9916 < $\mu$ < 1.0843], n=64, p>0.05) neurons did not show a statistically significant difference in smooth optomotor steering gain relative to control (Figure 5A, C, E, G). However, expression of Kir2.1 did impact the proportion of T2 and T3 flies that exhibited a bar tracking behavior. While control trials had a success rate of 25/48 (52.08%), T2 or T3 Kir2.1 expressing flies yielded success rates of 9/53 (16.98%) and 10/64 (15.62%), respectively (Figure 4A, C, D). Additionally, the relative frequency of an $\rho>0$ is significantly less in T2 or T3 Kir2.1 expressing flies relative to control, with 47.92% of trials yielding an $\rho=0$ for control, and 83.02% and 84.38% of trials yielding an $\rho=0$ for T2 and T3 Kir2.1 expressing flies, respectively (Figure 5B, F, H). T4/T5 Kir2.1 expressing flies showed no significant difference in bar tracking success rates (33/64 = 51.56%, Figures 4B) and relative frequency of $\rho=0$ (48.43%, Figure 5D) when compared to control. The robustness of smooth optomotor steering allowed us to notice a peculiar feature of T2 and T3 neurons and their potential contribution to triggering the initiation
of saccadic bar tracking. Kir2.1-induced inhibition of T2 or T3 neurons produced success rates for smooth optomotor steering approaching 100% for our experiments, as expected since these columnar neurons are not excited by wide-field motion (Keleș et al. in review). Yet, this result is contrasted by the performance index of bar tracking behavior by the same flies, which was reduced by nearly 70% when either T2 or T3 neurons were inhibited (Figure 4C, D and Figure 5B, D, F, H), relative to control. This suggests T2 and T3 neurons strongly contribute to the initiation of saccadic bar tracking events in Drosophila. We next decided to perform a thorough analysis of Kir2.1 expression on saccade dynamics in any of our candidate cell lines.

**Saccade dynamics: Kir2.1 expression reduced tracking saccade amplitude and duration, and increased peak angular velocity in T4/T5**

Phenotypic differences in saccade amplitude were present when Kir2.1 was expressed in T4/T5. The mean tracking saccade amplitude of control flies was 52.36° [49.3958 < μ < 55.3372] (n = 125), which was higher than mean tracking saccade amplitude in T4/T5-Kir2.1 (mean = 38.56° [36.74 < μ < 40.38], n = 242, p<0.05, Figure A). Further analysis suggests T4/T5-Kir2.1 also showed a decrease in tracking saccade duration. Mean tracking saccade duration for control flies was 253.1ms [245.4 < μ < 260.9] (n = 125), while mean tracking saccade duration for T4/T5-Kir2.1 was much lower, at 98.1ms [93.8 < μ < 10.23] (n = 242, p<0.05, Figure 7B). Lastly, a survey of tracking peak angular velocity yielded a mean for control flies of 207.78°/s [196.67 < μ < 218.90] (n = 125), which was significantly lower than mean tracking peak angular velocities for T4/T5-Kir2.1 (mean = 417.9534°/s [394.86 < μ < 441.03], n = 242, p<0.05), Figure 7C). This is expected as an increase in tracking saccade amplitude and a decrease in saccade duration equates to increases in tracking saccade peak
angular velocity. Data on tracking saccade dynamics for T2-Kir2.1 and T3-Kir2.1 were slightly different than those of T4/T5-Kir2.1 flies.

**Saccade dynamics: Kir2.1 had no effect on T3 tracking saccade dynamics while increasing amplitude and peak angular velocity in T2**

After analyzing the tracking saccade data, it became apparent that T3-Kir2.1 showed no statistically significant differences in saccade amplitude (mean = 51.80° [43.43 < μ < 60.17], n = 31, p>0.05, Figure 7A), duration (mean = 256.5ms [238.9 < μ < 274.0], n = 31, p>0.05, Figure 7B), and peak angular velocity (mean = 197.95°/s [171.45 < μ < 224.45], n = 31, p>0.05, Figure 7C). However, T2-Kir2.1 flies did have greater measured amplitude (mean = 38.56° [75.57 < μ < 93.48] n = 42, p<0.05) and peak angular velocity (mean = 330.20°/s [299.51 < μ < 360.89], n = 42, p<0.05, Figure 7A,C) relative to control flies. Despite this data, results remain inconclusive as the amount of tracking saccades made available to analyze was low (n=42) relative to the amount of trials tested (=53) due to the low success rate of bar tracking events (Figure 4C,D). Although we saw Kir2.1 expression in T2 and T3 reduce the proportion of trials with bar tracking saccades, we nonetheless were able to record and analyze the dynamics of spontaneous saccades.

**Saccade dynamics: Spontaneous saccade amplitude, duration and peak angular velocity were all altered in T4/T5-Kir, while T3-Kir2.1 flies showed differences in amplitude and peak angular velocity**

First, we looked at the effects of Kir2.1 expression in T4/T5 on spontaneous saccades. We saw that, compared to control flies, T4/T5-Kir2.1 had a decreased spontaneous saccade amplitude (mean = 46.30° [40.67 < μ < 51.93], n = 92, p<0.05), decreased duration (mean = 105.7ms [98.10 < μ < 11.33], n = 92, p>0.05), and increased peak angular velocity (mean =
453.14°/s [396.59 < μ < 509.68], n = 92, p>0.05) relative to control flies (mean control spontaneous amplitude = 65.47° [58.55 < μ < 72.38], n = 87; mean control duration = 25.33ms [24.44 < μ < 26.22], n = 87; mean control peak angular velocity = 254.31°/s [ 231.37 < μ < 277.25], n = 87, Figure 7A,B,C). Furthermore, the experimental evidence from our T3-Kir2.1 results suggest Kir2.1 expression decreases both spontaneous saccade amplitude (mean = 49.98° [36.14 < μ < 63.82], n = 22, p<0.05) as well as peak angular velocity (mean = 193.24°/s [143.40 < μ < 243.08], n = 22, p<0.05, Figures 7A,D) relative to controls. Interestingly, although T3-Kir2.1 seemed to show some differences in spontaneous saccade dynamics, T2-Kir2.1 did not show any significant differences in dynamics when compared to control flies (mean T2-Kir2.1 spontaneous saccade amplitude = 67.50° [57.94 < μ < 77.07], n = 73, p>0.05; mean T2-Kir2.1 duration = 25.78ms [24.30 < μ < 27.26], n = 73, p>0.05; mean T2-Kir2.1 peak angular velocity = 255.58°/s [226.84 < μ < 284.33], n = 73, p>0.05, Figure 7A,B,C). Lastly, we analyzed the impact of Kir2.1 expression in any of our candidate cell lines on integrated error and intersaccade intervals.

**Saccade dynamics: Baseline subtracted integrated error and intersaccade interval increased in T2-Kir2.1**

Kir2.1 expression significantly increased the saccade triggering threshold, computed using baseline subtracted integrated error, in T2-Kir2.1 (mean = 13.44°*s [8.28 < μ < 18.59], n = 38, p < 0) relative to control (mean = 4.56°*s [3.64 < μ < 5.49], n = 117). No difference was seen between control and T3 (mean = 5.35°*s [2.60 < μ < 8.11], n = 21, p > 0) or T4/T5 (mean = 3.93°*s [3.59 < μ < 4.27], n = 206, p > 0) (Figure 8C). Similarly, Kir2.1 expression increased intersaccade intervals in T2-Kir2.1 (mean = 567.39ms [442.01 < μ < 692.77], n = 38, p < 0) when compared to control (mean = 270.65ms [241.96 < μ < 299.34], n = 117). No difference
was seen between control and T3 (mean = 294.04ms [213.95 < μ < 374.13], n = 21, p > 0) or T4/T5 (mean = 290.83ms [277.72 < μ < 303.94], n = 206, p > 0) (Figure 8D).

In summary, Saccade dynamic analysis showed that inhibition of T4/T5 neurons decreases tracking saccade amplitude, the tracking saccades were smaller, however their duration also drastically decreases, resulting in a net increase in tracking saccade angular velocity (Figure 7). These variables are the ones that are pre-programmed by the visual motion dynamics that the fly experiences before a saccade is initiated (Mongeau and Frye, 2017). However, T4/T5 inhibition did not influence the integrated error of retinal slip that was experienced by the fly before a saccade was initiated, nor did it impact intersaccade intervals, suggesting that inhibition of T4/T5 neurons results in a greater amount of faster and smaller tracking saccades per unit of revolution (Figure 6A), with no difference in the threshold of visual motion that triggers a saccade (Mongeau and Frye, 2017). In conclusion, T4/T5 neurons might considerably contribute to the tuning mechanisms responsible for pre-programming size and duration of tracking saccades, variables that might be tuned to the strength of motion cues generated by the object. By contrast T4/T5 may play little role in contributing to the detection of the bar location on the retina, which triggers the saccade.

By contrast to T4/T5, expressing Kir2.1 in T3 resulted in similar saccade amplitude, duration, and angular velocity distributions as control flies (Figure 7). Therefore, considering T2 and T3 neurons both originate from the medulla and synapse into the second and third layers of the lobula to provide receptive field input onto LC11 (Keleş et al. in review), it would be expected that both show similar saccade dynamic phenotypes, however that is not the case. T2 Kir2.1 flies had a larger tracking saccade amplitude and angular velocity than control flies (Figure 7A, B). It is worth noting that due to the incredibly low success rate of bar tracking
events in T2 and T3 inhibited flies (16.98% and 15.63%, respectively), the population of tracking saccades available to analyse was significantly less than those available from control and T4/T5 experimental animals. Therefore, the results are inconclusive and require a greater population of saccades to confidently conclude whether inhibition of T2 neurons impacts dynamics of tracking saccades. In addition, it is worth noting that T2 Kir2.1 flies’ tracking saccade amplitude, duration and angular velocity is the same as their spontaneous saccade counterparts (Figure 7), and after considering the low success rate of tracking bouts, it is not unlikely that some of the saccades that passed our tracking bout criteria are actually spontaneous saccades, performed in the same direction and in the proximity of the stimulus, that are positively skewing our data. Thus, it is likely that Kir2.1 induced inhibition of T2 neurons should not significantly impact tracking saccade dynamics. Furthermore, T2 inhibition did present significant increases in both integrated error and intersaccade intervals (Figure 8B, C). This suggests T2 might play a determining role in programing saccade triggering threshold during bar tracking events. This is an interesting finding, however, considering spontaneous saccades have a larger saccade amplitude and larger intersaccade distances (Mongeau and Frye, 2017), it is not unlikely that our T2 tracking saccade sample might be substantially impacted by spontaneous saccades acting co-directional to the moving stimulus. This may result in larger than expected integrated errors and intersaccade intervals. Thus, it is likely that Kir2.1 induced inhibition of T2 neurons should not significantly impact integrated error and intersaccade interval recordings.

**DISCUSSION**

The “fixate and saccade” strategy of visual gaze stabilization to track moving objects and reject visual perturbation to stabilize gaze (upon a salient object) is common among flies and
humans (Breugel and Dickinson., 2012; Boeddeker and Egelhaaf, 2005; Xivry and Lefèvre, 2007). In flies, smooth fixational optomotor steering in response to steady-state wide-field motion is a robust and well documented reflex to reduce retinal slip during unintended perturbations to visual gaze (Duistermars et al., 2007; Heisenberg and Wolf, 1979; Götz, 1968; Geiger and Nässel, 1981). Despite its prevalence, it is still unclear which neurons are responsible for facilitating the transformation between visual input and behavior.

In this study, we provide evidence that corroborates prior published results literature and suggests T4/T5 serves a primary role in the circuitry responsible for computing the speed of a moving panorama across the fly retina and accurately matching it to smooth fixational optomotor tracking. Specifically, we demonstrate inhibition of T4/T5, through the expression of Kir2.1, resulted in a significant decrease in smooth fixational optomotor gain (Figure 5A, C, E, G). Our conclusion directly coincides with results published by Keleş et al., 2019, which demonstrated that a moving luminance-defined bar, such as a dark bar on a white background, elicited noticeable body movement between saccades in the direction of the moving bar. These noticeable body movements between saccades were characterized as similar to smooth optomotor steering responses seen during wide-field tracking. It was also demonstrated, through expression of a genetically encoded calcium indicator, that T4/T5 are highly responsive to movement of dark bars. Therefore, we hypothesis that T4/T5 neurons contribute directly to the smooth optomotor steering responses seen between dark bar tracking saccades. One interesting question that arises from this hypothesis is whether this smooth steering response is decreased, or completely eradicated, with inhibition of T4/T5 during solid dark bar tracking, in a similar fashion to seeing a reduction in smooth fixational optomotor gain in T4/T5-Kir2.1 flies. Two interesting future experiments to better understand T4/T5’s role in smooth steering response
modulation would involve optogenetic activation of T4/T5 neurons using the red-light activated channelrhodopsin Chrimson (Klapoetke et al. 2014). Chrimson activation in T4/T5 could be paired with wide-field motion studies to test tracking gain before and after optogenetic stimulation of T4/T5-Chrimson to see if a lower tracking gain is present when Chrimson is not activated. In addition, Chrimson activation in T4/T5 could be paired with solid bar tracking to test whether optogenetic stimulation of T4/T5-Chrimson increases the length and duration of the smooth optomotor steering response component seen between tracking saccades during dark bar tracking bouts. Both of these experiments would solidify T4/T5’s role in the neural circuitry responsible for smooth optomotor steering.

It is also known that T4/T5 neurons contribute specifically to a well-defined co-directional optomotor steering responses elicited by bar motion in tethered flight, however, the mechanism behind fourier bar driven counter-directional responses remains unknown (Keleş et al., 2019). It has been suggested by Keleş et al., in review, that T2/T3 neurons and their postsynaptic targets seem to be well suited for feature detection. And if the co-directional optomotor component of bar tracking is strongly driven by T4/T5 motion detectors (Keleş et al., 2019), then T2/T3 cells serve as suitable candidates underlying the mechanism for counter-directional orientation responses seen during fourier bar tracking. A suitable experiment to test this hypothesis involves a T2/T3-Kir2.1 inhibition experiment that seeks to address whether Kir2.1 expression impacts the initial co-directional steering response seen in fourier bar tracking. If the results show T2/T3-Kir2.1 has a statistically significant effect on the initial co-directional steering response, such as inhibition of T2/T3 causes a dramatic decrease in the strength of a co-directional steering response, then this could explain the striking results in our performance index analysis of bar tracking behavior of T2/T3-Kir2.1 (Figure 4C, D and Figure 5B, D, F, H).
Specifically, the initial co-directional steering response might be necessary for the target acquisition mechanism required for recognizing and initiating saccadic tracking bouts in response to a fourier bar. Such experiments are essential to furthering our understanding of T2 and T3 neurons and the mechanisms contributing to target acquisition.
FIGURES
**Figure 1:** (A) Highlights the T4 (ON), T5 (OFF), and T2/T3 upstream pathways leading up to the central brain. (B) Diagram of arena used to study wide-field tracking and bar tracking with the fly free to rotate in yaw. The fly is suspended within a magnetic field and illuminated from below with infrared lights. Visual display is made up of random “on” and “off” pixel columns.
**Figure 2**: Display is rotating at 112.5°/s, fly heading is designated as a blue trace, saccade start and end points are designated as green and red lines, respectively. (A) Raw trace of a control fly tracking the movement of a panorama across the visual display. (B) Raw trace of a control fly tracking the movement of a rotating bar across the visual display with tracking bouts expressed as a black trace overlapping the fly heading in blue. (C) Inset from figure (B) to highlight classification of tracking bouts, shape of saccades, and start and end points of a saccade.
Figure 3: Display is rotating at 112.5°/s, fly heading is designated as a blue trace, saccade start and end points are designated as green and red lines, respectively. Tracking bouts are expressed as a black trace overlapping the fly heading. (A) Unwrapped fly heading with position of rotating bar relative to the heading of the fly (PI = ~1). (B) Unwrapped fly heading with position of rotating bar relative to the heading of the fly (PI = ~0.8). (C) Wrapped fly heading with position of rotating bar relative to the heading of the fly (PI = 0).
**Figure 4:** Paired dotplots of wide-field drum and bar tracking events. Drum and bar for all experiments were rotated at at 112.5°/s. Qualitative analysis of wide-field utilized gain calculations. Qualitative analysis of bar tracking utilized performance index calculations. Red dot and line represents a trial with a non-zero drum gain and a zero performance index. Black dot and line represents a trial with a non-zero drum gain and a non-zero performance index. X-axis represents two experimental conditions (drum or bar), left y-axis represents drum gain, right y-axis represents bar performance index. Success rate describes the proportion of trials where a fly had a non-zero drum gain and a non-zero performance index. (A) Control paired drum and bar analysis. (B) T4/T5-Kir2.1 paired drum and bar analysis. (C) T2-Kir2.1 paired drum and bar analysis. (D) T3-Kir2.1 paired drum and bar analysis.
Relative Frequency Histograms

A. Control Drum

B. Control FB

C. T4/T5-Kir2.1 Drum

D. T4/T5-Kir2.1 FB

E. T2-Kir2.1 Drum

F. T2-Kir2.1 FB

G. T3-Kir2.1 Drum

H. T3-Kir2.1 FB

Legend:
- Red: Control Median
**Figure 5:** Relative frequency histograms of data represented in Figure 4. Control gain median (0.9737) is represented in plots A, C, E, G and performance index control median (0.1712) is represented in plots B, D, F, H. PI=0 is denoted as the first bin in plots B, D, F, H; rest of bins represent PI>0. (A) Relative frequency histogram of control gain. (B) Relative frequency histogram of control PI. (C) Relative frequency histogram of T4/T5-Kir2.1 gain. (D) Relative frequency histogram of T4/T5-Kir2.1 PI. (E) Relative frequency histogram of T2-Kir2.1 gain. (F) Relative frequency histogram of T2-Kir2.1 PI. (G) Relative frequency histogram of T3-Kir2.1 gain. (H) Relative frequency histogram of T3-Kir2.1 PI.
Figure 6: (A) The average amount of saccades performed per revolution during bar tracking trials with a PI>0. (B) The average duration of a saccade tracking bout (in seconds) during bar tracking trials with a PI>0. (C) The average total displacement (in revolutions) of a saccade tracking bout during bar tracking trials with a PI>0.
Figure 7: Tracking saccades dynamics for bar tracking trials with a PI>0 and spontaneous saccade dynamics are represented in plots A, B, and C. X-axis represents saccade dynamic, y-axis represents the sample of saccades (control vs. experimental and tracking vs. spontaneous). Control = C, T2-Kir2.1 = T2, T3-Kir2.1 = T3, T4/T5-Kir2.1 = T4/T5; tracking saccades = Tr, spontaneous saccades = Sp. Red “+” superimposed over each plot represents the sample median. (A) Plots the sample of tracking and spontaneous saccade amplitudes (in degrees for tracking and spontaneous saccades for control, T2-Kir2.1, T3-Kir2.1, and T4/T5-Kir2.1. (B) Plots the sample of tracking and spontaneous saccade duration (in milliseconds) for tracking and spontaneous saccades for control, T2-Kir2.1, T3-Kir2.1, and T4/T5-Kir2.1. (D) Plots the sample of tracking and spontaneous saccade peak angular velocity (in degrees/second) for tracking and spontaneous saccades for control, T2-Kir2.1, T3-Kir2.1, and T4/T5-Kir2.1.
**Figure 8:** (A) Raw trace of a control fly tracking the movement of a rotating bar across the visual display with tracking bouts expressed as a black trace overlapping the fly heading in blue. Saccade start and end points are designated as green and red lines, respectively. Position of bar relative to fly is represented as an orange line. Bar is rotating at 112.5°/s. (B) Top: fly heading (blue), bar position (black), and error angle (red) between the fly and the bar during bar fixation. Bottom: filtered velocity data. The horizontal dashed line indicates the threshold for detecting saccades; vertical lines indicate identified saccades. For plots B and C, control = C, T2-Kir2.1 = T2, T3-Kir2.1 = T3, T4/T5-Kir2.1 = T4/T5. (C) Baseline subtracted integrated error of tracking saccade bouts for bar tracking trials with a PI>0. X-axis represents the sample of saccades (control vs. experimental) and Y-axis represents integrated error (in degrees*seconds). (D) Intersaccade interval of tracking saccade bouts for bar tracking trials with a PI>0. X-axis represents the sample of saccades (control vs. experimental) and Y-axis represents intersaccade interval (in milliseconds).


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