Title:

Rest is Required to Learn an Appetitively-Reinforced Operant Task in *Drosophila*

Abbreviated Title:

Operant Conditioning of Food Reward Requires Rest

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The authors declare no competing financial interests.

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ABSTRACT

Maladaptive operant conditioning contributes to development of neuropsychiatric disorders. Candidate genes have been identified that contribute to this maladaptive plasticity, but the neural basis of operant conditioning in genetic model organisms remains poorly understood. The fruit fly *Drosophila melanogaster* is a versatile genetic model organism that readily forms operant associations with punishment stimuli. However, operant conditioning with a food reward has not been demonstrated in flies, limiting the types of neural circuits that can be studied. Here we present the first sucrose-reinforced operant conditioning paradigm for flies. Flies of both sexes walk along a Y-shaped track with reward locations at the terminus of each hallway. When flies turn in the reinforced direction at the center of the track, sucrose is presented at the end of the hallway. Only flies that rest during training show evidence of learning the reward contingency. Flies rewarded independently of their behavior do not form a learned association but have the same amount of rest as trained flies, showing that rest is not driven by learning. Optogenetically-induced rest does not promote learning, indicating that rest is not sufficient for learning the operant task. We validated the sensitivity of this assay to detect the effect of genetic manipulations by testing the classic learning mutant *dunce. Dunce* flies are learning impaired in the Y-Track task, indicating a likely role for cAMP in the operant coincidence detector. This novel training paradigm will provide valuable insight into the molecular mechanisms of disease and the link between sleep and learning.

SIGNIFICANCE STATEMENT

Operant conditioning and mental health are deeply intertwined: maladaptive conditioning contributes to many pathologies, while therapeutic operant conditioning is a frequently used tool in talk therapy. Unlike drug interventions which target molecules or mechanisms, it is not known how operant conditioning changes the brain to promote wellness or distress. To gain mechanistic insight into how this form of learning works, we developed a novel operant training task for the fruit fly *Drosophila melanogaster*. We made three key discoveries. First, flies are able to learn an operant task to find food reward. Second, rest during training is necessary for learning. Third, the *dunce* gene is necessary for both classical and operant conditioning in flies, indicating that they may share molecular mechanisms.

INTRODUCTION

Learning is a broadly conserved, highly regulated, and health relevant function of the nervous system. Learning updates the frequency of behaviors to reflect stimulus predictability in an animal's environment. The associative forms of learning transfer the value of an innately valued stimulus (an unconditioned stimulus or US) to an associated predictor, either a behavior or cue (Fanselow and Wassum, 2015). US association with internally-generated behavior (e.g. locomotion, static posture, lever press) produces "operant conditioning" across a wide range of animal species (Skinner, 1948; Kimble et al., 1955; Susswein et al., 1986). Operant conditioning allows the animal to modify its behavior to increase the likelihood of obtaining rewarding stimuli and decrease the likelihood of encountering aversive stimuli.

Operant conditioning to reward or relief from punishment incorporates a positive feedback loop – learning increases the generation of the behavior, which in turn increases reward frequency, which strengthens the learned association. This type of positive feedback loop is hypothesized to contribute to diverse neuropsychiatric disorders including childhood anxiety, compulsive behaviors, and chronic pain (Ollendick et al., 2001; Korff and Harvey, 2006; Chóliz, 2010; Gatzounis et al., 2012). Genome-wide association studies have identified candidate genes that increase susceptibility to these operant conditioning-associated disorders (Smith et al., 2016; Levey et al., 2020; Smit et al., 2020). However, despite the relevance to human health, the neural basis of operant conditioning in genetic model organisms remains incompletely understood. It is not currently possible to trace a neural circuit of operant conditioning in animals more complex than *Aplysia californica* (Nargeot and Simmers,

2011), nor has there been a genetic screen for molecular components of operant learning in model organisms. A promising system to address this gap in knowledge is the fruit fly *Drosophila melanogaster*. Much of the known molecular machinery underlying learning and memory was first discovered using genetics in the fly and these molecules have subsequently been shown to be essentially identical in humans (Greenspan and Dierick, 2004). Furthermore, a draft map of the neural connections in a fruit fly hemi-brain has been recently published which, along with advanced genetic tools, greatly facilitates mapping complex neural circuits (Pfeiffer et al., 2010; Xu et al., 2020).

Operant conditioning has been studied extensively in flies, but only limited progress has been made in understanding circuit-level mechanisms. There have been many operant conditioning paradigms reported in flies: geotaxis training (Murphey, 1967), leg position conditioning (Booker and Quinn, 1981), proboscis extension suppression (DeJianne et al., 1985), flight simulator heat avoidance (Wolf and Heisenberg, 1991), conditioned place preference (Wustmann et al., 1996), social freezing (Kamyshev et al., 1997), and left-right navigation in tethered ball-walking (Nuwal et al., 2012). However, a pair of landmark publications (Brembs and Plendl, 2008; Brembs, 2009) demonstrated that when predictive sensory cues are available, flies preferentially learn these sensory cues and block the formation of operant conditioning. This finding dramatically compromises a number of paradigms that claim to test operant learning in flies, since sensory information present during training may have inhibited operant learning. The remaining purely operant learning paradigms that are routinely used in flies, flight simulator heat avoidance and proboscis extension

suppression, have two important limitations. First, they use restrained fly preparations, which unavoidably alter animal behavior (Stowers et al., 2017). Second, they use an aversive US which may not recruit the full repertoire of US pathway neurons (Liu et al., 2012) and may use neurons outside the brain for learning (Booker and Quinn, 1981).

In order to extend the range of operant conditioning paradigms in flies, we developed a positively reinforced, self-paced, operant training task for untethered flies, which we call the Y-Track. Surprisingly, we found that this operant training paradigm only produces a change in behavioral frequency in the subset of experimental animals that rest during training. This surprising finding further reinforces the importance of rest for learning (Maquet, 2001) and opens a new avenue for measuring this link in a single-session paradigm.

MATERIALS AND METHODS

Experimental Animals

Flies were raised on cornmeal-dextrose-yeast food in bottles at room temperature or in a 25 C incubator with a 12 hour:12 hour light:dark cycle. Wild type flies were from the Canton-Special (CS) background. Transgenic flies were obtained from the Bloomington Drosophila Stock Center (BDSC) and Vienna Drosophila Resource Center (VRDC) as follows: *P{VT058968-GAL4}attP2* (VT058968-GAL4, VDRC# 204550), *P{w[+mW.hs]=GawB}104y* (104y-Gal4, BDSC# 81014), *PBac{y[+mDint2] w[+mC]=UAS-ChR2.XXL}VK00018* (UAS-ChR2.XXL, BDSC# 58374), and dnc^{1} (BDSC# 6020). Flies with Gal4 and UAS transgene insertions were

outcrossed to a CS background for several generations because we found that *white* knock-out backgrounds may be learning deficient in this task (*data not shown*).

Design of the Y-Track Apparatus

The Y-Track conditioning apparatus was designed as a 4 layered structure. The first (top) layer of the structure was a 3D printed holder for a USB camera (ELP-USBFHD01M) and 3.6mm S-mount lens facing downward toward the track. The second layer was a mount for a red filter (Tiffen #25 Red) to block light blue and green light from optogenetic activation and light landmark experiments. These top layers are supported by four 3D pillars on each side of the apparatus. Red LEDs (630nm, Vishay VLDS1235G) were attached to each pillar and illuminated the Y-Track area. The third layer of the apparatus was the Y-Track itself. Two versions of the Y-Track were tested: a square-walled track and a curved floor track. In the square-walled track, the width of the hallways was 3.5 mm and the height of the hallways was 2.5 mm. In the curved floor track, the track surface was described by a circular arc with a diameter of 9 mm. The width of the top of the hallways was 6.7 mm and the height at the middle of the hallways was 1.5 mm. In both tracks, each of the three hallways that made up the Y shape was 20mm long, the hallways met in the middle of the track, and the hallways had 120° radial spacing. At the end of each arm of the maze was a circular plastic holder for reward filter paper (“food circle”) securely screwed to a servomotor (Towerpro MG91). Each food circle had two filter paper slots, one for a sucrose-soaked filter paper and the other for a water-soaked filter paper. The top of the Y-Track was covered by a clear acrylic plate with a small hole for aspirating flies, machined by the Brandeis University

Machine Shop. A small 3D frame with 3 RBG LEDs (Broadcom HSMF-C114) was superglued below the Y-Track to deliver optogenetic stimulation and landmark location cues. The fourth (bottom) layer of the Y-Track apparatus is a frame that positions the servomotors correctly relative to the Y-Track and secures the entire apparatus to the base. Modelling of the 3D printed components was done using Autodesk Fusion 360 (San Rafael, CA). These components were fabricated from Polylactic Acid (PLA) filament in the Brandeis MakerLab.

This apparatus was controlled by a custom Java program running on an Udoo X86 Advanced Plus single-board computer. The JavaGrinders library was used to interface with the camera and servomotors (Donelson et al., 2012). Servomotors were controlled via a Phidget Advanced Servo (Phidgets, Calgary, CA). Red LEDs illuminating the Y-Track were powered by a BuckPuck (LuxDrive, 03021-D-E-700). The Y-Track apparatus and electronics were securely mounted inside a custom-built particle board box to provide environmental isolation. The internal walls of the box were painted white to reduce visual cues and a 120mm low-noise ventilation fan was installed to prevent overheating. Each Y-Track single-board computer was connected to a central control computer and controlled remotely via Virtual Network Computing (VNC). Code and 3D models are available on GitHub (https://github.com/Griffith-Lab).

Learning Assay

Flies were collected 0-1 days post-eclosion (dpe) and housed in mixed-sex vials for 24 hours to allow mating. The flies were then screened under light CO_2 anesthesia and stored in single-sex vials of up to 20 flies each. Flies were housed in a 25 C

187 incubator that was only accessed during the lights-on period for 7 days prior to the
188 experiment to ensure circadian entrainment. Each vial of flies was flipped onto fresh
189 food at 5 dpe (48 hours before training), flipped onto a food-deprivation vial at 6 dpe (24
190 hours before training), and trained in the Y-Track at 7 dpe. Food-deprivation vials were
191 made by inserting a kimwipe soaked with 1mL of tap water into an empty vial.

192 Prior to introducing the flies into the Y-Track, filter paper was prepared for the
193 food circles by pipetting 30μL of 2M sucrose solution (reward stimulus) or tap water
194 (neutral stimulus) and allowing the paper to dry overnight. The dried filter papers were
195 securely placed into the food circles, and the positioning of the servomotors was
196 adjusted to ensure that the flies could access only the intended stimulus and were not
197 able to escape. Following the final positioning of the motors, a reference image of the Y-
198 Track without a fly present was captured for background subtraction during the
199 experiment. Finally, the reward direction for the experiment and the sex of the
200 experimental animal was chosen based on the experimental design. In the square-wall
201 experiment, half of the flies were rewarded for turning right and the other half were
202 rewarded for turning left. We found no difference between training efficacy between the
203 reward directions, so subsequent experiments used left-turn rewards for all animals. In
204 the square-wall experiment, half of the animals were male and half were female. We did
205 not find a significant difference between male and female flies in this experiment, but we
206 did note that males died more rapidly during food deprivation. To reduce variability in
207 food deprivation, subsequent experiments used only female flies. Training was
208 performed during the lights-on period of the fly's circadian day, Zeitgeber time 0-9.

At the beginning of a standard training session, a single fly was aspirated out of the food deprivation vial into a Y-Track apparatus, and the lid of the track was secured in place to prevent escape. The Java control program was initialized to run the remainder of the experimental protocol, as follows: 1) The fly was given five minutes to acclimate to the maze with no sucrose presented. This acclimation time was a fixed interval and not dependent upon fly locomotion. 2) Block 0 began (Trials 1-20) and left/right turn decisions were recorded. No sucrose was presented. Block 0 was a locomotion-dependent acclimation period to ensure the fly is navigating the track. 3) Block 1 began (Trial 21). All servomotors turn to present the sucrose-soaked filter paper to the fly. A trial was initiated when the fly came within 6 mm of the center of the arena ("center zone"). If the fly back-tracked into the arm of arena it previously occupied, the servomotor turned and presented the water-soaked filter paper until the fly re-entered the center zone, but the trial continued. If the fly turned in the unrewarded direction, the servomotor turned and presented the water-soaked filter paper, ending the trial. If the fly turned in the rewarded direction, the servomotor did not turn, and the fly was given access to the sucrose-soaked filter paper, ending the trial. After a rewarded trial, the fly was given 10 seconds to consume sucrose. If the fly did not initiate a new trial by entering the center zone within 10 seconds, the servomotor turned and presented the water-soaked filter paper until the fly initiated a new trial. 4) After Trial 160, the fly was removed from the Y-Track.

In the open-loop, yoked-control experiment (Fig. 3), acclimation and trials were defined exactly as in the standard protocol. However, instead of trials being rewarded or unrewarded based on turn direction, trial outcome was determined by the reward/non-

reward sequence of a previously run fly. In the visual land-mark experiment (Fig. 4), the training protocol was the same as the standard experiment but a single green LED (525 nm peak) was illuminated under the Y-Track. In optogenetics experiments, flies were fed food supplemented with either 1.6 mM all trans retinal (ATR) dissolved in ethanol (4% final concentration), or ethanol alone as a Vehicle control. Food deprivation vials were also supplemented with ATR or Vehicle in the same concentration as the food. During ATR supplementation flies were housed in the dark to prevent premature activation of ChR2.XXL expressing neurons. During training, blue LEDs (470 nm peak) were illuminated for 5 minutes after the initiation of Trial 50 (Block 2).

Quantification of Activity and Rest

During training, the frame-by-frame coordinates of each fly, trial times, and trial outcomes were recorded. Coordinates were processed following training to remove incorrect detections, which were identified by fly coordinates outside the Y-Track region or a change in position faster than a fly could plausibly execute (Mendes et al., 2013). Gaps in data introduced by this error checking were filled by a linear interpolation of fly position. The position of the fly over time was used to determine when the fly was active: activity episodes were continuous periods of movement greater than 1 px / frame, in which the fly also exceeded 2 px / frame at least once. The activity/inactivity sequence was used to find rest episodes, which were then used to classify flies as drowsy, restless, or late-resting. Finally, the error corrected sequence of left/right turn directions was compared to the real-time turn direction determined during training. If more than 10% of the trial outcomes differed between the real-time and post-hoc

methods, the data from the fly was excluded from further analysis. Analysis code implementing this process is available on GitHub (https://github.com/Griffith-Lab).

Experimental Design and Statistical Analysis

Experiments were designed with change in Preference Index (ΔPI) as the primary measure of learning. Preference Index (PI) was defined as the preference for the rewarded turn direction and equal to (#correct turns - #incorrect turns)/#total turns. ΔPI was defined as the difference between the PI in Block 8 vs. Block 2. The one-sample Kolmogorov-Smirnov test was used to test for normality in our ΔPI data. A two-sample unpaired t-test was used to compare the drowsy and restless ΔPI groups. For the no-sucrose experiment in which there were not enough drowsy flies, a one-sample t-test was used to test for a significance difference from zero. Comparisons of behavior between flies in square-walled and curved-floor Y-Tracks were performed using Two-Factor ANOVAs and Tukey-procedure protected *post-hoc* tests. Within figures, groups that are not statistically different are identified by the same letter assignment. Coordinate data and statistics were calculated using MATLAB (MathWorks, Natick, MA) and 0.05 was used as the p-value for statistical significance.

RESULTS

Flies That Rest Learn the Operant Contingency in a Novel Sucrose-seeking Task

We used an ethology-informed approach to design a positive-valence operant conditioning paradigm. Flies locomote spontaneously while awake (Martin et al., 1999), forage for food in open fields (Hughson et al., 2018), and are adept at navigational tasks

(Warren et al., 2019). We therefore used food-seeking and navigation as the central features of the learning paradigm (Fig. 1A). Flies are individually loaded into a Y-shaped track (Y-Track). At the terminus of each arm of the track is a reward location that can be switched between a food reward and a neutral stimulus. Food reward is only available when the flies turn in the in the rewarded direction (*i.e.* left or right) relative to their previous location in the track. Because the rewarded choice is defined relative to the location of the animal, the location of the next rewarded location changes based on the previous behavioral choice and no single arm of the track is preferentially rewarded. Over many left/right choices ("trials"), the turn preference index (PI) is calculated for blocks of 20 trials as PI = (# correct turns – # incorrect turns) / # total turns. Because baseline left/right turn preference is idiosyncratic to individual flies (Buchanan et al., 2015), learning is measured as the change in PI across training to determine if the flies increase their preference for turning in the direction of food reward.

Implementing this task in a physical apparatus required satisfying several design constraints (Fig. 1B,C). First, the animal must be alert, healthy, and active to engage in spontaneous locomotion and learning. We included a loading port in a tightly fitted Y-Track lid that allowed us to load and remove flies without anesthesia using gentle aspiration. Second, the apparatus must include a detector element that records the performance of the reinforced behavior in real time. We used JavaGrinders real-time video tracking to measure locomotor behavior and turn choices (Donelson et al., 2012). Third, the apparatus must be able to actuate reward delivery based on the behavioral contingency. We used closed-loop control to allow the real-time tracker to activate servomotors at the terminus of each arm of the Y-Track and present either 10 seconds

of access to a food reward (filter paper pre-soaked with 2M sucrose) or a neutral stimulus (plain filter paper). Importantly, the servomotors turn to present sucrose at both termini while the fly is in the center of the Y-Track. The flies are not able to determine which arm is rewarded simply by smelling or seeing reward. In trials where the fly turns in the non-reinforced direction, the servomotor is actuated rapidly enough that the fly is never able to actually obtain food.

In order to validate the sensitivity of the real-time tracking, we compared long-term recordings of living flies to dead flies (*i.e.* flies that have no genuine locomotion; *n* = 2 per group; Fig. 1C). We found that the tracked position of the dead flies was contained within a radius of 1 pixel over several hours. Locomotor episodes were therefore defined as continuous sequences of frames in which the fly moved at least one pixel, with the requirement that the fly must exceed a speed of 2 pixels/frame (0.38 mm/s) for at least one frame. Flies frequently paused between locomotor episodes, sometimes for extended periods of time. We defined pauses of greater than 1 minute as "rest" (Fig. 1D).

Throughout the prototyping process, we evaluated the effectiveness of our apparatus in shaping wild type (WT) fly behavior. In pilot experiments (*n* = 15, WT flies, mixed sex), we found a small training effect of making sucrose available contingent upon turn direction in the center of the Y-Track. Interestingly, change in turn direction preference was correlated with time spent resting during training (Pearson's *R* = 0.50). In order to rigorously test the hypothesis that rest is correlated with learning in the Y-Track, we trained a large cohort of flies (*n* = 85 female, 87 male; Fig. 1F-I). Of this cohort, 11 (7%) rested early in training (3 minutes or more in Block #1-3). An additional

16 (9%) had high rest late in training (9 minutes or more in Block #4-6) and were excluded because WT flies reduce food seeking behavior during high sleep times (Donelson et al., 2012). Flies that did not rest showed no increase in turn preference in the direction of reward, indicating that they did not learn the task. However, consistent with our pilot results, flies with early rest had a significantly increased likelihood of turning in the direction of reward compared with flies that had low rest (Two-sample T-test; $t(148) = 2.1$; $p = 0.035$; Fig. 1G-I). These results indicate that WT flies learn a sucrose-rewarded operant contingency only when they rest in the first half of the training trials. Because of the behavioral importance of these rest-defined groups, we will refer to flies that rest early in training as “drowsy” flies, and flies with low rest as “restless” flies.

Y-Track Geometry Significantly Affects Thigmotaxis and Spontaneous Alternation

Operant conditioning paradigms designed for flies can be confounded by sensory cues; when presented with both an operant contingency and a classical prediction cue, flies preferentially attend to the classical cue (Brembs and Plendl, 2008). No classical cues were intentionally introduced into the Y-Track, but an examination of locomotor behavior of the in the apparatus revealed strong thigmotaxis behavior (Fig. 2A). This is consistent with the behavior of flies in open-arenas (Simon and Dickinson, 2010), but it is potentially problematic for the Y-Track task for three reasons. First, if flies maintain contact with the wall through the vertex of the Y-Track, turn direction is correlated with a unilateral touch stimulus, which may act as a classical predictor. Second, it is unclear where the “choice point” for choosing a turn direction is located – presumably at

whatever track location the flies "attach" to one of walls. Third, thigmotaxis may contribute to spontaneous alternation (Lewis et al., 2017), another behavior typical of unmanipulated WT flies. Spontaneous alternation would not independently result in flies preferring the rewarded turn direction, but, in simulated behavior, alternation magnifies small turn biases into large turn preference indices (Fig. 2C). If the effect of early rest is to modulate spontaneous alternation, it may be that restless and drowsy flies have the same mild change in "true" turn bias, and the difference in turn preference index is due to changes in alternation.

To address the sensory-motor confounds of thigmotaxis, we designed a second iteration of the Y-Track with gently curved floor, similar to open-field arenas (Simon and Dickinson, 2010). Thigmotaxis was dramatically reduced in the curved floor track compared to the square wall track in a heat map of location preference, including as the flies pass through the vertex of the track (n = 103 WT female; Fig. 2B). Median distance from the wall is significantly smaller in the square-wall design than it is in the curved floor design for both drowsy and restless flies (Two-way ANOVA; $F(1,1) = 2788$; $p < 0.0001$; Fig. 2D). There was no difference in thigmotaxis between drowsy and restless flies within experiments (Post-hoc test; all $p > 0.17$).

To quantify any link between thigmotaxis, spontaneous alternation, and revealed turn preference, we measured spontaneous alternation at the end of training for each of our groups of WT flies. Track geometry significantly affected alternation rate (Two-way ANOVA; $F(1,1) = 11$; $p = 0.001$; Fig. 2E) and drowsy flies have significantly lower alternation than restless flies (Post-hoc test; all $p < 0.005$). We therefore concluded that

while spontaneous alternation may be related to thigmotaxis, it changes in the wrong direction to contribute to the difference in learning between restless and drowsy flies.

Learning to Turn Toward Sucrose is Independent of Track Geometry but Dependent upon an Informative Operant Contingency

The curved floor track geometry dramatically reduces thigmotaxis (Fig. 2), so we repeated Y-Track conditioning in curved floor apparatus to determine if the sensory-motor experience of thigmotaxis contributes to Y-Track learning (Fig. 3A). Learning in the curved floor track (n = 103 WT female flies) was similar to learning in the square wall track. The percentage of drowsy flies was not different between the square wall and curved floor tracks (Fisher's Exact Test; p = 0.8). Drowsy flies in the curved floor track (8 of 103) significantly increased their likelihood of turning toward reward compared to restless flies (Two-sample T-test; t(100) = 2.5; p = 0.015; Fig. 3B-D).

In order to control for any confounding effect of time spent in the experimental apparatus on turn direction preference, we also performed an open-loop, "yoked" control in the curved floor track (n = 110 WT female flies). In the yoked control flies, the reward sequence of a previously trained fly was presented to a naïve fly independent of the turning behaviors of the naïve fly. The yoked control flies therefore had the same amount of sucrose/reward access as trained flies, but there was no behavioral contingency. As predicted, drowsy yoked control flies (8 of 110) did not show a significant change in turn direction compared with restless flies (Two-sample T-test; t(107) = 0.03; p = 0.98; Fig. 3E-H). These results allow us to conclude that the change in turn preference is due to learning of the operant contingency, and not dependent

upon sensory-motor feedback from thigmotaxis. The curved floor Y-Track was used for all subsequent experiments because learning is equivalent to the square wall track, without the potential confounding effect of thigmotaxis.

A World Orientation Cue does not Facilitate Learning the Operant Task

The curved floor Y-Track experiment (Fig. 3) removes the potential for an egocentric sensory-motor classical confound. We next considered the possibility that the flies were attending to an inadvertently introduced world-orientation cue rather than the operant contingency. In order to test this hypothesis, we trained flies in the presence of a strong orientation cue (n = 81 WT female flies). A green LED was illuminated beneath one arm of the Y-Track, creating a stable, mildly attractive, landmark (Fig. 4A). In the presence of this landmark, drowsy flies did not show a significant change in turn direction compared with restless flies (Two-sample T-Test; t(73) = 0.17; p = 0.86; Fig. 4B-D). This result indicates that the presence of a world orientation cue does not enhance learned change in turn preference. Together, the results of the thigmotaxis, alternation, and orientation cue experiments indicate that neither sensory inputs nor motor patterns explain the change in turn preference in the direction of reward observed in drowsy flies. We conclude that the learning produced in this paradigm is navigational and operant in character.

Sucrose Promotes Rest and is the Only US Attended to by Flies in the Y-Track

Operant conditioning is a learned association between behavior and a US, and the strength of the US influences the strength of the learned association (Rickard et al.,

2009). Rest is strongly regulated by feeding and nutritional state (Murphy et al., 2016), and the consumption and hedonic value of sugar is dependent upon the state of the fly (Krashes et al., 2009; Li et al., 2020). While we have shown that there is a correlation between early rest and learning, it is not clear how they are connected mechanistically. One possibility is that there may be a difference in the consumption of, or response to, the sucrose US that explains enhanced learning in drowsy flies. In order to test the hypothesis that drowsy flies receive a more rewarding US (either due to consumption quantity or reward value), we analyzed the relationship between time spent adjacent to sucrose and learning (Fig. 5A). We found that there is a significant main effect of early rest on time adjacent to sucrose (Two-way ANOVA; both $F(1,1) > 4.5$; both $p < 0.035$), but the difference between drowsy and restless flies is only significant at the group level in the flies trained in the square walled Y-Track (post-hoc test; $p = 0.016$). However, there was no correlation between time spent adjacent to sucrose and change in turn preference (Pearson correlation; Square Wall $R = 0.12$, Curved Floor $R = -0.01$; Fig. 5B). Increased sucrose consumption is associated with additional rest, but it is not associated with increased learning.

To validate the finding that sucrose consumption promotes rest, we trained flies in a Y-Track with no sucrose available ($n = 78$ WT female flies). Remarkably, only 2 flies in this cohort had early rest, a 3-fold reduction in drowsy flies compared to training with sucrose available (Fig. 5D). The restless flies in the no-sucrose experiment did not have a significant change in turn preference following training (One-sample T-test; $t(75) = 1.2$; $p = 0.25$; Fig. 5E-F). Together, increased time adjacent to sucrose in drowsy flies and the reduction in the number of drowsy flies when sucrose is removed from the Y-Track

438 indicate that sugar consumption promotes rest. The lack of residual learning in the Y-
439 Track when sucrose is removed also shows that sucrose is the learning-relevant US.
440
441 *Optogenetically-Induced Sleep is Not Sufficient to Enhance Learning*
442 Activation of several genetically-targetable cell types in flies is sufficient to induce
443 sleep (Donlea et al., 2011; Liu et al., 2016). Activation of these cells has the same effect
444 on sleep-dependent learning as spontaneous or pharmacologically-induced sleep
445 (Donlea et al., 2011; Dissel et al., 2015). We therefore tested the hypothesis that sleep
446 is sufficient to enhance learning of turn direction in the Y-Track by optogenetically
447 activating sleep-promoting neurons. Flies do not synthesize the cofactor of Channel
448 Rhodopsin (ChR), All-Trans Retinal (ATR), so ATR needs to be added to the food to
449 functionalize the channels (Zhang et al., 2006). We tested optogenetic sleep induction
450 using a dorsal Fan-Shaped Body driver (dFSB; 104y-Gal4; *n* = 39 ATR, 45 Vehicle), or
451 an Ellipsoid Body driver (EB; VT058968-Gal4; *n* = 56 ATR, 54 Vehicle) driving
452 expression of ChR2.XXL (Dawydow et al., 2014), and WT control flies (*n* = 43 ATR, 36
453 Vehicle) (Fig. 6A). Sleep was induced by turning on blue LEDs located under the Y-
454 Track for 5 minutes at the mid-point of Training Block 2. Lights were symmetrically
455 located in all arms of the Y-Track, so they did not provide a world orientation cue. Blue
456 light did not increase sleep in WT flies fed ATR or vehicle, but dramatically increased
457 sleep in the dFSB and EB driver flies (Fig. 6B). The drivers differed in both potency and
458 on/off kinetics: the dFSB driver rapidly induces sleep in all ATR fed flies, and flies woke
459 from sleep shortly after the light was turned off. In contrast, the EB driver induced a
460 lower level of sleep that persisted even after the blue light was removed (Fig. 6B). Both

drivers dramatically increased the % flies with early rest (Fig. 6C). Despite this robust rest induction, neither driver was sufficient to increase learning. Turn preference in early resting flies was not significantly different from non-resting flies in the dFSB induction experiment (Two-sample T-Test; t(77) = 0.71; p = 0.48; Fig. 6D,E) or in the EB induction experiment (Two-sample T-Test; t(96) = 0.65; p = 0.52; Fig. 6F,G). The effect of rest induction is not to increase learning, but instead to dilute the drowsy group with non-learning flies, effectively erasing the difference between the restless and drowsy groups. We conclude that while spontaneous early rest is associated with increased learning of the navigational task, rest itself is not sufficient to induce learning.

Y-Track Operant Learning is Dependent on cAMP Regulation

The Y-Track task we have developed is an operant, navigational, sucrose-reinforced learning paradigm, in which learning is revealed by changes in turn preference from baseline (Fig. 1,3). Many neurotransmitters, receptors, and second messengers necessary for classical conditioning in flies have been identified using genetic knockouts (Margulies et al., 2005). While cyclic adenosine monophosphate (cAMP) is important for the formation of classical conditioning (Zars et al., 2000), a previous study found that activity-regulated cAMP synthesis is not necessary for the formation of aversive operant conditioning (Brembs and Plendl, 2008). In order to determine if regulation of cAMP is necessary for formation of appetitive operant conditioning, we tested the performance of flies mutant for the *dunce* phosphodiesterase (*dnc*^{1}). *dnc*^{1} flies fail to learn in classical paradigms and fail to modulate cAMP in response to learning stimuli (Gervasi et al., 2010). In an independent

cohort (*n* = 209 WT female flies), we reproduced our earlier result that flies with early rest (*n* = 19) increase turn preference in the direction of reward (Two-sample T-test; t(200) = 2.1; *p* = 0.036; Fig. 7A-C). Flies carrying the *dnc*^{1} mutation failed to show learning (*n* = 95 female flies): the turn preference of drowsy and restless flies were not significant different (Two-sample t-test; t(88) = 0.95; *p* = 0.34; Fig. 7D-F).

DISCUSSION

The formal study of associative learning has been remarkably successful: experimentally-induced associative memory has been demonstrated across the animal kingdom and dozens of genes, neurotransmitters, second messengers, and neural structures have been implicated in its formation (Mayford et al., 2012). Within the context of fly learning, animals have been trained to texture (Platt et al., 1980), sound (Menda et al., 2011), color (Schnaitmann et al., 2010), location (Wustmann et al., 1996), and odor (Quinn et al., 1974), among other cues. The fly learning literature developed rapidly, with first reports of training paradigms for classical conditioning to an aversive US, operant conditioning to an aversive US, and classical conditioning to a rewarding US occurring within a decade of one another (Quinn et al., 1974; Booker and Quinn, 1981; Tempel et al., 1983). In this report, we present the first paradigm for operant conditioning of flies to a sucrose US: Y-Track conditioning (Fig. 1). The learning we observe is dependent upon rest (Fig. 1,3), does not depend on sensory cues (Fig. 2,4), and requires cAMP as a second messenger (Fig. 7). However, rest is not sufficient for learning as we see no learning enhancement in optogenetically induced rest (Fig. 6), indicating that rest does not indiscriminately promote learning in the Y-Track. Y-Track

training has implications for navigation, learning and memory, and the connection between sleep and learning in *Drosophila*.

Learning, Memory and Rest in Drosophila

Disorders of sleep and associative learning co-occur in several categories of neurological disease. Primary sleep disorders and sleep deprivation result in decreased cognitive and memory performance (Kessler et al., 2011; Shekleton et al., 2014; Zamarian et al., 2015). Conversely, plasticity disorders such as neurodevelopmental disability and post-traumatic stress disorder have co-morbid sleep abnormalities (Angriman et al., 2015; Gilbert et al., 2015). Finally, neurodegenerative disorders, including dementia, Huntington's disease, and Parkinson's disease, frequently disrupt both sleep and memory (Chaudhuri and Naidu, 2008; Morton, 2013; Robbins and Cools, 2014; Porter et al., 2015). The widespread connections between learning and sleep in human neurological disease indicate that there are neuronal circuits linking, or shared by, sleep and learning in humans. Similar to the sleep/learning connection in human disease, sleep and learning regulate one another in flies: sleep deprivation decreases learning (Seugnet et al., 2008) and learning increases time spent asleep (Ganguly-Fitzgerald et al., 2006). Remarkably, increased sleep is sufficient to rescue memory formation in learning mutant flies, aged flies, and in a fly model of Alzheimer's disease (Donlea et al., 2014; Dissel et al., 2015), demonstrating that the shared circuit can be therapeutically useful.

Learning in the Y-Track conditioning task we have developed depends upon rest during training (Fig. 1,3). We have described prolonged locomotor pauses as "rest"

rather than “sleep” because sleep has a precise, three-fold definition (quiescence, increased arousal threshold, homeostasis) and it is not possible to properly evaluate this definition on the Y-Track (Hendricks et al., 2000; Shaw et al., 2000). We hypothesize that the locomotor pauses that we classify as rest are “sleep-like.” In light of the extensive links between sleep and learning in flies, we proposed three hypotheses that could account for the correlation between rest and learning in the Y-Track: First, we hypothesized that learning could drive increased rest. This hypothesis is inconsistent with the similar amounts of rest observed in populations of flies that learn and those that do not (*i.e.* yoked controls and *dunce* mutants; Fig. 3,7). Next, we hypothesized that rest drives increased learning. Sleep is known to promote consolidation of memory both in mammals and flies (Buhry et al., 2011; Donlea et al., 2011). However, we do not find that optogenetically-induced rest promotes learning (Fig. 6). This failure to promote learning could be due to induced rest not promoting the learning-associated sleep state (Liu et al., 2019; Wiggin et al., 2020), or it could also be the case that the precise timing of rest is important to its learning-associated function. We refined this hypothesis to instead propose that rest acts as a gate to learning. In this model, a coincidence between behavior and US must be detected, presumably by a cAMP-dependent mechanism (Fig. 7). Following this coincidence detection event, sleep is required within a tight temporal window in order to consolidate the memory and prevent locomotion-related forgetting (Berry et al., 2015). While this rest-as-a-gate model is consistent with our behavioral characterization, we cannot eliminate the alternative explanation that both rest and learning are driven by a covert common factor, such as a low frequency genetic variant in wild type *Drosophila* (Croze et al., 2017).

553

554 *Neural Circuits of Navigation as a Locus for Operant Plasticity*

555 Identifying plastic neuronal circuits that are responsible for learning is a subject of
556 intense interest in the effort to understand learning, memory, and cognition. In flies, the
557 mushroom bodies are the best studied locus of learning-related plasticity. Learning of
558 sensory cues in flies, including odors and visual cues, is mediated by mushroom body
559 circuits (Aso et al., 2014; Vogt et al., 2014). The identification of the mushroom body as
560 an important learning center proceeded primarily from neuroanatomy, including their
561 connections to sensory projection neurons (Davis, 1993). Because operant conditioning
562 is not primarily a sensory-driven behavior, a sensory-first search for neural circuits is
563 unlikely to uncover the locus of plasticity that underlies operant learning. In fact, operant
564 conditioning in the fly is mushroom body independent (Wolf et al., 1998; Brembs, 2009),
565 while behavioral output circuits in the ventral nerve cord, such as motor neurons, have
566 been implicated instead (Booker and Quinn, 1981; Colomb and Brembs, 2016).
567 However, motor neurons themselves are unlikely to be the location of behavior/US
568 coincidence detection (Talay et al., 2017). Motor planning circuits in the fly central
569 complex, such as those responsible for navigation, are therefore an interesting potential
570 locus for operant plasticity.

571 Control of turn direction on the Y-Track is determined by a mix of innate motor
572 preferences and goal-directed search strategies. Innate handedness is strongly
573 influenced by the activity of PB-FB-No neurons (PFN) (Buchanan et al., 2015). While
574 the synaptic partners of PFN neurons implicated in innate handedness are not yet
575 mapped, the brain structures innervated are all heavily involved in orientation and

576 navigation in the fly (Giraldo et al., 2018; Shiozaki et al., 2020). Fly orientation circuits
577 show rapid plasticity and features of short-term memory (Fisher et al., 2019) and are
578 strongly responsive to visual stimuli (Seelig and Jayaraman, 2015). If fly orientation
579 circuits are part of an operant conditioning pathway, this mix of plasticity and visual
580 responses would account for both behavioral plasticity and our finding that a strong
581 visual stimulus inhibits learning rather than promoting it (Fig. 4).

582 In addition to innate preferences, flies display strong learned place preference
583 and goal directed search behaviors (Ofstad et al., 2011; Kim and Dickinson, 2017).
584 Development of a location preference is capable of overriding innate preferences
585 (Baggett et al., 2018) and foraging flies modify their innate locomotor preferences to
586 repeatedly visit remembered sites of food and search for nearby food sources (Kim and
587 Dickinson, 2017). The formation of spatial memories has been previously linked to
588 cAMP as a coincidence detector (Zars et al., 2000), consistent with our finding that
589 cAMP regulation is necessary for Y-Track conditioning (Fig. 7). Our behavioral results
590 are congruent with either plasticity happening directly in orientation/innate preference
591 circuits, or in a foraging/place preference circuit. Further characterization of the neural
592 components of spatial memory in flies is necessary for these possibilities to be
593 distinguished.

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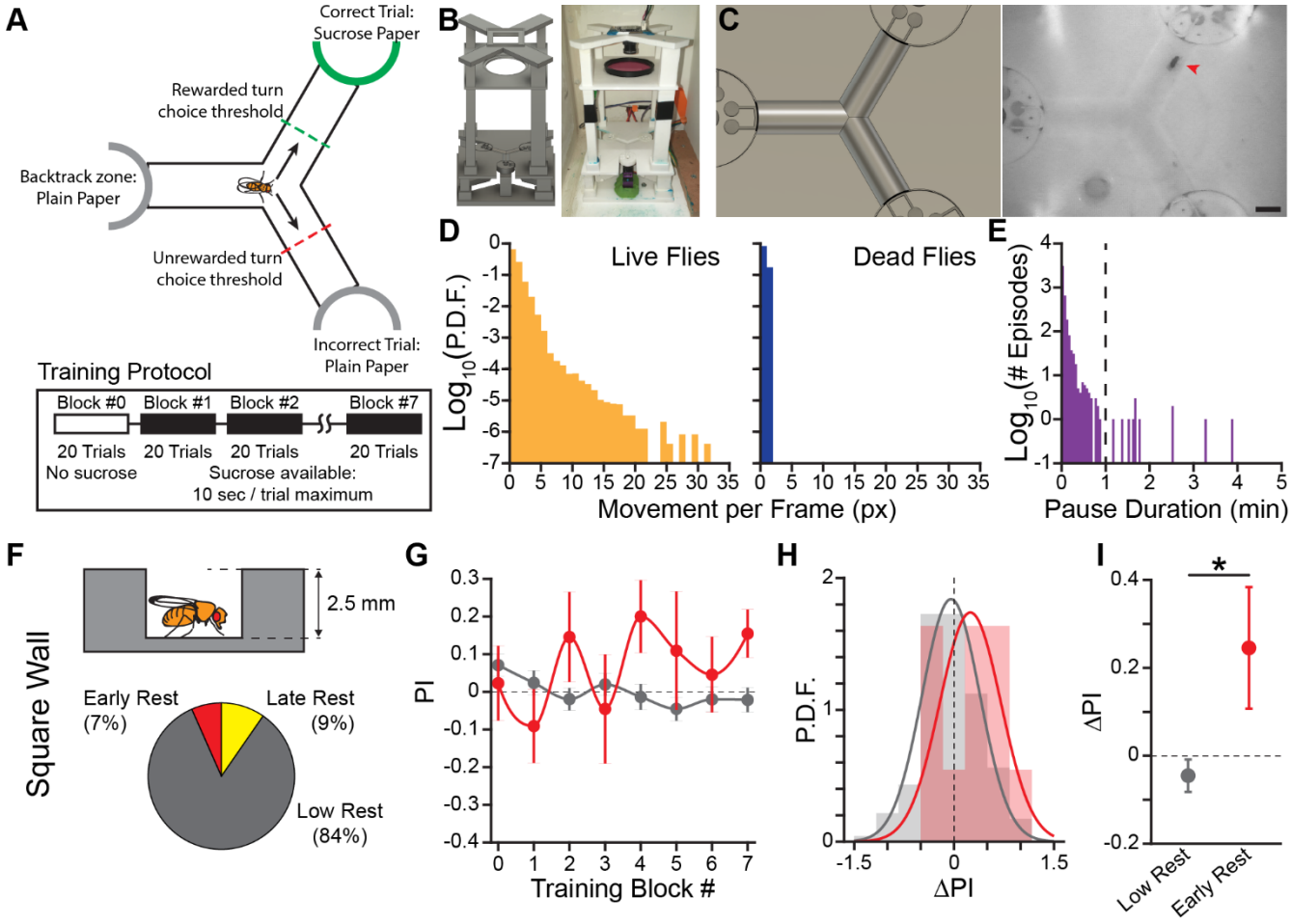

804 **Figure 1 – Flies That Rest Learn the Operant Contingency in a Novel Sucrose**
805 **Seeking Task** (A) Rendering of the 3D model of the novel apparatus used for 3D
806 printing (*Left*) and a photo of a fully assembled apparatus (*Right*). (B) Field of view of
807 overhead camera in the 3D render (*Left*) and a video frame of a fly navigating the Y-
808 Track (*Right*). Red arrowhead indicates the position of the fly. Scale bar: 5 mm. (C)
809 Probability Distribution Function (P.D.F.) histogram of movement per frame (in pixels)
810 for live flies (*Left*) and dead flies (*Right*). One pixel of movement is approximately 90
811 µm. (D) Histogram of locomotor pause durations from 12 hour recordings of locomotor
812 behavior (*n = 2* flies, 4127 episodes). Dashed line indicates the 1 minute threshold to
813 distinguish short pauses from rest episodes. (E) Diagram of operant conditioning

814 paradigm. Sucrose is presented at the end of the track in the reinforced direction. A trial
815 is completed after the fly crosses the choice threshold leaving the center of the track.
816 Each training block is 20 trials, reward presentation begins in training block #1. Sucrose
817 is made available for 10 seconds following the fly crossing the choice threshold. (F)
818 Schematic of hallway geometry with representative fly for scale (*Top*), Fraction of flies
819 with each rest phenotype (*Bottom*). (G) Mean turn direction Preference Index (PI) for
820 each training block. Points plotted in color are flies with early rest (drowsy), points
821 plotted in grey are flies with low rest (restless). (H) Probability Distribution Function
822 (P.D.F.) histograms of change in turn preference index (∆PI) between training block #1
823 and #7. The normal distribution fitted to each distribution is superimposed as a line on
824 the plot. Drowsy flies are plotted in red, restless flies are plotted in grey. (I) Mean ∆PI of
825 low rest (restless) and early rest (drowsy) flies. Error bars are standard error of the
826 mean, * significantly different groups.

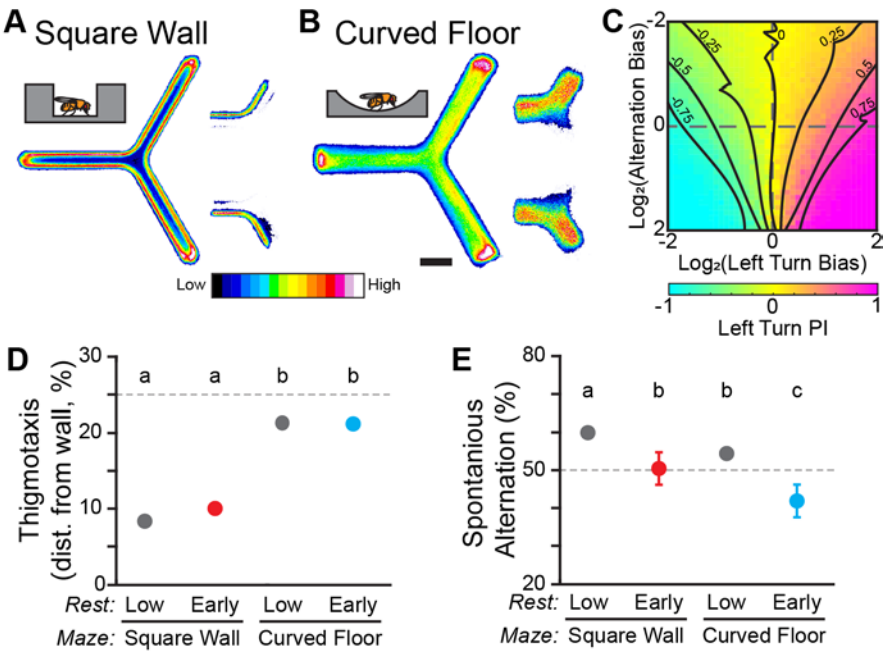

Figure 2 – Y-Track Geometry Significantly Affects Thigmotaxis and Spontaneous Alternation (A-B) Position heatmaps of flies in the square wall (A) and curved floor (B) Y-Mazes. Insets (*right*) show the heatmaps of trajectories through the center of the maze for flies walking from the left zone to the upper zone vs. the lower zone. Scale bar: 5 mm. Color shows relative occupancy of each pixel from low to high. (C) Heatmap of left turn Preference Index (PI) from a population of *in silico* flies. Each heatmap position is the mean of 1000 simulated trials of a fly with a range of left-turn bias and alternation bias. Dashed lines indicate zero bias on an axis. Solid lines are smoothed contours of constant PI. (D,E) Mean thigmotaxis (D) and spontaneous alternation (E) for WT flies in square wall, curved wall, and yolk control experiments. Groups with the same letter are not significantly different from one another. (D) Thigmotaxis is defined for each fly as the median distance from the wall of walking locations. The dashed line indicates the median distance from the wall if walking locations were distributed uniformly across the hallway. (E) Spontaneous alternation is measured in the final

841 training block. The dashed line indicates the random rate of alternation. Error bars are

842 standard error of the mean.

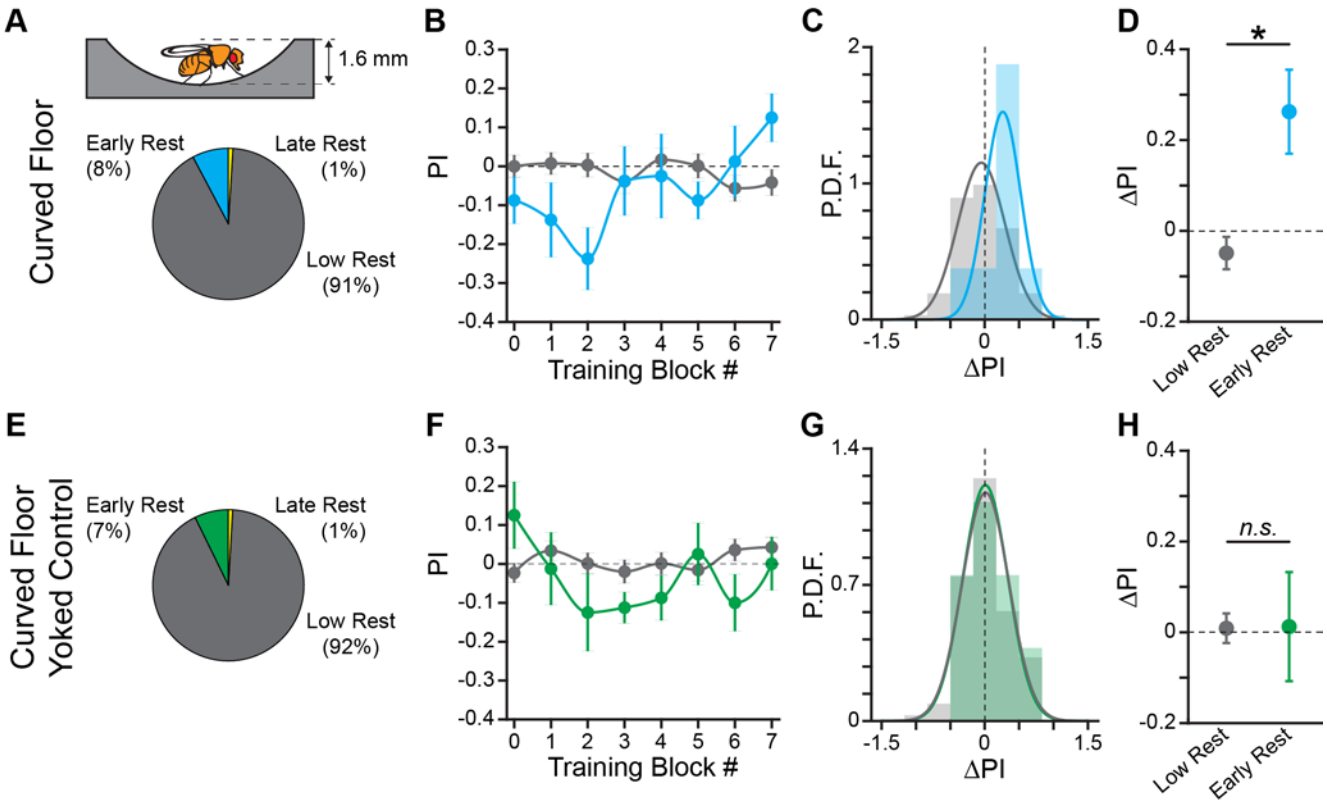

Figure 3 – Learning to Turn Toward Sucrose is Independent of Track Geometry but Dependent upon an Informative Operant Contingency (A) Schematic of hallway geometry with representative fly for scale (*Top*), Fraction of flies with each rest phenotype (*Bottom*). (B, F) Mean turn direction Preference Index (PI) for each training block for trained (B) and yoked control (F) flies in the curved floor Y-Maze. Points plotted in color are flies with early rest (drowsy), points plotted in grey are flies with low rest (restless). (C,G) Probability Distribution Function (P.D.F.) histograms of change in turn preference index (ΔPI) between training block #1 and #7 for trained (C) and yoked control (G) flies. The normal distribution fitted to each distribution is superimposed as a line on the plot. Drowsy flies are plotted in color (*cyan*: trained, *green*: yoked), restless flies are plotted in grey. (D,H) Mean ΔPI of low rest (restless) and early rest (drowsy) flies trained in curved floor Y-Maze (D), and yoked control flies (H). Error bars are

855 standard error of the mean, *n.s.*: groups are not significantly different, * significantly

856 different groups.

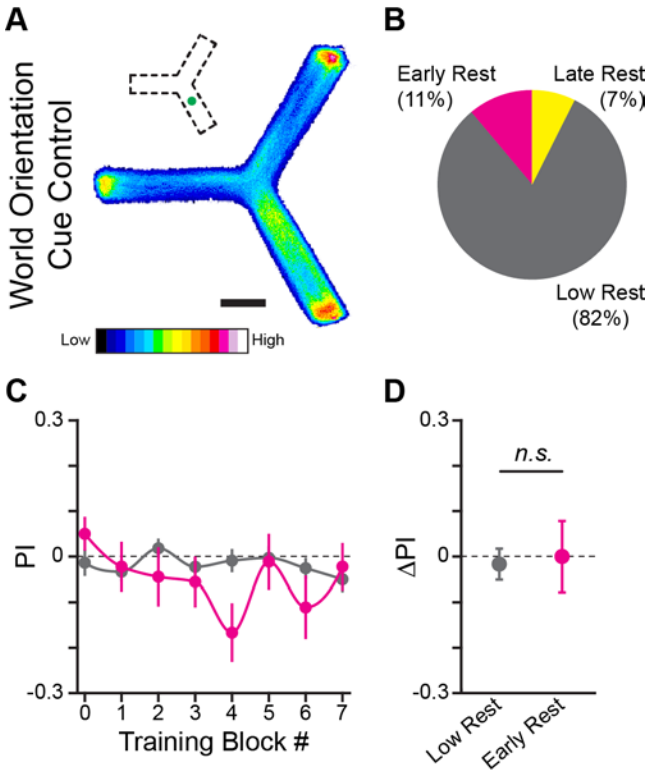

Figure 4 – A World Orientation Cue does not Facilitate Learning the Operant Task. (A) Position heatmaps of flies trained with an LED spatial orientation cue. Scale bar is 5 mm. Color shows relative occupancy of each pixel from low to high. (B) Percentage flies with each rest phenotype. (C) PI by training block for early rest (magenta) and low rest (grey) flies. (D) Mean ΔPI of early rest and low rest flies. Error bars are standard error of the mean, *n.s.*: groups are not significantly different.

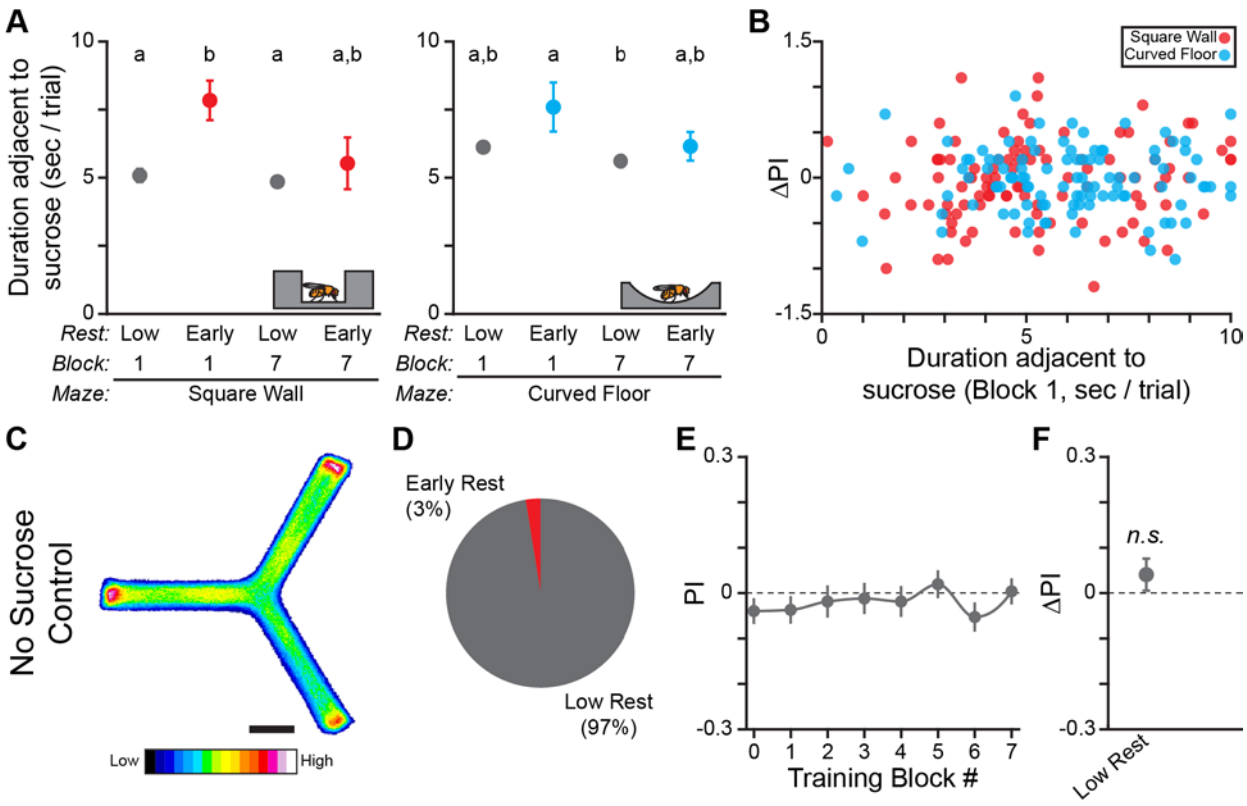

Figure 5 – Sucrose Promotes Rest and is the Only US Attended to by Flies in the Y-Track. (A) Mean time per trial WT flies spend adjacent to sucrose in the square wall and curved floor experiments. Time per trial is capped at 10 seconds, because sucrose is automatically removed 10 seconds after it is made available. Groups with the same letter are not significantly different from one another. (B) Scatter plot of change in turn preference index (ΔPI) between training block #1 and #7 by time adjacent to sucrose in training block #1 for flies in the square wall experiment (red dots) and curved floor experiment (cyan dots). (C-F) Results of Y-Maze training with no sucrose available. (C) Position heatmaps of trained flies. Scale bar is 5 mm. Color shows relative occupancy of each pixel from low to high. (D) Percentage flies with each rest phenotype. PI by training block (E) and mean ΔPI (F) for low rest flies. Only 2 flies had early rest, which is

874 insufficient to calculate a standard error, so these flies are not plotted. Error bars are

875 standard error of the mean, *n.s.*: group is not significantly different

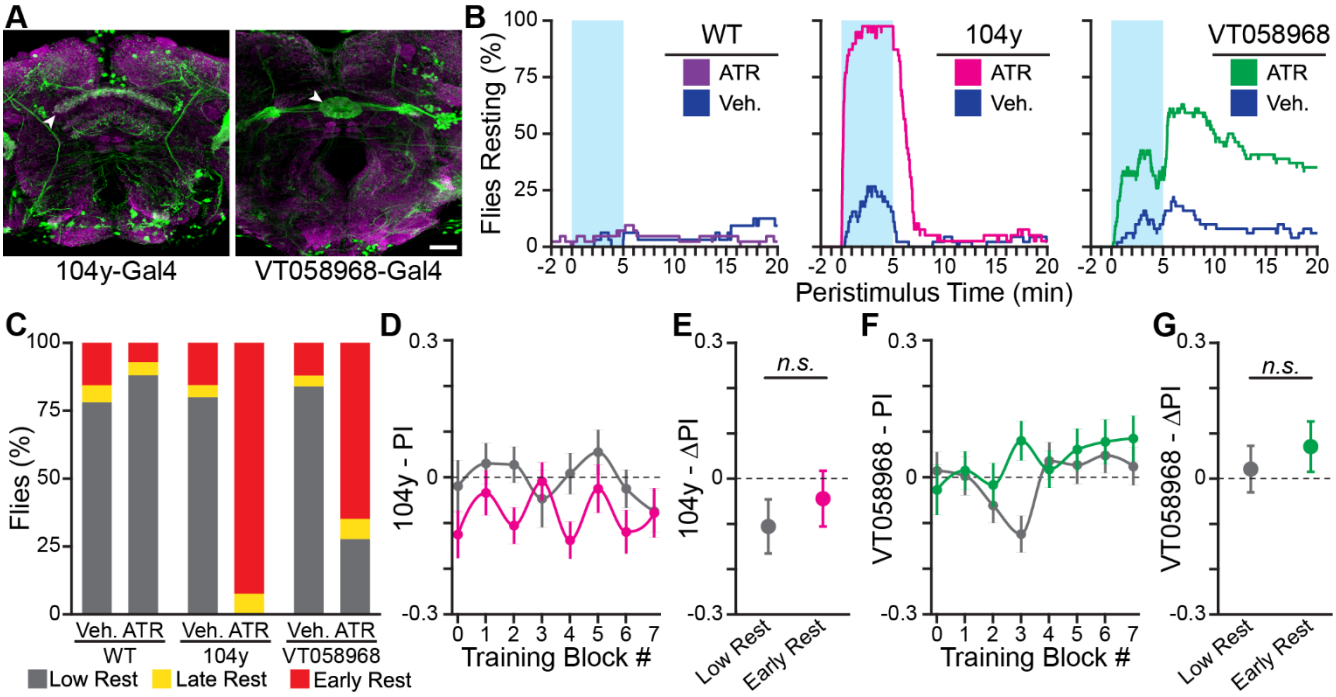

Figure 6 – Optogenetically-Induced Rest is Not Sufficient to Enhance Learning.

(A) Confocal maximum intensity projections of fly brains expressing 104y-Gal4;UAS-GFP (*left*) and VT058968-Gal4;UAS-GFP (*right*) stained for GFP (*green*) and Bruchpilot (nc82, *magenta*). Scale bar is 20 µm. White arrowheads indicate the sleep-promoting neuropil for each Gal4 driver: the dorsal Fan Shaped Body for 104y-Gal4 and the Ellipsoid Body for VT058968-Gal4. (B) Peri-stimulus rest plots for WT (*left*), 104y (*center*), and VT058968 (*right*). Blue shaded area shows when the blue LED is on. The plots show 20 minutes of behavior following light onset - this time is not linked to trial times, which vary between animals, and does not encompass the entire duration of the experiment. (C) Percent of each rest phenotype present in each experiment group. (D-G) Results of training 104y (D,E) and VT058968 (F,G) flies. Early and low rest flies for each genotype were pooled from the ATR and vehicle groups. Turn preference index (PI) (D,F) and change in PI (ΔPI) (E,G) are plotted for each genotype. Colorful points

889 are flies with early rest (*magenta*: 104y, *green*: VT058968). Error bars are standard

890 error of the mean, *n.s.*: groups are not significantly different.

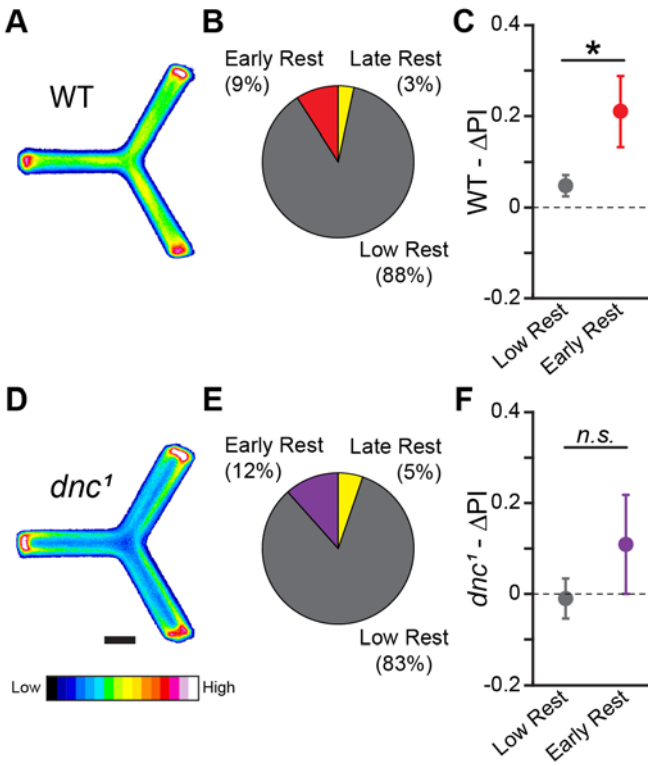

Figure 7 – Y-Track Operant Learning is Dependent on cAMP Regulation. (A,D) Position heatmaps of trained WT (A) and *dnc*^{1} (D) flies. Scale bar is 5 mm. Color shows relative occupancy of each pixel from low to high. (B,E) Fraction of WT (B) and *dnc*^{1} (E) flies with each rest phenotype. (C,F) Mean ΔPI of low rest (restless) and of early rest (drowsy) WT (C) and *dnc*^{1} (F) flies. Error bars are standard error of the mean, *n.s.*: groups are not significantly different, * significantly different groups.