Rapid Communication

Drosophila learn efficient paths to a food source

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A R T I C L E   I N F O
Article history:
Received 7 December 2015
Revised 23 March 2016
Accepted 25 March 2016
Available online 5 April 2016

Keywords:
Drosophila
Flies
Behavior
Skinner box
Feeding task
Learning assay
Drug screening

A B S T R A C T
Elucidating the genetic, and neuronal bases for learned behavior is a central problem in neuroscience. A leading system for neurogenetic discovery is the vinegar fly Drosophila melanogaster; fly memory research has identified genes and circuits that mediate aversive and appetitive learning. However, methods to study adaptive food-seeking behavior in this animal have lagged decades behind rodent feeding analysis, largely due to the challenges presented by their small scale. There is currently no method to dynamically control flies’ access to food. In rodents, protocols that use dynamic food delivery are a central element of experimental paradigms that date back to the influential work of Skinner. This method is still commonly used in the analysis of learning, memory, addiction, feeding, and many other subjects in experimental psychology. The difficulty of microscale food delivery means this is not a technique used in fly behavior. In the present manuscript we describe a microfluidic chip integrated with machine vision and automation to dynamically control defined liquid food presentations and sensory stimuli. Strikingly, repeated presentations of food at a fixed location produced improvements in path efficiency during food approach. This shows that improved path choice is a learned behavior. Active control of food availability using this microfluidic system is a valuable addition to the methods currently available for the analysis of learning, memory, addiction, feeding, and many other subjects in experimental psychology.

1. Introduction

Learning and memory are fundamental brain functions that are important to all aspects of the human experience by allowing us to adapt to a challenging, changing environment. The molecular pathways underlying learning & memory are involved in both aspects of the ‘genes + environment’ sum, influencing our adaptability as individuals and serving as a conduit as we are shaped by experience. A better understanding of learning will be valuable to better treatment of addiction disorders and other forms of dysfunctional learning. Addiction disorders include food addiction, binge eating, and binge eating disorder (Marcus & Wildes, 2014), behavioral disorders that contribute to the worldwide obesity epidemic (Finkelstein & Strombotne, 2010). Obesity is a major risk factor for heart disease, stroke, type II diabetes, osteoarthritis, and some forms of cancer; public health policies have failed to reverse the epidemic and anti-obesity drugs have weak efficacy, problematic side effects, or both (Finkelstein & Strombotne, 2010; Gautron, Elmquist, & Williams, 2015). Finding better ways to treat obesity will require multidisciplinary efforts including basic research to connect dysfunctional reward learning with the neuroscience of hunger and satiety.

An important model system for understanding the fundamental molecular and neural mechanisms of learning is Drosophila melanogaster (Keene & Waddell, 2007). Landmarks include the discovery of the first learning mutants (Dudai, Jan, Byers, Quinn, & Benzer, 1976), cloning of the associated genes, identification of the brain region that stores olfactory memories (Han, Levin, Benzer, 1976), cloning of the associated genes, identification of the brain region that stores olfactory memories (Han, Levin, Reed & Davis, 1992; Zars, Fischer, Schulz, & Heisenberg, 2000) and the circuitries that mediate aversive (Claridge-Chang et al., 2009) and appetitive (Burke et al., 2012; Liu et al., 2012) conditioning signals. In addition to memory research, Drosophila genetics has emerged as a powerful system to study other basic brain and metabolic functions, including food seeking (Sokolowski, 1980), food receptiveness (Deak, 1976), fat accumulation (Pospisil...
et al., 2010), alcohol susceptibility (Moore et al., 1998), alcohol reward (Kaun, Azanchi, Maung, Hirsh, & Heberlein, 2011), and feeding regulation (Pool & Scott, 2014). Existing Drosophila assays have enabled major advances, but none currently give detailed information about behavior in response to single packets of food. There are a range of methods to measure various aspects of feeding behavior in larval and adult flies (Deshpande et al., 2014; Itskov et al., 2014; Ro, Harvanek, & Pletcher, 2014; Smith, Thomas, Liu, Li, & Moran, 2014), but none enable the automated control of food availability in freely moving adult flies. Active control of food availability is a long-established method in rodents (Skinner, 1930), but the adult male mouse weighs about 30 g while the adult male vinegar fly weighs about 0.6 mg, a 50,000-fold difference in size.

We developed a microfluidic feeder for Drosophila that delivers meal-sized, nanoliter-scale portions to a behavior chamber with visual and auditory stimuli. With repeated presentations, flies learned to approach food via more direct paths.

2. Methods and materials

2.1. Drosophila

D. melanogaster flies (a yw stock) were cultured in plastic vials at 22 °C, 60–70% relative humidity, under 12:12 h light and dark cycles.

2.2. Design of the SNAC microfluidic chip

Chips were designed with SolidWorks 2013 CAD software (Dassault Systemes, USA). The chip’s external dimensions were 33 mm × 30 mm × 4 mm; the behavior chamber was 20 mm × 15 mm × 2 mm (Fig. 1A). The food channel delivered liquid to a feeding alcove; the volume of food delivered from this channel on actuation was 80 nl [range 60, 100]. We refer to the chip as the Small-animal Nutritional Access Control (SNAC) chip. A fly’s head is ~1 mm wide and its proboscis is <400 μm wide. The design aimed to control the liquid food delivery dynamically, allow video recording of both behavior and the microfluidic food channel. The design incorporated a feeding alcove that required that a fly insert its head to drink from a feeding channel (Fig. 1B). The channel was 200 μm wide and 50 μm deep, while the alcove was designed to be 400 μm wide. Completed chips showed a ~20 μm divergence from the design dimensions for channel and ~50 μm for the feeding alcove (Fig. 1C).

2.3. Chip fabrication and assembly

Chips were fabricated with optically clear thermoplastic cast polymethyl methacrylate (PMMA, Professional Plastics, Singapore). Computer numerical control machining was used to fabricate the chip layers. Valves and interconnects were made from polydimethylsiloxane (PDMS), cast from a pre-fabricated PMMA mold. The chip layers were bonded by thermal fusion as follows. The two chip layers (each 2 mm thick) were aligned in an L-shaped guide under an inverted microscope (Fig. S1A). A small amount of acrylic glue was applied to the layer sides to hold them during thermal bonding (Fig. S1B). The layers were sandwiched between 3 mm thick borosilicate glass sheets and tightened with binder clips (Fig. S1C). This assembly was placed in a hot air bonding oven at 125 °C for 45 min, with a 1 h cooling time. The channel dimensions and bonding fidelity were measured with a 3D optical profiler (Zeta-20, Zeta Instruments). The chips were also tested with food dyes (Winner Brand, Thailand) for flow and leaks. Twenty chips were measured with the optical profiler at 40× objective to assess how closely they conformed with the design. After bonding, the alcove width was 446 ± 8 μm; the channel width and depth had the dimensions 181.4 ± 3.4 μm and 56.4 ± 6.3 μm, respectively. Error values are given as standard deviations.

2.4. Pumps and controllers

The SNAC chip system is shown in Fig. S1F. A liquid food solution containing 5% sucrose (Sigma-Aldrich) and food dye (Winner Brand) was pushed and retracted through a microfluidic channel with custom syringe pumps (not shown). Each pump was constructed from a 10 μl precision glass syringe (80300, Hamilton, USA) to a 100 mm linear actuator (L12 NXT, Fergelli, Canada). Each syringe was connected by flexible tubing (Tygon S-54-HL AAQ04103, OD 1/16", Professional Plastics, Singapore) to a 4 mm long 21 G stainless steel tube inserted into the chip inlet. The tube was filled with mineral oil (Sigma M8410, U.S.) before adding sucrose solution from the chip-facing end. A microcontroller (NXPLPC1768, mbed, USA) was used to drive the actuators with 5-volt digital pulses; pump speeds were adjusted by varying the pulse duty cycle. An H-driver circuit (SN754410, Texas Instruments, USA) was used to control pump direction. The microcontrollers were controlled with custom C++ firmware. During the experiment, fluid was alternately dispensed into the food channel and retracted back to a ‘standby’ position. The fluid’s extent was detected by software that monitored color changes at distinct positions along the food channel (Fig. S1G).

2.5. System integration and sensory stimuli

Experiments were conducted with an eight-chip array (Fig. S1H) in a temperature-controlled incubator (MIR-154, Sanyo, Japan). For light stimulus control, the chips were positioned on two LCD screens (µLCD-43, 4D systems, Australia) mounted on an aluminum stand. For sound stimuli, a 0.5 W (8 ohm) speaker (COM-09151, Sparkfun.com) was mounted next to each screen. The chips were illuminated with white LED strips (ST-6500-CT, Inspired LED, U.S.A.) at 600 lux (measured on the chip surface). Two color cameras (A601fc, Basler, Germany) monitored animal activity and fluid location on all chips. All devices were controlled with a custom program in LabVIEW (National Instruments, USA).

2.6. Experimental protocols

Four to seven day-old flies of both sexes were starved in batches of 10 for 24 h in vials containing water-soaked tissue (Kimwipes). Flies were maintained in a 12:12 h light and dark cycle at 22 °C during starvation. Flies were anesthetized on ice for less than a minute and transferred individually to chips. Two protocol variants were used. In Experiment 1, the flies were tracked 30 min before delivery and 60 min after delivery of a single food bolus. In Experiment 2, food was repeatedly delivered along with sound and light stimuli. Each 100 s epoch contained a 2 s 300 Hz 82 dB sound signal followed immediately by a white to blue screen change (Fig. 1D). A food bolus of ~80 nl [range 60–100] was delivered ~3 s (range 1–5) after sensory cue onset. The screen was kept blue until the fly’s head was detected to be in the feeding alcove, upon which the food was retracted and the screen returned to white. Six food/stimulus epochs were presented over 30 min (Fig. 1E) in a 4 min cycle, with a 140 s wait between epochs.

2.7. Tracking, feeding metrics and data analysis

The animals were tracked with computer vision code in LabVIEW using standard background subtraction and centroid methods. For tracking in changing blue/white light conditions, the red or blue plane was extracted from the color video when the screen...
was white or blue, respectively. The alcove width in each video was used to rescale tracking data to millimeters. Behavior data were plotted with Matlab; summary statistics were means or median with relevant confidence intervals shown as error bars. ‘Time to alcove’ measured the time the animal spent after food presentation and before detection of a fly’s head in the food alcove. The path efficiency was calculated as the distance of the most direct path to the food from their location at the start of an epoch, with a figure around a feed event.

3.1. Flies briefly increased their locomotion around food intake

We examined behavior before and after consumption of a single bolus of a 5% sucrose solution. An example of the behavior observed is illustrated in Fig. 2A and in Supplementary Video 1. Baseline median walking speed was less than 0.1 mm/s, but increased sharply when the food bolus was discovered by the fly and walking speed remained high for several minutes after feeding (Fig. 2C and D). We conclude that flies undergo locomotor arousal around a feed event.

3.2. Flies were able to discriminate accessible from inaccessible food

We aimed to identify learned aspects of food approach after repeated presentations. Starved flies were subjected to a six-epoch regime with food, a screen color change and a 2 s audio cue (Fig. 1E). In this regime, when food was made accessible in the alcove, flies entered it an average of 3.6 out of a possible 6 epochs [95CI 3.3, 3.9] (Fig. S2A). To investigate the cues that flies used in making a food approach, liquid food was pumped along the channel but stopped just before the food port. The latency to alcove entry (time to alcove) for accessible food was 30.5 s [95CI 27.9, 33.1] and 39.1 s [95CI 28.4, 49.8] for inaccessible food (Hedges’ g = 0.34, p = 0.10; Fig. S2B). In each epoch, a fly may be near or far from the food alcove, and in each case the direct path to the alcove is a straight line. The path efficiency was also similar for both conditions (Fig. S2C). These results indicate that flies could usually discriminate inaccessible food from accessible, but that in the minority of cases where they approach the alcove, they do so with similar speed and efficiency.

3.3. Background color changes and an auditory signal increased food approach

In conditioning chambers for vertebrate experiments, some protocols use one or more sensory stimulus to cue reward delivery.
We asked whether flies were using the screen color change and the audio signal as cues for the alcove approach. Four experiments omitting either the color switch, the audio pulse, or both were performed: both blue light and a 300 Hz tone, light-only, sound-only, or neither stimulus. Path efficiency and time-to-alcove were largely unchanged by the presence of sensory cues (Fig. S2E–G). When both stimuli were presented together, the number of alcove entries per fly was higher by at least one entry relative to either single-stimuli or no-stimulus conditions, (Fig. S2D) (ANOVA $p = 1.6 \times 10^{-7}$). Thus, only the visual + auditory stimuli combination promoted increased food approach.

3.4. Over repeated presentations, food approach and time to food were unchanged

Two behavioral metrics, the proportion of flies entering at the alcove and the time to the food alcove, were analyzed over epoch number (Fig. 3A and B). Only modest, non-statistically significant differences in the proportion of alcove entries were observed (Fig. 3A). Similarly, there was little to no change in alcove approaches for flies in the light-only, sound-only and no-stimuli regimes (data not shown). A modest dip in the time to alcove was observed by the third epoch, but there was no statistical change in this metric by the sixth epoch (Fig. 3B). These results indicate that the flies’ frequency of food approach and the time taken to approach a food source undergo little or no adaptation during repeated presentations. Both male and female flies were tested in these experiments, no substantial differences in the proportion of alcove entries were observed between these groups (data not shown).

3.5. Flies learn to improve their food approach path efficiency

Flies generally did not follow direct paths to the food after the epoch commenced, but displayed more or less circuitous paths during each food presentation epoch (Fig. 2B). Flies’ alcove approach path efficiency increased progressively over repeated presentations, from 0.18 in the first epoch to 0.34 by the sixth epoch, a 0.16 path efficiency increase [95CI 0.10, 0.22] (Fig. 3D). The standardized effect size indicated that the flies made a large improvement in path efficiency (Hedges’ $g = 1.06$, $p < 0.0001$). These data indicate that flies learn to follow more direct paths to a food delivery location over repeated presentations.

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**Fig. 2.** Fly walking activity responds to a food bolus. A. Five video frames of a fly in a single food presentation. Before the food delivery, the fly is walking around a white-illuminated chamber (−6 s). Blue light is activated and food is extruded, the fly has not approached the alcove at 33 s. At 60 s the fly enters the alcove and feeds, which triggers the software to retract the food and switch the screen back to white. The fly stays eating residual food before leaving and returning to walking around the chamber (81 s). B. Cumulative traces of five flies; blue dots indicate the location of the fly at the start of the epoch, ‘+’ symbols indicate the alcove location. Each colored trace represents the path taken during one epoch period and only epochs where the fly entered the feed alcove are shown. C. Individual fly walking speeds before and after feeding on a bolus of sucrose liquid food. Tracking data timelines were re-centered around feeding events. D. Median walking speed of flies fed sucrose food. Light blue error band indicates confidence intervals of the median. Walking speed is affected by food intake. The pale vertical red line indicates the time of feeding events. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)
3.6. Path efficiency is weakly related to walking speed

Path efficiency increased despite a largely unchanged time take to enter the alcove (Fig. 3B, suggesting that the flies move more slowly towards the alcove in more efficient epochs. A plot of mean alcove approach speed over epoch confirmed that flies moved slower in the later, more efficient epochs (Fig. 3C). To investigate how closely path efficiency was related to walking speed, we performed a linear regression of the two metrics, finding that there was a relationship, albeit a weak one, $R^2 = 0.12$ (Fig. S3A). Path efficiency was also somewhat related to path length, $R^2 = 0.27$ (Fig. S3B). Thus, in successive food presentation epochs, flies tend to walk shorter paths towards the alcove more efficiently and more slowly.

4. Discussion

The tiny size of the genetically tractable insect *D. melanogaster* means that some tools development has lagged behind some available for rodent species. A number of innovative methods to study fly feeding are currently available, including CAFE (Ja et al., 2007), which uses food capillaries to provide a precise quantification of food intake, and flyPAD (Itskov et al., 2014) and FLIC (Ro et al., 2014), which use electrical methods to detect food contact events with high temporal precision, but there are no methods to dynamically control access to defined quantities of food in fly. Here we show that the SNAC microfluidic chip enables the delivery of small quantities of liquid food (~80 nl) to flies while simultaneously tracking animal locomotion, allowing the system to capture animal behavior when food is presented. The utility of the SNAC chip system can be further enhanced by the addition of components that enable computer vision feedback to control food access in response to the animal's behavior, in a similar manner to a ' Skinner' conditioning apparatus.

While we found no evidence for behavioral adaptation for the fraction of flies entering the alcove to feed or the time taken to reach the alcove, we found that flies learn to walk along more efficient paths to a transient food source. Surprisingly, there is no relationship between path efficiency and time-to-alcove, and flies walk more slowly towards the alcove in later epochs. These results indicate that, on average, flies slowly follow more direct paths to the feeder during later epochs, rather than the rapid exploration that occurs in the earlier epochs. That they learn to take more efficient paths shows that flies associate food with a location within an enclosed space. This result is compatible with results showing that flies can associate food with odors (Krashes & Waddell, 2008), and are capable of visual place learning (Ofstad, Zuker, & Reiser, 2011).
Previous studies on larval foraging behavior showed that genetic functions are shared between foraging and learning (Mery, Belay, So, Sokolowski, & Kawecki, 2007). Path efficiency learning may be relevant to foraging adaptation in wild Drosophila adults. The development of a microfluidic dynamic feeder device for feeding and learning analysis opens new possibilities in the study of learned foraging behaviors.

Acknowledgements

This work received support from A*STAR Joint Council Office Grant 1231AFG030 awarded to ACC at IMCB and ZPW at SIMTech. ACC received support from Duke-NUS Medical School. Grant 1231AFG030 awarded to ACC at IMCB and ZPW at SIMTech.

Appendix A. Supplementary material

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.nlm.2016.03.019.

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