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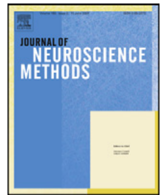

Automated measurement of *Drosophila* jump reflex habituation and its use for mutant screening

Punita Sharma[1,2,3], John Keane[4], Cahir J. O'Kane**, Zoltan Asztalos*

Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK

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ABSTRACT

In habituation the probability of a behavioral response decreases with repeated presentations of a stimulus. This is a simple kind of learning since it involves an adaptive change in behavior due to experience. The present study describes a high-throughput semi-automated system to track movement of individual flies and score their jump response to repeated presentations of an odor. We find a decreased response on repeated presentations of odor, which a number of criteria suggest to be habituation. Tracking of up to sixteen flies simultaneously allows analysis of large numbers of flies for mutant screens. We demonstrate the use of the Autojump system for large-scale screens by conducting a pilot-scale screen of 150 *P* insert lines for habituation mutants.

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1. Introduction

High-throughput genetics and genomics have become major driving forces in biology. However, the ability to analyze behavioral phenotypes that may result from genome-wide mutagenesis lags behind. The recognition that phenotypic characterization is crucial has led to first the Mouse Phenome Project (Paigen and Eppig, 2000; Bogue, 2003) and now the Human Phenome Project (Freimer and Sabatti, 2003). Fulfillment of a demand for sophisticated and rapid behavioral phenotyping tools will broaden the perspective of behavioral analysis, both conceptually and technologically. In particular, the ability to measure learning in high-throughput screens should allow the study of learning and memory to take advantage of the explosion in genome-wide information and reagents.

Habituation is a kind of non-associative learning that involves an adaptive change in behavior due to experience. In habituation, the probability of a response decreases with repeated presentations of a stimulus. A variety of reflexes in *Drosophila* undergo habituation, including the proboscis extension reflex to sucrose solution (Duerr and Quinn, 1982), the cleaning reflex to stimulation of thoracic microchaetae (Corfas and Dudai, 1989), the landing response of extension of the front legs after presentation of a moving dark horizontal band (Rees and Spatz, 1989), increase in walking speed in response to a novel odor (Wolf et al., 2007), and avoidance response to electric shock (Acevedo et al., 2007). Flies also exhibit a jump response to high concentrations of benzaldehyde odor (McKenna et al., 1989). This response wanes on exposure to repeated pulses, and meets several criteria of habituation (Thompson and Spencer, 1966; Boynton and Tully, 1992; Asztalos et al., 2007). However, these habituation paradigms are labor-intensive, and the response of the fly is scored by human eye, hence limiting the number of flies tested and consequently, the rate at which lines can be tested. This in turn makes them unfavorable for high-throughput screens.

Here we present an apparatus (which we name the Autojump machine) that can simultaneously test 16 flies for their jump responses to repeated olfactory stimuli. It uses a camera, movement-tracking software and a custom-made analysis software to detect jump, calculate habituation indices and apply statistical comparisons between mutant and control lines. With this apparatus we analyzed how parameters like odor concentration, age of flies, and circadian cycle could affect the response and interpretation of results from the screen. We also demonstrate successfully the use of this machine to screen 150 insertional mutant lines for defects in habituation of the olfactory jump response.

* Corresponding author. Present address: Aktogen Ltd., Department of Genetics, University of Cambridge, Downing Street, Cambridge CB2 3EH, UK.
Tel.: +44 7800818483; fax: +44 1223 333992.
** Corresponding author.
E-mail addresses: c.okane@gen.cam.ac.uk (C.J. O'Kane), zoltan@aktogen.com (Z. Asztalos).
[1] Present address: Gladstone Institute of Neurological Diseases, 1650 Owens Street, San Francisco, CA 94158, USA.
[2] Present address: Department of Neurology, University of California, 505 Parnassus Avenue, San Francisco, CA 94143, USA.
[3] Present address: Department of Physiology, University of California, 505 Parnassus Avenue, San Francisco, CA 94143, USA.
[4] Present address: School of Biological Sciences, University of Sussex, Brighton BN1 9QG, UK.

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2. Materials and methods

2.1. Fly strains and genetics

Flies were of the Canton-S wild-type strain (de Belle and Heisenberg, 1994, 1996; Dura et al., 1993) unless otherwise noted. The strains isogenized for the 2nd and 3rd chromosome were generated from the Canton-S wild-type background by us (Bellen et al., 2004; Sharma et al., 2005) and characterized in various behavioral, physiological and developmental assays including their olfactory responses to benzaldehyde and jump reflex habituation (Sharma et al., 2005). The *P* element insert lines were generated by the FlySeq *P* Element Mapping Project (Department of Genetics, University of Cambridge, Cambridge, UK, http://www.flyseq.org.uk/) in isogenized autosome combinations 2C + 3J (also designated 1817 in the FlySeq stock lists), 2C + 3I (also designated 1839) and 2A + 3A (also designated 4147) derived from the Canton-S background (Sharma et al., 2005) and the *P*{*GT1*} dual-tagging gene-trap vector (Lukacsovich et al., 2001). The white eyed version of isogenized 2A + 3A was created by crossing 2A + 3A males to w^{118}/Y; *Sp*/*CyO*; *Sb*/*TM6B* females (Dura et al., 1993). All strains were raised in either half-pint glass bottles or 4 in. glass vials on standard corn meal/yeast/agar medium (18 g agar, 30 g dried yeast, 150 g dextrose, 170 g maize powder, 50 ml Nipagin, and 1700 ml water). All cultures were maintained at 25 °C and 50–60% relative humidity on a 12/12 h light-dark cycle.

To breed flies for behavioral experiments, 10 virgin females were crossed with at least 20 males in 4-in. glass vials. These flies were transferred into fresh vials twice a week. Flies eclosing from these vials were collected and separated by gender under light CO_2 anesthesia on the same day and stored in 4-in. vials in groups of 20. Since a mixed population of male and female flies increased variation in the habituation index (data not shown), only males were used in the assay 2 days after the collection.

All behavioral experiments were performed in a dedicated room maintained at 24 °C and 60–75% relative humidity. Purchasing information for all parts and equipment is provided in Supplementary Table 1. The air in the room was kept clean and non-static using a VIVA air purifier with HEPA filter and ionizer. A Weather Monitor II was used to monitor temperature, humidity and atmospheric pressure changes. The interior of the room was painted matt-black to reduce light reflection that might startle the flies.

2.2. Autojump machine

Fig. 1 shows a schematic of the Autojump apparatus. 16 individual flies were housed in cylindrical glass chambers each of length 150 mm, inner diameter 12.6 mm, and outer diameter 15 mm. The ends of the cylindrical chambers were fitted with removable PTFE (polytetrafluoroethene) plugs. A sintered glass disc was inserted inside these plugs to hold the flies within the chambers and allow free flow of odorants. Nitrile rubber O-rings were used to seal the gap between the PTFE plugs and chambers. The chambers were held in place in an aluminum scaffold (not shown) by a removable aluminum frame (not shown). Having two such frames allowed us to prepare the next group of flies for testing while a test was in progress. A PTFE odor delivery manifold delivered odor pulses via PTFE tubing to each chamber controlled by a 3-way solenoid valve that switched between benzaldehyde diluted in heavy mineral oil and heavy mineral oil housed in 250-ml Drechsel bottles. A vacuum pump generated airflow of 1 l/min to each chamber via flow meters mounted on the aluminum scaffold. A PC computer with a valve-control card switched the valve using a custom-made valve driver software MSV-16, to deliver a 4-s odor pulse every minute for 30 min. The glass chambers holding the flies were illuminated using two flicker-free vertical and horizontal lights and a uniform, low intensity, flicker-free flat light at the back. The flies were viewed by a Sony SSC-M370CE monochrome CCD camera (1/2 in. CCD; 0.4 mega pixel; 25 Hz) fitted with a 16 mm cosmicar manual iris lens. The analogue image from the camera was viewed on a 9 in. monochrome monitor before being fed into a VCR, which allowed the experiment to be taped if required. The VCR sent this image to a EureCard Picolo Pro video capture board in a PC computer. The image was processed using EthoVision tracking software to determine the position of each fly and record its (*x*,*y*) coordinates 12.5 times per second for the entire 30 min of the experiment.

2.3. Testing procedure

Aliquots of mineral oil, containing appropriate benzaldehyde dilutions in heavy mineral oil, were prepared in advance and stored at −140 °C to minimize oxidation of benzaldehyde to benzoic acid, which has a distinct odor different from that of benzaldehyde. The aliquots were thawed and poured into Drechsel bottles on the day of the experiment.

Flies were allowed to acclimatize for at least 30 min in the behavior room prior to an experiment. 16 clean glass chambers were held in position in the aluminum frames and a single fly was mouth-aspirated into each chamber and secured using the PTFE plugs. Before the frames were inserted into the scaffold to begin recording, flies were allowed to acquaint to the surroundings of the chamber for 15–20 min. At the end of a 30-min experiment, the frames were removed, the flies were aspirated out and new flies added.

After the last experiment of the day, odorants were discarded and the Drechsel bottles and chambers were washed with odor-free detergent, Tween-20 and rinsed with ethanol and deionized water. The plugs were rinsed with ethanol and washed with deionized water in a sonicating water bath. The PTFE tubing carrying the odor pulse, the valve and the odor delivery manifold were washed with a stream of ethanol followed by a stream of deionized water and allowed to air-dry overnight.

2.4. Data analysis and statistics

2.4.1. Calculation of habituation index

In a habituation experiment, 16 flies housed in individual chambers receive a 4-s benzaldehyde (5% in heavy mineral oil) odor pulse at predefined intervals. Since at this concentration benzaldehyde acts as a repellent (Ayyub et al., 1990), the flies jump (McKenna et al., 1989). Sometimes the flies give a late response (late jump), and therefore we recorded the response to each odor pulse for a total of 7 s. Response to the first odor pulse was used to select flies that had wild-type responses to the habituating odor concentration. *P* insertion lines with mean olfactory responses significantly lower than the control isogenized line ($P < 0.05$, unpaired Student's *t*-test) were excluded from further analysis.

EthoVision software was used to generate the (*x*,*y*) co-ordinates of each fly every 0.08 s. The analysis software (implemented in Perl, www.cpan.org) first selected the 7 s of data within which a response was to be scored, and then calculated the speed of each fly within the 0.08-s intervals. The analysis software then computed the maximum velocity of the fly within this 7-s window, compared it to a threshold jump velocity (100 pixels/s) and scored the response of the fly as "jump" or "no-jump". The threshold was determined by manual observation, as the minimum value that produced no false positive "jumps" during 480 stimuli with 2% benzaldehyde and 960 stimuli with 5% benzaldehyde. Using these responses, the habituation index for each fly was calculated as the last jump before four consecutive "no-jumps" (Boynton and Tully, 1992; Asztalos et al., 2007).

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