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Increased throughput assays of locomotor dysfunction in *Drosophila* larvae

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ABSTRACT

Larval locomotion is a sensitive readout of a range of nervous system deficits in *Drosophila*, and has been utilised to quantify modulation of the disease phenotype in models of human disease. Single larvae are typically analysed in series using manual quantification of parameters such as contraction rate, or grouped together and studied en-masse. Here, we describe the development of tests for the analysis of several spatially isolated third instar larvae in parallel. We rapidly quantify larval turning rate and velocity during wandering behaviour in a 4 plate assay. In a second test, larvae are recorded as they race along five parallel lanes towards a yeast stimulus. This allows increased throughput analysis of comparative genotypes simultaneously, video archiving, and detection of exacerbation or rescue of defective locomotion in a *Drosophila* model of tauopathy, as we demonstrate genetically and through delivery of candidate therapeutic chemicals in fly food. The tests are well-suited for rapid comparison of locomotion capability in *Drosophila* mutants or candidate modulation screens in *Drosophila* models of human disease.

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1. Introduction

Behavioural assays in disease model organisms are desirable as they offer a functional phenotypic readout in the context of an intact, living organism. Despite this, complex behavioural traits can be difficult to quantify and adapt to genetic or chemical modulation screens of the pathological phenotype. Although several locomotion assays that test a variety of quantifiable behavioural parameters in rodents are now routinely employed (Brooks and Dunnett, 2009), invertebrates such as *Drosophila* or *Caenorhabditis elegans* offer additional advantages over these experimental paradigms for readouts of cellular function. Rapid development to testable age allows for generation of larger numbers of animals and increased statistical strength when analysing complicated behavioural traits, and offers the opportunity to measure a greater number of behavioural readouts. These factors have contributed to recent efforts towards increased throughput quantification of a range of behavioural phenotypes in *Drosophila* (Min and Condron, 2005; Sharma et al., 2009; Murakami et al., 2010).

Several tests have been applied to study locomotion deficits in both *Drosophila* adults (Sherwood et al., 2004; Tamura et al., 2009) and larvae (Wang et al., 1997; Min and Condron, 2005; Khurana et al., 2010). Wandering-stage third-instar larvae, which possess an accessible and well-described peripheral nervous system (Budnik and Gramates, 1999), move via successive telescoping peristaltic body wall contractions as they roam outside of their food medium (Wang et al., 1997). Mutations affecting a range of nervous system components give rise to defective locomotion in *Drosophila* larvae (Heiman et al., 1996; Wang et al., 1997; Caldwell et al., 2003), and larval locomotion defects have been described in several *Drosophila* models of prevalent neurodegenerative diseases, such as Alzheimer's disease (Folwell et al., 2010) and poly-glutamine disorders including Huntington's disease (Funderburk et al., 2009; Sinadinos et al., 2009). We previously described a *Drosophila* model of tauopathy in which an abnormally phosphorylated form of the human tau protein is expressed in larval motor neurons, leading to neuronal and synaptic dysfunction that is reflected in a range of locomotion defects. These were assessed using several previously characterised behavioural tests (Mudher et al., 2004, Fig. S1). These include counting the number of lines crossed over a square grid or the number of body wall contractions by a single larva as it crawls on an agarose surface for 30 s (line crossing and contractions assays, Fig. S1A and B), timing how long it takes for a larva to turn back onto its ventral surface (upon which it crawls) after being inverted (righting assay, Fig. S1C), and timing how long it takes for a larva to crawl 3 cm along a narrow lane in an alternative 'lane crawling' environment (crawling assay, Fig. S1D). Although these tests robustly detect a locomotion deficit in tau larvae and its genetic or chemical modulation (Mudher et al., 2004, Fig. S2), they suffer from certain limitations with regard to candidate pharmacological/genetic modulation screening. As in the case of a *C. elegans* model of tauopathy, which exhibits a reduced thrashing rate upon pan-neuronal expression of human tau (Kraemer et al., 2003), testing is partially subjective to the investigator. Furthermore, animals are tested one-by-one and in series. This limits the throughput of the assay when a large number of animals are available and ready

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for testing, and increases the chance of introducing unintended time-dependent environmental variables between comparisons.

One way to address these limitations is to record several moving larvae at once and use an image tracking system to individually identify and track the larvae at the same time in separate test areas. Ethovision tracking software (Noldus et al., 2001) has been utilised to study open field locomotion in a range of organisms (Krober and Guerin, 1999; Dunne et al., 2007; Wong et al., 2010), including *Drosophila* (Funderburk et al., 2009). The ability to track several objects in parallel, yet to be applied to *Drosophila* larvae, allows for greater assay throughput, the testing of comparative genotypes/treatments simultaneously, and yields several different locomotion parameters, such as velocity and turning rate, from a single experimental run. Temporal separation of video recording and quantification reduces any confounding effects of experimenter bias and allows completion of a more procedurally complex assay without the distraction of time spent in immediate quantification. Prior video recording is also applicable to the crawling assay, making it possible for a single experimenter to 'race' several larvae in parallel.

Here, we describe the development of *Drosophila* larval locomotion assays in these two experimental settings, which we name the '4 plate' and '5 lane' assays. Utilising simple video recording apparatus and image analysis software, these assays increase throughput, reduce bias, and yield several parameters pertaining to different components of open-field or directed locomotion. Using a model of human tauopathy, we obtain new information about the nature of open-field locomotion in these mutants. We also use the assays to detect a genetic exacerbation, and both a pharmacological rescue and worsening of the locomotion defect in tau larvae using a chemical tau kinase inhibitor and the antioxidant curcumin, demonstrating their utility for medium throughput candidate therapeutic testing.

2. Materials and methods

2.1. Drosophila genotypes, treatments and development analysis

Transgenic expression of human three-repeat tau was directed to *Drosophila melanogaster* motor neurons as described previously (Mudher et al., 2004). Male flies homozygous for human three-repeat tau under the UAS promoter (+; +; UAS-h-tau^{0N3R}) were crossed to female virgins homozygous for the motor neuron-specific GAL4 driver, D42, yielding progeny that express three-repeat tau in motor neurons (3Rtau larvae). Oregon R males not carrying a pUAS insert were crossed to D42 driver virgins as a driver control (OreR larvae). Flies doubly transgenic for 3Rtau and a constitutively active allele of the *Drosophila* GSK-3β, *shaggy* (+; UAS-h-tau^{0N3R}; UAS-*sgg**) were generated as previously described (Mudher et al., 2004) and also driven with D42 for motor neuron expression (3Rtau/*sgg** larvae). HttEx1Q20 and HttEx1Q93 larvae were raised at 29 °C and generated as previously described (Sinadinos et al., 2009). Otherwise, stocks and transgenic crosses were kept at 23 °C on a 12 h light/dark cycle and raised on standard fly food medium unless otherwise indicated. Basic food consisted of (*w/v*) 3.5% soy flour, 1.5% yeast, 7.5% granulated sugar, 2.5% powdered agarose, and 0.25% 4-hydroxybenzoate delivered in 2.5% *v/v* ethanol. The GSK-3β inhibitor, AZ-A014418, was delivered to basic fly food at a final concentration of 20 μM as previously described (Mudher et al., 2004). Solid curcumin (Sigma) was dissolved in the ethanol stock used to deliver 4-hydroxybenzoate to basic food (4 mM curcumin stock for 100 μM final concentration). Basic food was prepared without this stock, and 250 μl of the stock added to 10 ml of fly food prior to pouring vials. To chart development, age-matched virgin females were crossed to males in OreR or 3Rtau crosses (as above) on curcumin-treated food, and the number of pupae and adult emergences quantified daily. F1 L3-stage larvae were selected by size and wandering behaviour for all behavioural analyses, and moved or manipulated with a fine brush wetted with *Drosophila* saline (2 mM KCl, 128 mM NaCl, 4 mM $MgCl_2[6H_2O]$, 1.8 mM $CaCl_2[2H_2O]$, 360 mM sucrose, 5 mM HEPES). The brush was instead wetted with distilled water for manipulation of larvae in the 5 lane crawling assay. All behaviour assays were conducted in a temperature and light controlled room at 23 °C and at the same time of day during the first half of the 12 h light period for transgenic crosses.

2.2. Manual line crossing, contractions, righting and crawling assays

Manual locomotion assays, shown schematically in Fig. S1, were conducted as previously described (Mudher et al., 2004), except that each larva was acclimatised to the test plate for 2 min prior to testing. Larvae were tested individually in three trials for each test, and the measurement for the third trial used for statistical analysis with the un-paired Student's *t*-test in Microsoft excel.

2.3. '4 plate' open field larval locomotion assay

Equipment: The 4 plate assay was conducted on 1% agarose plates dyed with 0.1% *w/v* alcian blue dye (Hopkin and Williams, UK). Dye was dissolved into one volume of *Drosophila* saline over gentle heat whilst stirring for 1 h to ensure dye solubilisation, and then mixed with one volume of pre-dissolved molten 2% agarose prior to pouring of plates. Set plates were stored at 4 °C and warmed to room temperature for at least 30 min prior to the assay.

The assay was conducted on four precisely positioned blue agarose plates (arenas 1–4) with constant light source, as shown in Fig. 1A.

Larval activity was recorded with an Ikegami digital video camera and 5 mm digital video camera lense (Tracksys, UK). The camera was positioned above assay plates (Fig. 1A) with a Benbo Trekker camera tripod (Tracksys, UK) and larval movement viewed live on a connected monochrome monitor (JVC 66W 15″ TM1500PS). Assays were recorded on a Panasonic Diga digital video recorder (HDMI DMR-EZ27, Tracksys, UK) to R+ writable DVDs (Mediastar gold). Lamps ('L' in Fig. 1) were 2.5A 250 V halogen (Ring Lighting, UK).

Recording: An initial 10 s recording of empty plates was made prior to the introduction of larvae. This allowed background subtraction in Ethovision and was repeated every few recordings. A larva was positioned in the centre of each plate and allowed to acclimatise for 2 min. Larvae were then replaced to the centre of the plate and their open field activity recorded for 2 min. This DVD chapter constituted trial 1 for this cohort of larvae. Further 2 min recordings were made for trials 2 and 3. The four larvae were then replaced with the next cohort of animals for testing in the same way. In one experiment, recordings of only 30 s per trial were made for comparative purposes. Wherever possible, genotypes and treatments were randomised between adjacent plates.

Video analysis: Videos of larval crawling were analysed in Ethovision 3.0 software (Noldus Information Technology, kindly supplied by P. Newland, see Acknowledgements). An image of empty plates devoid of larvae was used as a background reference image. Four separate arena boundaries were defined above each plate in this image (Fig. 2D–G). After larval introduction, the image was thresholded to maximise contrast between larvae and the background for consistent tracking of larvae. To help this, the reference image was refreshed on each occasion that a 'blank' recording devoid of larvae was made. The identity of larvae for each trial was specified as independent variables prior to commencing trials. Larval movement was analysed for each trial using a frame

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