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Original article

An automated method to assay locomotor activity in third instar *Drosophila melanogaster* larvae

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ABSTRACT

Introduction: The purpose of these studies was to describe a novel application of an automated data acquisition/data reduction system, DanioVision™ by Noldus. DanioVision™ has the ability to detect changes in locomotor activity in third instar *Drosophila melanogaster* larvae. The noncompetitive $GABA_A$ receptor antagonist picrotoxin (PTX), was used as a pharmacologic agent to decrease locomotor activity.
Methods: Two strains of *Drosophila* were used in these studies; *wild-type* flies and flies with a mutation in the *Rdl* gene ($Rdl^{MD\text{-}RR}$). $Rdl^{MD\text{-}RR}$ *Drosophila* are naturally occurring mutants that express an aberrant form of the $GABA_A$ receptor, which has a lower affinity for PTX, but not GABA itself. Larvae, extracted from food in 20% sucrose, were randomly placed into vials containing vehicle or PTX (0.03–3 mM). After incubation of 2–24 h, individual larvae were put in each well of a 6-well culture plate previously coated with 2% agar, the plate was then placed in the DanioVision™ apparatus. The activity of individual larva was recorded for 5 min, digitized and analyzed using Ethovision® XT software.
Results: Incubation of third instar *wild-type* larvae in 1 mM PTX for 4 or 24 h decreased activity; whereas, a 2 h incubation in PTX was without effect. PTX caused a concentration-dependent decrease in activity as demonstrated by consistently reduced locomotor activity with 1.0 and 3.0 mM: 0.3 mM resulted in variable decreases in locomotor activity and 0.03 mM yielded no effect. By contrast, PTX did not affect activity in $Rdl^{MD\text{-}RR}$ larvae even at the highest concentration, 3.0 mM.
Discussion: Using an automated data acquisition system, it was found that PTX decreases activity in third instar *Drosophila* larvae due to a selective blockade of the $GABA_A$ receptor. The method will reduce the likelihood of human error and bias, as well as increase the speed and ease of data collection and analysis.

1. Introduction

Several methods currently exist to track and quantify the activity of *Drosophila melanogaster* larvae. Some of the most commonly used methods include grid-based approaches coupled with manual counting of grid passage per minute (Nichols, Becnel, & Pandey, 2012), video recording and digitizing followed by export and analysis into Photoshop (Stilwell, Saraswati, Littleton, & Chouinard, 2006), image enhancement to improve the low contrast of the translucent larvae against the background (Khurana, Li, & Atkinson, 2010), and most recently, the use of the TriKinetics *Drosophila* activity monitor (McParland, Follansbee, & Ganter, 2015). All of these methods have proven to be successful in the context of mobility assessment; however, each has caveats related to length of assay and time needed set-up and/or data analysis.

In order to enhance the speed of an assay and eliminate the need for image enhancement by adding dye to the food, or by post-hoc image processing, we employed DanioVision™. Originally designed to track the activity and behavior of zebrafish larvae (*Danio rerio*) in multi-well plates, we adapted it to video track, digitize, and quantify the activity of third instar *Drosophila* larvae in 6-well culture plates with no image enhancement using Ethovision® XT software.

To validate this novel application of DanioVision™, third instar *Drosophila* were exposed to the non-competitive $GABA_A$ receptor antagonist picrotoxin, PTX, a known chemical convulsant. We were able to reproduce the report of Stilwell et al. (2006), which showed PTX decreasing activity in *wild-type* larvae, but not $Rdl^{MD\text{-}RR}$ larvae. $Rdl^{MD\text{-}RR}$ are a mutant strain of fruit flies with a point mutation in the *Rdl* gene, rendering them 10–50-fold less sensitive to PTX (Buckingham et al., 1996; ffrench-Constant et al., 1991; Lees et al., 2014)). Thus, we conclude that DanioVision™, in conjunction with Ethovision® XT software, can be employed to accurately detect changes in the locomotor activity of *Drosophila* larvae. In addition to the logistic benefits of this method, we are also able to decrease the likelihood of human error

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and potential for bias, thereby improving the validity, objectivity and reproducibility of larval locomotor activity assessments.

2. Materials and methods

2.1. Fly stocks and husbandry

Rdl$^{MD\text{-}RR}$ mutant *Drosophila*, stock number 35492, were purchased from Bloomington *Drosophila* Stock Center (Bloomington, IN, USA). *Wild-type* flies were obtained from Carolina Biologic Supply (Burlington, NC, USA). Flies were housed in 28.5 × 95 mm vials and fed with blue Formula 4–24® Fly Food prepared according to the instructions (Carolina Biologic Supply, Burlington, NC, USA). Flies were housed in an incubator at 25 °C on a 12 h light/dark cycle.

2.2. Larval isolation

Adult male and female *Drosophila* were transferred into vials with freshly prepared food and mated for 24 h before being passed. After approximately 4 days, third instar larvae were harvested from the food by floating them in a 20% sucrose solution (Nichols et al., 2012) and then transferred to a 50 mL Falcon™ tube containing 20% sucrose.

2.3. Drug administration

Approximately 15 larvae were randomly distributed to vials containing vehicle or PTX. PTX (0.03 to 3.0 mM) was initially dissolved in a small volume of DMSO, and then the appropriate volume of 20% sucrose was added. This was followed by gentle heating and vigorous stirring to ensure that the PTX was in solution. The final concentration of DMSO was 0.3%, which is lower than the effect-inducing threshold for this solvent (Nazir, Mukhopadhyay, Saxena, & Chowdhuri, 2003). Larvae were incubated in drug or vehicle (20% sucrose with 0.3% DMSO) for 2 to 24 h. Picrotoxin (PTX) and dimethyl sulfoxide (DMSO) were obtained from Sigma-Aldrich (St. Louis, MO, USA).

2.4. Data collection, reduction and analysis

The activities of six larvae were simultaneously and individually tracked using an infrared analog camera installed in the DanioVision™ Observation Chamber. Five mL of 2% agar (EMD Millipore, Billerica, MA, USA) was added to each well of a non-sterile 6-well tissue culture plate. Larvae were randomly selected from each treatment vial and placed in a well assigned in pseudorandom order. The plate was placed on a back-lit holder suitable for standard multi-well tissue culture plates with an infrared light source below the plate. The camera was connected to a USB port on the EthoVision® XT (Noldus Information Technology, Leesburg, VA) computer to digitize the analog signal.

In addition to converting the analog signal to a digital signal, the EthoVision® XT software was used to define the arenas (i.e. wells) to allow for the tracking of the movement of the larvae only while they were in their assigned well, and not if/when they escaped by crawling out of the well. The arena size was calibrated in cm using the "scale arenas" function, and the sampling rate for data digitization was 25 Hz. Trial control software was used to adjust the detection settings to allow for the accurate quantitative determination of movement in the X and Y directions in individual larvae. Larvae were tracked for 5 min with the DanioVision White Light set at 50% illumination in the closed observation chamber at ambient temperature.

Using the same software, the 5 min of data for an individual larva was reduced into 5, 1-min values. The software also reported missing data which would occur if the larva had escaped its well or if the detection settings were not adjusted with complete accuracy. When missing data for any 1 min of data exceeded 10%, that minute's data was discarded and not included in the final calculation. If the missing data was less than 10% but greater than 0%, the missing points were filled in using an interpolation function in the software. The video was then reviewed on the same screen as the data were replayed to be sure that the interpolated data points fit the observed path taken by the larva. If there were no missing data points there was no data review. The 1-min values were averaged to obtain a single distance traveled for each larva. When no review was required, which occurred very frequently, all of this data reduction was complete in less than 1 min and the data for each of the 6 larvae were exportable in a CSV file format. Additional and variable time was required for data review depending on the amount of missing data.

2.5. Statistics

All data are presented as the mean ± 1 standard deviation of the mean. The number of animals in each treatment group is indicated in the tables and figure legends. Data were initially analyzed by one-way or two-way analysis of variance (ANOVA), as appropriate. Only when a significant interaction term was reported, differences between treatment groups and their appropriate vehicle-treated group were determined using Tukey's post-hoc test. In those studies in which the data were analyzed by two-way ANOVA, differences among individual treatment groups were determined only if there was a significant interaction term. Significant main effects (treatment and time) are not indicated on figures, but are noted in figure captions. A p-value of ≤0.05 was considered significant in all analyses.

3. Results

3.1. PTX reduces locomotor activity independent of time

Locomotor activity in *Drosophila* larvae can be used to analyze the effects of anti-epileptic drugs. Studies were conducted to demonstrate that the DanioVision™ Observation Chamber can be employed to accurately assess locomotor activity in 3rd instar larvae, while simultaneously reducing experimenter bias. To that extent we chose to conduct experiments analogous to those of Stilwell et al. (2006) with slight variation in PTX concentration from 5 mg/mL (0.8 mM) to 1.0 mM PTX. In order to determine whether locomotor activity decreased in a time-dependent manner, third instar *wild-type* larvae were treated for 2, 4 or 24 h in vehicle or 1.0 mM PTX (Fig. 1). While PTX significantly reduced locomotor activity, there was no significant interaction between treatment duration and activity suggesting that the decrease was not time-dependent. Due to the lack of time-dependency, a single incubation time, 4 h, was chosen to use in further studies because it subjectively appeared to produce the greatest effect of PTX and was convenient with respect to completing an entire experiment in a given day.

3.2. PTX decreases locomotor activity in a concentration-dependent manner

In order to establish a working concentration for locomotor assays using DanioVision™, *wild-type* larvae were exposed to vehicle or varied concentrations of PTX (0.03, 0.3, or 3.0 mM) for 4 h (Fig. 2, upper panel). PTX treatment at 3.0 mM caused a significant decrease in locomotor activity, whereas lower concentrations produced no significant effect. To refine the concentration dependency of the response, an intermediate concentration, 1.0 mM, was tested. Larvae were treated for 4 h in vehicle, or 0.3 mM, 1.0 mM or 3.0 mM PTX (Fig. 2, lower panel), which all yielded significantly decreased activity. Based on these results, a 4 h treatment in 1.0 mM PTX was used in all subsequent experiments with *wild-type* larvae.

3.3. PTX requires normal Rdl activity to reduce larval locomotion

Similar to *wild-type* larvae, there were no significant time × dose interactions of PTX, 3.0 mM on locomotion in *Rdl*$^{MD\text{-}RR}$ larvae (Fig. 3).