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Measuring Movement to Determine Physiological Roles of Acetylcholinesterase Classes in *Caenorhabditis elegans*

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Abstract: A difference in movement has been hypothesized to exist between *Caenorhabditis elegans* strains lacking one of two main genes for acetylcholinesterase (AChE), *ace-1(+)* and *ace-2(+)*. We explored the precision of movement as an endpoint by measuring and comparing the movements of these strains (VC505 and GG202, respectively) and of N2 (wild-type). The order of movement of the strains is: N2 > VC505 > GG202; therefore, loss of the *ace-2(+)* gene is more detrimental to movement. We then compared the sensitivities of the three strains to an AChE inhibitor (propoxur) by generating movement-concentration curves, identifying effective concentrations that decreased movement by 50% (EC_{50}), and comparing them. EC_{50} show an order of: N2 ≈ GG202 < VC505. Therefore, the enzymes encoded by *ace-1(+)* were more susceptible to propoxur than those of *ace-2(+)*, suggesting that the innate difference in the AChE classes' contributions to movement will not always determine the strain sensitivity. Measuring movement was sufficiently precise to record differences following genetic manipulation and further chemical exposure.

Key words: acetylcholinesterase, behavior, *Caenorhabditis elegans*, genetics, inhibitor, method, movement, physiology, technique.

We are investigating whether measuring the movement of individuals from different populations of *Caenorhabditis elegans* following chemical exposure and/or genomic modification could be a useful endpoint to study the physiological roles of biomolecules of interest or an endpoint in chemistry-to-gene screens. Many studies have used *C. elegans*' movement to assess phenotypic behavior following genomic modification and/or chemical treatment. Examples include subjective observation, timing the worms' ability to escape from a chemo-repellant and measuring the frequency and amplitude of the trail left by individual worms on an agar plate. Technological advances now make it possible for our lab to measure the individual movements of up to 400 worms simultaneously, increasing the power and precision of these types of measurements. Our lab, as well as pharmaceutical companies (Williams et al., 2000; Dengg and van Meel, 2004; Jones et al., 2005) and the National Toxicology Program (http://ntp.niehs.nih.gov), are investigating the free-living nematode as a potential high-throughput toxicological organism. We are currently evaluating compounds affecting the acetylcholinesterase (AChE) enzymes of *C. elegans*.

Caenorhabditis elegans has three functioning classes of AChE: A, B and C. Of these, AChE A, which is encoded by the gene *ace-1(+)* on the X chromosome (Johnson et al., 1981), and AChE B, which is encoded by the *ace-2(+)* gene on chromosome I (Culotti et al., 1981), are responsible for most of the hydrolysis of acetylcholine in *C. elegans*. AChE C, which is encoded by the gene *ace-3(+)* on chromosome II (Johnson et al., 1988), accounts for <5% of the total AChE activity measured in the N2 wild-type strain, but is not required for normal function (Combes et al., 2000). A fourth gene also exists, *ace-4(+)*, which is thought to encode a non-functional protein (Johnson et al., 1988). Early histochemical staining showed a wide overlap of AChE A, B and C distribution in *C. elegans*' tissues (Culotti et al., 1981; Johnson et al., 1988). Later studies using Green Fluorescing Protein (GFP) expression found *ace-1(+)* in all body-wall and vulval muscle cells (Culetto et al., 1999), *ace-2(+)* almost exclusively in neurons and *ace-3(+)* in several muscle cells of the pharynx and in the two Canal Associated Neurons (CAN cells) (Combes et al., 2003). Despite the distinct areas of expression, however, loss of either the *ace-1(+)* or *ace-2(+)* gene produces a worm with no observable movement defect, whereas loss of both *ace-1(+)* and *ace-2(+)* produces a mutant whose movement is clearly impaired (Culotti et al., 1981; Johnson et al., 1988). Additionally, AChE activity in the strain VC505 (lacks *ace-1(+)* activity) and the strain GG202 (lacks *ace-2(+)* activity) are roughly equivalent (Culotti et al., 1981). Possible explanations for the functional redundancy between *ace-1(+)* and *ace-2(+)* despite differing areas of expression include: (i) acetylcholine escapes from a synapse and migrates to a muscle cell and is hydrolyzed, or vice versa, or (ii) AChE migrates between muscle cells and neurons (Johnson et al., 1988). It has been hypothesized that subtle differences in locomotion might also exist between the VC505 and GG202 strains (Culotti et al., 1981; Johnson et al., 1981), although it has never been proven.

In this study, we explored the utility of measuring the endpoint of movement by noting whether we could measure a difference in movement between two mutant strains, VC505 and GG202, the *ace-1(+)* and *ace-2(+)* deficient mutants, respectively. Next, we explored how the functional redundancy of *C. elegans*' AChE classes affects the precision of measuring movement by exposing the mutant and N2 wild-type strains to an AChE inhibitor, the carbamate pesticide propoxur, and noting differences in sensitivity.

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Materials and Methods

Culture of nematodes: We obtained *C. elegans* N2 wild-type strain, *ace-1(+)* deficient strain VC505 and *ace-2(+)* deficient strain GG202 from the *Caenorhabditis* Genetics Center (Minneapolis, MN). We raised all developmental stages of all strains of *C. elegans* in 115-mm-diam. petri dishes with solid-medium K-agar (0.032 mM KCL, 0.051 mM NaCl, 0.1 mM $CaCl_2$, 0.1 mM $MgSO_4$, 2.5% Bacto-peptone, 0.17% Bacto-agar and 0.01% cholesterol), a modification of Williams and Dusenbery (1988). We seeded the plates with *Escherichia coli* strain OP50 as a food source and incubated at 20°C for 24 hr (Brenner, 1974). To age-synchronize the populations, we harvested eggs from adult populations. Two-day-old J populations (L4 larvae) were transferred to a plate containing a fresh lawn of OP50 to maintain high nutritional status before testing on d 3 (Boyd et al., 2003). All exposures were carried out using 3-d-old adults.

Exposure chemicals and conditions: Stock solutions of propoxur (Sigma-Aldrich, St. Louis, MO) were made in K-medium, and further dilutions were made by adding additional K-medium (0.032 mM KCl, 0.051 mM NaCl in dH_2O) (Williams and Dusenbery, 1990). Exposures for assessment of movement used a 12-well sterile tissue culture plate, on which we loaded 5 µl (approximately 100 worms) into a single 1-ml well containing either 1 ml of propoxur or K-medium for the control well. We prepared a control population for each exposure. All exposure plates were then placed in an incubator at 20°C for 4 hr in the absence of food (Anderson et al., 2001; Anderson et al., 2004). To control for any daily variation between worm populations collected from different egg populations and raised in separate cultures, we normalized all replicates to their control by dividing the movement of a single exposure concentration by its control's measured value, creating a percent of control.

Movement Tracking: We patterned the movement tracking after Boyd et al. (2000) and Dhawan et al. (1999). Immediately following the 4-hr exposure, we transferred the worms with a Pasteur pipette into 2-ml glass centrifuge tubes. They were washed by allowing the worms to gravity settle into a pellet, removing the supernatant, adding 1.5 ml of fresh K-medium, gently mixing the worms by creating bubbles with a Pasteur pipette and repeating the process for a total of three washes. We then transferred 5 µl of the settled pellet (~50–80 worms) to a cooled, 2-ml 1% agar pad (1% Bacto-Agar, 0.3% NaCl in dH_20) on a clear glass slide measuring 100 mm × 200 mm. The worms were allowed to disperse on the agar pad inverted over a petri dish filled with water to avoid desiccation. We began movement tracking at exactly 1 hr after the end of exposure for each replicate. We placed the individual glass slides in a tracking chamber with a gentle stream of humidified air. Using a video camera interfaced with a Macintosh computer that contained a modification of the NIH image, v1.59 computer tracking program (Georgia Institute of Technology, Atlanta, GA), the individual worm movements were tracked and recorded to an Excel spreadsheet. We used a macro to calculate the average distance of movement per worm per second in µm.

pH measurement: pH values were measured to ensure that they were in the range of 5.8 (K-medium alone) to 3.5. No effects of pH on *C. elegans*' movement have been observed in this pH range (Cole et al., 2004). For this reason, to facilitate comparisons with historical data, and to prevent any interaction between the buffer and propoxur, we did not use a buffer. We measured the pH values for the highest and lowest concentrations of propoxur using an Orion Z20A pH meter at room temperature (22–24°C) (Orion Research, Beverly, MA).

Statistical Analysis: Normality of VC505 and GG202 distributions was tested using Shapiro-Wilk. We modeled the relationships between movement and concentration for each strain using nonlinear regression, PROC NLIN (SAS Inc., Cary, NC), and we generated an EC_{50} value (effective concentration that decreases movement by 50%) along with its 95% confidence interval for each strain.

Results and Discussion

We measured the movements of unexposed samples of the VC505 and GG202 strains to determine whether AChE type differentially affects movement. Because all three functional AChE genes are independently expressed and *ace-3(+)* constitutes <5% of AChE activity of the wild-type N2 strain, the double mutant strain (GG201) was not considered in movement analyses. By using deletion mutants lacking one of the two major forms of AChE, we determined that strain VC505, possessing class B AChE expressed almost exclusively in neurons, moved at 83% the rate (2.91/3.52 µm/sec) of N2 wild-type, while strain GG202, possessing class A AChE expressed almost exclusively in body-wall and vulval muscle cells, moved at 54% the rate (1.90/3.52 µm/sec) of N2 wild-type (Table 1). Therefore, loss of the *ace-2(+)* gene (class B AChE) is more deleterious to *C. elegans'* movement than loss of the *ace-1(+)* gene (class A AChE). To our knowledge, this is the first attempt to

Table 1. Mean average rates of movement of N2 wild-type, VC505 and GG202 strains.

Strain	Mean movement rate (µm/sec)	95% Confidence interval
N2	3.52[a]	(3.41, 3.62)
VC505	2.91[a]	(2.53, 3.30)
GG202	1.90[a]	(1.55, 2.25)

[a] Difference significant to $p < 0.001$

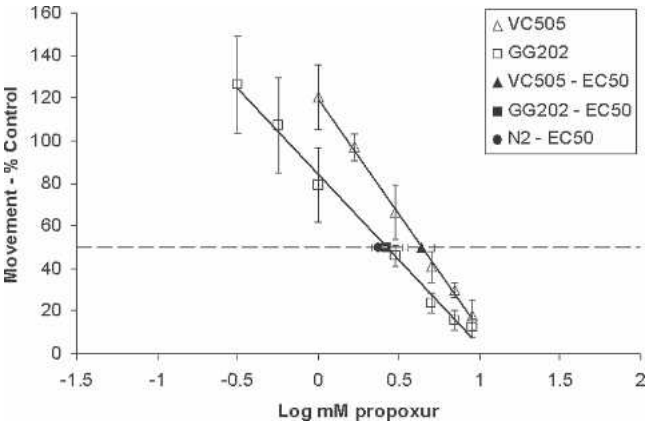

FIG. 1. Movement rate as a percent of control is shown for VC505 and GG202 strains vs. log mM propoxur. The effective concentration required to reduce movement by 50% (EC_{50}) of GG202 (■) shows greater sensitivity to propoxur than the EC_{50} of VC505 (▲) and is nearly identical to the N2 wild-type EC_{50} value (●).

quantitatively determine whether a difference exists between the movements of these two mutant strains.

We also compared the changes in movement of the N2, VC505 and GG202 strains following exposure to an AChE inhibitor, propoxur, by generating movement-concentration curves (Fig. 1). EC_{50} values were indistinguishable between N2 and GG202 populations and least among VC505 populations when movement values were normalized to each strain's respective control values (Table 2). Therefore, EC_{50} values show the movement of worms possessing only the neuronal class B AChE (VC505) to be less sensitive to inhibition of movement by propoxur than worms possessing only the class A AChE (GG202), despite the absence of AChE B having a greater effect on movement than the absence of AChE A. In fact, the decline in the movement rate of the GG202 strain was approximately equal to that of the N2 wild-type strain, while a decline in the movement rate of the VC505 strain did not occur until approximately 40% inhibition of the N2 strain's movement. Therefore, inhibition of the class A AChE likely drove the initial decrease in movement of the N2 stain.

Several conclusions can be drawn from these data. First, the data suggest that a difference exists between the movement rates of the *ace-1(+)* and *ace-2(+)* deletion mutants. Second, in the case of *C. elegans*' AChE classes, the different innate contributions to movement will not always dictate which class has a greater affect on movement decrease when exposed to inhibitors. In this respect, the innate difference in contributions to movement is less important than the absorption, distribution and binding affinity of the chemical inhibitor. Therefore, although the precision of movement as an endpoint for compounds affecting the AChE enzymes of *C. elegans* is limited by the functional redundancy of this enzyme system, the extent to which it is limited is a function of the preferential affinity of an inhibitor towards one AChE class over the other, but not the innate difference in movement. The significance of this preferential affinity is very small with propoxur, as a difference in estimated EC_{50} of ~2 mM is well within the error tolerance for a toxicological screen. In past studies, we examined the effects of reversible and irreversible inhibitors (24 compounds in total) on *C. elegans*' AChE enzymes and found significant correlations to mammals (Anderson et al., 2004; Cole et al., 2004; Melstrom and Williams, 2007). This suggests that the preferential affinities of inhibitors towards one AChE class over the other are not significant for many compounds.

TABLE 2. Effective concentrations of propoxur required to decrease movement of N2 wild-type, VC505 and GG202 strains by 50% (EC_{50}).

Strain	EC_{50} (mM)	95% Confidence interval
N2	2.35	(2.00, 2.77)
VC505	4.34[a]	(3.58, 5.24)
GG202	2.66	(2.14, 3.30)

[a] Difference significant to $p < 0.05$

In summary, measuring average rates of movement across *C. elegans* populations was sufficiently precise to show a difference in the contributions of the two main classes of AChE towards movement in *C. elegans*. In the case of the inhibitor, propoxur, its absorption, distribution and binding affinity were more influential on *C. elegans'* movement than this innate difference. From this data, we were able to discern the effects of propoxur on each enzyme class. We believe that using movement as a measured endpoint may be a useful technique to gain information about the physiological roles of other biomolecules or in other chemistry-to-gene screens using *C. elegans*.

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