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Planarian cholinesterase: molecular and functional characterization of an evolutionarily ancient enzyme to study organophosphorus pesticide toxicity

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Abstract

The asexual freshwater planarian *Dugesia japonica* has emerged as a medium-throughput alternative animal model for neurotoxicology. We have previously shown that *D. japonica* are sensitive to organophosphorus pesticides (OPs) and characterized the *in vitro* inhibition profile of planarian cholinesterase (DjChE) activity using irreversible and reversible inhibitors. We found that DjChE has intermediate features of acetylcholinesterase (AChE) and butyrylcholinesterase (BChE). Here, we identify two candidate genes (*Djche1* and *Djche2*) responsible for DjChE activity. Sequence alignment and structural homology modeling with representative vertebrate AChE and BChE sequences confirmed our structural predictions, and show that both DjChE enzymes have intermediate sized catalytic gorges and disrupted peripheral binding sites. *Djche1* and *Djche2* were both expressed in the planarian nervous system, as anticipated from previous activity staining, but with distinct expression profiles. To dissect how DjChE inhibition affects planarian behavior, we acutely inhibited DjChE activity by exposing animals to either an OP (diazinon) or carbamate (physostigmine) at 1μM for 4 days. Both inhibitors delayed the reaction of planarians to heat stress. Simultaneous knockdown of both *Djche* genes by RNAi similarly resulted in a delayed heat stress response. Furthermore, chemical inhibition of DjChE activity increased the worms' ability to adhere to a substrate. However, increased substrate adhesion was not observed in *Djche1/Djche2 (RNAi)* animals or in inhibitor-treated day 11 regenerates, suggesting this phenotype may be modulated by other mechanisms besides ChE inhibition. Together, our study characterizes DjChE expression and function, providing the basis for future studies in this system to dissect alternative mechanisms of OP toxicity.

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Keywords

acetylcholinesterase; planarians; organophosphorus pesticides; behavior; heat stress

Introduction

Organophosphorus pesticides (OP) are among the most agriculturally important and common pesticides used today. In the United States, 20 million pounds of OPs were used in 2012, accounting for 33% of all insecticides used (Atwood and Paisley-Jones 2017). Similarly, in 2014, Spain, France, Italy, Germany, and Poland, which together make up 72.7% of the European Union's pesticide sales (EUROSTAT 2016), had a combined usage of 4642 tonnes (~10 million pounds) of OPs according to the Food and Agriculture Organization of the United Nations (http://www.fao.org/faostat/en/#data/RP). The primary shared mode of action of these pesticides is to inhibit the enzyme acetylcholinesterase (AChE), an essential regulator of cholinergic nerve transmission (Russom et al. 2014; King and Aaron 2015; Taylor 2017). Inhibition of AChE, which catalyzes the hydrolysis of the neurotransmitter acetylcholine (ACh), results in increased levels of synaptic ACh and subsequent overstimulation of nicotinic and muscarinic ACh receptors. Cholinergic toxicity is clinically manifested by decreased heart and respiration rates, increased secretions (sweating, lacrimation, and salivation), muscle tremors, and eventually paralysis and death (Eleršek and Filipic 2011; Russom et al. 2014; King and Aaron 2015; Taylor 2017). Because of its key role in regulating cognitive, peripheral autonomic, and somatic motor functions, AChE is also a common pharmacological target. For example, Alzheimer's disease, glaucoma, and myasthenia gravis have been treated with carbamate AChE inhibitors, such as physostigmine, and OPs, such as phospholine (echothiophate) iodide (Giacobini 2000; Pope et al. 2005; Taylor 2017).

At high doses, OPs are lethal to both insects and humans due to inhibition of AChE and subsequent cholinergic toxicity. However, there have been growing concerns that chronic, low dose exposure to these pesticides can also cause harm. Epidemiological studies have suggested a correlation between pesticide exposure and neurodegenerative diseases (Sánchez-Santed et al. 2016). Similar correlations have also been found linking prenatal and early life OP exposure to cognitive impairment in children (Muñoz-Quezada et al. 2013; Shelton et al. 2014; González-Alzaga et al. 2014).

In addition to inhibiting AChE function, studies have suggested that some chronic (Ray and Richards 2001; Terry 2012) and/or developmental (Timofeeva et al. 2008a; Timofeeva et al. 2008b) toxic outcomes may be independent of OPs' effects on AChE. This idea is corroborated by findings in *in vivo* and *in vitro* rat studies showing that OPs can have effects on a variety of cellular processes, such as cell signaling, oxidative stress, and axonal growth, at concentrations which do not significantly inhibit AChE (Slotkin and Seidler 2007; Yang et al. 2008). However, the degree that these secondary effects relate to specific toxic endpoints remains unclear.

Ach can also act as a neuromodulator to dynamically regulate the state of neurons, including but not limited to cholinergic neurons, in response to changing conditions (Picciotto et al.

2012). For example, feedback loops exist to regulate the levels of ACh synthesis, release, uptake, and receptor binding. Thus, chronic exposure to OPs may trigger compensatory mechanisms to adapt to chronically elevated Ach levels. The extent that adaptive mechanisms modulate specific toxic outcomes, and whether these mechanisms can be affected by secondary effects of OPs (Pope et al. 2005), warrant further investigation.

The freshwater planarian *Dugesia japonica* has recently emerged as a valuable *in vivo* model for neurotoxicity studies, with particular focus on neurodevelopment (Hagstrom et al. 2015; Hagstrom et al. 2016). This asexual species naturally reproduces through transverse fission. Herein, the worm splits itself into two pieces which, due to a large population of adult stem cells (Rink 2013), subsequently regenerate all missing body structures, including the central nervous system (CNS). In these animals, regeneration is the sole mechanism of neurodevelopment and shares fundamental processes with vertebrate neurodevelopment (Cebrià et al. 2002b; Cebrià et al. 2002a; Cebrià and Newmark 2005; Umesono et al. 2011; Cowles et al. 2013). Distinct from other animal models, the similar sizes of full and regenerating planarians allows for a direct comparison of the effects of neurotoxicants on brain development and function with the same behavioral assays (Hagstrom et al. 2015; Hagstrom et al. 2016). Using a custom planarian screening platform (Hagstrom et al. 2015), we showed that planarians are sensitive to OPs as subchronic exposure to sublethal concentrations of dichlorvos (10–500nM) caused reduced rates of locomotion, with greater effects on regenerating rather than adult animals.

Furthermore, using activity measurements in planarian homogenates, we have recently demonstrated that planarian cholinesterase, DjChE, has intermediate characteristics of AChE and the closely related butyrylcholinesterase (BChE) (Hagstrom et al. 2017). Moreover, DjChE underwent similar rates of inhibition by OPs and carbamates as mammalian AChE, suggesting similar levels of sensitivity. We predicted that the enzyme(s) responsible for DjChE activity would be defined by a conserved catalytic triad and choline binding site, an active site gorge that is larger than that of AChE but smaller than BChE, and a disrupted peripheral anionic site. However, these predictions remained to be verified through structural analysis, and the *in vivo* expression profile of the enzyme(s) was unknown. Moreover, a direct link between *in vivo* inhibition of DjChE activity and the functional consequences on planarian behavior is still missing. Herein, we verify our *in vitro* predictions by identifying and characterizing the expression and function of two candidate genes responsible for DjChE activity *in vivo*.

Using RNA interference (RNAi), we further compared the effects of simultaneous knockdown of both *Djche* genes with those induced by ChE inhibitors (diazinon and physostigmine) on planarian locomotion, the animals' response to heat stress, and substrate adhesion. These endpoints were chosen based on our previous results that OPs can cause decreased planarian locomotion (Hagstrom et al. 2015), findings in nematodes that increased Ach levels caused heat stress tolerance (Furuhashi and Sakamoto 2016), and the use of hyper-secretions as one of the clinical hallmarks of cholinergic toxicity (Eleršek and Filipic 2011; Taylor 2017). Comparison of acute and subchronic developmental exposure of these endpoints suggests the existence of secondary effects on non-ChE targets to modulate the functional outcomes of OP toxicity.

Together, we structurally and functionally characterize DjChE and demonstrate a direct link between *in vivo* inhibition of DjChE activity and functional consequences on planarian behavior. This work therefore lays the foundation for the dissection of the mechanisms underlying OP toxicity in planarians.

Materials and Methods

Planarian culture

Freshwater asexual planarians of the species *Dugesia japonica* were used for all experiments. For behavioral experiments, animals used were 5.4 ± 1.1 mm (mean ± standard deviation) in length. Planarians were maintained in 1x Instant Ocean (IO, Blacksburg, VA) in Tupperware containers at 20°C in a Panasonic refrigerated incubator in the dark. Animals were fed organic chicken or beef liver 1–2x/week and cleaned twice a week when not used for experiments. Animals were starved for at least 5 days before experiments.

Identification and cloning of Djche

D. japonica homologs of acetylcholinesterase (AChE) were found using NCBI tBLASTn to query the deduced amino acid sequence of *Schistosoma mansoni* AChE (GenBank AAQ14321.1) (Bentley et al. 2003) against a *D. japonica* transcriptome. The transcriptome was assembled *de novo* from published sequencing data (Qin et al. 2011) using EBARDenovo (Chu et al. 2013). Two potential *ache* homologs were identified in *D. japonica* and crosschecked against the ESTHER protein database (Lenfant et al. 2013) to align most closely with acetylcholinesterase. Since we recently determined that *D. japonica* cholinesterase activity has characteristics of a hybrid AChE/BChE (Hagstrom et al. 2017), we termed these candidate sequences as *Djche1* and *Djche2*. The deduced amino acid sequences were determined from the longest open reading frame found using NCBI ORF finder (https://www.ncbi.nlm.nih.gov/orffinder/). Sequence alignments were performed in JalView (Waterhouse et al. 2009).

RNA was extracted from recently amputated *D. japonica* head fragments using an RNeasy Mini Kit (Qiagen, Germantown, MD). Head cDNA was created using a SuperScript III First Strand Synthesis Reverse Transcriptase Kit (Invitrogen, Carlsbad, CA). Approximately 700 and 1000 bp fragments of *Djche1* and *Djche2*, respectively, were amplified from this cDNA by PCR using the following primers: Djche1_F: TCGAAACGCTATAATGGAATCCG, Djche1_R: AGGTTGGCAATGTTACTGTACG, Djche2_F: TTGGCAAGCTGATGGAAGTG, Djche2_R: CCAGCCGGTTATAGTTGAAGG. These fragments were subsequently cloned into the pPR-T4P vector.

Homology modeling of DjChE structure

Individual amino acid sequences of the two candidate DjChEs were submitted to Swiss-Model, a homology based 3D structure creation server (https://swissmodel.expasy.org/). The server searched its template library for evolutionary related structures matching the target sequence resulting in identification of several hundred potential templates. Template quality has been predicted from features of the target-template alignment and three of those with the highest quality were then selected for model building (Arnold et al. 2006; Benkert et al.

2011; Biasini et al. 2014). For both DjChE structures, *Torpedo californica* AChE was selected as the template (2cek and 2w6c, respectively). For comparisons in Figure 2, the 2w6c structure is shown. Additional details on model building can be found in Supplementary Materials.

In situ hybridization

Anti-sense digoxigenin (DIG) or fluorescein labeled probes were synthesized using T7 RNA polymerase essentially as described in (King and Newmark 2013). Planarian fixation and subsequent *in situ* hydridization were performed as in (King and Newmark 2013) with a few modifications: initial mucus removal was performed by treating with 2% hydrochloric acid in phosphate buffered saline (PBS) for 45 seconds with hand-inversion; animals were bleached overnight in 6% hydrogen peroxide in methanol under bright white light and subsequently rehydrated in 50% MeOH/50% PBSTx (0.3% Triton-X 100 in PBS); and hybridization was performed at 60ºC overnight.

For co-localization experiments, a double fluorescent *in situ* hybridization (FISH) was performed using a combined POD-based tyramide development and AP-based Fast Blue development (Brown and Pearson 2015). Briefly, hybridization was performed concurrently with both DIG- and fluorescein-labeled riboprobes. Following post-hybridization washes, the samples were blocked in 5% horse serum and 0.5% Roche Western Blocking Reagent (RWBR, Roche, Indianapolis, IN) in MABT (150mM NaCl, 100mM Maleic Acid, 0.1% Tween 20, pH 7.5) at room temperature for 3–4 hours and treated overnight at 4˚C with a mix of anti-fluorescein-POD and anti-DIG-AP antibodies (both from Roche and diluted 1:2000 in 5% horse serum/0.5% RWBR). Following fluorescein tyramide development of the POD antibody, the samples were washed four times for 5–10 minutes with MABT. An AP-based Fast Blue development was then performed for colorimetric and fluorescent (far red) detection of the DIG-labeled riboprobe, as described in (Brown and Pearson 2015). Samples were mounted on glass slides and imaged on an inverted IX81 spinning disc confocal microscope (Olympus DSU) using an ORCA-ER camera (Hamamatsu Photonics) and Slidebook software (version 5, Intelligent Imaging Innovations, Inc).

Chemical Exposure

To analyze the effects of inhibition of ChE catalytic activity, planarians were exposed to 1μM physostigmine (eserine) or diazinon (both from Sigma-Aldrich, Saint Louis, MO). These concentrations were chosen because preliminary experiments determined they were not systemically toxic or lethal. Lack of systemic toxicity was demonstrated by the absence of lethality or morphological abnormalities for up to 12 days of exposure (Fig. S1) and by the absence of regeneration delays (Fig. S4). Exposure solutions were prepared in IO water from 200X stocks solution in dimethyl sulfoxide (DMSO, Sigma-Aldrich) to have a final concentration of 0.5% DMSO. While others have suggested DMSO concentrations used with planarians should not exceed 0.1% (Pagán et al. 2006), we found 0.5% did not have a significant effect on planarian behavior (Hagstrom et al. 2015). Control animals were treated with 0.5% DMSO. Solutions were replaced daily to keep concentrations constant. During exposure, worms were kept in 12-well plates (Genesee, San Diego, CA) containing one worm and 1ml of chemical per well and stored in the dark at room temperature. Gliding and

heat stress assays were performed on day 4 of exposure and stickiness assays on day 5. For experiments with regenerating animals, intact planarians were decapitated with an ethanol-sterilized razor blade. The tail pieces were placed in 12-well plates and exposed to inhibitor solutions within 1 hour of amputation. Gliding and heat stress assays were performed on day 11 of exposure/regeneration and stickiness assays on day 12. Experiments were performed in IO water.

Cholinesterase activity assays

Qualitative detection of ATCh or BTCh catalysis in fixed worms was performed as previously described (Zheng et al. 2011; Hagstrom et al. 2017), except staining incubation was decreased to 3.5 hours to gain the sensitivity needed to detect differences in activity in inhibitor-treated and knockdown animals.

To quantify the extent of ChE inhibition in inhibitor-treated planarians, 30 planarians were exposed to either 0.5% DMSO, 1μM diazinon, or 1μM physostigmine for 5 or 12 days, as described above. At the end of exposure, the planarians were washed three times with IO water and homogenized in 100μl 1% Triton X-100 (Sigma-Aldrich) in PBS as previously described (Hagstrom et al. 2017). Levels of acetylthiocholine (ATCh) catalysis (ChE activity) were determined by an Ellman assay (Ellman et al. 1961) using 1mM ATCh (Sigma-Aldrich) as a substrate, as previously described (Hagstrom et al. 2017). Activity measurements were performed with at least 3 technical replicates per condition. Activity levels were normalized by protein concentration, determined by a Bradford Assay, and compared to the mean of the normalized levels in the DMSO controls in the same experiment (set as 100% activity). Data are shown as the mean and standard deviation of two independent experiments (biological replicates).

RNA interference (RNAi) experiments

Expression of *Djche1* and *Djche2* were knocked down in combination by feeding planarians organic chicken liver mixed with *in vitro* transcribed dsRNA mixed with food coloring, per standard protocols (Rouhana et al. 2013). Negative control populations, denoted as *control (RNAi),* were fed organic chicken liver mixed with dsRNA of the *unc22* gene, a non-homologous *C. elegans* gene. All RNAi treated populations were fed twice per week and cleaned three times per week. To speed up knock down, some RNAi worms were injected directly with the respective dsRNA (1μg/μl per gene). Injections were performed on intact animals daily for 4 consecutive days (Takano et al. 2007) using a Pneumatic PicoPump, Model PV 820 (World Precision Instruments, Sarasota, FL). One day after the last injection, the planarians were decapitated using an ethanol-sterilized razor blade. Animals were allowed to regenerate for 11 days before behavioral analysis.

Behavioral assays

Gliding—Six contact lens containers (Wöhlk Contactlinsen, Schönkirchen, Germany) containing one planarian each and 1.5 mL IO water were placed on a LED panel (Amazon, Seattle, WA). The planarians were allowed to glide undisturbed for 10 minutes while imaging from above using a Basler camera (A601f-2, Basler, Germany), mounted on a ring stand. Assays were typically run with n=12 (2 sets of 6) animals per experiment for each

condition. At least 2 independent experiments were run per condition. Gliding movies were analyzed as previously described in detail in (Hagstrom et al. 2015).

Heat stress—A single planarian was pipetted into 2 mL IO water into a 35 mm petri dish (CELLTREAT Scientific Products, Pepperell, MA). Of note, we also tried Falcon (Corning, NY) 35 mm petri dishes, but found that planarians in the CELLTREAT brand were easier to image because they spent relatively less time at the container edges. To create a high temperature environment, we used a peltier plate (TE Technology Inc., Traverse City, MI), which was controlled by a temperature controller (TE Technology, Inc.) and powered by an AC to DC power supply (Amazon). The plate was set to 52ºC and six dishes, with one planarian each, were heated for 10 minutes starting from room temperature. Thermistors were used to determine the dynamics of the aquatic temperature in the dishes over the course of the experiment (Fig. S2). The aquatic temperature stabilized after about 3 minutes to 30ºC and was consistent across all dishes and across multiple trials. The dishes were imaged from above using a Basler camera mounted to a ring stand. Lighting was provided via a red LED string light (Amazon) from above and surrounding the edges of the peltier. Assays were typically run with n=12 (2 sets of 6) animals per experiment and condition. At least 2 independent experiments were run per condition.

Analysis was performed using a custom MATLAB center-of-mass (COM) tracking script. The displacement of each worm across 12 second intervals was calculated in MATLAB. Displacement was scaled by body length and displacements under 1 body length were empirically determined to correspond to movements which were primarily body shape changes. The proportion of displacements under 1 body length to all tracked displacements was determined and binned across one minute intervals. The median value for each condition is shown, with error bars representing the 25 and 75% quantiles.

Worm Substrate Adhesion (“Stickiness”)—The stickiness of planarians was determined based on the worms’ ability to adhere to a substrate as described in (Malinowski et al. 2017). In brief, an individual planarian was placed into a 3D printed plastic arena filled with 25ml of IO water and allowed to acclimate for approximately 2 mins. We then introduced a water flow and tested whether it was able to displace the worm from a fixed distance (~ 25mm). If displaced, the current flow rate was recorded with a Hall sensor (Amazon). If not displaced, the flow rate would be increased in discrete steps until displacement occurred.

Regeneration assay—The rate of blastema growth was determined as described in (Hagstrom et al. 2015). For chemical treatment, exposure began immediately (within 1 hour) after decapitation.

Statistical Analysis—Since all data for gliding, heat stress, and substrate adhesion experiments were not normally distributed, statistical analysis was done using the Wilcoxon rank sum test (Mann Whitney test) in MATLAB.

Results

D. japonica has two candidate genes encoding cholinesterase

We assembled a *D. japonica* transcriptome *de novo* using published sequencing data (Qin et al. 2011) as described in Materials and Methods. Two putative transcripts encoding cholinesterase were found using NCBI tBLASTn to query the deduced amino acid sequence of *Schistosoma mansoni* AChE (GenBank AAQ14321.1) (Bentley et al. 2003) against the *D. japonica* transcriptome. We named the two corresponding candidate genes *Djche1* and *Djche2.* The deduced amino acid sequences of these genes were aligned with representative amino acid sequences for vertebrate AChE and BChE from *Torpedo californica* (TcAChE) and human (HsBChE), respectively (Fig. 1).

Both DjChE amino acid sequences contain essential catalytic residues for cholinesterase function, including the esterase-type catalytic triad (Ser200, Glu327, His440, numbering corresponding to TcAChE, per convention) and choline binding site (Trp84, Glu199, Phe330, Phe331). In agreement with our predictions based on *in vitro* inhibitor data (Hagstrom et al. 2017), both DjChE sequences seem to have intermediate characteristics between AChE and BChE (acyl pocket consisting of one phenylalanine, and an undefined peripheral anionic site at the rim of the gorge).

We further evaluated the protein structure of the candidate planarian cholinesterases by performing homology modeling using the published structure of TcAChE (Paz et al. 2009) (Fig. 2, Supplementary Material). The homology-based structures of DjChE1 and DjChE2 similarly agree with our previous structural predictions (Hagstrom et al. 2017). Particularly, in both DjChE1 and DjChE2 structures, the catalytic triad and choline binding site are well conserved. Conversely, with only one (F288) of two commonly found phenylalanines and the substitution of the Arg289 “anchor” with a smaller side chain, the acyl pocket volume is much larger and more flexible than that of TcAChE. Lastly, several of the largely aromatic residues that define the vertebrate peripheral anionic site (Tyr70, Asp72, Tyr 121, Trp279) have been substituted with smaller aliphatic side chains in the planarian structures resulting in a wider gorge opening. In summary, both planarian cholinesterase candidate genes have hybrid features of both AChE and BChE, similar to other invertebrate cholinesterase (see Discussion) (Sanders et al. 1996; Bentley et al. 2005; Pezzementi et al. 2011).

Djche1 and 2 are expressed in the planarian nervous system

Whole-mount fluorescent *in situ* hybridization (FISH) was performed to determine the expression patterns of *Djche1* and *Djche2* (Fig. 3). Similarly to the cholinergic marker, *Djchat* (Fig. 3A), *Djche1* is expressed widely throughout the planarian nervous system in both the anterior cephalic ganglion and ventral nerve cords (Fig. 3B). This mRNA expression profile agrees with cholinesterase activity stains which have shown cholinesterase enzymatic activity distributed throughout the planarian CNS (Hagstrom et al. 2017). *Djche2,* however, was found to be more ubiquitously expressed throughout the planarian body in a punctate pattern, with concentration of some puncta in the head region and along the nerve cords (Fig. 3C).

Next, we performed multi-color FISH to determine the extent that these important regulators of the cholinergic system co-localize (Fig. 4). As expected from the single FISH, expression of *Djche1* extensively overlapped with expression of *Djchat* (Fig. 4A). *Djche2* also showed partial co-localization with both *Djchat* and *Djche1*, particularly in the medial arc of the cephalic ganglion (Fig. 4B-C).

Inhibition of cholinesterase activity decreases sensitivity to heat stress

It has been previously shown in the nematode *Caenorhabditis elegans* that exogenous ACh exposure promotes thermo-tolerance. In these experiments, worms pre-cultured for 24 hours on plates containing ACh solution demonstrated increased survivability compared to controls after 10h incubation at 35°C (Furuhashi and Sakamoto 2016). Therefore, we assayed whether inhibition of ChE, which would increase synaptic ACh levels, affects planarians' response to heat stress. To this end, the animals' aquatic environment was slowly heated from room temperature to 30°C (Fig. S2) and the animals' reactions were monitored through video recordings (see Materials and Methods). Being higher than planarians' normal comfortable temperature range, 15–25 °C (Inoue et al. 2014), 30°C should induce heat stress while not induce scrunching, a planarian escape gait induced at 34–36°C (Cochet-Escartin et al. 2015). Solvent control animals (treated with 0.5% DMSO) reacted to the heat stress through frequent turns and head flailing, followed by decreased movement and eventual paralysis (Fig. 5A, Supplemental Video). This reaction was quantified by the fraction of time that the animals exhibited body shape changes rather than normal gliding behavior (see Materials and Methods). In control animals, the fraction of body shape changes gradually increased over time as the temperature rose and leveled out at approximately 0.9 once the temperature plateaued at 30° after 3 minutes (Fig. 5B).

To acutely inhibit DjChE activity, planarians were treated for 4 days with 1μM diazinon, an OP whose oxon metabolite efficiently inhibits DjChE activity *in vitro* (Hagstrom et al. 2017). Diazinon treated animals exhibited decreased sensitivity to heat stress, manifested in less body shape changes for a longer time (Supplemental Video). They eventually reached control levels by 10 minutes of heat exposure (Fig. 5A-B). To determine whether this phenotype was specific to inhibition of ChE activity, we also exposed worms to physostigmine, a carbamate ChE inhibitor that has been previously shown to inhibit planarian ChE activity *in vitro* (Hagstrom et al. 2017). Moreover, acute exposure to at least 3μM physostigmine has been shown to cause planarians to contract (Nishimura et al. 2010). Similarly to diazinon, 4 day exposure to 1μM physostigmine caused a delayed reaction to heat stress (Fig. 5A-B). Activity stains confirmed that under these exposure conditions, diazinon and physostigmine significantly inhibited DjChE activity (Fig. 5C). Quantitative measurements of DjChE in homogenates of exposed planarians further confirmed significant inhibition of DjChE compared to solvent controls (Fig. S3).

To verify that differences in the heat stress response were not due to general motility differences, we assayed the unstimulated locomotion of these animals. At the used concentrations, physostigmine and diazinon caused a significant decrease in gliding speed (Fig. S4A). Notably, we previously observed a decrease in gliding speed of full planarians

after exposure to dichlorvos for 8 days (Hagstrom et al. 2015), suggesting that this may be a shared phenotype of ChE inhibition.

Despite moving at a slower speed, the ChE inhibitor-treated animals had generally higher activity under heat stress than controls. Therefore, the heat stress phenotype is independent of the gliding phenotype.

Knockdown of both Djches causes decreased sensitivity to heat stress

To determine whether the toxic outcomes of the ChE inhibitors were specific to their action on ChE, RNAi was used to simultaneously knockdown expression of both *Djche1* and *Djche2.* At first, RNAi was administered through feeding of dsRNA mixed with chicken liver. However, this technique remained inefficient at establishing consistent knockdown even after prolonged feedings (greater than 1 year). To increase the efficiency of knockdown, planarians which were previously fed RNAi liver were injected with dsRNA for both genes for 4 consecutive days. The animals were decapitated 1 day after the last injection and allowed to regenerate for 11 days before being assayed for behavioral phenotypes. This protocol was followed, because amputation and subsequent regeneration following dsRNA injection has been shown to increase knockdown efficiency in the newly regenerated tissue in planarians (Takano et al. 2007).

Djche1/Djche2 (RNAi) animals did not display any defects in regeneration when compared to *control (RNAi)* populations (Fig. S4). However, similarly to chemical inhibition of DjChE activity, *Djche1/Djche2 (RNAi)* animals were less sensitive to heat stress. They underwent dramatically less body shape changes as the temperature increased compared to *control (RNAi)* animals (Fig. 5D). Although the fraction of body shape changes did gradually increase over time, it never reached the same extent as in *control (RNAi)* animals (Fig. 5E). In contrast to acute chemical inhibition of DjChE, *Djche1/Djche2 (RNAi)* animals did not display noticeable differences in normal locomotion/gliding speed (Fig. S4). Knockdown of *Djche1* and *Djche2* mRNA were confirmed by whole-mount ISH (Fig. S5). We further confirmed that knockdown of the two putative *Djche* genes is sufficient to functionally knockdown DjChE activity through staining of cholinesterase activity (Fig. 5F) and an Ellman assay of homogenized RNAi animals (Fig. S3).

Inhibition but not knockdown of Djche increases worm stickiness

When handling diazinon or physostigmine treated worms, we observed the animals tended to be “stickier” and often adhered to their substrate more strongly than control animals. Planarians secrete mucus for self-defense and locomotion, the latter of which is accomplished by cilia beating in a layer of secreted adhesive mucus (Martin 1978). Increased mucus secretion or changes in mucus composition in response to environmental stimuli can increase mucus production (Cochet-Escartin et al. 2015) and the worm’s adhesion to its substrate (“stickiness”) (Malinowski et al. 2017). To quantify the animals’ stickiness, we dispelled a controlled stream of water at the animal and measured the flow rate necessary to dislodge the worm (Malinowski et al. 2017). In agreement with our qualitative assessment of increased stickiness, planarians which had been treated with 1μM diazinon or physostigmine for 5 days required larger flow rates to be dislodged, indicating

increased stickiness and adhesion (Fig. 6A). Of note, although the distributions were significantly different from controls, the stickiness of inhibitor-treated planarians was much more variable than that of controls, possibly due to inter-worm variability in uptake or metabolism.

We next assayed whether *Djche1/Djche2 (RNAi)* animals also displayed increased stickiness to determine if this phenotype is specific to decreased DjChE activity. Unlike animals treated with the chemical inhibitors, *Djche1/Djche2 (RNAi)* animals did not demonstrate increased stickiness compared to *control (RNAi)* animals (Fig. 6B), suggesting that this effect may be modulated, in part or total, by mechanisms other than solely decreased DjChE activity.

In summary, while acute chemical inhibition of DjChE activity causes effects on gliding speed, heat stress response, and substrate adhesion, knockdown of *Djche* gene expression only caused effects on the heat stress response. We therefore assayed whether absence of some behavioral effects in *Djche1/Djche2 (RNAi)* animals could be due to adaptation to decreased DjChE activity over time. To this end, we repeated our behavioral analysis on regenerating planarians exposed to either 1μM diazinon or physostigmine for 11–12 days. As with acute chemical inhibition and RNAi treatment, inhibitor-treated regenerating planarians exhibited a less pronounced heat stress response compared to control animals (Fig. S6) and had substantially less DjChE activity than control animals (Fig. S3). However, in contrast to acute inhibition, inhibitor-treated regenerating planarians were not significantly stickier than control animals (Fig. 6C). Particularly for diazinon-treated animals, the flow required to unstick the worms was significantly lower in regenerating animals compared to day 5 full (intact) animals. In addition, inhibitor-treated regenerating animals did not have reduced gliding speeds (Fig. S6) or any regeneration defects (Fig. S4). Thus, chemical inhibition of regenerating planarians recapitulated the effects seen with regenerating RNAi animals, but not those of acutely inhibited animals. Together, these data suggest that planarians may develop adaptive mechanisms to mitigate the effects of long-term cholinergic stimulation.

Discussion

Enzymatic properties of DjChE: sequence and structure

In this study, we have identified two potential gene sequences (*Djche1* and *Djche2*) responsible for cholinesterase activity in *D. japonica.* Our previous work characterizing the catalytic properties and inhibition profile of cholinesterase activity in planarian homogenates demonstrated that DjChE activity has hybrid properties of both AChE and BChE (Hagstrom et al. 2017). Both potential DjChE sequences identified in this study contain the features we previously predicted, namely: (a) classic esterase-type catalytic triad (Ser200, Glu 327 and His440), (b) an acyl pocket containing only one of two Phe (295 and 297), (c) a choline binding site containing Trp84, (d) disruption of a peripheral anionic site defined by Trp286, Tyr72, and Tyr124, and (e) fewer aromatic side chains lining the active center gorge compared to AChE (Figs. 1 and 2). Together these characteristics result in planarian cholinesterases with a larger acyl pocket and a wider gorge opening than vertebrate AChE. This is consistent with our previous observations that DjChE can catalyze the larger

butyrylcholine substrate (although less efficiently than acetylcholine) and does not undergo substrate inhibition (Hagstrom et al. 2017). These qualities are common among cholinesterases from many invertebrates, including *Drosophila* (Gnagey et al. 1987), *C. elegans* (Arpagaus et al. 1994), *Schistosoma* (Bentley et al. 2003; Bentley et al. 2005), and some vertebrate species (Pezzementi et al. 2011) and may represent an ancestral cholinesterase before separation into the distinct AChE and BChE enzymes found in vertebrates (Pezzementi and Chatonnet 2010).

In planarian homogenates, we could not distinguish more than one distinct cholinesterase activity (Hagstrom et al. 2017). However, here we have identified two potential genes responsible for DjChE activity which are both actively expressed in *D. japonica*. Both genes contain all the key enzymatic features described above, though sometimes achieved in different ways. Thus, they likely have similar catalytic properties and inhibitor affinities preventing us from distinguishing the two activities in crude homogenates. We cannot exclude the possibility, however, that one *Djche* may be much more highly expressed than the other and may account for the majority of the activity. Future experiments using planarian recombinant DNA expressed enzymes would help answer whether there are any significant differences in the enzymatic properties of translated proteins expressed from these two genes.

Expression profiles of Djche

In vertebrates, AChE is encoded by a single gene but is alternatively spliced to produce different isoforms, differing only in their C-termini regions, each with distinct expression profiles and possibly different functions (Li et al. 1991; Taylor and Radić 1994; Soreq and Seidman 2001; Camp et al. 2010) Conversely, nematodes have three genes encoding AChE (*ace-1, −2, −3*) with distinct expression profiles and mostly non-redundant functions (Combes et al. 2003; Selkirk et al. 2005). Similarly to nematodes, *Djche1* and *Djche2* were found to have mostly non-overlapping expression profiles. D*jche1* is primarily expressed in the planarian nervous system with extensive co-localization with *Djchat*, suggesting this gene is expressed in cholinergic neurons. Conversely, *Djche2* is much more ubiquitously expressed throughout the planarian body with less spatial compartmentalization than *Djche1.* This spatial segregation could hint that the different *Djche* genes perform distinct functions, such as modulating ACh in the central versus the peripheral nervous systems, or discretely in synapses versus extra-synaptic release.

Interestingly, both *Djche* genes and *Djchat* were found to co-localize in neurons located in the medial arc of the planarian brain (Fig. 4). Several important regulators of planarian neurogenesis and patterning are expressed in this region, including netrin (Cebrià et al. 2002b; Cebrià and Newmark 2005), hedgehog (Rink et al. 2009), and homeodomain transcription factors (Currie et al. 2016). In *Schmidtea mediterranea, hedgehog* and the homeodomain transcription factors *arx* and *nkx2.1* were all found to be expressed specifically in ventromedial cholinergic neurons. Knockdown of *arx* reduced the number of ventromedial cholinergic neurons specifically in adult animals, suggesting arx and the hedgehog machinery are necessary for maintenance of these neurons (Currie et al. 2016). Together, these data suggest that ventromedial neurons, such as those that co-express *Djchat*

and the *Djche* genes, may be important for formation, patterning, and maintenance of the planarian brain. Studies in several animal models and cell culture systems have suggested that AChE may have morphogenic functions during neurodevelopment, which may or may not depend on catalysis of ACh (Bigbee et al. 2000; Biagioni et al. 2000; Paraoanu et al. 2006; Yang et al. 2008; Sperling et al. 2012; Layer et al. 2013). Consistent with the possibility that DjChE activity may regulate planarian brain formation, we previously found that subchronic exposure to high concentrations of the OP chlorpyrifos led to decreased brain size in regenerating but not full worms (Hagstrom et al. 2015). In this study, we did not observe any regeneration defects in either RNAi worms or chemically-treated worms. However, it is still possible that brain size defects were present, since this would have likely not been picked up by gross analysis of the blastema size. Thus, the role, if any, of DjChEs in planarian neurodevelopment remains to be discovered.

Functional consequences of decreased DjChE activity

Acute toxicity of OPs is primarily due to over-activation of the cholinergic system due to increased synaptic ACh levels and overstimulation of the nicotinic and muscarinic ACh receptors in the central and peripheral nervous systems (Pope et al. 2005; Russom et al. 2014; King and Aaron 2015; Taylor 2017). However, it has long been recognized that these chemicals have other direct and indirect effects. For example, OPs have been shown to directly interact with other targets, including other esterases (e.g. neurotoxic esterase (NTE), carboxylesterase, etc.), and a host of other hydrolase enzymes (e.g. lipases, proteases, acyl peptide hydrolase, etc.) (Clarke et al. 1994; Pope et al. 2005; Pancetti et al. 2007; Eleršek and Filipic 2011). These targets may also modulate the extent of cholinergic toxicity elicited by OP exposure by up- or down-regulating pre- and post-synaptic components involved in ACh synthesis, uptake, and binding (receptors) (Liu and Pope 1998; Pope et al. 2005). Importantly, actions on these secondary targets can vary dramatically between different OPs and can occur even at concentrations lower than necessary to inhibit AChE. Thus, it has been suggested that these secondary effects may play an important role in modulating the subacute and chronic effects of OPs, which can vary greatly depending on the inhibitor (Pope 1999; Casida and Quistad 2004; Pope et al. 2005; Eleršek and Filipic 2011). The manner and extent that these secondary effects play in the manifestation of specific toxic endpoints, however, are unclear.

We have previously reported that physostigmine and diazinon-oxon, the oxon metabolite of diazinon, are efficient inhibitors of DjChE activity *in vitro* (Hagstrom et al. 2017). However, *in vitro* inhibition concentrations are not necessarily predictive of *in vivo* inhibition capacity as other factors, such as the amount of inhibitor taken up by the animal, may modulate the actual concentration seen by the enzyme. In this study, we found that planarians acutely exposed for 4–5 days to 1μM diazinon or physostigmine had substantial inhibition of DjChE activity, as seen qualitatively by activity staining (Fig. 5) and quantitatively by Ellman assays in homogenates of exposed worms (Fig. S3). Efficient *in vivo* inhibition by diazinon suggests that planarians are capable of bioactivation by cytochrome P450 of diazinon to diazinon-oxon, which is the active metabolite responsible for AChE inhibition (Mutch and Williams 2006). Of note, in our hands, quantification of physostigmine-induced inhibition in homogenates underestimated the levels of inhibition when compared to the qualitative

activity stains. This is likely due to the instability of the carbamylated enzyme as it can undergo rapid decarbamylation in the absence of physostigmine (Dawson 1994). For example, it has been reported that single dose exposure to physostigmine in Alzheimer's patients has a BChE inhibition half-life of 84 minutes (Asthana et al. 1995). Thus, during the preparation of the homogenized sample, which takes approximately 1.5 hours, inhibited DjChE may be partially reactivated before activity measurements are made. However, in the activity stain, in which animals are fixed immediately after exposure, inhibition by physostigmine can be accurately captured and demonstrated that substantial loss of DjChE activity had occurred (Fig. 5). Reactivation was likely a concern in both the day 5 full worms and day 12 regenerating worms, with differences between the two due to increased inhibition in the regenerates compared to the acute exposure (compare Fig. 5C and Fig. S3D).

Despite significant loss of activity (greater than 95% in 1μM diazinon treated animals), inhibitor-treated planarians were alive and generally healthy for up to 12 days of exposure with no overt morphological or regenerative defects (Fig. S1, S4). Although AChE inhibition of 70–80% has been shown to be associated with lethality in birds, fish, and mammals (Russom et al. 2014), a similar absence of systemic toxicity or lethality despite significant inhibition of AChE has been previously demonstrated. Exposure of zebrafish larvae for 5 days to varying concentrations of chlorpyrifos, diazinon, or parathion decreased AChE activity by more than 50–80% without inducing significant lethality (Yen et al. 2011). Moreover, in *C. elegans*, double mutants with nonfunctional *ace-1* and *ace-2*, which together account for approximately 95% of AChE activity, are not lethal (Selkirk et al. 2005). Thus, in these species, as well as in planarians, it seems that very low levels of cholinesterase activity are sufficient to maintain viability.

In this study, both inhibitor-treated and *Djche1/Djche2 (RNAi)* animals displayed delayed and less reactive responses to heat stress, suggesting that increased thermo-tolerance is specific to loss of DjChE activity and subsequent overstimulation of the cholinergic system. This agrees with previous studies in *C. elegans*, which showed that excess ACh, either through exogenous ACh exposure or inhibition of AChE by neostigmine, led to increased thermo-tolerance, which was mediated by activation of a muscarinic receptor (Kalinnikova et al. 2013; Furuhashi and Sakamoto 2016).

Normal planarian locomotion is achieved through beating of cilia in a layer of mucus (Martin 1978). Changes in mucus secretion or composition can change the adhesive properties of the worm, as observed during physiological events such as fission (Malinowski et al. 2017) or in response to noxious stimuli to trigger an escape gait (Cochet-Escartin et al. 2015). Therefore, generally, an increase in worm stickiness would be considered an adverse effect on worm physiology and behavior. In this study, we found that while worms that were acutely treated with diazinon or physostigmine had increased stickiness, *Djche1/Djche2 (RNAi)* animals did not. This suggests that this endpoint may be modulated in part or total by some other mechanism besides decreased ChE activity. We have previously shown that the detergent Triton-X 100 increases mucus secretion and planarian stickiness (Malinowski et al. 2017) raising the possibility that increased mucus secretion and subsequent increased stickiness may be a nonspecific defense response to external toxicants. However, subchronic

exposure (11–12 days) of regenerating planarians to the same concentrations of these ChE inhibitors did not elicit increased stickiness, compared to control animals (Fig. 6). Thus, as this effect could be modulated, it is unlikely to be a general toxicant response. Additionally, inhibitor-treated regenerating planarians and RNAi animals did not show defects in gliding speed, although acute inhibitor-treated animals did. In our previous screen, we found that regenerating planarians were more sensitive than full worms to effects on gliding speed when treated with chlorpyrifos or dichlorvos (Hagstrom et al. 2015). Additionally, only dichlorvos, but not chlorpyrifos, caused a gliding speed defect in full animals (8 day exposure), suggesting that different effects beyond ChE inhibition may modulate how different OPs affect planarian locomotion. It is worth noting, however, that in the current study, we exchanged the chemical solutions daily to keep exposure conditions constant, while we did not exchange them in the previous screen. Together, these data suggest potential compensatory mechanisms may be activated in the regenerating animals to mitigate the long-term effects on stickiness and gliding speed.

Therefore, we propose that long-term overstimulation of the developing planarian cholinergic system may lead to adaptive mechanisms to gain tolerance to certain aspects of cholinergic toxicity, particularly increased stickiness and decreased gliding. In rats, down-regulation of the nicotinic and muscarinic ACh receptors has been proposed to be responsible for long-term tolerance to diazinon treatment (Ivanović et al. 2016). Moreover, down-regulation of muscarinic receptors has been proposed to at least partially explain the surprisingly mild phenotypes of AChE knockout mice (Li et al. 2003). Increased secretions, including increased sweating, lacrimation, and salivation, due to overstimulation of muscarinic receptors are a hallmark of cholinergic toxicity (Pope et al. 2005; Eleršek and Filipic 2011; Taylor 2017). We have previously shown that increased planarian stickiness is correlated with an increase in mucus secretion (Malinowski et al. 2017). Therefore, we speculate that, while being induced through ChE inhibition, increased worm stickiness may not be correlated directly with decreased ChE activity as compensatory mechanisms may allow planarians to adapt to long-term cholinergic overstimulation by down-regulating muscarinic receptors. The role of planarian muscarinic receptors in this process, whether modulation is due to direct or indirect effects of ChE inhibitors, and whether adaptation is specific to regenerating planarians remains to be verified.

Understanding of the potential role of non-ChE targets and effects in modulating ChE inhibitor toxicity is an important regulatory concern. Currently, levels of AChE inhibition are the gold standard biomarker to determine significant OP exposure (Kapka-Skrzypczak et al. 2011). However, growing evidence suggests that toxic outcomes may manifest from exposure to OP concentrations below those needed to inhibit AChE. This is of particular concern for chronic, low dose exposure and for prenatal exposure to the developing fetus (Pancetti et al. 2007). Here, we show that the wide repertoire of planarian morphological and behavioral endpoints, combined with accessible molecular biology techniques, enables us to dissect potential mechanisms underlying specific phenotypes of ChE inhibitor exposure. So far, however, we have only assayed a small subset of accessible behaviors based on endpoints that have previously been published to be affected by OP exposure in planarians or other systems. Thus, other effects of OP exposure may also exist that are not captured in this study. A comprehensive map quantifying the wide range of possible behaviors in

planarians will be necessary for future studies aimed at elucidating the differential actions of OPs on neuronal function and behavior. As rates of OP inhibition of ChE are similar to mammals (Hagstrom et al. 2017) and the planarian brain contains many of the same important genes as the vertebrate brain, these mechanisms are likely to be conserved and could be further investigated in mammalian models. Together, these characteristics make planarians a well-suited model system to analyze OP toxicity.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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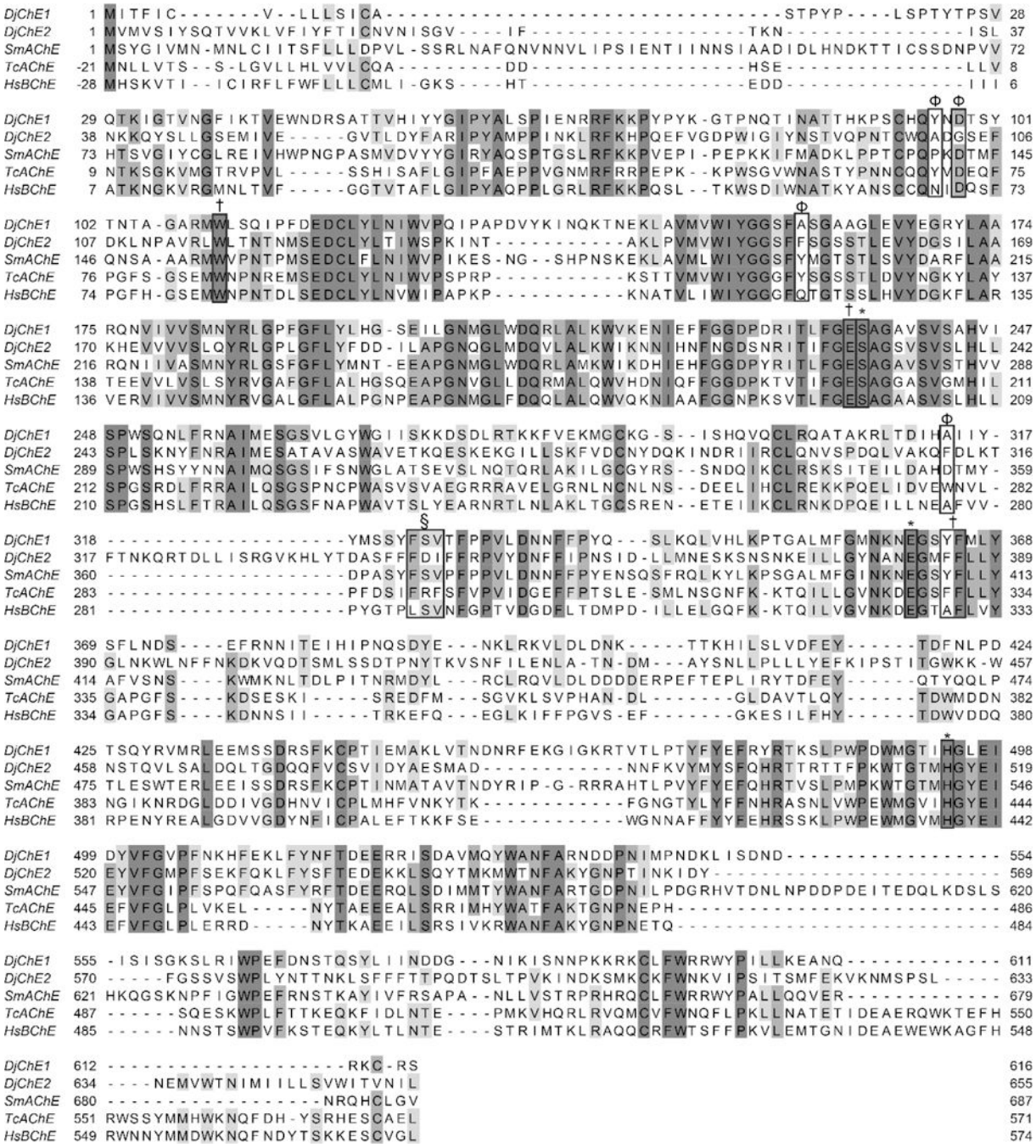

Fig. 1.

Candidate DjChEs show characteristics of both AChE and BChE. Alignment of deduced amino acid sequences of DjChE1 and DjChE2 with a representative vertebrate AChE (TcAChE), vertebrate BChE (HsBChE), and AChE from a related parasitic flatworm, *S. mansoni* (SmAChE). Note: for TcAChE and HsBChE, the leader signal peptide is shown but is not included in the numbering since it is not found in the mature polypeptides. Shading indicates level of conservation. Important structural residues are boxed and labeled: catalytic triad (*), acyl pocket (§), choline binding site (†), and peripheral anionic site (ϕ)

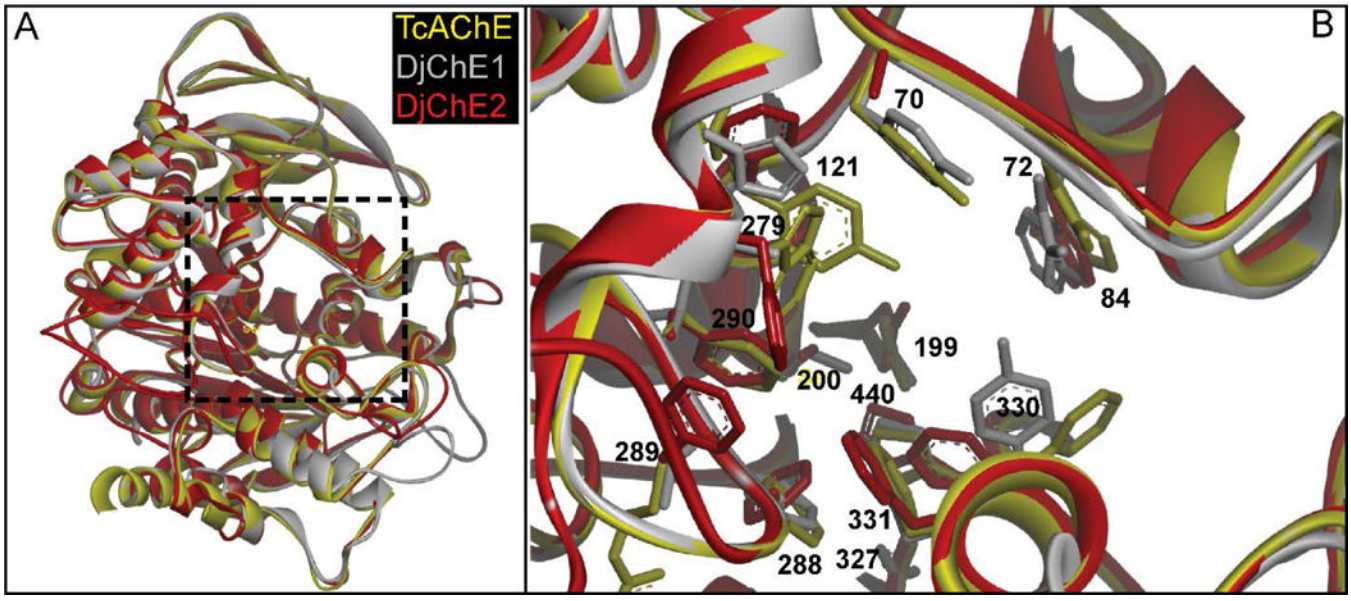

Fig. 2.

Homology modeling of planarian cholinesterase protein structure. **a** Whole protein structures of DjChE1 (grey) and DjChE2 (red) are overlaid with TcAChE (2w6c, yellow). Boxed area denotes the catalytic gorge. **b** Magnified view of boxed area in a. Important structural residues are labeled, with numbering based on TcAChE

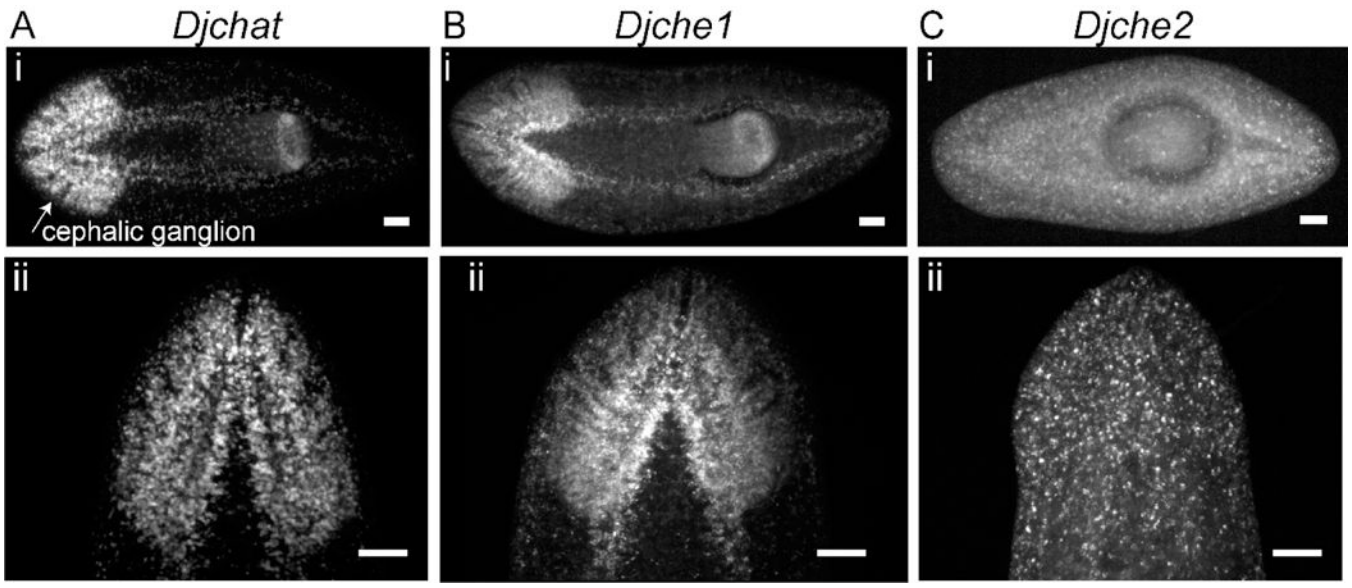

Fig. 3.

Planarian cholinesterases are expressed in the nervous system. Fluorescent *in situ* hybridization of *Djchat* (**a**), *Djche1* (**b**), and *Djche2* (**c**) showing the whole animal (i) or a maximum intensity projection of multiple planes in the head region (ii). Scale bars: 100μm

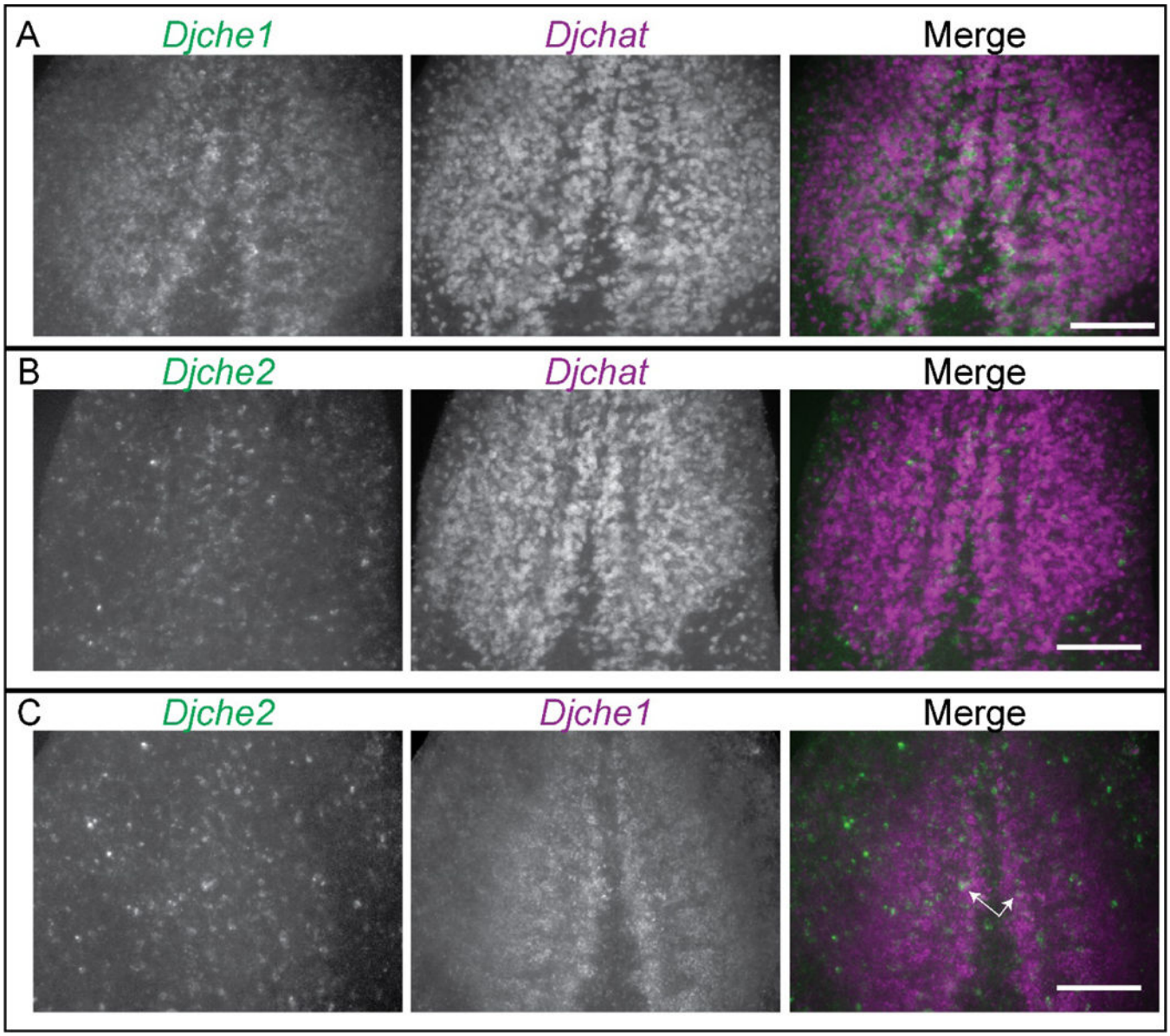

Fig. 4.

Planarian cholinesterases co-localize with each other and *Djchat* in the medial arc of the brain. Multicolor FISH for *Djche1* (green) and *Djchat* (magenta) (**a**), *Djche2* (green) and *Djchat* (magenta) (**b**), and *Djche2* (green) and *Djche1* (magenta) (**c**). Co-localization is indicated by a lighter color where the two channels overlap. Arrows denote examples of co-localization in the medial arc domain. Scale bars: 100μm

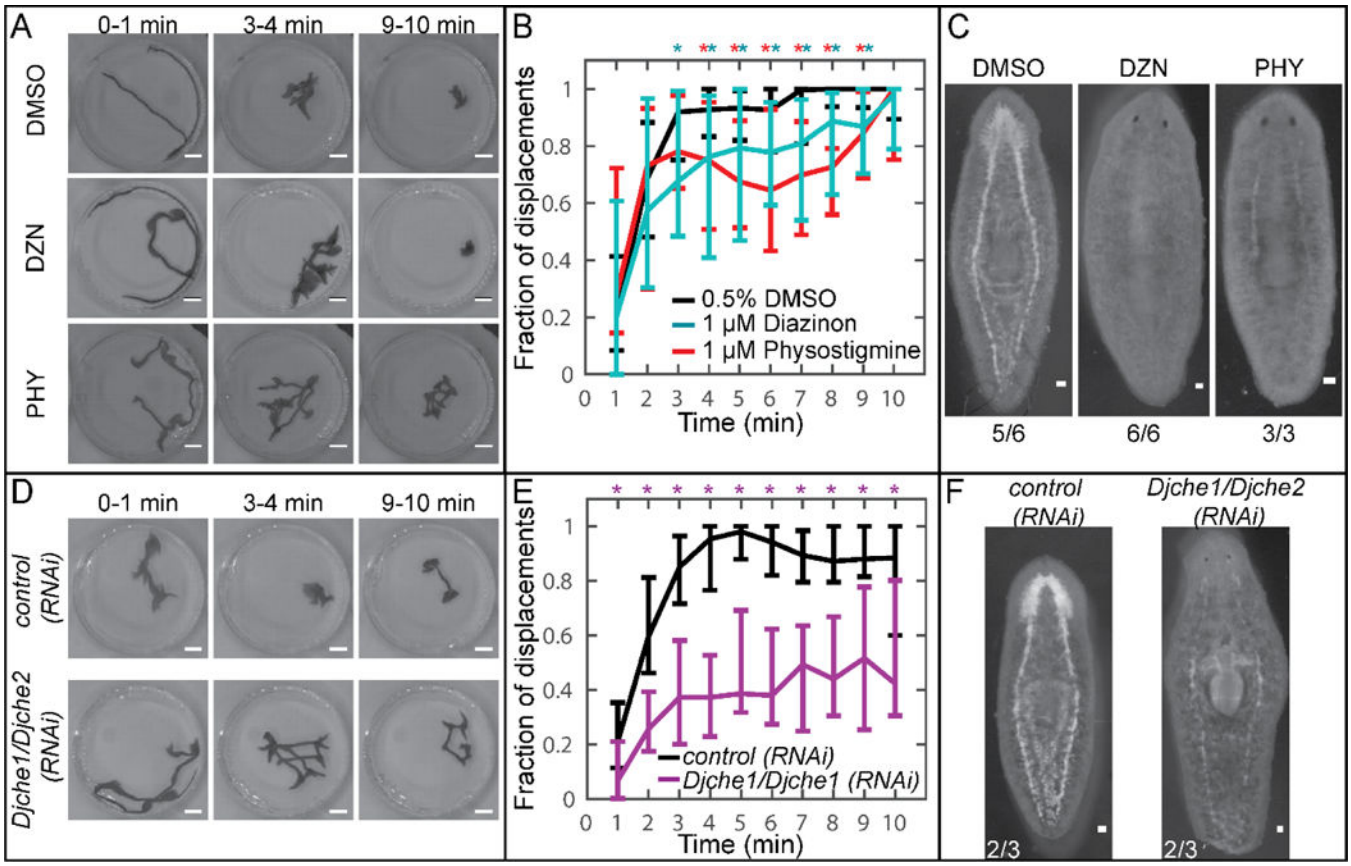

Fig. 5.

Inhibition of DjChE decreases sensitivity to heat stress **a** Representative minimum intensity projections over 1 minute intervals to show worm tracks of a 0.5% DMSO (DMSO) (top), 1μM diazinon (DZN, middle), and physostigmine (PHY, bottom) treated worm in response to heat stress. Note how during minutes 3–4, the DMSO-treated worm stays in one location with frequent turning (fan-like pattern in track) whereas the DZN and PHY-exposed planarians explore a larger area and have wider turns. **b** Diazinon and physostigmine treated animals undergo fewer and delayed body shape changes (as a fraction of all displacements tracked) than DMSO controls (n= 39, 46, 24 for DMSO, diazinon, and physostigmine, respectively). **c** ChE activity stains show inhibition of DjChE activity in 1μM diazinon and physostigmine treated animals. Numbers indicate how representative the staining is out of the number of animals assayed. **d** Representative minimum intensity projections over 1 minute intervals to show worm tracks of a *control (RNAi)* and *Djche1/Djche2 (RNAi)* animal in response to heat stress. **b** *Djche1/Djche2 (RNAi)* animals undergo fewer and delayed body shape changes (as a fraction of all displacements tracked) than *control (RNAi)* animals (n= 20 and 29 for *control (RNAi)* and *Djche1/Djche2 (RNAi)*, respectively). **c** ChE activity stains show loss of DjChE activity in *Djche1/Djche2 (RNAi)* animals. Numbers indicate how representative the staining is out of the number of animals assayed. Scale bars: 5mm (A, D), 100 μm (C, F). * indicates significant differences at the 5% level

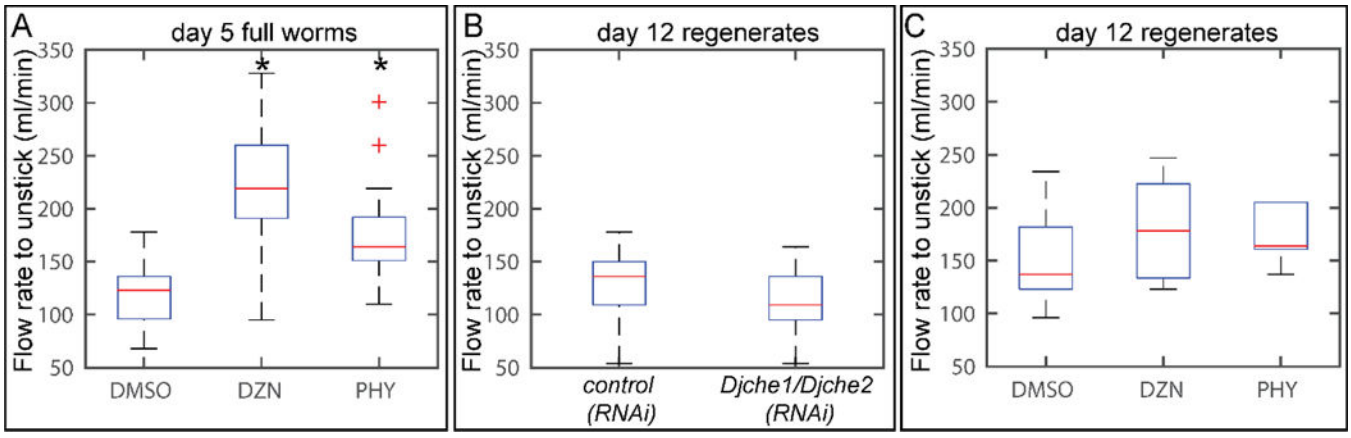

Fig. 6.

Diazinon and physostigmine, but not DjChE knockdown, increase worm adhesion ("stickiness"). Boxplot of the flow rate necessary to unstick worms from a substrate comparing worms exposed for 5 days to either **a** 0.5% DMSO (DMSO, n=46), 1μM diazinon (DZN, n=46), or 1μM physostigmine (PHY, n=23), **b** *control (RNAi)* (n=18) and *Djche1/Djche2 (RNAi)* (n=24) animals, or **c** regenerating tails exposed for 12 days to either 0.5% DMSO (DMSO, n=11), 1μM diazinon (DZN, n=9), or 1μM physostigmine (PHY, n=9). * indicates significant differences at the 5% level