Published in final edited form as:

Toxicon. 2010 December ; 56(8): 1398–1407. doi:10.1016/j.toxicon.2010.08.005.

A novel approach for *in vivo* screening of toxins using the *Drosophila* Giant Fiber Circuit

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Abstract

Finding compounds that affect neuronal or muscular function is of great interest as potential therapeutic agents for a variety of neurological disorders. Alternative applications for these compounds include their use as molecular probes as well as insecticides. We have developed a bioassay that requires small amounts of compounds and allows for unbiased screening of biological activity *in vivo*. For this, we paired administering compounds in a non-invasive manner with simultaneous electrophysiological recordings from a well-characterized neuronal circuit, the Giant Fiber System of *Drosophila melanogaster*, which mediates the escape response of the fly. The circuit encompasses a variety of neurons with cholinergic, glutamatergic, and electrical synapses as well as neuromuscular junctions. Electrophysiological recordings from this system allow for the detection of compound-related effects against any molecular target on these components. Here, we provide evidence that this novel bioassay works with small molecules such as the cholinergic receptor blocker mecamylamine hydrochloride and the potassium channel blocker tetraethylammonium hydroxide, as well as with venom from *Conus brunneus* and isolated conopeptides. Conopeptides have been

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Disclosure statement

The authors declare that there no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

developed into powerful drugs, such as the painkillers PrialTM and Xen2174. However, most conopeptides have yet to be characterized, revealing the need for a rapid and straightforward screening method. Our findings show that mecaminylamine hydrochloride, as well as the α -conotoxin ImI, which is known to be an antagonist of the human $\alpha 7$ nicotinic acetylcholine receptor, efficiently disrupted the synaptic transmission of a *Drosophila* $\alpha 7$ nicotinic acetylcholine receptor-dependent pathway in our circuit but did not affect the function of neurons with other types of synapses. This demonstrates that our bioassay is a valid tool for screening for compounds relevant to human health.

Keywords

Neuromodulation; conotoxins; ImI; *in vivo* drug screening assay; acetylcholine receptor antagonists; neuronal circuit function; electrophysiology; *Drosophila*

1. Introduction

Cone snails (family Conidae, genus *Conus*) are venomous marine gastropods that prey on worms, other mollusks or small fish depending on the species. Their venom is comprised of a diverse mixture of small peptides (6–40 amino acids), most of which bioactive (Olivera, 1997). Conopeptides contain several posttranslational modifications, including disulfide bonds. Conopeptides with two or more disulfide bonds are designated as conotoxins (Buczek et al., 2005; Bulaj and Olivera, 2008; Jones and Bulaj, 2000; Kaas et al., 2010). Depending on the species, the number of different conopeptides in the venom ranges from 20 to over 1000 (Davis et al., 2009; Olivera and Teichert, 2007). In addition, there are more than 700 identified cone snail species worldwide formulating over 100,000 different bioactive conotoxins that can be isolated; however, only about 200 of these have been characterized to date (Kaas et al., 2010). Previous studies have demonstrated that these isolated conotoxins can elicit a wide variety of effects with high affinity and specificity on neuronal and muscular components. Although these gastropods prey on invertebrates and small vertebrates, their conotoxins have also been shown to affect mammalian components required for neuronal communication demonstrating that numerous aspects of the nervous system have been evolutionarily highly conserved. Already identified targets of conopeptides are ligand-gated ion channels such as glutamate receptors, voltage-gated ion channels (K^+ , Na^+ , Ca^{2+}), G protein-coupled receptors (vasopressin, neurotensin), and neurotransmitter transporters (Layer et al., 2004; Lewis, 2004). These conopeptides proved to be helpful tools to study the physiological functions of neuronal and muscular circuits. In addition, several of these conotoxins have been developed as powerful pharmacological agents (Kaas et al., 2010). Clearly, the isolation and characterization of conopeptides is of great interest with regard to the development of neuropharmacological agents or biomarkers to study and treat numerous neuronal and muscular diseases. This is especially important since there are several conotoxins that are very abundant in the cone snail venom but their biological effects or the molecular targets are still unknown (Kaas et al., 2010).

In the past few decades the major approaches to test for bioactivity of conotoxins involved techniques such as patch clamp on neurons and oocytes (Carter and Mynlieff, 2004; Wang et al., 2009) or *in vivo* drug injections into vertebrates (Terlau and Olivera, 2004). Patch clamp is a difficult technique and limits the testing mostly to a single known *in vitro* target. In contrast, injections into mice, rats, and fish revealed interesting behavioral phenotypes such as hyperactivity, sleep induction and paralysis but this method requires large amounts of the conopeptides and the characterization of the molecular target can be difficult. *In vivo* microinjection system in arthropods has been described before, but the method allowed for observation of lethal or paralytic effects of the toxins (Drummond and Wiener, 1956; Escoubas et al., 1995). Here, we show a novel bioassay for screening conopeptides and other compounds

that unites many of the advantageous features and avoids several of disadvantages of the previous assays.

We took advantage of the fruit fly's open circulatory system by injecting nanoliter quantities of a compound into the living animal while simultaneously obtaining recordings from a well-characterized neuronal circuit, the Giant Fiber System (GFS, Figure 1), which mediates the escape response of the fly (Allen et al., 2006) and has been previously used to study axonal guidance and synapse formation and function (Allen et al., 1999; Blagburn et al., 1999; Fayyazuddin et al., 2006; Godenschwege et al., 2009; Godenschwege et al., 2006; Godenschwege and Murphey, 2009). The *Drosophila melanogaster* GFS is composed of several cholinergic synapses, electrical connections (GAP junctions) and glutamatergic neuromuscular junctions (NMJs), with some receptors and ion channels being evolutionarily conserved while others are not. For example, the cholinergic synapses between the Peripheral Synapsing Interneurons (PSI) and the Dorsal Longitudinal motoneurons (DLMn) was shown to be dependent on the previously cloned $\alpha 7$ nicotinic acetylcholine receptor (nAChR) subunit and the *Drosophila* $\alpha 7$ gene (*Da7*) is the homolog of the *CHRNA7* gene in humans (Fayyazuddin et al., 2006; Grauso et al., 2002). The GAP junctions present between the Giant Fiber (GF) and the PSI as well as the (TTMn) synapses are dependent on the *Drosophila* *shaking-B* gene (Phelan et al., 1998b), which belongs to the innexin family and homologues have been reported in several organisms, including vertebrates (Baranova et al., 2004; Panchin et al., 2000). Interestingly innexins, also termed pannexins, are structurally conserved analogues to the mammalian connexins, which are not present in invertebrates (Panchin et al., 2000; Phelan et al., 1998a; Phelan et al., 2008). Unlike vertebrates, the major neurotransmitter of the insect neuromuscular junctions (NMJs) is glutamate (Jan and Jan, 1976a,b; Usherwood et al., 1968). Therefore, the NMJs on the flight muscles (DLMs) and the jump muscles (TTMs) of the *Drosophila* GFS are of glutamatergic nature with the ionotropic receptors DGluRIIA, DGluRIIB, DGluRIII, DGluRIID and DGluRIIE present on the muscles (Marrus et al., 2004; Petersen et al., 1997; Qin et al., 2005; Schuster et al., 1991). Conservation and divergence is also reflected among these receptors, with the Ca^{2+} permeable pore region of DGluRIIA receptor being identical to vertebrates and the DGluRIIB receptor being less preserved (Petersen et al., 1997; Schuster et al., 1991). Other examples in the fly are the metabotropic glutamate receptor DmGluRA, which is highly homologous to its mammalian counterpart or the glutamate-gated Cl^- channel DrosGluCl- α which has no identified vertebrate equivalents (Cully et al., 1996a; Cully et al., 1996b; Parmentier et al., 2000; Parmentier et al., 1996). There is an array of ion channels present in *Drosophila*. They included including approximately 30 potassium channels some of which are homologous to the eag, erg, elk subfamily or similar to the K_V channels in vertebrates in addition to a dozen ligand-gated and voltage-gated calcium channels of L, T, N -type as well as TTX-sensitive voltage-gated sodium channels, and 3 voltage-gated chloride channels (Elkins and Ganetzky, 1988; Littleton and Ganetzky, 2000; O'Dowd and Aldrich, 1988; Wu and Ganetzky, 1980). Again, when compared to vertebrate some channels or aspects of channels are conserved while others are not (Elkins and Ganetzky, 1988; Littleton and Ganetzky, 2000), suggesting that some toxins affecting the function of the GF circuit could be developed into insect-specific pesticides while other may have the potential to be developed into therapeutic agents to treat human neurological disorders.

2. Material and methods

2.1 Fly stocks

We maintained *w¹¹¹⁸* and P{GawB}OK307 flies (Stock #6326 and #6488, Bloomington Stock center) at room temperature in vials containing standard media. 1–6 day old flies were used in our assays.

2.2 Crude venom extraction

The venom machinery (venom duct, venom bulb and radular sac) was extracted from *Conus brunneus*. 15–20 venom ducts were placed in 0.1% TFA in H₂O. The venom ducts were then homogenized and centrifuged at 10,000 RPM for 10 minutes. The supernatant, which contained extracted venom, was transferred to a new tube and kept on ice. 5 mL of 0.1% TFA in H₂O were then added to the first tube and the sample homogenized and centrifuged again, collecting the venom. These steps were repeated at least one more time. The sample was frozen at –80° C and dried in a lyophilizer. 1.6 mg of dry crude venom was dissolved in 200 µl of 0.7% NaCl as a stock solution for the electrophysiology/nanoinjection bioassay.

2.3 Paired electrophysiology/nanoinjection bioassay

2.3.1 Electrophysiology—Flies were immobilized on dental wax with the dorsal side exposed. We used standard methods to obtain electrophysiological recordings from the Giant Fiber System (Allen and Godenschwege, 2010). Two sharp stimulating tungsten electrodes were placed into the brain through each of the fly's eyes. The GFs were stimulated with 40–50 mV pulses given for the duration of 0.03 ms (S48 stimulator, Grass instruments, Warwick, RI, U.S.A.). The stimulating electrodes were also placed in the anterior end of the thorax to bypass the Giant Fiber and stimulate the motor neurons directly. A third tungsten electrode in the animal's abdomen served as the ground. Recording glass electrodes (80–100 MΩ, 1.0 mm OD, 0.58mm ID borosilicate glass capillaries, World Precision Instruments, Sarasota, FL, U.S.A.) were pulled with a vertical pipette puller (Electrode Puller Model 700c, David Kopf Instruments, Tujunga, CA, U.S.A.) and filled with saline (NaCl 101 mM, CaCl₂ 1 mM, MgCl₂ 4 mM, KCl 3 mM, glucose 5 mM, NaH₂PO₄ 1.25 mM, NaHCO₃ 20.7 mM, pH 7.2) (Gu and O'Dowd, 2006). The glass recording electrodes were placed into the Tergo Trochanteral Muscle (TTM) and the Dorso Longitudinal Muscle (DLM, Figure 1). The recordings were amplified (5A amplifier, Getting Instruments Inc., San Diego, CA, U.S.A.) and stored in a computer using pCLAMP software and a Digidata 1440A interface (Molecular devices®, Silicon Valley, CA, U.S.A.).

Before compound injection, the GF system of the fly was tested electrophysiologically in order to have a baseline for the drug effects. Here, we only chose flies with wild type responses defined as having a response latency ≤ 1 ms for TTM and ≤ 1.6 ms for DLM as well as having the ability for both pathways, GF-TTM and GF-DLM, to follow one-to-one when stimuli were given at 100Hz. In order to monitor any immediate effects on the function of the GF system, the Giant Fibers were stimulated with single pulses given at 1 Hz just before, during and continuing after drug injection for a total of 100 sweeps (see 2.3.2), and the TTM and DLM responses were recorded and stored. Immediately after the 1 Hz period (approximately 1 minute after injection), the functions of the GF-TTM and the GF-DLM pathways were tested for reliability with 10 sweeps of 10 stimulus trains at 100 Hz with 1 second between each sweep resulting in a total of 100 stimuli. The ability to follow the trains was calculated as a percentage and compared to the average of train responses before compound injections and to control flies injected with saline or DMSO control solution. In addition, any change in the amplitude and shape of the responses, as well as changes in the response latencies were noted.

2.3.2 Dye/Drug injections—Injection glass micropipettes (Nanoliter2000 borosilicate glass capillaries, World Precision Instruments, Sarasota, FL, U.S.A.) were pulled with a vertical pipette puller (Kopf instruments, Tujunga, CA, U.S.A.) and beveled to an 11–17µm opening with a K.T. Brown Type micropipette beveller (Sutter Instrument Co., Novato, CA, U.S.A.). The injection micropipette was then mounted on a Nanoliter2000 automatic injector (World Precision Instruments, Sarasota, FL, U.S.A.) controlled by a micromanipulator and was inserted into the fly's head via the ocelli to inject the drug (Figure 1).

For dye injections, 23–46 nl of blue food color (McCormik®) was injected into the head capsule of the fly ($n = 30$, w^{1118} or P{GawB}OK307 flies were used) via the ocelli to measure the time of dispersion to the thorax.

For mecamlamine hydrochloride injections (MP Biomedicals, LLC, Salon, Ohio, U.S.A.), flies ($n = 15$) were injected with 23 nl of different doses of mecamlamine hydrochloride (4, 8, 13, 16, 22, 39 and 104 ng/mg) in the head capsule via the ocelli. The quantities in nanomoles per gram, were 21, 41, 64, 80, 106, 209, and 509 nmol/g and the mecamlamine hydrochloride was dissolved in saline solution (NaCl 101 mM, CaCl₂ 1 mM, MgCl₂ 4 mM, KCl 3 mM, glucose 5 mM, NaH₂PO₄ 1.25 mM, NaHCO₃ 20.7 mM, pH 7.2). We used female (1.2 mg) and male (1.0 mg) flies and calculated the average weight based on number of females and males used. In all experiments we did not notice any difference between female and male flies after drug injection. The electrophysiological recordings (see method 2.3.1) of flies injected with mecamlamine hydrochloride were compared to control flies ($n = 15$), which were injected with 23 nl of saline solution.

For tetraethylammonium hydroxide (TEA, Sigma-Aldrich, St. Louis, MO, U.S.A.) injections, flies ($n = 8$) were injected with 23 nl of 2.7 ng/mg (or 18 μ mol/g) of TEA dissolved in 0.7% NaCl solution. The electrophysiological responses from the DLM and TTM were recorded as described above. The GF circuit was tested with 10 sweeps of 10 stimulus trains at 100 Hz before and after injection, starting immediately after the 1 Hz stimulation period, and every minute thereafter up to a total of 5 minutes. Muscle responses were compared to control flies ($n = 10$), which were injected with 23 nl of 0.7% NaCl solution.

For venom injections, flies ($n = 11$) were injected with 46 nl of *Conus brunneus* dissected crude venom (368 ng in 0.7% NaCl solution in DI water, see method 2.2). In addition, the functions of the GF-TTM and GF-DLM pathways were tested with 10 sweeps of 10 stimulus trains at 100 Hz before and after injection immediately after the 1 Hz stimulation period, 5 minutes after and 10 minutes after. Experimental flies were compared to control flies ($n = 15$), which were injected with 46 nl of 0.7% NaCl solution in DI water.

For ImI injections, flies ($n = 8$) were injected with 23 nl of synthetic ImI, which was prepared as previously described (Armishaw et al., 2009; Armishaw et al., 2010). Briefly, the peptide was assembled using *tert*-butyloxycarbonyl solid phase synthesis on a 4-methylbenzhydrylamine (MBHA) resin. The peptide was then cleaved from the resin using hydrogen fluoride / *p*-cresol / *p*-thiocresol (18:1:1). The crude peptide was oxidized in 0.1M ammonium bicarbonate, pH 8.2 containing 50% isopropanol, followed by purification using RP-HPLC. Different doses of ImI 15 and 53 ng/mg (or 11 and 39 nmol/g) were dissolved in 5% DMSO in DI water and injected into the fly's head capsule to study a dose dependent effect. Control flies ($n = 10$) were injected with 23 nl of 5% DMSO in DI water. In addition, the functions of the GF-TTM and GF-DLM pathways were tested with 10 sweeps of 10 stimulus trains at 100 Hz before as well as after injection at immediately after the 1 Hz stimulation period as well as 5 minutes, 10 minutes, 15 minutes and 20 minutes after the 1 Hz stimulation period.

3. Results

3.1. Rapid dye dispersion through the whole animal after injections into the head

The Giant Fiber (GF) cell bodies and dendrites are localized in the brain, which receive visual input. A behavioral output, the escape response, is mediated by the GFs via the jump (Tergo Trochanteral Motoneuron, TTMn) and the flight motor neurons (Dorsal Longitudinal Motoneuron, DLMn) in the thorax of the fly (Figure 1A). As an arthropod, the fly has an open circulatory system. In order to test how fast a compound injected into the heads of the animals

would reach the thorax, we injected blue food coloring to visualize the dispersal. We found that the vast majority of the flies had very rapid dispersion throughout their bodies within a few seconds (Figure 1B). In only 6 out of 30 animals were we unable to visualize from outside the body the complete dispersion of the blue, however dissections performed on these flies revealed that dye did reach the thorax.

3.2. Mecamylamine hydrochloride blocked the cholinergic GF-DLM pathway of the *Drosophila* GF circuit

In order to test whether our bioassay is susceptible to chemical agents we chose mecamylamine hydrochloride, as it is a non-selective and non-competitive nicotinic acetylcholine receptor (nAChR) antagonist. The Peripheral Synapsing Interneuron, PSI (Figure 1C), between the GF and the DLMn has been previously shown to be cholinergic (Fayyazuddin et al., 2006; Gorczyca and Hall, 1984). The GF-PSI and GF-TTMn connections (Figure 1A) are a mixed electrical and chemical synapse (Blagburn et al., 1999; Phelan et al., 1996). Although the chemical transmitter of the Giant Fiber has been shown to be acetylcholine (Allen and Murphey, 2007), the synaptic transmission of the electrical synapse is dominant over the chemical synapse (Allen et al., 1999). Therefore, mecamylamine hydrochloride should affect the function of the GF-DLM pathway but not of the GF-TTM pathway. Indeed, we found that mecamylamine hydrochloride disrupted the GF-DLM pathway but not of the GF-TTM pathway in its ability follow 10 stimuli given at 100 Hz (Figure 2A1 and 2B1, compare traces before and after injection). We injected different amounts of mecamylamine (4, 8, 13, 16, 22, 39 and 104 ng/mg, n= 15 for each dose) and saline as a negative control (n= 15). We conducted a Wilcoxon signed-rank test to analyze the significance in changes from the GF-DLM pathway and the GF-TTM pathway before and after injections of mecamylamine. Our data revealed that the disruptive effect of mecamylamine on the GF-DLM but not the GF-TTM pathway, was dose dependent (Figure 2A2 and 2B2). The maximum effect was reached with about 39 ng/mg of mecamylamine, when stimulation of the GF in the brain did not result in any response output at the DLM muscle (Figure 2A2). However, in some of these animals we re-placed the stimulation electrodes to stimulate the motoneurons directly in the thorax (Figure 1C). In all cases we found the DLMn to follow stimuli given at 100 Hz one to one demonstrating that the glutamatergic neuromuscular junction was unaffected in the drug-treated animals. In addition, injection of saline into the flies had no effect on the GF-DLM or GF-TTM pathways, confirming a drug specific effect for mecamylamine hydrochloride (Figure 2A2 and 2B2). Finally, aside from the lack of responses, we did not notice any other alterations, such as changes in response latency or shape of the TTM and DLM responses, when experimental groups to control groups were compared.

3.3. Tetraethylammonium hydroxide disrupted the function of muscles and neurons in the *Drosophila* GF circuit

In order to test a drug that affects the function of neurons and muscles, we chose tetraethylammonium hydroxide (TEA), which is a non-specific K⁺ channel blocker. Potassium channels are crucial for returning the membrane of a cell to its resting potential following an action potential (Armstrong, 1971; Choi et al., 1991; Yellen, 2002). After injections of 23 nl of TEA (2.7 ug/mg), we saw effects that are dramatically different from those seen with mecamylamine hydrochloride. Immediately after injection and for as long as we tested (up to 5 minutes) we found a disruption of both the GF-TTM and the GF-DLM pathways in all flies tested (Figure 3). In most cases we were not able to record any responses from muscles when the GF were stimulated in the brain (Figure 3 After, top). However, occasionally upon brain stimulation we did observe a few responses, which usually had an abnormal shape (Figure 3 After, bottom, asterisks). Our findings show that after TEA injections the duration of the muscle responses were dramatically increased (Figure 3, asterisks), and are consistent with earlier studies (Elkins and Ganetzky, 1988). Using voltage and current clamps from the DLM muscles,

the Ganetzky lab showed that TEA blocks voltage-gated and Ca^{2+} activated potassium channels at the DLM muscles thereby preventing the repolarization of the DLM spikes, which results in the prolonged DLM responses (Elkins and Ganetzky, 1988). Thus, while the change of the muscles response shape is due to drug specific effects on molecular targets on the DLM and TTM muscles, the absence of any response upon brain stimulation of the GF circuit is likely due to blocking potassium channels on neurons and muscles of the circuit.

3.4. Crude venom from *Conus brunneus* immediately disrupts the function of the *Drosophila* GF circuit

In order to test whether our system is suitable to screen for novel conopeptides with modulatory effects on neuron or muscles, we injected 46 nl of crude venom (368 ng in 0.7% NaCl solution in DI water) from *C. brunneus* into the flies (n= 11) while recording from the GFS and compared them to flies injected with 46 nl of 0.7% NaCl solution in DI water. The disruptive effects were highly variable whereby some flies were unaffected and in others the GF-DLM but not the GF-TTM pathway was disrupted. However, in 6 out of the 11 flies, immediately after the crude venom injection during the 1 Hz stimulation monitoring, we were unable to record any muscle responses when the GF was stimulated in the brain (Figure 1C). In addition, the function of the circuit was fully recovered after 5 minutes in all but two flies. No changes in the GF-DLM or GF-TTM pathway's ability to follow stimuli given at 1 HZ or at 100 Hz (tested at 1 minute and 5 minutes after the 1 Hz testing period) were found in 14 of 15 control flies injected with 46 nl of 0.7% NaCl solution in DI water at any time after the injection.

3.5. ImI blocked the $\text{D}\alpha 7$ nAChRs in the DLM pathway of the *Drosophila* GF circuit

In order to test whether we can obtain specific and reliable results with isolated conopeptides, we tested the effects of ImI on the GF circuit. ImI is a well-characterized α -conotoxin from *Conus imperialis* that has been shown to be a potent $\alpha 7$ nAChR antagonist (McIntosh et al., 1994). In addition, synaptic transmission of the PSI-DLM synapse has been shown to be depended on the *D. melanogaster* $\alpha 7$ receptors (Fayyazuddin et al., 2006).

We tested control flies (injected with 23 nl of 5% DMSO, n=10) and flies injected with 23 nl of ImI dissolved in 5% DMSO in DI water with two different dosages (15 and 53 ng/mg). Similar to mecamylamine we found that ImI disrupted the ability of the GF-DLM pathway but not the GF-TTM to follow stimuli given at 100 Hz (Figure 4A1 and 4B1). We used the Wilcoxon signed-rank test to analyze any significant difference in either GF pathways before and after injections of ImI and found that the disruptive effect on the GF-DLM pathway was dosage dependent (Figure 4A2 and 4B2). Interestingly, ImI reaches its maximum effect only after 20 minutes at low concentrations (15 ng/mg) and 10 minutes at higher concentrations (53 ng/mg). Finally, DMSO control flies showed no significant disruptive effect on either GF pathways and thoracic stimulation revealed that the glutamatergic synapses of the neuromuscular junctions were unaffected by ImI, demonstrating a selective and drug specific effects of ImI on the GF circuit. In contrast to TEA but similar to mecamylamine hydrochloride, we did not observe any changes in response latencies or EJPs shapes after injection of ImI in either muscle when we compared experimental flies with control flies.

4. Discussion

Screening natural products to identify novel compounds for the development of powerful therapeutic agents for disorders and diseases has been successful in the past. For example, Prialt™ (or Ziconotide) is a conopeptide originally found in *Conus magus* that is a painkiller 10,000-fold more powerful than morphine, and is used to treat chronic pain in cancer patients (Jones and Bulaj, 2000, Miljanich, 2004). In addition, other conopeptides are in early clinical trials to be developed into drugs to treat heart arrhythmia, epilepsy and deafness among other

conditions but the bioactivity of several hundred thousand conopeptides remains to be discovered (Brust et al., 2009). In order to be able to efficiently screen the vast amount of compounds present in nature, a cost-effective and fast screening assay is needed that allows the detection of bioactivity with small quantities. Here, we described a novel bioassay that is likely to be useful for the identification of novel potential drugs in the future.

We simultaneously paired *in vivo* nanoinjections of compounds with electrophysiological recordings, to instantly detect compound bioactivity on various molecular targets on different neurons and muscles along the well characterized simple neuronal circuit of the *Drosophila melanogaster*. Due to the open circulatory system of the fly, we showed with blue dye that injection of compounds into the head capsule via the ocelli allowed a rapid dispersal throughout the head and thorax usually within seconds. While some of the injected solution may have entered the ensheathed central nervous system (CNS) directly via the axonal (Lima and Miesenböck, 2005), the majority of animals tested in our assay clearly ends up in the hemolymph of the fly in which the CNS, peripheral nervous system (PNS) and the muscles are immersed in. Similar to vertebrates, the glia sheath around the CNS serves as blood-brain barrier (BBB) in *Drosophila melanogaster* (Stork et al., 2008). However, with small molecules (mecamylamine hydrochloride and TEA) and a small peptide (ImI), we saw a fast compound-specific and selective effect on the function of the cholinergic PSI-DLM synapse in the CNS or on the entire circuit. Although we do see almost instant effects with ImI, it only reached its maximum effect after approximately 10 minutes suggesting that the fly's BBB may be the cause of the delayed effect. In summary, our results show that injections into the head capsule via ocelli and simultaneous recording from the Giant Fiber Circuit is a suitable bioassay to monitor for bioactivity of compounds that have an effect on the function of neurons in the CNS and PNS as well as on muscle physiology.

Injections of venom from cone snails into mice have been used to characterize the pharmacology and toxinology of the venom (Kohn et al., 1960; Olivera et al., 1999). While these studies have been very useful they are hampered by the amount of venom required. One advantage of our system is that it allows for *in vivo* screening of bioactivity of a chemical or biological compound and only requires small quantities. Another advantage of our novel bioassay is that it is a much easier and less expensive technique than patch/current clamp from neurons or oocytes. Although our assay does not allow seeing the direct effects on a particular channel, it does allow the detection of any molecular target that is critical to the function of the neurons and muscles in the circuit. Hence, it allows us to find novel conopeptides with new functions and therefore is an excellent system for an unbiased screen for any bioactivity on cholinergic or glutamatergic neurons, electrical synapses or muscle physiology. Although the limitation of the system is that it does not allow to instantly identify the actual molecular target for any conopeptides with bioactivity, it does however provide strong clues about their molecular target in some cases (e.g. α -conotoxins specifically disrupting the GF-DLM pathway but not the GF-TTM pathway). The change in other electrophysiological aspects of the recordings, such as change in the shape or size of the response amplitude of both muscle responses or an increase of the response latency in the GF-TTM pathway allows us to categorize the conopeptides into groups with different bioactive properties that may indicate an effect on potassium channels (as seen with TEA), glutamatergic receptors or gap junctions, respectively (Allen et al., 2006; Baird et al., 1990; Blagburn et al., 1999; Thomas and Wyman, 1984). In addition, there are mutants available for almost any gene in *Drosophila melanogaster* and an enhancement or suppression of bioactivity in mutants could be used to provide further evidence for a potential molecular target. However, it is clear that in many cases other assays such as patch clamp will be required for the final identification of the molecular target. Finally, some compounds with bioactivity in *Drosophila* may not have any bioactivity in vertebrates. However, there is an increasing need for powerful and effective pesticides that do not harm humans and their pets or farm animals (Centers for Disease Control and Prevention, 2010;

Jacobsen et al., 2010; Riccio et al., 2010) and hence compounds found to be insect-specific may be further developed into a pesticide.

When we tested the crude venom from the invertebrate hunter *Conus brunneus* in our bioassay we found that the ability to record responses from the escape response circuit of the flies diminished instantly in most flies but had variable effects in individual flies that wore off within 1–5 minutes. In contrast, besides ImI presented here, we also found that several isolated conopeptides from the venom of *Conus brunneus* injected individually into the flies, had reproducible and long-lasting effects up to at least 20 minutes. However, the results of these novel conopeptides identified in our ongoing screen from the venom of *Conus brunneus* using our assay and their strong but distinct effects on the function of the circuit will be presented elsewhere. A discrepancy of effects between venom and single conotoxins has also been observed in other model systems including mammals and the final concentration of individual conopeptides or even a cocktail of multiple conopeptides (and proteases) being lower than the crude venom is a possible explanation for different effects of the injection of single concentrated conopeptides isolated from the same venom in the fly. Thus, our assay could be used to test and compare venoms from various species but also in the view of the many potential molecular targets in the circuit, it is more suitable for testing single compounds or single conopeptides isolated from venom, which results in more conclusive data.

5. Conclusions

In conclusion we have developed a novel, easy, reliable and fast bioassay suitable to screen for bioactivity of chemical agents or compounds isolated from a biological source including, but not limited to, marine cone snails. The bioassay allows testing compounds in an *in vivo* system that only requires nanogram amounts to elicit effects on a wide range of molecular target on different types of neurons and muscles. Identified compounds with bioactivity in *Drosophila melanogaster* may be further tested and developed into drugs for therapeutic treatment of disorders and diseases in humans or alternatively into pesticides that are of no harm to vertebrates.

Abbreviations

GF	Giant Fiber
DLM	Dorsal Longitudinal Muscle
TTM	Tergo Trochanteral Muscle
EJP	Excitatory Junction Potential
NMJ	Neuromuscular Junction
nAChR	nicotinic Acetylcholine Receptor
CNS	Central Nervous System
PNS	Peripheral Nervous System

Acknowledgments

We would like to thank Felipe Mejia for assistance with the graphics as well as Daniel J. Tobiansky and Dr. Jana Börner for their comments on the manuscript. Finally, we thank Marcus Allen and Robin Konieczny for the artwork in Figure 1A. This work was funded by the National Institute for Neurological Disorders and Stroke grant R21NS06637 to F.M. and T.A.G. and A.B. was funded by National Science Foundation award #0829250, URM: Integrative Biology for future researchers.

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Figure 1. Bioassay for paired drug injections and GFS electrophysiological recordings

A. Depiction of the Central Nervous System (CNS) and the Giant fiber circuit in the fly with electrophysiological arrangement. Tungsten electrodes were placed through the eyes into the brain of the fly to stimulate the Giant Fibers (GF) as well as in the abdomen of the fly to serve as a ground (not shown in figure). In addition, glass electrodes were placed in the Dorsal Longitudinal Muscle (DLM) and Tergo Trochanteral Muscle (TTM) to record the muscle responses to GF stimulation. The compounds were injected through the ocellus while simultaneously stimulating the GF circuit. **B.** Blue food color was injected via the ocellus into the head capsule of the fly. A rapid dispersion throughout the animal due to its open circulatory system was observed. In this particular case the dye dispersed in less than one second. **C.** Top illustration: Illustration of one lateral half of the GF circuit (Allen et al., 2006). The GF (in red) cell bodies and dendrites are localized in the brain and each extends a single axon into the second thoracic neuromere, where it makes a mixed electrical (GAP junction) and chemical (acetylcholine neurotransmitter) synapse onto the Tergo Trochanteral Motorneuron (TTMn in yellow), which innervates the jump muscle (TTM in brown). The GF makes an electrical and chemical synapse onto the Peripheral Synapsing Interneuron (PSI in green) however, this drawing only depicts the GAP junction because without the electrical synapse present no response can be recorded from the DLM when the GFs are stimulated in the brain (Allen et al., 2006; Baird et al., 1990; Blagburn et al., 1999; Thomas and Wyman, 1984). Finally, the PSI makes a cholinergic synapse onto the Dorsal Longitudinal Motorneurons (DLMn in blue) that innervate the flight muscle (DLM in purple). Both the TTMn and the DLMn neuromuscular junctions are of glutamatergic nature. Bottom illustration: Traces of single DLM and TTM responses when stimulated at 1 Hz before and after crude venom injection. The lack of response for both muscles after injection is illustrated by the asterisks (*).

Figure 2. Electrophysiological recordings from the GFS before and after injection of mecamlamine hydrochloride

A1. Traces of DLM responses to a 10 pulse train at 100 Hz before and after the injection of mecamlamine hydrochloride (8 ng/mg). Failures in muscle responses are illustrated here by asterisks (*). **A2.** Effects of mecamlamine hydrochloride on the GF-DLM pathway with increasing dosage. *** $p < 0.001$, Wilcoxon signed-rank test. **B1.** Traces of TTM responses to a 10 pulse train at 100 Hz before and 1 minute after the injection of mecamlamine (8 ng/mg). No effect of mecamlamine hydrochloride on the ability of the GF-TTM pathway to follow stimuli at 100 Hz was seen. **B2.** Effects of mecamlamine hydrochloride on GF-TTM pathway with increasing dosage (4, 8, 13, 16, 22, 39 and 104 ng/mg). There was no significant difference before and after injection at any drug dosage. Control flies (saline injections) are displayed as 0 ng/mg injected.

Figure 3. Electrophysiological recordings from the GFS before and after injection of tetraethylammonium hydroxide

Superimposed Traces of TTM and DLM responses of ten trains to ten stimuli given at 100 Hz before and after the injection of tetraethylammonium hydroxide (2.7 ug/mg). After injection most responses are completely lacking from both pathways (top traces). Occasionally, few responses are observed, which in most cases are significantly increased in duration (bottom traces, prolonged responses marked with the asterisks) when compared to responses before injection.

Figure 4. Electrophysiological recordings from the GFS before and after of injection of ImI

A1. Traces of DLM responses to a 10 pulse train at 100 Hz before and after the injection of ImI (53 ng/mg). Failures of muscle responses are illustrated here by asterisks (*). **A2.** Effects of ImI on the GF-DLM pathway with increasing dosage and over time after injection (1, 5, 10, 15, 20 minutes). * $p < 0.05$, ** $p < 0.01$, Wilcoxon signed-rank test. **B1.** Traces of TTM responses to a 10 pulse train at 100 Hz before and after injection of ImI (53 ng/mg). ImI had no effect on the ability of the GF-TTM pathway to follow stimuli at 100 Hz. **B2.** Effects of ImI on GF-TTM pathway with increasing dosage and over time (1, 5, 10, 15, 20 minutes after injection). There was no significant difference before and after injection at any dosage. Control flies (5% DMSO in DI water injections) are displayed as 0 ng/mg injected.