A Pharmacological Approach to Dissecting the Neuromodulation of Locomotary Circuits in *Caenorhabditis elegans* First-Stage Larvae (L1)

by

Shagun Gupta

A thesis submitted in conformity with the requirements
for the course MGY480Y

Supervisor: Dr. Andy Fraser

April 11, 2016

Table of Contents

Shagun Gupta

Abstract

Despite having a nervous system that consists of only 302 neurons, *Caenorhabditis elegans* contains molecular and cellular signaling pathways that are comparable to higher organisms, making it an excellent organism to model complex biological behaviors. Deciphering the logic of the motor circuit is an essential component of such models. In C. elegans, the motor circuit undergoes major developmental restructuring at the end of the L1 larval stage. In the adult worm, both ventral and dorsal body wall muscles are innervated in alternating manner by cholinergic and GABAergic motor neurons which act in concert with each other to drive locomotion. In the L1 larvae, neither cholinergic nor GABAergic motor neurons that innervate ventral muscles exist and yet the worms generate undulation patterns similar to that adult worms. This robustness in motor output indicates the presence of an intricate network of signaling relationships that can be modulated to provide optimal motor performance. In particular, G-protein coupled receptors regulate motor circuit activity by mediating various feedback inhibition pathways. Previously, the GAR-3 muscarinic receptor has been implicated in providing alternative feedback inhibition on cholinergic motor neurons. The goal of this research was to further investigate whether muscarinic receptors play a role in modulating output of the GABAergic motor neuron via feedback mechanisms in the L1 motor circuit. Instead of using traditional genetic interrogation tools, chemical perturbation was employed. It was found that larval worms recover from sustained GABAergic stimulus after a period of rapid paralysis. Muscarinic receptors do not play a role in mediating this recovery; however, activation of the GAR-3 muscranic receptor via arecoline, a muscarinic agonist, leads to enhanced recovery from GABA induced paralysis suggesting that muscarinic receptors may play a key role in the feedback mechanisms that modulate the interplay between the cholinergic and GABAergic system in the L1 motor circuit.

Abbreviations

GABA	γ-aminobutyric acid
VNC	Ventral nerve cord
NMJ	Neuromuscular Junction
ACh	Acetylcholine
AChR	Acetylcholine receptor
mAChR	Muscarinic acetylcholine receptors
nAChR	Nicotinic acetylcholine receptors
GPCR	G-protein coupled receptor
CGC	Caenorhabditis Genetic Centre
NGM	Nematode Growth Medium
rpm	Revolutions per minute

Introduction

In the late twentieth century, Sydney Brennar introduced *Caenorhabditis elegans*, a simple soil nematode, as a model organism to study development and neurobiology (Kaletta and Hengartner, 2006). Over time, the simplicity and experimental amenability of *C. elegans* has transformed it into a powerful system to study the interactions between the nervous system and the external environment. Consisting of only 302 neurons and 5000 chemical synapses, the small size of its nervous system allowed for the construction of a precise map of its neural origins and connections. This, in conjunction with a vast repertoire of mutant libraries and tools for optical imaging and interrogation of circuit electrophysiology, makes *C. elegans* an excellent model in which to dissect the genetics of circuit development and function (Kawano et al., 2011).

The Anatomy and Wiring of the C. elegans Motor Circuit

Animal movement is a complex, coordinated choreography between the nervous system and the musculoskeletal system. In the simplest sense, muscles controlling movement are innervated by motor neurons that reside in the axial nerve cord. These motor neurons receive input from the anteriorly located ganglia (for example, the brain) that extend their processes into the nerve cord and synapse with them. Although these networks are significantly larger and more complex in vertebrates than in non-vertebrates, the basic architecture and arrangement of components in the motor circuit is largely conserved in animals with bilateral symmetry (Von Stetina et al., 2005). As such, C. elegans provides a capable model organism in which to dissect the underlying features of motor circuit development and function. Defining these fundamental circuits provides the basic framework to further develop complex behavioural models that span sensory input to motor output (Gjorgjieva et al., 2014).

C. elegans typically exhibits rhythmic, undulatory locomotion. In the adult motor circuit, this locomotion is driven by a several classes of motor neurons and a small number of "command interneurons". There are five pairs of premotor, command interneurons: AVB, PVC, AVA, AVD and AVE, that integrate input from sensory and upper layer interneurons. These interneurons reside within the head (AVB, AVA, AVD and AVE) or tail (PVC), and extend their processes along the length of the ventral nerve cord (VNC) where they form extensive chemical and electrical with synapses with motor neurons. The five distinct classes of motor neurons, distributed along the VNC, include: A, B, D, VC and AS. The A, B, and D- type motor neurons are further divided into dorsal and ventral subclasses, which innervate either dorsal (DA, DB, DD) or ventral (VA, VB, VD) muscles. The A and B-type motor neurons form excitatory cholinergic neuromuscular junctions (NMJs) with muscle cells. The D-type GABAergic neurons, which are postsynaptic to the A and B-type motor neurons that innervate muscles on the opposite side, form inhibitory NMJs with muscle cells (Figure 1A). As a result, locomotion in the adult worm is driven by the cholinergic motor neurons exciting muscles cells on either side of the body (DB, VB for forward motion and DA, VA for backward motion) while indirectly resulting in contralateral inhibition via excitation of the GABAergic motor neurons (DD and VD) (Figure 1B). Interestingly, these D-type motor neurons are not necessary and in their absence adult worms appear to display normal locomotion (Zhen and Samuel, 2005; Cohen and Sanders, 2014).

Unlike the adult worm, the motor circuit newly hatched L1 larvae (first of four larval stages) has distinct functional differences and is not as well understood. Consisting of only 22 motor neurons, the L1 larvae contains only DA, DB and DD motor neurons. Similar to the properties of the adult circuit, the DB and DA motor neurons are excitatory and are associated

with forward and backward movement, respectively (Zhen and Samuel, 2005). The physiological nature of DD motor neurons is less clear. They are thought to be thought to be inhibitory like in adults, and their activation is crucial for generation of both forward and backward movement (Figure 1B). Additionally, L1 DD neurons exhibit reversed synaptic polarity compared to DDs in adults, forming synapses with the ventral muscles (White et al., 1978). These differences in the nature of D-type neurons in adults and L1s are a result of a neuronal remodeling process that begins in the motor circuit at the end of the L1 developmental stage. Although such remodeling processes are not uncommon, this process remains poorly understood. Hence while cholinergic motor neurons have dedicated roles from birth, the GABAergic D-type motor neurons exhibit extensive developmental plasticity, allowing them to modulate locomotion patterns via different mechanisms at different stages of life (Zhen and Samuel, 2005).

Neuromodulation of the motor circuits via metabotropic signaling

If the L1 motor circuit as currently described is correct, where DA and DB provide excitatory innervation on the dorsal muscles while DD is inhibitory to ventral muscles, it leaves us with an asymmetric model of the motor system. Despite this, the L1 exhibits an unchanged locomotion pattern with similar biophysical properties compared to the adult worm (Backholm et al., 2013). Furthermore, even upon drastic perturbation of the inhibitory versus excitatory input balance to muscles, C. *elegans* only displays subtle changes in motor patterns. This robustness in motor output indicates the presence of an intricate network of signaling relationships throughout the motor circuit that can be modulated to allow normal motor performance in response to a variety of mutant backgrounds and environmental conditions (Zhen and Samuel, 2005).

Signalling networks are largely driven by two major classes of receptors: ionotropic receptors and neurotransmitter-activated, extra synaptic G-protein coupled receptors (GPCRs).

The ionotropic receptors are ligand gated ion channels that open and desensitize rapidly, making them ideal for fast, short-term signaling required for behaviors such as locomotion (Bargmann, 1998). The C. elegans genome contains twenty-nine AChR subunits and four GABA receptor subunit- encoding genes. These subunits make form receptors that function both at motor neurons and body wall muscles to produce excitatory and inhibitory signals converge at muscles to affect body bending (Zhen and Samuel, 2005).

Although a great deal is known about the role of these ionotropic receptors in locomotion, much less is known about how neurotransmitter-gated GPCRs regulate activity in the motor circuit. GPCRs generate slower, longer lasting changes in neuronal excitability than ionotropic receptors, and further extend the signaling capabilities of motor neurons by mediating feedback inhibition (Dittman and Kaplan, 2008). The C. elegans motor circuit contains four known GCPRs: two $GABA_B$ receptor subunits and three muscarinic acetylcholine receptors (mAChR). These receptors help maintain locomotion patterns by dynamic feedback modulation loops between excitatory and inhibitory inputs to muscles. For example, the GAR-2 muscarinic receptor which is expressed on both cholinergic and GABAergic motor neurons contributes to a feedback inhibition of the cholinergic motor neuron activity when acetylcholine levels are elevated (Dittman and Kaplan, 2008; Zhen and Samuel, 2005).

To help define these dynamic signalling relationships mediated throughout the motor circuit, methods to measure neuronal activity in moving animals with high sensitivity are required. Traditional studies have relied on tools such as RNAi perturbation and cell abalation studies. Although these single perturbation methods are effective for identifying essential components of a system, they fail to elucidate the functional connections that modulate motor circuit dynamics (Lehár et al., 2008). These functional connections are best identified by direct

interaction data. In particular, perturbations via drugs provide information that is both distinct and complementary to the information provided by genetic mutations. The advantages of chemical perturbations are that they (i) can produce a quick response in the system, (ii) result in transient perturbation, (iii) allow for control in severity via varying drug concentrations and (iv) various drugs can be used to increase or decrease target activity. Furthermore, drugs can be used to target single domains of multidomain proteins. Small molecules also lend themselves more readily to combination interventions, making them especially useful for integrating systems (Lehár et al., 2008).

In order to study these transient drug responses, the lab developed a simple imaging assay to track the acute effects of chemical perturbation on *C. elegans* mobility. Using this approach, it was demonstrated that aldicarb, an acetylcholinesterase inhibitor, caused paralysis in adult worms. However, L1 worms treated with aldicarb seemed to recover in a dose dependant manner (Figure 2). Further investigation revealed a feedback mechanism unique to L1s mediating this recovery: activation of ionotroptic AChRs via increased synaptic ACh caused rapid paralysis. At the same time, the activation of the slow acting *gar-3* muscarinic AChR (mAChR) actually mediated a slow dose-dependant recovery (Mark Spensley, unpublished observations/data). These data form the underlying framework of my thesis work. Given the crucial role of GABAergic signalling in the L1 larvae, I investigated whether muscarinic receptors also modulate the GABAergic motor output in the L1 motor circuit.

Materials and Methods

Strain Maintenance and Worm Handling

The C. elegans wild-type strain used was N2, Bristol variety. Mutant strains used were JD217 *gar-3(vu78)* V, VC657 *gar-3(gk305)* V, RB756 *gar-2(ok520)* III, RB896 *gar-1(ok755)*, TQ225 *trp-1(sy690)* III. All strains were obtained from the Caenorhabditis Genetic Centre (CGC), University of Minnesota, USA. The strains were maintained at 20°C on Nematode Growth Medium (NGM) agar plates seeded with Escherichia coli (OP50) according to standard protocols (Stiernagle, 2006). To maintain stocks, small chunks of agar were removed from a plate using a knife sterilized under flame and transferred to a fresh plate with food.

Isolation of L1 Larvae

To obtain L1 worms, plates with mixed-stage populations were observed under a microscope to ensure a high ratio of L1's were present. Worms were washed off plates in M9 buffer and pelleted by centrifugation at 1000 rpm for 1 minute. Most of the supernatant was aspirated, leaving behind 3mL. The worms were resuspended and pipetted into a 10µm nylon mesh (Millipore Corporation, Bedford) placed on top of a 96-deep-well plate. L1 worms were passed through the mesh by running the centrifuge to 800 rpm. The filtrate was transferred to a clean mesh and centrifuged again. Synchronized L1s were collected and diluted to a concentration of approximately 12 L1s per 10 µl M9 buffer.

Locomotion Assay

L1 worms in M9 buffer were exposed to varying range drug concentrations in a 96-well plate. Each well contained a total of 100uL worms in M9 and 100uL drug solution. Each well was imaged twice, 0.5 seconds apart, in 5 minute intervals for 3 hours. A fractional mobility score

was then calculated by determining which worms moved between consecutive images. For each drug concentration a minimum of 4 replicate wells were analyzed. This assay is diagrammed in Figure 3.

Preparation of Drug Solutions

In locomotion assay worms were either subject to a single drug (GABA) or to a combination of two drugs (GABA and another drug). Drug combinations and strains used are outlined in Table 1.0.

For assays GABA alone, working concentrations were made at 2X final concentration in M9 and DMSO (1.6% final concentration). Each final well contained 100uL drug and 100uL worms in M9, pipetted using a multichannel pipette.

For assays with a two drugs, working concentrations for each drug were made at 4X final concentration M9 and DMSO (1.6% final concentration). Each final well contained 50uL drug A, 50uL drug B and 100uL worms in M9, pipetted using a multichannel pipette.
Final drug concentrations used were as follows:

- GABA (Sigma-Aldrich; A2129): 500mM, 375mM, 250mM, 187.5mM, 125mM, 62.5mM, 31.25mM.
- Nicotine: 10mM, 5mM, 2.5mM, 1.25mM, 625uM, 312.5uM, 156.3um
- Arecoline: 10mM, 5mM, 2.5mM, 1.25mM, 625uM, 312.5uM, 156.3Um
- Aldicarb: 4mM, 3mM, 2mM, 1mM, 500uM, 250uM, 125Um
- Atropine: 4mM, 2mM, 1mM, 500uM, 250uM, 125uM, 62.5Um

Results

C. elegans L1 worms display recovery from GABAergic stimulus

In order to dissect the pharmacodynamics of the GABAergic system, the response of L1 worms to sustained GABAergic stimulus was examined. L1 worms were exposed to varying concentrations of GABA and their mobility was tracked over a period of 180 minutes. A period of rapid paralysis was observed in the worms following which they exhibited a dose dependent recovery response i.e. worms exposed to lower concentrations of GABA recovered better than those exposed to higher concentrations of GABA (Figure 4a). At higher concentrations of GABA this recovery response was supressed.

The same experiment when performed in adult worms revealed a delayed recovery response, highlighting the existence of inherent differences in the organization of GABAergic motor neurons in the adult motor circuit (Figure 4b). A longer period of paralysis was observed compared L1s, followed by a steady dose dependent recovery response.

Muscarinic receptors are not required for recovery from GABAergic paralysis

The C. elegans genome contains three muscarinic receptors: GAR-1, GAR-2 and GAR-3. L1 response to GABA was examined in strains mutant for muscarinic receptors to investigate whether muscarinic receptors play a role in the recovery from GABA induced paralysis. Since previous data from the Fraser lab demonstrated that the GAR-3 muscarinic receptor is responsible for mediating recovery from paralysis induced via sustained cholinergic stimulus (Mark Spensley, unpublished observations/data), it was hypothesized that the absence of muscarinic receptors would decrease recovery from GABA induced paralysis. Response to

GABA paralysis was unchanged in *gar-1* and *gar-2* mutants (Figure 5), i.e. no different than the rapid paralysis followed by distinct recovery pattern seen in the wild-type N2 strain. This suggests that GAR-1 and GAR-2 muscarinic receptors are not involved in mediating recovery from GABA paralysis. As a positive control, mutant response to aldicarb exposure was measured in the same experiment.

However, unlike *gar-1* and *gar-2* receptor mutants which did not show a remarkable change in response to GABAergic stimulus, *gar-3* mutants appeared to show greater recovery over time, to GABA paralysis than wild-type (Figure 6). As a positive control, mutant response to aldicarb exposure was measured in the same experiment. Although there may be complex interactions in the motor nervous system that could explain why *gar-3* mutants show better recovery to GABA paralysis, it is possible that it is a result of unknown effects from mutation itself which is a substitution in exonic region of the protein, resulting in a missense mutation (WormBase) and prompts the need for further investigation.

Activation of muscarinic GAR-3 receptor enhances recovery from GABA paralysis

Pharmacological screens for identifying feedback interactions between cholinergic and GABAergic signaling were carried out by examining combinations of cholinergic modulators and GABA. It was reasoned that since the GABAergic and cholinergic system act in concert with one another to modulate movement in L1, modulating cholinergic receptor activity would impact recovery to GABA.

Using an 8x8 array, eight different concentrations of GABA were compared against each of the 8 different concentrations of a cholinergic modulator. To analyze the results of the array, an expected mobility value was calculated by multiplying the fractional mobility value of the

individual drugs. Each experiment was performed four times such that each combination of drug concentrations had four replicates.

Affect of Nicotine exposure on GABA paralysis

Response to GABA in the presence of nicotine, an nicotinic acetylcholine receptor (nAChR) agonist, was examined. C. elegans genome possesses an extensive twenty-nine nAChR encoding genes. These receptors are expressed at both cholinergic and GABAergic motor neurons, and as well as muscle cells within the motor circuit (Jones et al., 2007). The results were found to be as expected, i.e. fractional mobility scores corroborated with calculated expected values (Figure 7). Activation of nAChR via nicotine did not significantly impact rate of recovery to GABA paralysis.

Affect of Aldicarb exposure on GABA paralysis

L1 response to GABA in the presence of aldicarb was examined. Aldicarb is a cholinesterase inhibitor that leads to elevated levels of ACh that can cause paralysis due to prolonged muscle contraction (Nguyen et al., 1995). The results were found to be as expected, i.e. fractional mobility scores corroborated with calculated expected values (Figure 8).

Atropine has no effect on GABA paralysis

Response to GABA in the presence of atropine, a muscarinic antagonist, was examined (Lee et al., 2000). Atropine on its own, without GABA, displayed no visible phenotype in the worm, i.e. mobility was unaffected. When combined with GABA, it had no effect on GABA induced paralysis at any concentration. These data are presented in Figure 9.

Arecoline enchances recovery from GABA paralysis

Combinations of GABA and arecoline, a muscarinic agonist, was examined. Here, the results revealed data that significantly differed from expected mobility values. At high concentrations of GABA which usually supress recovery, arecoline was able to significantly enhance recovery. These data are presented in Figure 10. The recovery occurred in a dose dependant manner, i.e. at higher concentrations of arecoline, worms recovered quicker and better (increased mobility). This rescuing effect by arecoline suggests the muscarinic receptors may play a key role in the feedback mechanisms that modulate the interplay between the cholinergic and GABAergic system in the L1 motor circuit. Previous studies have indicated that the GAR-3 muscarinic receptor is an important target of arecoline (Steger, 2004). To confirm that arecoline induced recovery from GABA paralysis is mediated via the GAR-3 muscarinic receptor, the effect of arecoline on GABA paralysis was examined in *gar-3* mutant L1 worms. Mutation of *gar-3* suppressed the rescuing effect of arecoline exposure on GABA paralysis was significantly diminished compared to wild-type (Figure 11).

Discussion

Significant progress has been made to elucidate the connectivity of the motor circuit in C. elegans; however, the nature of functional connectivity and the signaling mechanisms involved remain less well understood. While ion gated channels mediate the fast synaptic transmission in the motor circuit, neurotransmitter-gated GPCRs play a crucial role in modulating and fine-tuning these responses via feedback mechanisms (Dittman and Kaplan, 2008). The goal of this research was to identify roles, if any, for muscarinic receptors in modulating response of GABAergic motor neuron output in the L1 larvae.

Results showed that an increase of synaptic GABA induced rapid flaccid paralysis. This is likely to be mediated via activation of the $GABA_A$ receptor in the muscle cells which leads to decreased muscle tone (Sujkowski, 2010). Following the period of rapid paralysis, a dose-dependent recovery pattern is observed. Unlike the GAR-3 mediated recovery seen in worms treated with aldicarb, absence of either of the three muscarinic receptors did not worsen recovery from GABA paralysis. The GAR-3 receptor mutant demonstrated a slight but noticeable improvement in recovery response to GABA. Although there may be complex interactions in the motor circuit that could explain why *gar-3* mutants show better recovery to GABA paralysis, it is probable that the effect is a result of unknown effects from the nature of the mutation itself. This is further supported by the fact in the presence of atropine, a muscarinic antagonist, there is no improvement in recovery from GABA paralysis. Therefore, it can be concluded that muscarinic receptors do not play a direct role in in recovery from sustained GABAergic stimulus. Instead, it is possible that recovery is mediated via the GBB-1/GBB-2 heterodimeric $GABA_B$ receptor which is already known to play a role in modulating feedback inhibition on cholinergic motor neurons in adult worms (Dittman and Kaplan, 2008; Schultheis et al., 2011). If in L1 worms the

$GABA_B$ receptor localizes on cholinergic neurons as seen in adults, detection of excess GABA could lead to feedback inhibition of cholinergic stimulus which in turn would down regulate GABAergic output. This would suggest that GABAergic neurons are postsynaptic to cholinergic neurons in L1s and that modulation of GABAergic output is regulated via cholinergic output.

Further evidence for cholinergic regulation of GABAergic motor neuron activity comes from the fact that although although muscarinic receptors are not directly involved in mediating GABA recovery, activation of the GAR-3 muscarinic receptor via arecoline leads to enhanced recovery from GABAergic stimulus. However, it is important to note that recovery from GABAergic stimulus is likely directed via a different mechanism than arecoline mediated recovery from GABAergic due to their varying response dynamics. At high concentrations of GABA, recovery from paralysis is diminished; however, upon arecoline exposure the worms are able to recover fully. Currently it is unknown where gar-3 localizes; identification of localization patterns could shed light on the mechanism of action by which arecoline enhances GABAergic recovery. Possible localization on the GABA motor neuron may suggest a biochemical mechanism by which either GABA signalling is down regulated or expression of GABA transaminase, the enzyme responsible for GABA degradation, increased. A previous study in the rat brain demonstrated muscranic agonists such as arecoline induce a rapid decrease of GABA levels and an increase of its destruction via GABA transaminase, although the mechanism of action was not identified (Shevtchenko et al., 1978). In contrast, localization on cholinergic neurons would suggest electrical transduction (Philbrook et al., 2013).

Lastly, expression patterns of GAR-3 expression change across development; it is highly expressed in L1 larvae but almost nonexistent in the adult worm (Park et al., 2003). This would suggest the mechanism modulating recovery from GABA paralysis upon muscarinic receptor

activation is unique to L1s and could play a role in fine-tuning cholinergic and GABAergic motor outputs to provide optimal locomotion in the absence of VD, VB and VA motor neurons found in adults.

In summary, this report presents preliminary data suggesting that cholinergic motor neurons play a role in modulating GABAergic output via muscarinic receptors. Through this and ongoing studies by other members of the lab, we hope to gain insights on complex signaling relationships that maintain homeostatic states in biological systems.

Future Directions

Future experiments will be focused on first identifying the receptor mediating recovery from sustained GABAergic stimulus. This could be performed by targeting the GABAA and GABAB receptors with various antagonists bicuculline and saclofen respectively.

Identifying the localization of the GAR-3 receptor in the motor circuit would help frame and guide further research questions. Currently the lab is working on expressing a GFP tagged GAR-3 in the L1 larave to visualize areas where it localizes.

Additionally, live imaging of neuronal activity using genetic calcium or voltage sensors provides a real-time measurement of the activity patterns of the neural network in behaving animals (Kawano et al., 2011). This approach could be used to further dissect which neurons get activated when arecoline is introduced into a system with excess GABA.

Beyond these experiments, further systems remain to be explored. Although GABA and ACh are the two primary motor neurons that drive locomotion in the worm, these rely on input from upstream interneurons. These interneurons rely on serotonin and dopaminergic systems to regulate their activity and disruption in normal activity of these interneurons can have a significant impact on the locomotion of the worm.

Acknowledgements

I wish to thank Dr. Andy Fraser for providing me the opportunity to complete my fourth year project in his lab and and as well as Mark Spensley for providing me with continual guidance, direction and help with this project.

In addition, I would like to thank all members of the Fraser Lab for their help and advice throughout the year.

References

Backholm, M., Ryu, W. and Dalnoki-Veress, K. (2013). Viscoelastic properties of the nematode Caenorhabditis elegans, a self-similar, shear-thinning worm. *Proceedings of the National Academy of Sciences*, 110(12), pp.4528-4533.

Bargmann, C. (1998). Neurobiology of the Caenorhabditis elegans Genome. *Science*, 282(5396), pp.2028-2033.

Cohen, N. and Sanders, T. (2014). Nematode locomotion: dissecting the neuronal–environmental loop. *Current Opinion in Neurobiology*, 25, pp.99-106.

Dittman, J. and Kaplan, J. (2008). Behavioral Impact of Neurotransmitter-Activated G-Protein-Coupled Receptors: Muscarinic and GABAB Receptors Regulate Caenorhabditis elegans Locomotion. *Journal of Neuroscience*, 28(28), pp.7104-7112.

Gjorgjieva, J., Biron, D. and Haspel, G. (2014). Neurobiology of Caenorhabditis elegans Locomotion: Where Do We Stand?. *BioScience*, 64(6), pp.476-486.

Han, B., Bellemer, A. and Koelle, M. (2015). An Evolutionarily Conserved Switch in Response to GABA Affects Development and Behavior of the Locomotor Circuit of Caenorhabditis elegans. *Genetics*, 199(4), pp.1159-1172.

Jones, A., Davis, P., Hodgkin, J. and Sattelle, D. (2007). The nicotinic acetylcholine receptor gene family of the nematode Caenorhabditis elegans: an update on nomenclature. *Invertebrate Neuroscience*, 7(2), pp.129-131.

Kaletta, T. and Hengartner, M. (2006). Finding function in novel targets: C. elegans as a model organism. *Nature Reviews Drug Discovery*, 5(5), pp.387-399.

Kawano, T., Po, M., Gao, S., Leung, G., Ryu, W. and Zhen, M. (2011). An Imbalancing Act: Gap Junctions Reduce the Backward Motor Circuit Activity to Bias C. elegans for Forward Locomotion. *Neuron*, 72(4), pp.572-586.

Lee, Y., Park, Y., Nam, S., Suh, S., Lee, J., Kaang, B. and Cho, N. (2000). Characterization of GAR-2, a Novel G Protein-Linked Acetylcholine Receptor from Caenorhabditis elegans. *Journal of Neurochemistry*, 75(5), pp.1800-1809.

Lehár, J., Stockwell, B., Giaever, G. and Nislow, C. (2008). Combination chemical genetics. *Nature Chemical Biology*, 4(11), pp.674-681.

Park, Y., Kim, S., Shin, Y., Choi, B. and Cho, N. (2003). Alternative splicing of the muscarinic acetylcholine receptor GAR-3 in Caenorhabditis elegans. *Biochemical and Biophysical Research Communications*, 308(4), pp.961-965.

Philbrook, A., Barbagallo, B. and Francis, M. (2013). A tale of two receptors. *Worm*, 2(3), p.e25765.

Rakowski, F., Srinivasan, J., Sternberg, P. and Karbowski, J. (2013). Synaptic polarity of the interneuron circuit controlling C. elegans locomotion. *Front. Comput. Neurosci.*, 7.

Schultheis, C., Brauner, M., Liewald, J. and Gottschalk, A. (2011). Optogenetic analysis of GABAB receptor signaling in Caenorhabditis elegans motor neurons. *Journal of Neurophysiology*, 106(2), pp.817-827.

Shevtchenko, A., Bobrova, N. and Shevtchenko, N. (1978). Regulation of gamma-aminobutyric acid metabolism in different brain structures by cholinergic and adrenergic drugs. *Ann Ist Super Sanita*, 14(1), pp.133-8.

Steger, K. (2004). The GAR-3 Muscarinic Receptor Cooperates With Calcium Signals to Regulate Muscle Contraction in the Caenorhabditis elegans Pharynx. *Genetics*, 167(2), pp.633-643.

Sujkowski, Alyson L., "GABA(A) receptor homeostasis at the C. elegans neuromuscular junction" (2010). Theses and Dissertations. Paper 986

Von Stetina, S., Treinin, M. and Miller, D. (2005). The Motor Circuit. *International Review of Neurobiology*, pp.125-167.

White, J., Albertson, D. And Anness, M. (1978). Connectivity changes in a class of motoneurone during the development of a nematode. *Nature*, 271(5647), pp.764-766.

Williams, D., El Bejjani, R., Ramirez, P., Coakley, S., Kim, S., Lee, H., Wen, Q., Samuel, A., Lu, H., Hilliard, M. and Hammarlund, M. (2013). Rapid and Permanent Neuronal Inactivation In Vivo via Subcellular Generation of Reactive Oxygen with the Use of KillerRed. *Cell Reports*, 5(2), pp.553-563.

Zhen, M. and Samuel, A. (2015). C. elegans locomotion: small circuits, complex functions. *Current Opinion in Neurobiology*, 33, pp.117-126.

Tables

Table 1.0. Major experiments performed and final setup in each well imaged for those experiemnts

Experiment #	Strain	Drug A	Drug B	Final setup in each well
1	N2	GABA		100uL GABA + 100uL worms in M9
2	N2	GABA	Nicotine	50uL GABA + 50uL Nicotine + 100uL worms in M9
3	N2	GABA	Aldicarb	50uL GABA + 50uL Aldicarb + 100uL worms in M9
4	N2	GABA	Arecoline	50uL GABA + 50uL Arecoline + 100uL worms in M9
5	N2	GABA	Atropine	50uL GABA + 50uL Atropine + 100uL worms in M9
6	JD217	GABA		100uL GABA + 100uL worms in M9
7	VC657	GABA		100uL GABA + 100uL worms in M9
8	RB896	GABA		100uL GABA + 100uL worms in M9
9	RB756	GABA		100uL GABA + 100uL worms in M9
10	JD217	GABA	Arecoline	50uL GABA + 50uL Arecoline + 100uL worms in M9

Figures

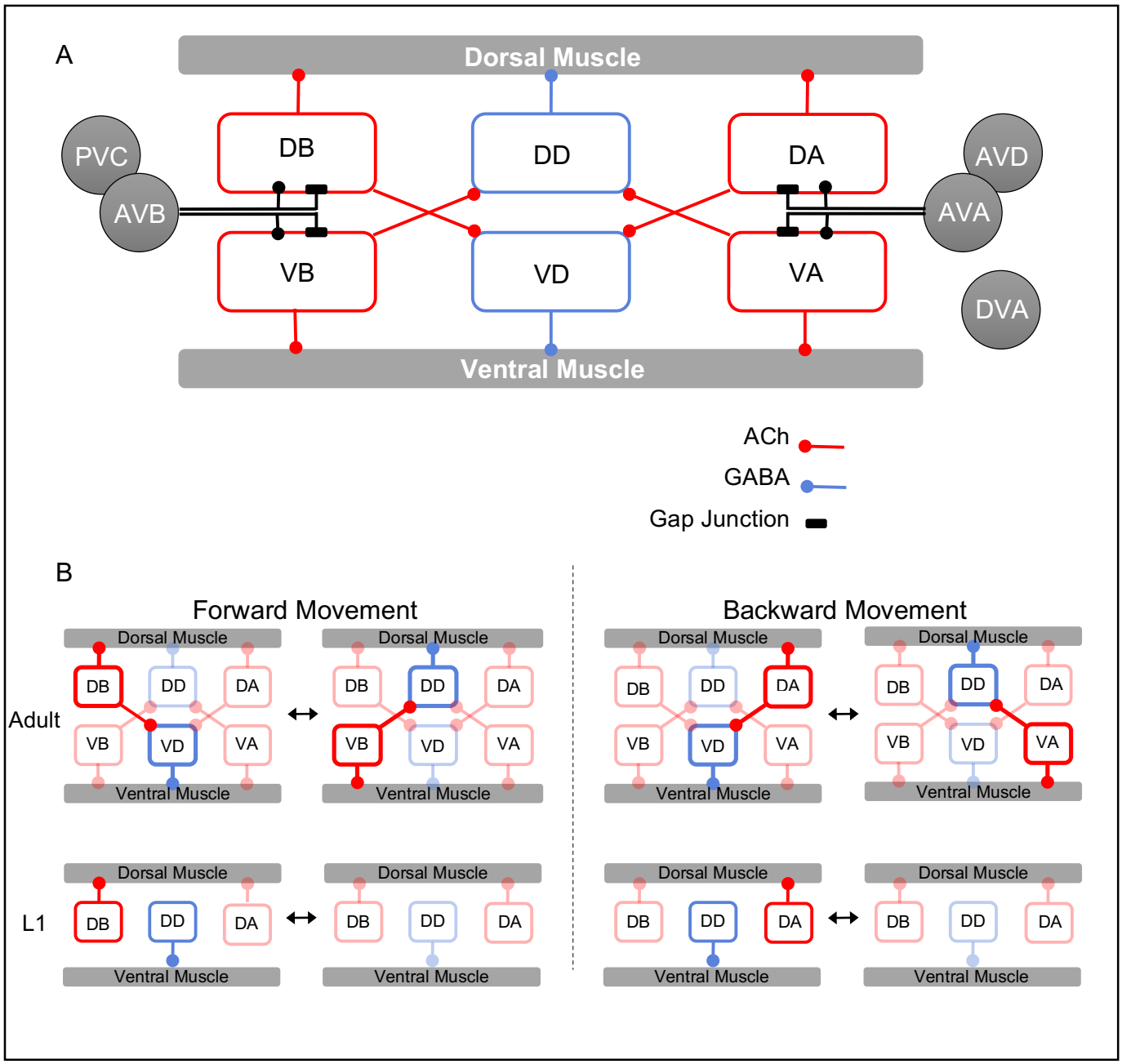

Figure 1. The C. elegans motor circuit. A) A simplified representation of the anatomic connections between the cholinergic motor neurons (B-type, for forward movement; A-type, for backwards movemet), GABAergic neurons (D-type) and muscle cells in the adult motor circuit. B) Inherent differences between the L1 and adult motor circuit as a result of developmental remodelling. During adult locomotion, alternating excitation and inhibition of dorsal and ventral muscles is mediated by B-type and D-type motor neurons during forward movement and is mediated by A-type and D-type motor neurons during backward movement. In L1, only the DA, DB, and DD motor neurons are present, and the DD motor neurons innervate the ventral muscle. Adapted from (Zhen and Samuel, 2015).

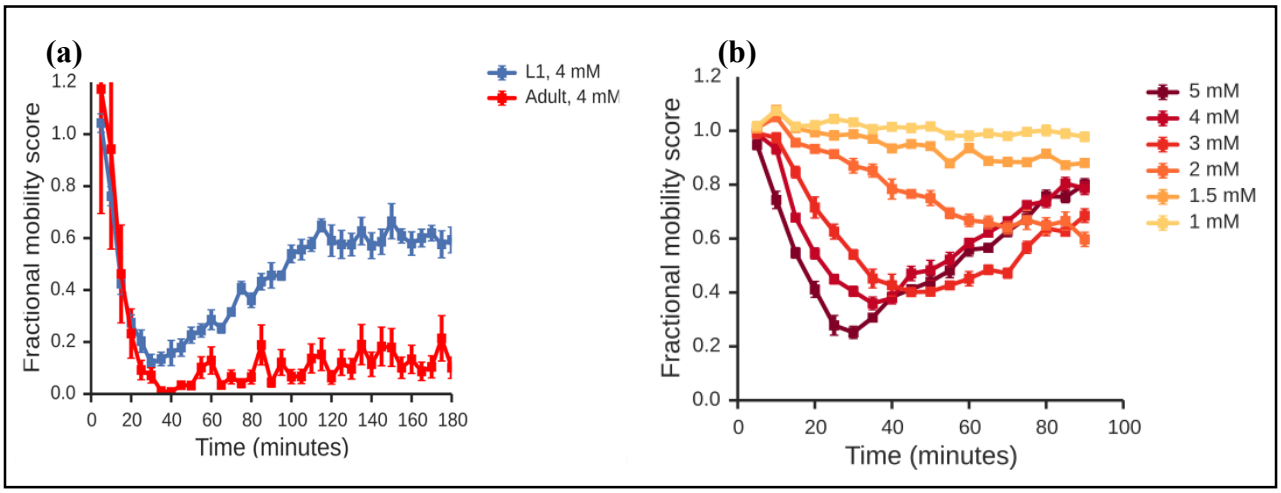

Figure 2 Effect of aldicarb on C. elegans. (a) Dose response over time showing the effects of aldicarb on C. elegans. Adult worms do not recover while L1 larvae show recovery. (b) Dose-dependant recovery of L1 larvae. L1 exposed to lower concentrations. Image adapted from (Mark Spensley, unpublished observations/data).

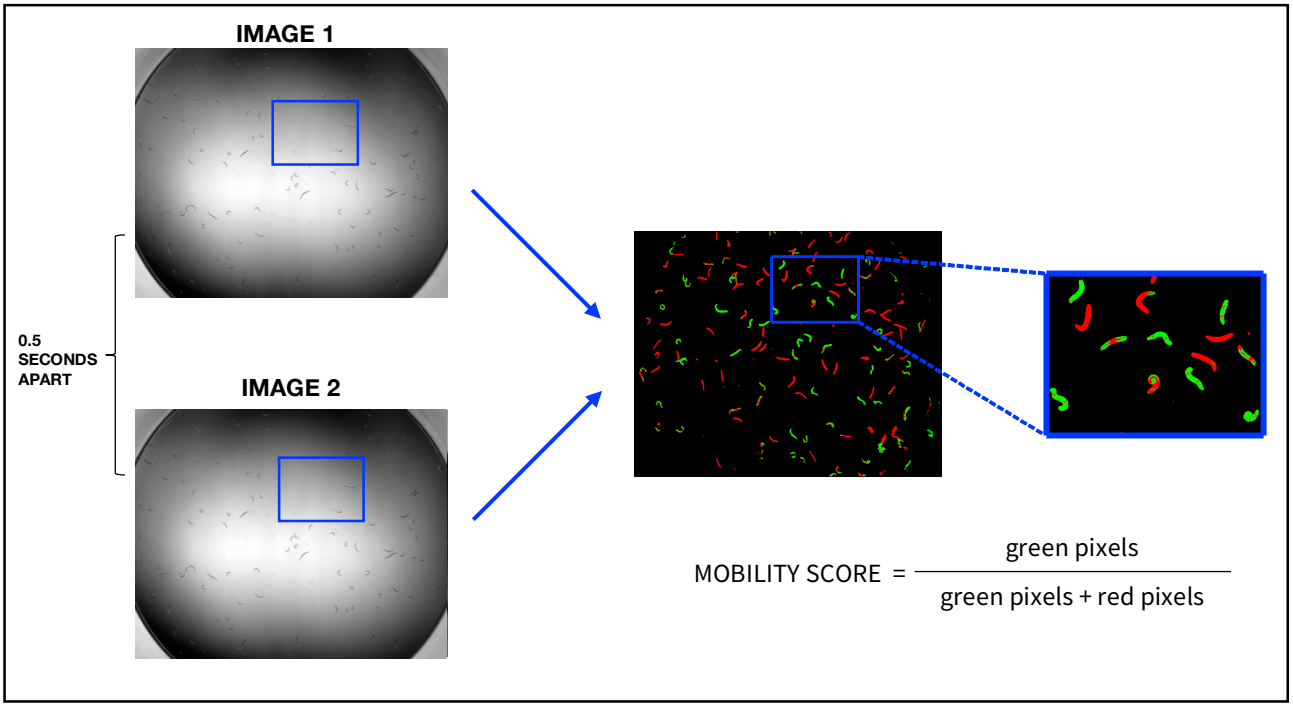

Figure 3. Imaging Assay. L1 larvae in M9 buffer were exposed to a range of drug concentrations in a 96-well plate. Two images of each well are taken 0.5 seconds apart. A fractional mobility score is then calculated by determining which worms moved between the two images. This is repeated every five minutes for 180 minutes to produce a drug-response over time. Image adapted from **(Mark Spensley, unpublished observations/data).**

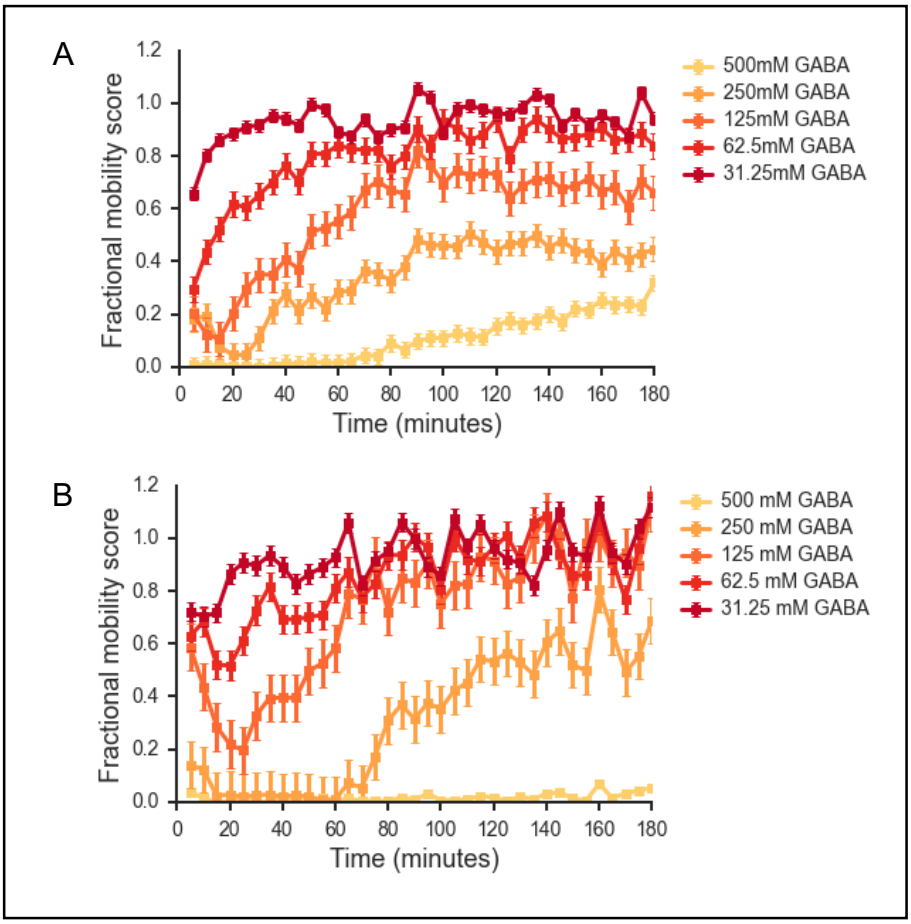

Figure 4. Drug response over time for GABA in A) L1 larval worms. L1's display fast paralysis followed by a steady, dose-dependent recovery B) Adult worms exhibit a similar fast paralysis to L1 worms however recovery is delayed.

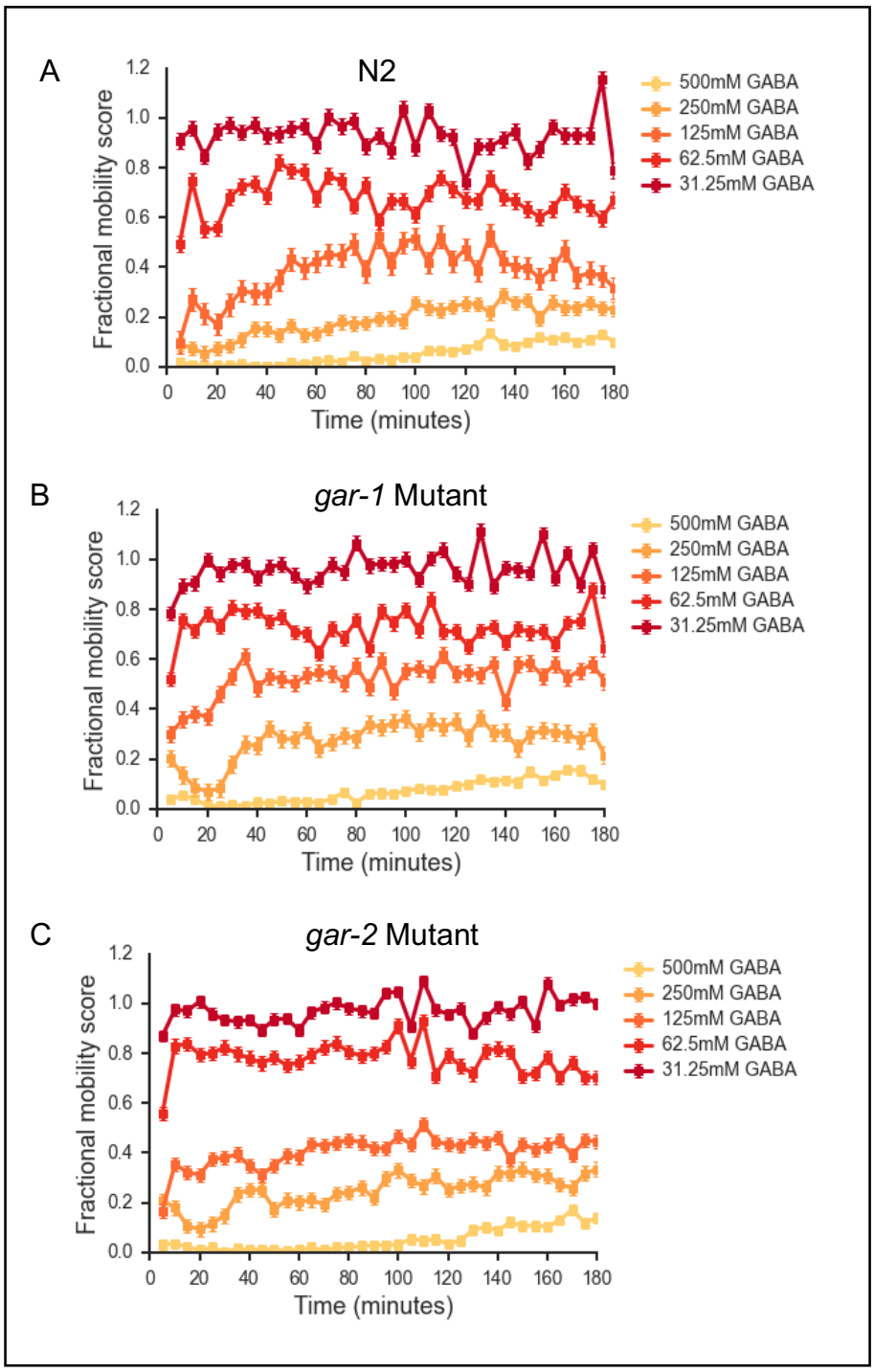

Figure 5. Drug response over time for GABA in A) N2 wild-type L1 larval worms. B) *gar-1* mutant worms C) *gar-2* mutant worms. Response to GABA stimulus is unchanged in *gar-1* and *gar-2* mutants compared to N2 wild-type

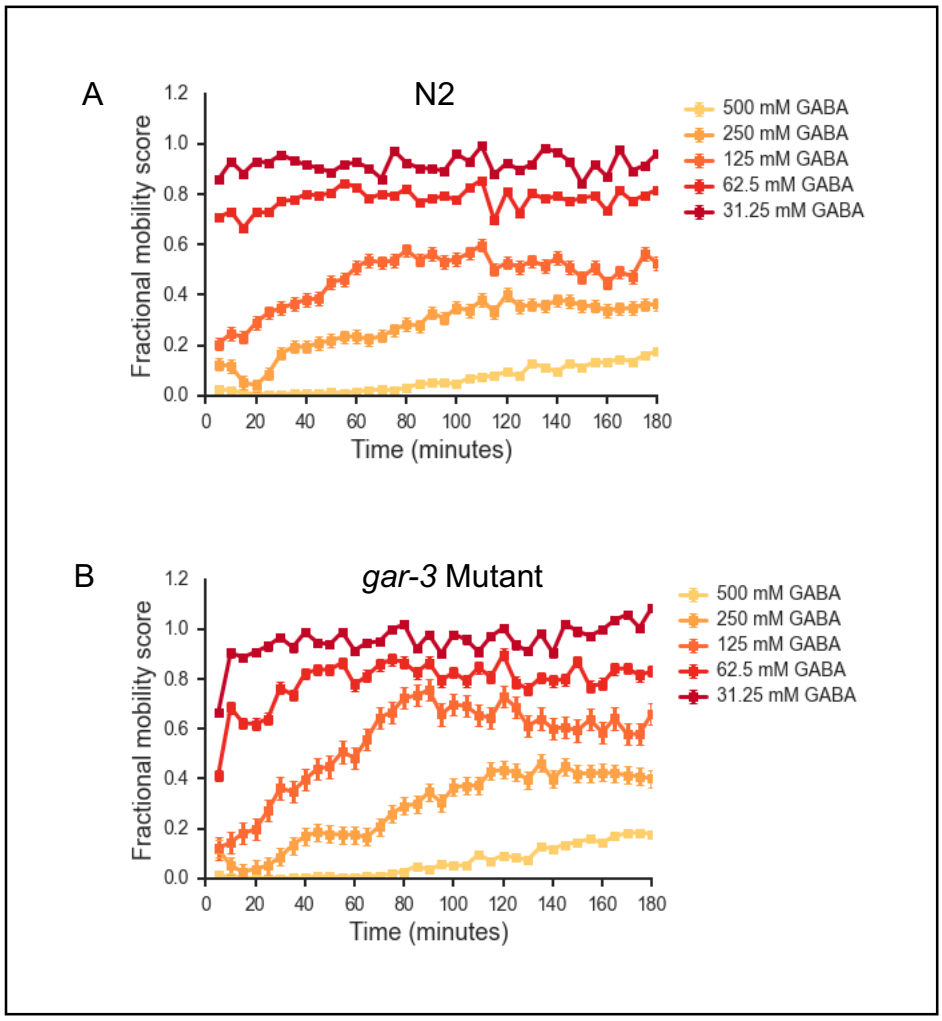

Figure 6. Drug response over time for GABA in A) N2 wild-type L1 larval worms. B) *gar-3* mutant worms. Recovery in *gar-3* mutant is enhanced compared to N2 wild-type.

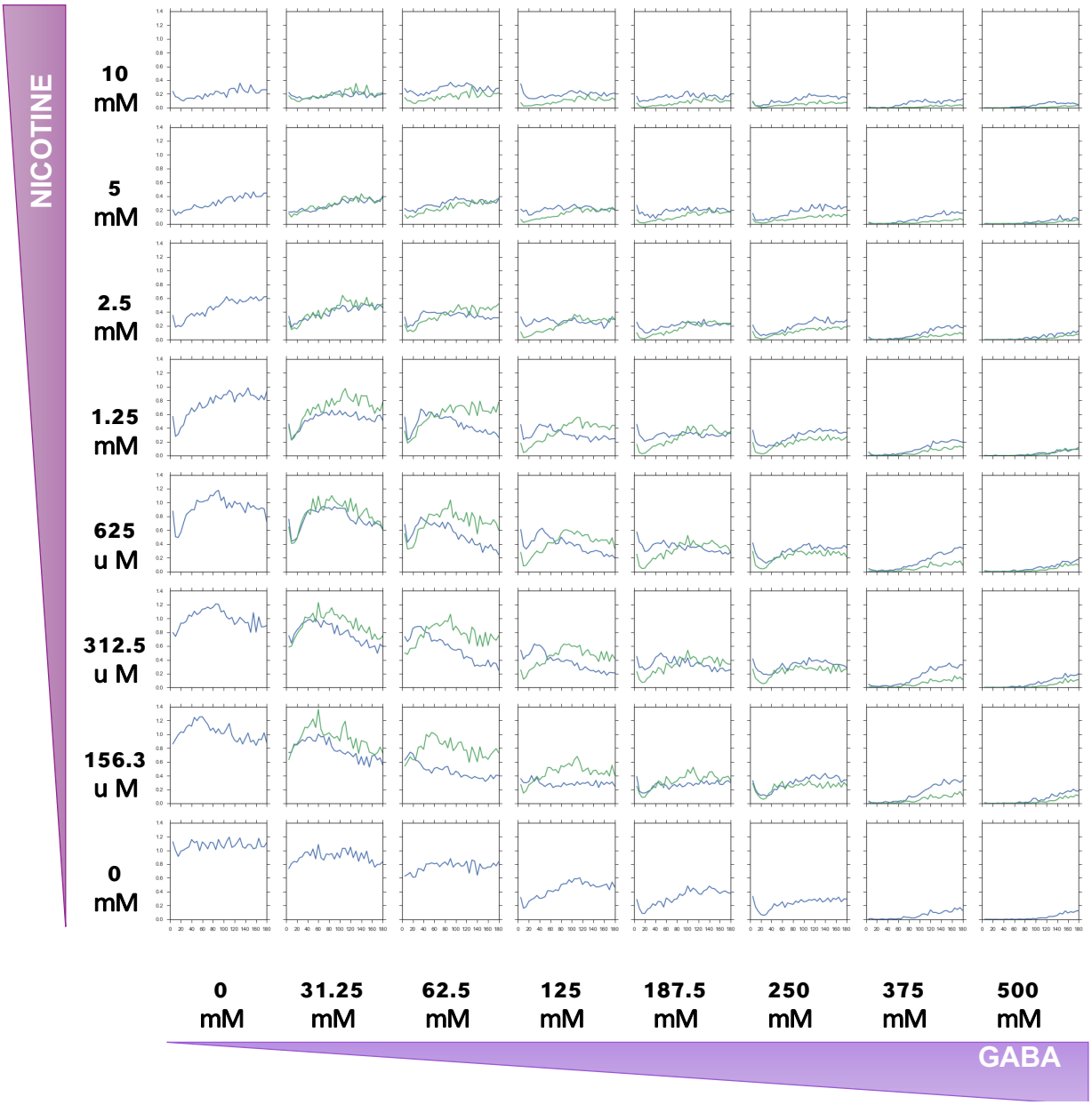

Figure 7. An 8x8 array of drug combinations. Each cell above contains the dose-response over 180 minutes for the particular combination of drugs. Eight different concentrations of GABA were compared against each of the 8 different concentrations of nicotine, a nAChr agonist in L1 larvae. To analyze the results of the array, an expected mobility value was calculated by multiplying the fractional mobility value of the individual drugs. Overall, actual fractional mobility scores match predicted mobility scores suggesting no interactions. Green: Predicted fractional mobility score. Blue: Experimental fractional mobility score.

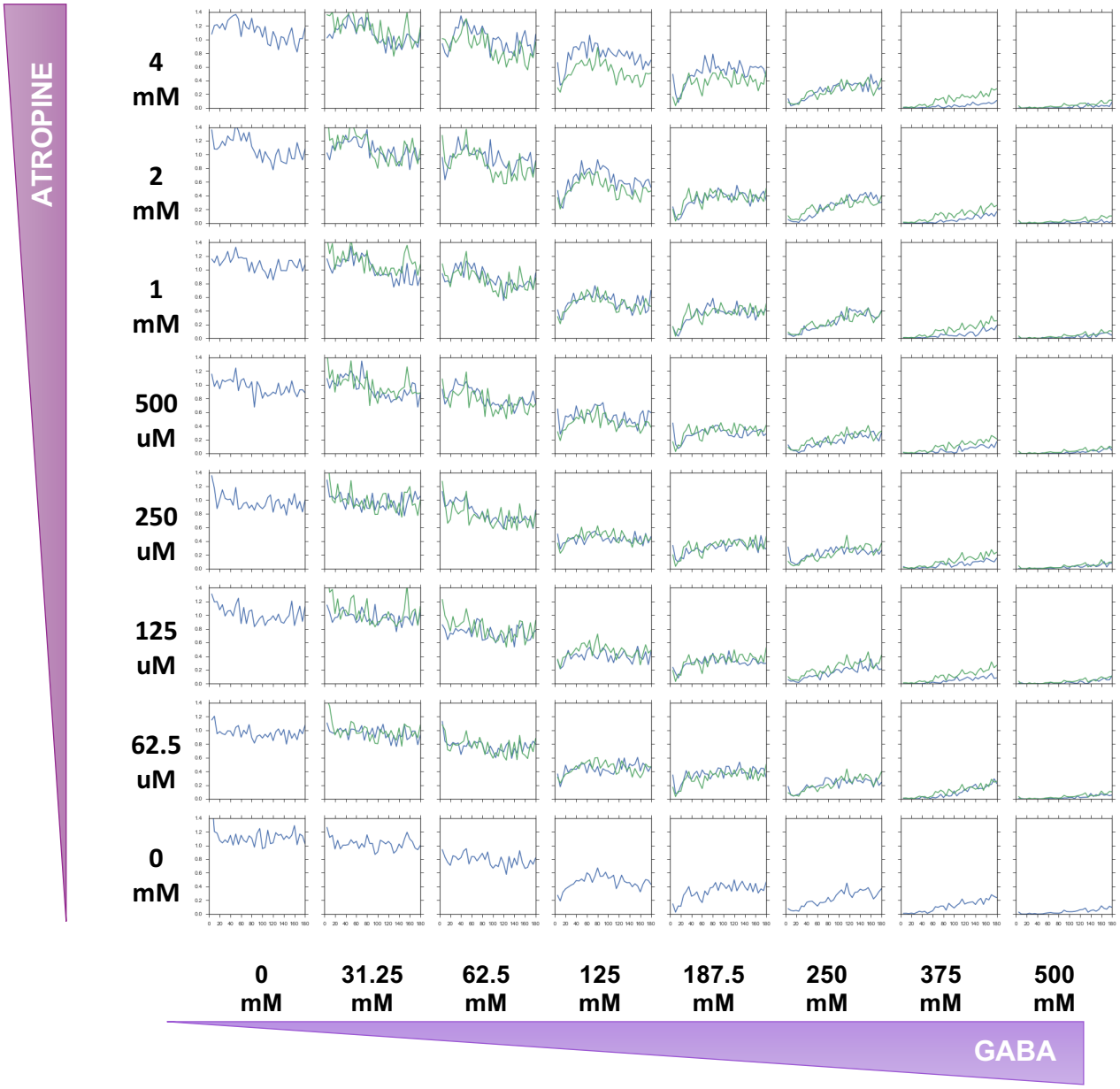

Figure 8 An 8x8 array of drug combinations. Each cell above contains the dose-response over 180 minutes for the particular combination of drugs. Eight different concentrations of GABA were compared against each of the 8 different concentrations of atropine, a muscarinic antagonist, in L1 larvae. To analyze the results of the array, an expected mobility value was calculated by multiplying the fractional mobility value of the individual drugs. Overall, actual fractional mobility scores match predicted mobility scores suggesting no interactions. Green: Predicted fractional mobility score. Blue: Experimental fractional mobility score.

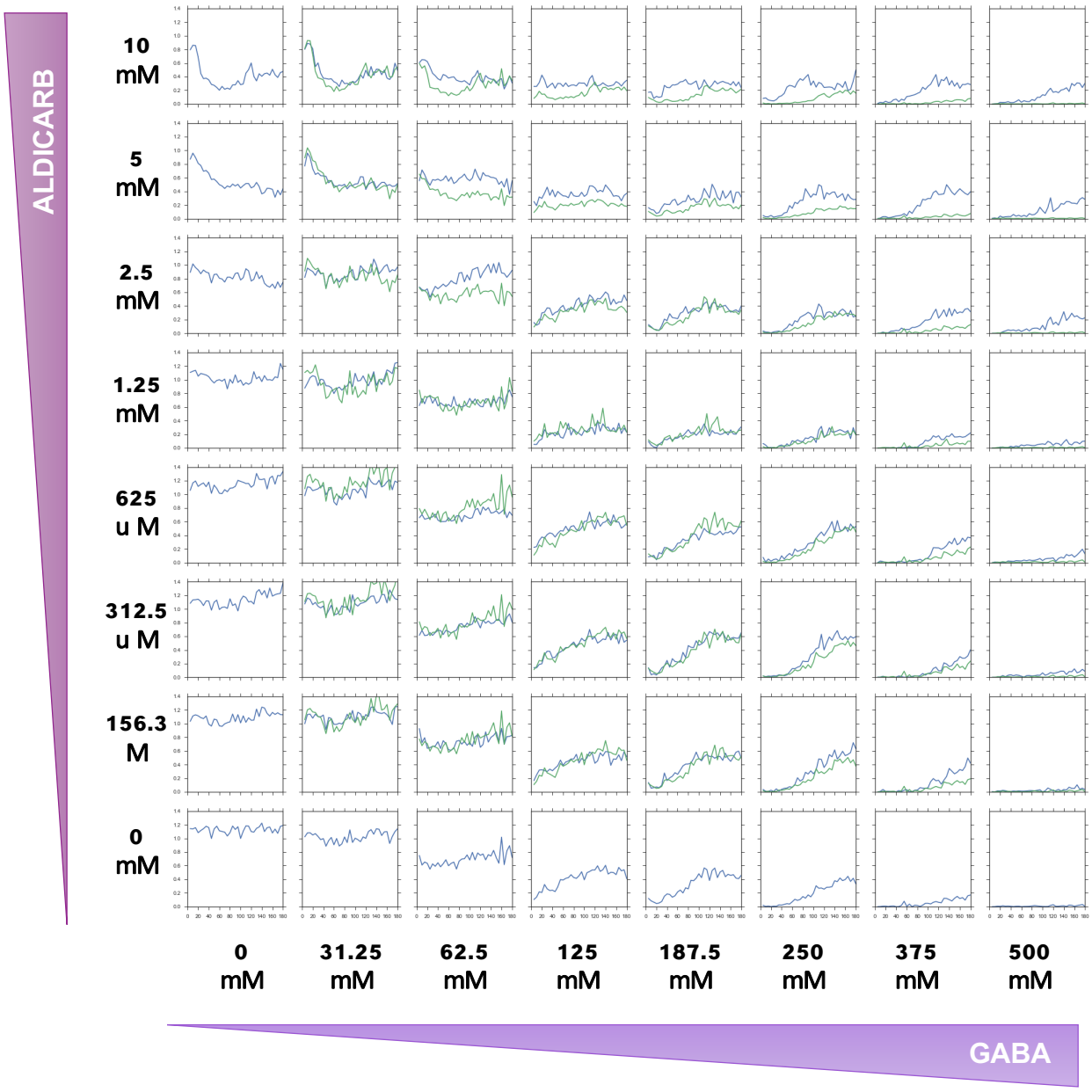

Figure 9. An 8x8 array of drug combinations. Each cell above contains the dose-response over 180 minutes for the particular combination of drugs. Eight different concentrations of GABA were compared against each of the 8 different concentrations of alidcarb, a cholinesterase inhibitor, in L1 larvae. To analyze the results of the array, an expected mobility value was calculated by multiplying the fractional mobility value of the individual drugs. Overall, actual fractional mobility scores match predicted mobility scores, however, high concentrations of aldicarb, some improvement was seen in recovery from GABA paralysis at GABA concentrations of 500mM. Green: Predicted fractional mobility score. Blue: Experimental fractional mobility score.

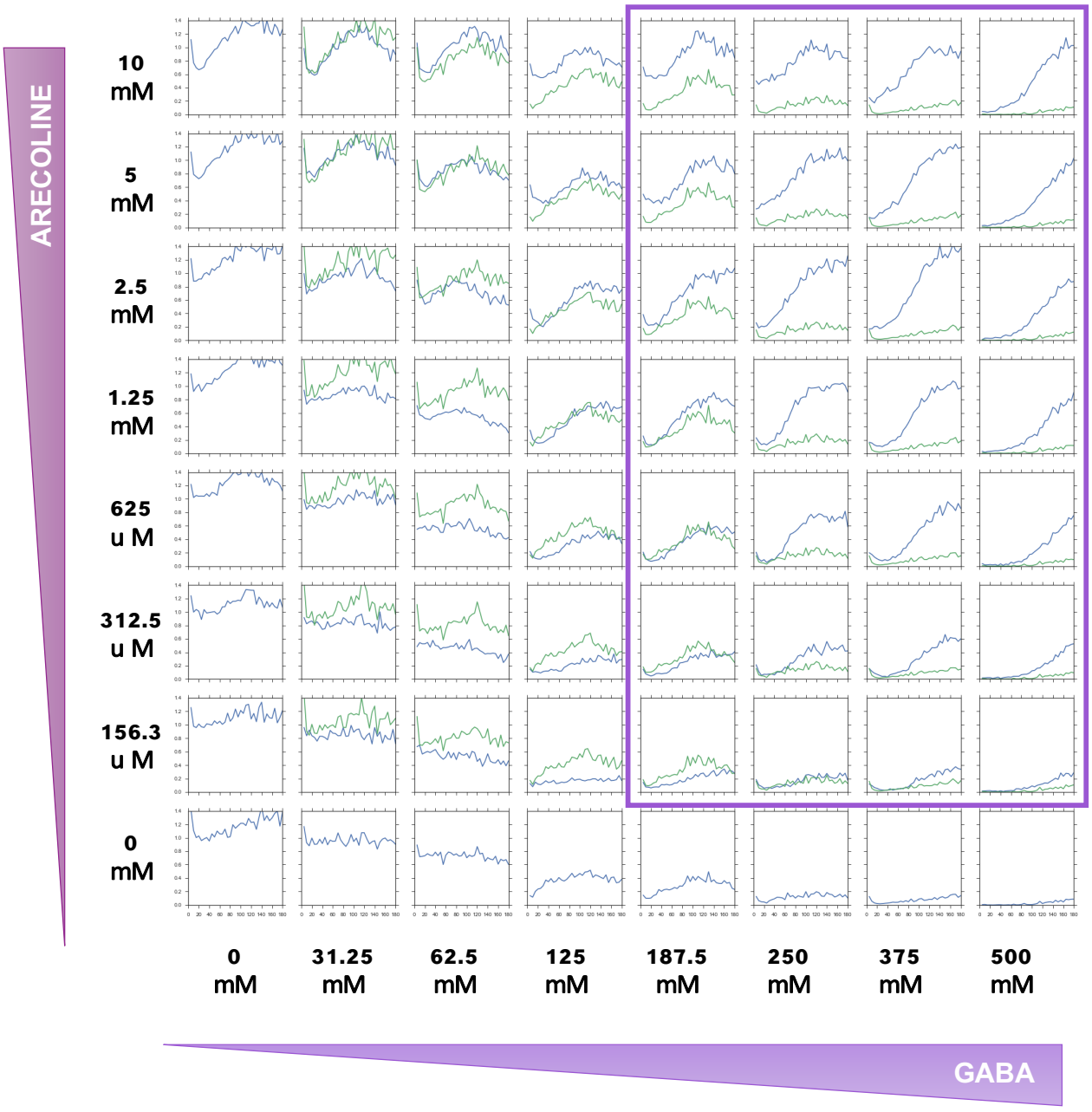

Figure 10. An 8x8 array of drug combinations. Each cell above contains the dose-response over 180 minutes for the particular combination of drugs. Eight different concentrations of GABA were compared against each of the 8 different concentrations of arecoline, a muscarinic agonist, in L1 larvae. To analyze the results of the array, an expected mobility value was calculated by multiplying the fractional mobility value of the individual drugs. Actual mobility scores differed significantly from expected scores (outlined in purple) suggesting a positive interaction. Green: Predicted fractional mobility score. Blue: Experimental fractional mobility score.

.

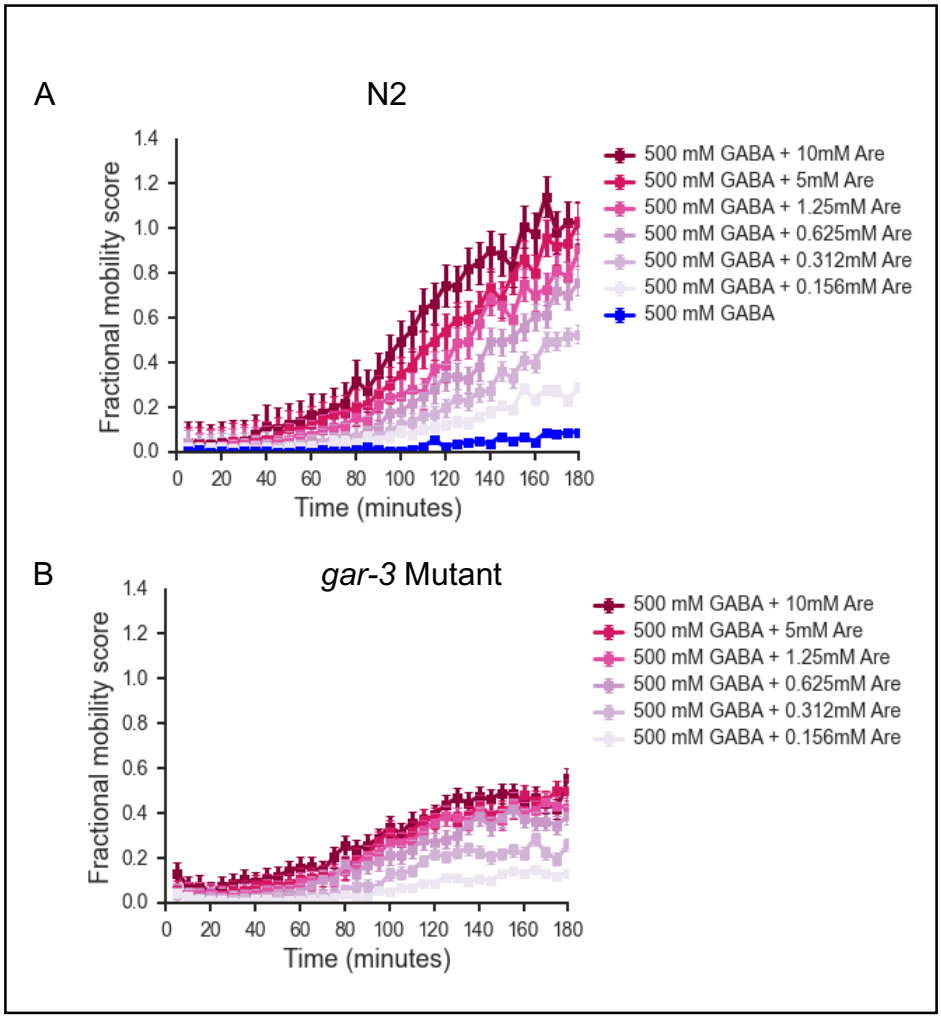

Figure 11. Drug-response over time for exposure to GABA (fixed, 500Mm) and arecoline (varying concentrations) in A) wild-type N2 L1 larvae B) *gar-3* mutant L1 larvae. Arecoline rescues wildtype worms exposed to GABA in a dose-dependant manner. Worms lacking a functional *gar-3* receptor are unable to recover from paralysis suggesting that arecoline mediates recovery from GABA induced paralysis via the GAR-3 muscarinic receptor.