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Reverse genetic screening of innexin gap junction proteins in drosophila neurons

Shannon P. Fox
James Madison University

Reverse Genetic Screening of Innexin Gap Junction Proteins in *Drosophila* Neurons

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FACULTY COMMITTEE:

Project Advisor: Susan Halsell, Ph.D.,
Associate Professor, Assistant Dept. Head, Biology

Reader: Timothy A. Bloss, Ph.D.,
Associate Professor, Biology

Reader: Corey L. Cleland, Ph.D.,
Associate Professor, Biology

HONORS PROGRAM APPROVAL:

Bradley R. Newcomer, Ph.D.,
Director, Honors Program

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Abstract

The reflexive response and perception of pain (nociception) is an evolutionarily conserved process in animals. Pain can be a major health concern and current treatments often prove insufficient, especially in regards to chronic pain. Greater understanding of the molecular processes underlying pain sensation could lead to new and more effective treatments. The aim of this study is to investigate the molecular mechanisms of cold nociception in *Drosophila melanogaster.* A specific subset of peripheral sensory neurons (Class III dendritic arborization (da) neurons), are implicated in *Drosophila* larvae's response to noxious cold.

Previous literature has associated a family of gap junction protein, termed Innexins, to be responsible for various roles in the structure of the central nervous system and the giant fiber system in *Drosophila*. It is unknown if the Innexin family also functions in the Peripheral Nervous System (PNS).

This study focused on Innexin family members as potential mediators of noxious cold-evoked sensory behavior within multidendritic neurons of the PNS. A cold behavioral assay was used to investigate the role of two *innexin* family member genes: *ogre* and *shaking-B.* The analyses revealed that Shaking-B may be required for nociceptors to react properly to a cold stimulus. Ogre was revealed to have an apparent inverse effect, in which *Drosophila* larvae responded more strongly to a cold stimulus when lacking the gene producct. These studies indicate that Innexins probably do play a role in the peripheral nervous system and in reacting to noxious cold stimuli.

Background and Significance

Introduction

Nociception refers to an organism's perception and reaction to potentially damaging noxious stimuli. Nociception is clearly beneficial, but debilitating chronic pain occurs in humans when pain signals abnormally persist months after any form of trauma, injury, or infection (NINDS 2015). Due to the lack of understanding behind the perpetuation of chronic pain, understanding the mechanisms underlying neuropathic pain has become very important (Mogil 2004). Better understanding of nociceptive processes should aid in developing treatments for chronic pain. Evolutionary similarities between mammalian and Drosophila nociception makes the fruit fly an ideal organism to study the molecular components of nociceptor neurons.

Model System: *Drosophila* Nociception

Drosophila larvae have two primary types of peripheral sensory neurons: Type I and Type II. Type I neurons innervate bristle and chordotonal sensory organs on the external surface; presumably, they are associated with mechanosensory function, such as an external light touch (Im and Galko 2011). Type II peripheral sensory neurons are also known as dendritic arborization (da) sensory neurons, or multidendritic sensory neurons (Fig. 1; Im and Galko 2011). These da neurons have naked dendritic projections that extend to nearly every cell in the epidermis of the larva. The naked dendritic extensions found in da neurons for *Drosophila* are structurally similar to mammalian nociceptors. These da neurons play a role in sensing various noxious stimuli including extreme heat or cold and strong touch (Tracey *et al.* 2003; Zhong *et al.* 2010; Sulkowski *et al.* 2011; Armengol, 2015; Williamson, 2015).

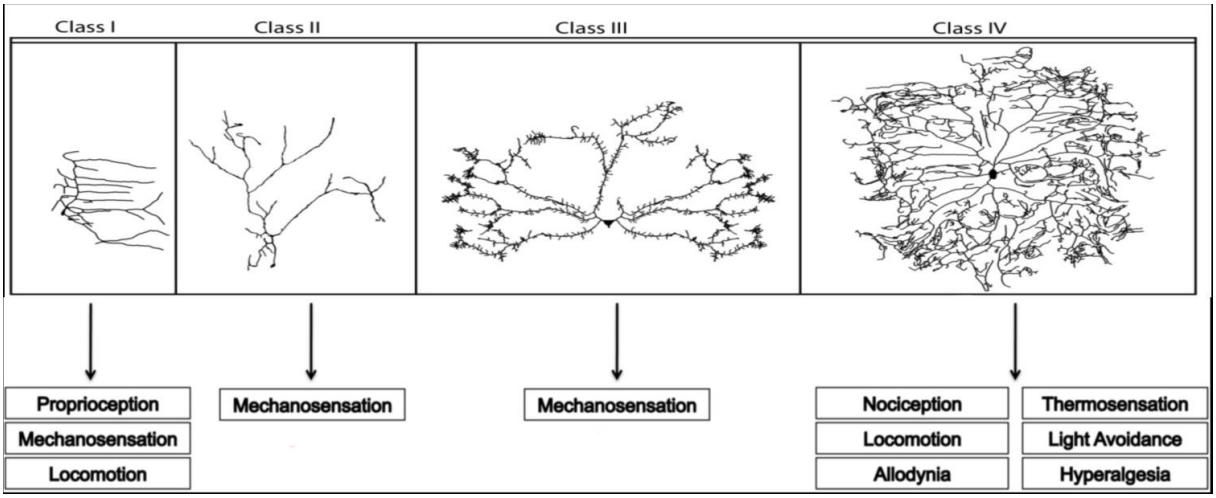


Figure 1. The four classes of peripheral neurons, which are known as dendritic arborization (da) neurons, matched with their functional role in nociceptive responses. The classes are grouped by the increasing complexity of their dendritic arborizations. Response to noxious cold is mediated primarily by Class III da neurons (Armegol, 2015). In this study, RNAi was expressed in all of the da neurons. (Figure is personal communication from Dan Cox and is based on a figure in Sullivan, *et al.* 2013).

The da neurons within *Drosophila* are classified into four subgroups (Fig. 1). Each subgroup is categorized based on the amount of branching, and the group class number increases with the complexity of the branching of the naked dendrites. The projections of these da neurons can function as nociceptors, as they are found underneath the epidermis and are both structurally and functionally similar to that of vertebrate nociceptors. Each class of da neurons is responsible for different nociceptive functions for different noxious situations. For example, class IV da neurons are found to be responsible for several nocifensive responses (Tracey *et al.* 2003; Hwang *et al.* 2007). It is still unknown whether each neuronal class has its own particular nociceptive function or whether each class makes a slight contribution to the perception of each modality (Im and Galko 2011). However, studies performed in Daniel Cox's lab implicate Class

III da neurons as primarily responsible for cold nociception (personal communication; Armengol, 2015).

In response to noxious cold temperatures, the reflex of the *Drosophila* larvae is to cringe, (Fig 2). The normal temperature range for *Drosophila* ranges between 19ºC-29ºC. The characteristic of 'cringe' behavior begins when temperatures reach 10ºC (Armengol, 2015). Maximum 'cringing' of larvae occurs consistently at 6ºC. Changes in this cringe behavior can be assessed to determine the requirement of a gene's function in larvae in which the function of the gene has been knocked-down. By quantifying the length of the larva over time, the extent of cringing for a mutant can be compared to that of a control. The less a larvae cringes during a behavioral assay, the more likely the gene product being studied is involved in cold nociception.

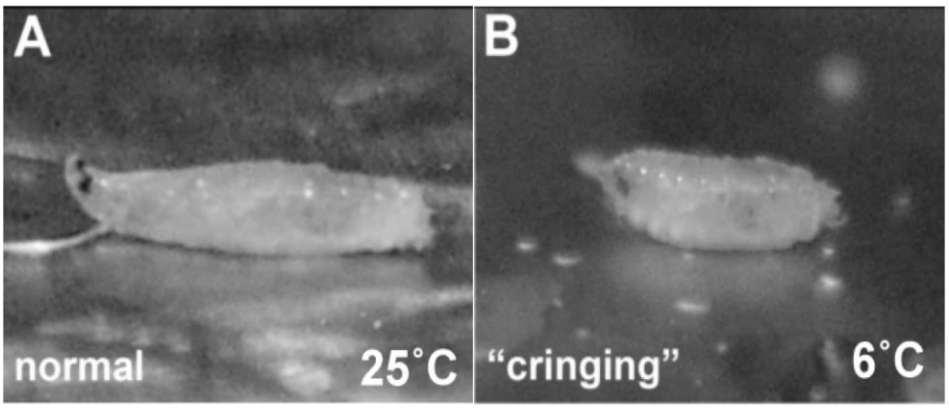


Figure 2. *Drosophila* third instar larva's response to noxious cold observable by the contraction of their body. A) Wild type larval behavior when exposed to a temperature of 25.6ºC. B) When exposed to noxious cold, wild type larvae exhibiting a contraction in body length: the 'cringing' behavior. From Sullivan, *et al.* (2013).

Drosophila Cold Nociception: Molecular Contributors

For an organism to sense and respond to noxious stimuli, the epithelium must have nociceptors that detect the stimulus then propagate the signal. This signal is then transferred through the nervous system to responsive motor neurons and also the brain. By using reverse genetic screening (see Methodology and Experimental Approach below) molecules that function in cold nociception can be identified. Ben Williamson (Williamson 2015; S. R. Halsell unpublished) focused on the role of degenerate epithelial sodium channels (DEG/NaC) family members within class III da neurons. Three members of the *pickpocket* family were identified as partially responsible for transduction (*ppk23)* and propagation (*ppk12 and ppk25)* of noxious cold stimulation in class III da neurons (Williamson 2015). This study focuses on the synaptic transmission and continuation of the electrical signal caused as a result of noxious cold. One candidate class of molecules that may play a role in the transmission of electric signals are Innexins.

Drosophila Innexin Gap Proteins

Innexins are protein trans-membrane structures that create gap junctions in invertebrates. The structure of these gap junction proteins are conserved in humans, found in proteins called Connexins (Phelan and Starich 2001). There are eight *innexin* genes known in *Drosophila* (Fig 3; Bauer *et al.* 2005), and functions have been identified by mutant analysis for *innexin 1 (ogre*), *innexin 2* (*kropf*), *innexin 4* (*zero population growth*) and *innexin 8* (*shaking-B*) (Bauer et al 2005). *Kropf* functions during embryonic gut formation (Bauer *et al.* 2002) and with *zero population growth* is required in differentiating germ cells (Tazuke *et al.* 2002). Of particular

interest for this study are the Ogre and Shaking-B functions within the nervous system (Lipshitz and Kankel, 1985; Krishnan *et al.* 1993; Phelan *et al.* 1996; Trimarchi and Murphey, 1997).

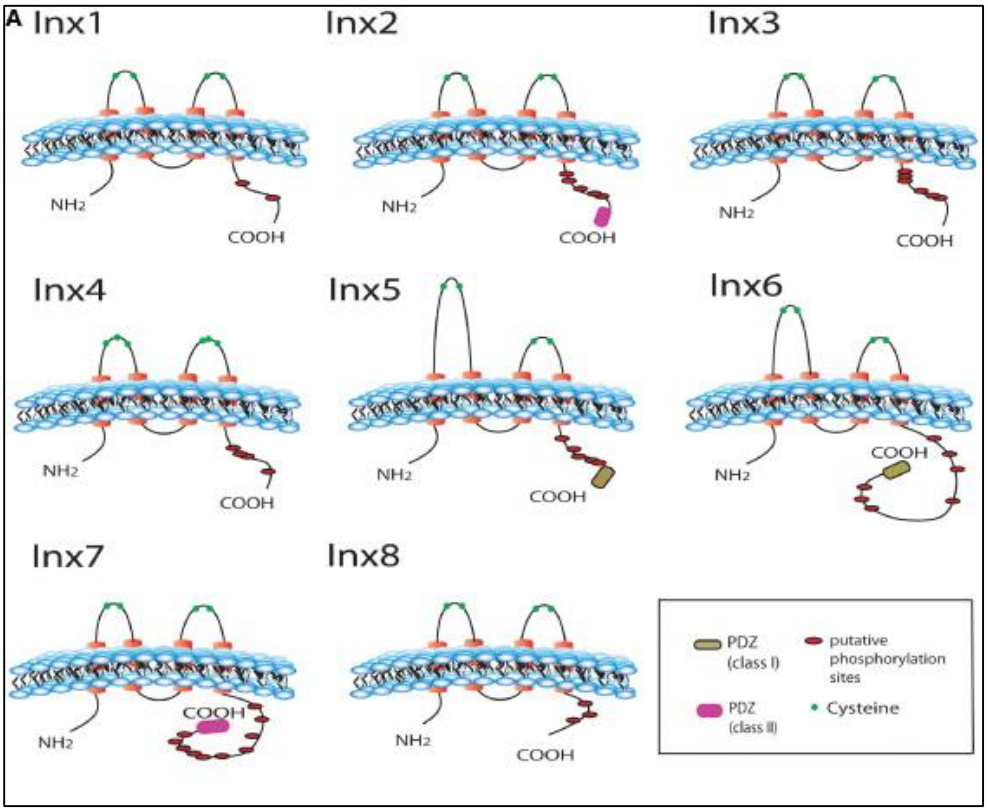


Figure 3. Representations of the hypothesized structure of the eight known Innexins found in *Drosophila*. This study focused on Ogre (Innexin 1) and Shaking-B (Innexin 8). Figure from Bauer *et al.* 2005

Some Connexins in humans form electrical synapses in neurons. Such connections between neurons allows direct passage of an electrical signal across the synapse. This bypasses the need for chemical transmission at synapses between neurons and results in a faster transmission of a signal (Pereda 2014). This would be an ideal system for a nociceptive response because the nociceptor signal can be rapidly transmitted to subsequent neuron in the pathway. By analogy to vertebrate Connexins, *Drosophila* Innexins could function similarly in the PNS. In fact, previous research analyzing Innexin function in the *Drosophila* Central Nervous System

revealed its requirement in the Giant Fiber System (GFS). This pathway mediates escape behavior in a fly, and inhibition of Shaking-B (Innexin 8) caused a lack of escape behavior in flies in response to light stimuli (Krishnan *et al.* 1993; Phelan *et al.* 1996). It is required also in additional flight responses outside of the GFS (Trimarchi and Murphey 1997).

Drosophila Cold Nociception: Specific Gene Targets for Study

This study examined two Innexins to determine whether or not any member of the *Drosophila* innexin family functions during cold nociception. Dr. Halsell's lab will continue to screen the rest of the Innexins in the future. The Innexin known as Ogre (Optic Ganglion Reduced, Innexin 1) has been determined in previous research to be a gene essential for postembryonic neurogenesis (Lipshitz and Kankel 1985; Holcroft *et al.* 2013). This stage of neurogenesis begins in the early larva stage, where embryonic neuroblasts reactivate and divide into new adult specific neurons. In a normal fly, this period of neurogenesis remodels the neural architecture to form the adult central nervous system. Flies lacking the Ogre gene were significantly smaller than their wild-type counterpart, the central neural architecture was highly disorganized, and the optic lobes were significantly smaller (Lipshitz and Kankel 1985). Previous research has identified that the loss of *ogre* function in surviving larva also leads to defective locomotor and sensory-motor activity. This behavioral failure of the mutant *Drosophila* is presumed to be a result of the failure of the adult nervous system to develop and is similar to defects seen in neural degeneration (Holcroft et al. 2013). The defective sensory-motor activity of mutants for *ogre* may thus prove to have an effect on the ability of the mutant to respond to noxious sensory stimuli, such as cold. The role of Ogre in postembryonic development of the nervous system suggests it is a good candidate for examining the mutant's effect on a fly's ability to react to noxious stimuli.

The second mutation that was studied is known as *shaking-B* (innexin 8), previously known as *passover*. This gene is constitutively expressed in the adult central nervous system and in pupa during Giant Fiber synapse formation (Krishnan et al 1993). The Giant Fiber System (GFS) in *Drosophila* transmits visual and mechanosensory information from the brain to the thorax. *Drosophila* lacking *shaking-B* also lack the escape behavior to visual stimuli (Krishnan *et al.* 1993; Phelan *et al.* 1996). Another study pinpointed the function of *shaking-B* in the GFS, determining that it was responsible for mediating the electrical, not chemical, synapses (Blagburn et al. 1999). It was also shown that *shaking-B* was responsible for mediating the electrical synapses in more than just the GFS. Mutant flies of *shaking-B* eliminated the electrical synapses between haltere afferents and a flight motorneuron, indicating that the expression of *shaking-B* may be required throughout the entire *Drosophila* nervous system for most electrical synapses (Trimarchi and Murphey 1997).

In this study I examined whether Ogre and Shaking-*B* play a role in the mediation of electrical synapses in response to noxious cold. Due to previous research suggesting it was required in most electrical synapses, *shaking-B* was chosen as the best candidate from the Innexin family to have any impact on the larva's nociceptive behavior. The cold behavioral assay would assist in determining if ogre and/or shaking-*B* play a role the da neurons of the Peripheral Nervous System. Specifically, the effect of tissue specific down-regulation of *ogre* and *shaking-B* gene expression on the larval cold behavioral response was studied.

Methodology and Experimental Approach

Summary of Experimental Approach

This project aimed to determine any possible roles that Ogre (Innexin 1) and Shaking-B (Innexin 8) might play in cold nociception of *Drosphila melanogaster*. Gene expression of *ogre* or *shaking*-B was down-regulated in all da neuron classes. Cold-plate behavioral assays were performed on third instar larvae expressing RNAi for *ogre* and *shaking-B*. A functional role in cold nociception was determined by analysis of data obtained from the cold assays.

Tissue Specific Expression of *innexin* RNAi Transgenes

Experimentally induced RNA Interference (RNAi) can decrease or eliminate expression of endogenous mRNAs. Transgenic RNAi lines for *ogre* and *shaking-B* are available from the Bloomington Drosophila Stock and Vienna Drosophila Resource Centers (Table 1). Tissue specific expression of these RNAi transgenes was driven in da neurons with the Gal-4/UAS system. The GAL4/UAS system is a tool developed to target gene expression in *Drosophila* (Fig. 4; Duffy 2002). This technique repurposes a *Saccharomyces cervisiae* gene expression system. The yeast GAL4 encodes an 881 amino acid protein that acts as an activator protein for galactose metabolism gene expression. GAL4 activates transcription of a target gene by binding the target genes upstream activating sequence (UAS; Fig. 4). In 1988, induced GAL4 expression was seen to be capable of stimulating the transcription of a gene associated with the UAS promotor in *Drosophila* and the GAL4/UAS system itself has no deleterious phenotypic effects (Duffy 2002). In this study, the genes downstream of the UAS are either *ogre* or *shaking-B* RNAi constructs.

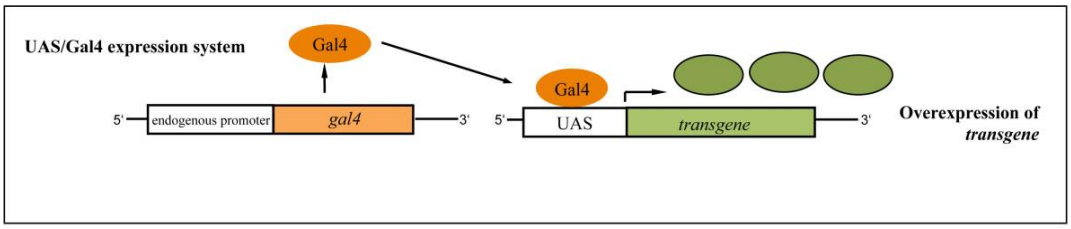


Figure 4. The *Drosophila* UAS/Gal4 Expression system. Transcription of the yeast GAL4 activator protein is controlled by *Drosophila* tissue specific regulatory elements. In turn, the GAL4 binds its UAS target. As a result, any UAS-transgenes present in the genome are expressed only in those specific cells. Figure modified from Prübing, Voigt, and Schulz 2013.

RNA interference evolved as a cellular system to protect cells against retrovirus infection (Obbard *et al.* 2009). Retroviruses inclue a double-stranded RNA as a phase in their infection cycle. In order to prevent retroviral mRNA from being used to produce viral protein, cells have learned to recognize the double stranded (dsRNA) mRNA as a foreign body (Fig. 5). A protein called Dicer binds to dsRNAs and breaks them up into 20-base pair fragments called small interfering RNAs (siRNA) or micro RNAs (miRNA; Wilson and Doudna 2013). A protein complex known as the RNA-induced silencing complex (RISC) then binds to individual si- or miRNAs and uses the fragmented RNA to complementary base pair endogenous mRNA. Once RISC complex binds the mRNA, the mRNA is targeted for degradation.

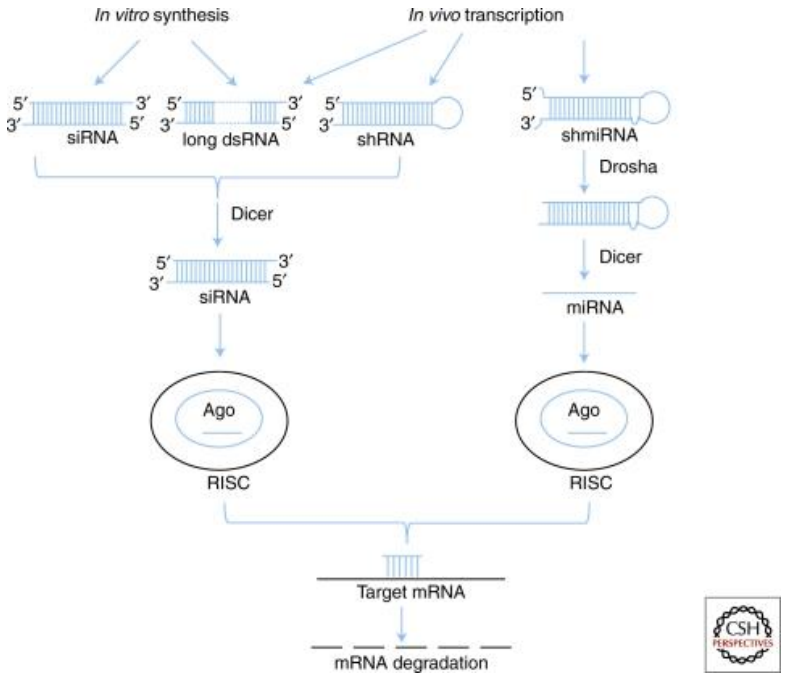


Figure 5. Mechanism of mRNA degradation by RNA interference. In the cell double stranded RNAs are cleaved into small fragments by the Dicer protein. The resulting small interfering (si) or micr (mi) RNAs complex with the proteins including Argonaute to form a RNA-induced silencing complex (RISC). This complex complementary base pairs its target mRNA and leads to its degradation. Figure taken from Perrimon *et al.* 2010.

Experimentally, the endogenous RNAi mechanism can be used to study the knockdown effects of specific, individual genes. In *Drosophila* RNAi transgenes specific for any gene can be generated (Perrimon *et al.* 2010). Using standard cloning techniques, the gene of interest is modified so 'sticky ends' RNA will be produced when it is expressed under the control a yeast UAS. These sticky ends will give rise to hairpin structures, thus creating double strand RNA recognized by Dicer. The RNAi transgenes are stably inserted into the *Drosophila* by standard procedures. When expressed under the control of a GAL-4 driver, these transgenes can down-regulate wild type endogenous gene expression via the RNA interference pathway. In this study, expression of the innexin RNAi transgenes was driven by a pan-da neuron GAL-4 driver construct, 21-7. This driver bears a *Drosophila* enhancer for expression in all four class of da

neurons. This driver was chosen to ensure that only in da neurons would the RNAi construct be expressed, making the knock-out tissue specific. Flies of the appropriate 21-7 GAL-4; UAS-*innexin* RNAi genotype will be generated by crossing and then examined in the cold behavioral assay (see Methods).

It is important to note that this system only knocks down the expression of an *innexin* gene, and it does not necessarily completely eliminate expression of the gene. Therefore, some mRNA may still be able to produce a few working *innexin* channels. When analyzing the results, the fact that the gene may still retain some functionality must be taken into account when determining the significance of certain data. However, this study is a first step in determining whether or not *innexins* play a role in cold nociception, so any slight difference in larval cringe is significant to the study.

Methods

Fruit Fly Husbandry and Crosses

The 21-7 GAL- 4 stock was provided by Dan Cox from Georgia State University. RNAi stock was obtained from the Bloomington Drosophila Stock and Vienna Drosophila Resource Centers (Table 1). Oregon-R was the wild type stock used to generate control larvae. Stocks were maintained on standard molasses/cornmeal/yeast media at room temperature. Each stock was transferred weekly to maintain healthy flies. Most stocks were maintained at the smaller scale vial level. However, 21-7 GAL4 homozygous flies and Oregon-R flies were be maintained at a large scale bottle level to facilitate virgin collection.

Table 1. Innexin RNAi Stocks

innexin Gene	Stock Identifier	Genotype
innexin 1 [ogre]	JF02595 (TRiP)	y,v; +; UAS-ogre-IR
innexin 8 [shaking-B (shakB)]	JF02603 (TRiP)	y,v; +; UAS-shakB-IR
	GD24578	w; +; UAS-shakB-IR

21-7 GAL4 virgin females were collected and crossed to UAS RNAi males. All progeny of these crosses were heterozygous for the GAL-4 driver and the UAS-RNAi construct of interest. These crosses generated the experimental larvae. Control larvae were generated by crossing the UAS-RNAi line to Oregon-R, resulting in progeny flies lacking a driver for RNAi expression. Within twelve days from the initiation of the cross, third instar larva were seen on the sides of the vials. These larvae were collected for the cold behavioral assay.

Cold Behavioral Assay

A thermocycler was repurposed for the behavioral assays (Figure 6). A wooden support beam was placed over the thermocycler to hold a Nikon 5200 digital camera mounted directly above. To reduce the amount of glare picked up by the video, a fiber optic light was placed behind the thermocyler at an angle to ensure that only the larva were visible when the lights were turned off. The wells of the thermocylcer are filled with water and the machine was set to 5ºC.

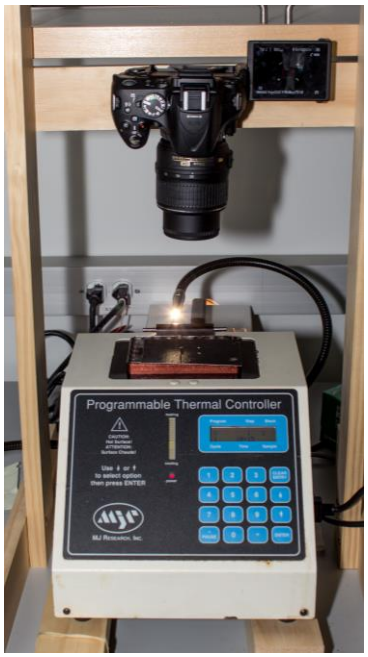


Figure 6. Experimental set up for the cold behavioral assays. Larvae were placed on a black metal plate and the plate was then placed on top of the chilled thermocycler block. Larval behavioral responses were video-recorded with the Nikon Camera.

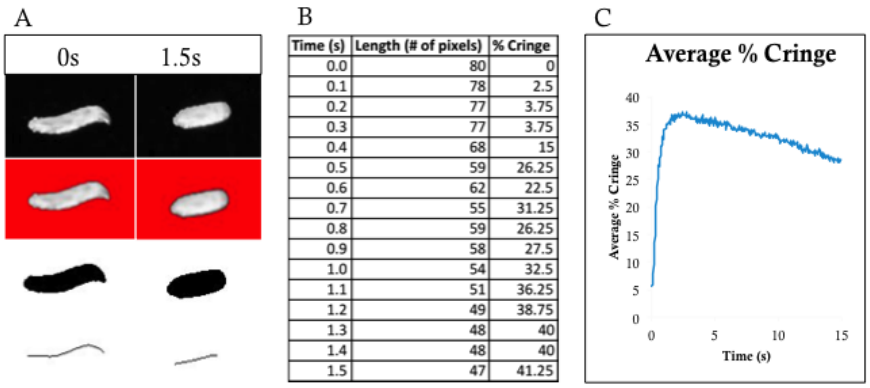
For each experimental trial, four larva from the same cross are transferred from the sides of the cross bottles to aluminum plates. The aluminum plates were spray-painted black to reduce any glare and make the larva more visible to the camera. A water bottle was used to mist the plate to keep it moist for the larva. Each larva was set up within a quadrant on the aluminum

plate. Later, the computer would be able to determine each larva as an individual animal so long as they remained within one of each of the four quadrants on the plate.

Once the larva were placed in their designated quadrant, the aluminum plate was placed on the cold section of the thermocycler. As soon as the plate made contact with the wells of the thermocycler, the camera started running on video mode. The cold temperature is transferred from the thermocycler to the metal plate, causing a sudden change in temperature in the environment of the larvae. This induced a cringe behavior in the larvae as the video was recording. The recorded videos were recorded for 45 seconds at 10 frames per second. The length of the videos were chosen to ensure that when the videos were quantified, there would be enough frame to accurately describe the cringing of the larva. At least 100 larvae were assayed for each genotype.

Digitization of Video and Quantification of Cringe Behavior

Videos were initially downloaded in MOV format, and were converted into AVI with a size limit of 640 x 380 pixels. The AVI file is then compatible with the program Image J. A macro written by Ben Williamson of Dr. Halsell's lab automated the image processing. First, the video was converted to greyscale. This significantly reduced any background glare not related to an actual larva. Next, the Image J threshold function was applied twice in order to create a single silhouette of each larva against the background of the plate. This was a second method to reduce the influence of glare in the videos. By using the threshold function multiple times, the glare was sufficiently reduced so that the only the larval bodies was processed further. To quantify larval length the skeletonize function was applied to transform the larvae into linear forms. Length was measured at the number of pixels in each line. This allowed the percent cringe for each larva to be calculated.



Time (s)	Length (# of pixels)	% Cringe
0.0	80	0
0.1	78	2.5
0.2	77	3.75
0.3	77	3.75
0.4	68	15
0.5	59	26.25
0.6	62	22.5
0.7	55	31.25
0.8	59	26.25
0.9	58	27.5
1.0	54	32.5
1.1	51	36.25
1.2	49	38.75
1.3	48	40
1.4	48	40
1.5	47	41.25

Figure 7. A visual representation using Image J to calculate percent cringe in single larvae. Panel A describes the process of taking the image from the raw video of a single larva and converting it into a linear form. Panel B shows the percent cringe calculated for a single larva after calculation from pixel data. Panel C displays the percent cringe of the larvae over 15 seconds. Figure from Williamson. 2015.

This numerical data was exported into an excel document then used to create a graph of percent cringe as a function of time. Percent cringe was calculated as follows. The longest length of the larva within the first second of the video was set as the baseline length of the larva. This baseline length was compared to each frame from the video later on of that same larva. Percent cringe was calculated by the difference between the baseline length and the length of the new frame divided by the baseline length of the larva. The percent cringe at each second was then averaged for about 100 larva sharing the same genotype. The averages were then plotted in line graph form to show the trend of cringe in larva of the same genotype.

Results

Cold Behavioral Assay

A cold behavioral assay was utilized to determine the functional role of Ogre and Shaking-B in the perception of noxious cold. Each Innexin was individually tested using RNAi to downregulate each gene in question. A negative control was run for each Innexin by crossing Oregon R stock to the flies with UAS paired to an RNAi template.

d The average cringe response for all experimental and control larva was graphed over time (Figs. 7, 8). Therefore, any small change in the percent cringe may be important. The negative control cross (OR x UAS) was used to compare any abnormalities in the experimental cross (UAS x Driver)

Results of Cold Assay: Ogre (Innexin 1)

The control larvae for *ogre* (*innexin 1*) depicted an exponential curve ($r^2 = 0.62$) that eventually peaks around 27.4% average cringe at 5.2 seconds in the behavioral assay (Fig. 7). Thereafter, the amount of cringing gradually declined to 22.2% at 15 seconds. This curve represented the behavior in *Drosophila* with normally functioning Ogre. In contrast, the experimental larvae containing both the UAS RNAi complex and the Driver protein cringed 25.1% at 5.2 seconds and peaked around 28.19% cringe at 7.8 seconds into the behavioral assay. Both the experimental and negative control larvae exhibited a similar trend in cringing until 6 seconds after the larva are exposed to noxious cold. From there, the experimental larvae had a larger response ($p=<0.001$) than the negative control, which lasted for the remaining 9 seconds.

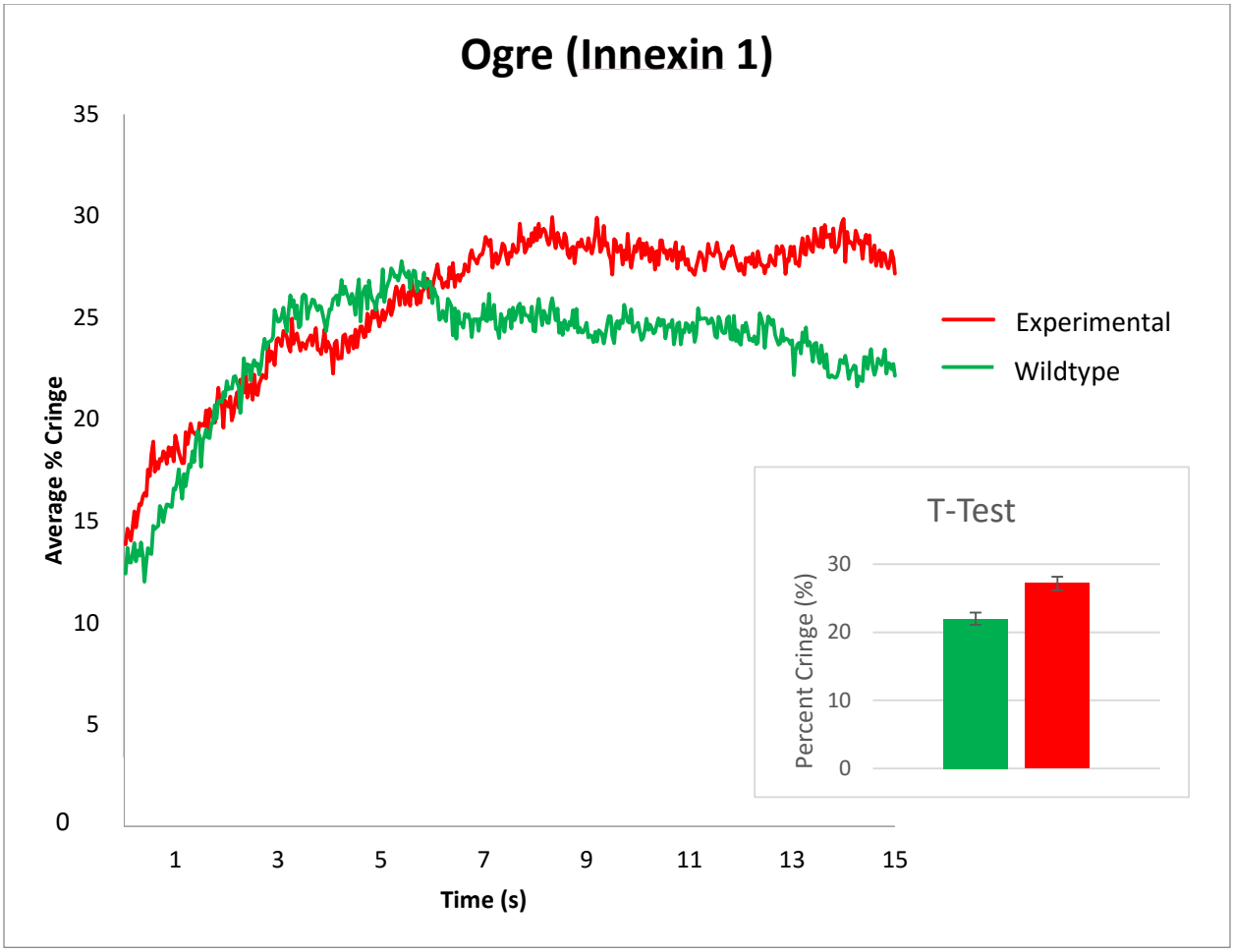


Figure 8. Cold behavioral assay results for ogre expressed as the average percent cringe over time. 80-100 larvae were used for each trial, while a cold temperature of 5°C was used as the stimulus. The red line represents the experimental larvae. The green line represents the control larvae. The data takes place for 15 seconds after direct contact with the cold stimulus. A t-test was done between the final frames of the experimental and wildtype curves ($P<0.001$) and represented as a bar graph.

Results of Cold Assay: Shaking-B (Innexin 8)

Control larvae (green) for *shaking-B* (*innexin 8*) gave rise to an exponential curve ($r^2=0.80$), and cringed 28.2% at five seconds and eventually exhibited a maximum cringe of 33.5% at 8.9 seconds into the behavioral assay (Fig. 8). The wild type curve was used to depict the nociceptive levels of *Drosophila* with normally functioning *shaking-B*. In contrast, the larvae showed an average cringe of 23.9% at 5 seconds and a maximum cringe of 26.2% cringe at 8.9

seconds. From the start of the assay to two seconds in, both experimental and wild type larvae exhibited a similar trend in percent cringe. The difference between the percent cringe for both datasets expands quickly from 2 seconds onward. Consistently thereafter, experimental larvae cringed less than the wild-type control larvae ($p<0.001$).

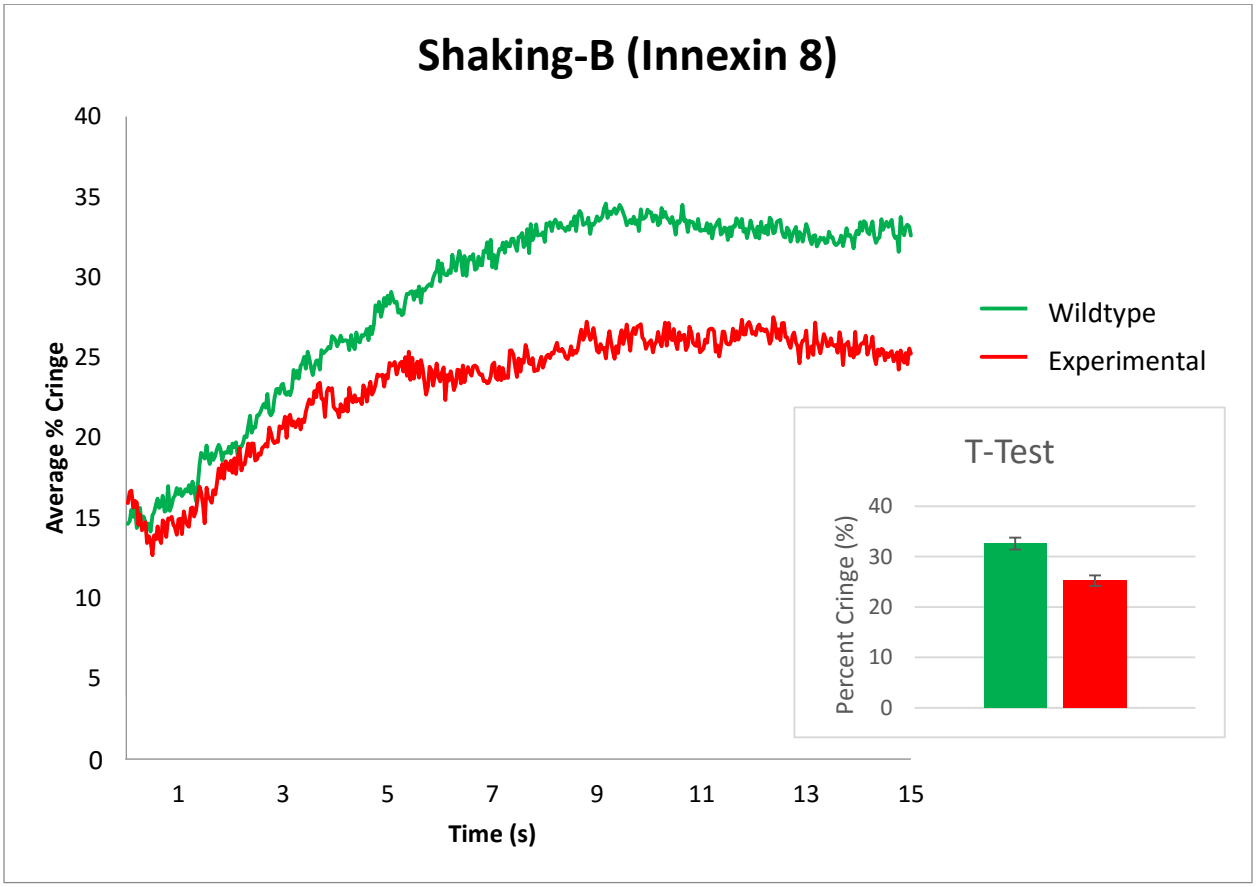


Figure 9. Cold behavioral assay results for shaking-B expressed as the average percent cringe over time. 80-100 larvae were used for each trial, while a cold temperature of 5°C was used as the stimulus. The red line represents the experimental larvae. The green line represents the Control larvae. The data was collected for 15 seconds after direct contact with the cold stimulus. A t-test was done between the final frames of the experimental and wildtype curves ($P<0.001$) and represented as a bar graph.

Discussion

Interpretation of Experimental Results

The Innexin family had been chosen as a focus of this study due to previous research demonstrating Ogre and Shaking-B played a role in the normal functioning of the central nervous system. Therefore, there was a chance that these Innexins could also have an important function in the peripheral nervous system. These two genes were down-regulated with RNAi transgenes to observe any effect on larval response to the nociception of cold. Any observationally significant inhibition in the cringing response from RNAi expression could be interpreted as the presence of a functional version of the gene being important for cold nociception. Statistical analysis will eventually be run for all results, in the form of a two-tailed t test.

Cold Behavioral Assay

While there was a change in the response of the experimental flies while compared to the wildtype, *ogre* flies displayed an unexpected response. Rather than having a diminished cringe due to the lack of Ogre, there was actually an enhanced cringe in the larva after 6 seconds. Therefore, we can see Ogre did effect the larva's response to noxious cold, but instead of lowering the larva's perception of the noxious stimuli, it increased the severity of their cringe response. This hyperalgesia displayed in only the experimental larva indicates that the down-regulation of *ogre* may cause this behavior in the *Drosophila* larvae, making them more sensitive to the noxious stimuli. One hypothesis for why this may be the case is that in *Drosophila* with normal levels of Ogre, the protein product acts as a negative feedback system and actually helps to regulate the response of the larva to noxious stimuli. Another hypothesis for this unexpected result would be that there was something in the genetic background of the UAS-*ogre* RNAi line caused a diminished response from the wild-type cross, making the experimental larvae seem to

have a higher cringe in comparison. A second experimental assay identical to this one with a different UAS-*ogre* RNAi line might be able to differentiate between these two possibilities.

The results for Shaking-B showed a more expected trend. When graphed with the experimental cross, the control displayed a higher average percent cringe than the experimental. This trend indicated that the down-regulation of *shaking-B* resulted in an inhibition of normal cringe response to noxious cold. Previous research had determined that a loss-of-function for *shaking-B* in *Drosophila* resulted in a lack of synapse formation and electrical signal transmission in the central nervous system (Trimarchi and Murphey 1997; Blagburn et al. 1999). It can be inferred, due to the results of this study, that this lack of electrical synapse function may also inhibit the larval response to noxious cold stimuli.

Future Directions

Due to the fact that Ogre provided unexpected results, it should be studied in further detail with possibly more RNAi constructs. This would help to determine if Ogre really does act as a negative feedback system for nociception, and rule out other reasons for this unexpected result. To further expand on the research done with both Ogre and Shaking-B, the complete loss of function of each of these genes could be studied to complement this study. This study only focused on two Innexins; there are six other Innexins that can be studied. Further research could be done by determining if any of the rest of the Innexins play a role in cold nociception in *Drosophila* larva.

Significance

This study had determined that the Shaking-B and Ogre maybe involved in noxious cold detection. Previous studies had only linked these two to the central nervous system and giant fiber system, respectively. However, this study indicates they may play a further role in other parts of the nervous system in *Drosophila*. By understanding the role of these Innexins further in nociception, the understanding of the molecular components behind nociception increases and may lead to new potential targets for the treatment of chronic pain.

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