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Growing Pains: Development of the Larval Nocifensive Response in *Drosophila*

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Abstract

The ability to perceive and avoid harmful substances or stimuli is key to an organism's survival. The neuronal cognate of the perception of pain is known as nociception, and the reflexive motion to avoid pain is termed the nocifensive response. As the nocifensive response is an ancient and evolutionarily conserved behavioral response to nociceptive stimuli, it is amenable to study in relatively simple and genetically tractable model systems such as *Drosophila*. Recent studies have taken advantage of the useful properties of *Drosophila* larvae to begin elucidating the neuronal connectivity and molecular machinery underlying the nocifensive response. However, these studies have primarily utilized the third-instar larval stage, and many mutations that potentially influence nociception survive only until earlier larval stages. Here we characterize the nocifensive responses of *Drosophila* throughout larval development and find dramatic changes in the nature of the behavior. Notably, we find that prior to the third instar, larvae are unable to perform the characteristic "corkscrew-like roll" behavior. Also, we identify an avoidance behavior consistent with a nocifensive response that is present immediately after larval hatching, representing a paradigm that may be useful in examining mutations with an early lethal phenotype.

Introduction

Nociception, the process of encoding and transmitting noxious stimuli within the nervous system, is essential for survival because it provides information about hazards that could cause irreversible damage. In humans, rapid movement away from the source of the noxious stimulus often accompanies nociception, which constitutes a pattern of behavior known as the withdrawal reflex (Bromm and Treede, 1980). Similar patterns of behavior, termed nocifensive responses, have been studied in both vertebrate and invertebrate animals (Le Bars *et al*., 2001; Smith and Lewin, 2009). Moreover, researchers using model systems have begun to elucidate the cellular and molecular underpinnings of the nocifensive response to thermal, mechanical, and chemical nociceptive stimuli.

As nearly all animals must avoid harmful interactions, the nocifensive response is believed to be an ancient and innate behavior. For example, a recent study found that TRPA1

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(transient receptor potential A1) proteins serve as the receptors for noxious reactive electrophiles in diverse animals, from humans to insects (Kang *et al.*, 2010). Researchers have therefore exploited the evolutionary conservation of nociception by performing studies in relatively simple and genetically tractable animals like *Drosophila melanogaster* and *Caenorhabditis elegans* (Tobin and Bargmann, 2004).

Different animals display diverse nocifensive responses. *Cepaea nemoralis*, a species of land snail, reacts to being placed on a heated surface by lifting the anterior portion of the extended foot (Sneddon, 2004). Injection of harmful substances into the hind-paws of mice elicits a variety of nocifensive responses including licking, biting, flinching, and guarding (Meseguer *et al.*, 2008). *Drosophila* has emerged as an exceptionally powerful genetic and behavioral model system in which to investigate the molecular underpinnings of nociception and nociceptive sensitization (Tracey *et al.*, 2003; Manev and Dimitrijevic, 2004; Babcock *et al.*, 2009). Third-instar *Drosophila* larvae have a distinct response to thermal nociception —when lightly touched by a heated probe, the larvae perform a “corkscrew-like” roll in the direction of the noxious stimulus (Tracey *et al.*, 2003), whereas in adults there is a characteristic jump response or thermal avoidance behavior (Xu *et al.*, 2006; Neely *et al.*, 2010). These behavioral responses to noxious thermal stimuli have been used in genetic screens to identify genes required for normal nocifensive behavior (Tracey *et al.*, 2003; Babcock *et al.*, 2009; Neely *et al.*, 2010). For example, a novel type of transient receptor potential ion channel, encoded by the *painless* gene, was discovered in a forward genetic screen for mutant larvae with delayed response to noxious thermal stimuli. *Painless* mutants were shown to have a diminished nocifensive response and a reduction of sensory neuron spiking as measured by electrophysiological recordings (Tracey *et al.*, 2003). Moreover, *Drosophila* Painless has been demonstrated to be directly activated by noxious heat, acting as a calcium-dependent channel (Sokabe *et al.*, 2008).

Although advances have been made in understanding the biochemical and electrophysiological properties of nociceptive signals, relatively little is known about the neuronal networks that encode this information even in the relatively simple nervous systems of invertebrates. However, a recent study identified a subclass of peripheral nervous system sensory neurons that function as nociceptors in *Drosophila* larvae. Genetic analyses reveal that class IV dendritic arborization (da) neurons are both necessary and sufficient to elicit nocifensive response in third-instar larvae (Hwang *et al.*, 2007).

On the basis of the growing importance of *Drosophila* larvae as a model for studies of nociception, we sought to characterize the development of the nocifensive response at each stage of larval development. Using both noxious thermal stimuli and optogenetic stimulation of nociceptive neurons, we assay the development of the nocifensive response of *Drosophila* larvae over time. Importantly, we find that larvae do not develop the ability to perform the characteristic “corkscrew roll” until the late second-instar stage. However, optogenetic assays indicate that activation of class IV da neurons elicits an avoidance behavior consistent with a nocifensive response in newly hatched first-instar larvae, indicating that these neurons appear to function as nociceptors from the onset of larval development. Interestingly, the mature corkscrew-roll larval nocifensive behavior in response to noxious thermal stimuli is observed at an earlier developmental time point than is observed *via*

optogenetic activation of class IV da neurons alone, suggesting that additional thermal nociceptors may function in *Drosophila* larvae. Overall, this study provides a baseline for future studies using *Drosophila* as a model organism for nocifensive response, particularly in studies focused on dissecting the molecular mechanisms underlying the age-dependent maturation or alteration of the pain response.

Materials and Methods

Drosophila strains and culture

The following *Drosophila* strains were used for behavioral assays: *UAS-ChR2::eYFP* (Hwang *et al.*, 2007) crossed to the class IV dendritic arborization (da) neuron driver *GAL4[477],UAS-mCD8::GFP* served as the experimental strain in the presence or absence of all-*trans* retinal for the optogenetic experiments. Wild-type *Oregon-R* served as the experimental strain for thermal nociception assays. All larvae were kept at 25 °C in the dark during all developmental stages. For optogenetic behavior assays, yeast paste containing 500 μmol l^{-1} all-*trans* retinal (Sigma-Aldrich) was added to the medium on which the *UAS-ChR2::eYFP* (x) *GAL4[477],UAS-mCD8::GFP* larvae and their parents were grown. As a control, other larvae from the same cross were grown with yeast paste that contained no all-*trans* retinal.

Thermal nociception assays

Thermal nociception assays were performed as previously described (Tracey *et al.*, 2003; Hwang *et al.*, 2007). All response assays were performed on larvae of specific ages (hours after egg laying) in increments of 8 h. The thermal responses were divided into three categories: no response, which includes all turning on the long axis or “rolling” less than 180°; ≥180°, which includes all rolling greater than or equal to 180° but less than 360°; and ≥360°, which includes all rolling of greater than or equal to 360° and constitutes the characteristic nocifensive “corkscrew-like” roll. Each larva that was tested was placed on a grape agar plate. The larva was left to adjust to the new plate for 1 min or more and was then touched lightly on the side between abdominal segments A4 and A6 with a thermal probe set to a noxious thermal stimulus of 45 °C.

Optogenetic nociception assays

Optogenetic nociception assays were performed under a Leica MZF16A automated stereofluorescent microscope equipped with a Roper CoolSnap CCD camera allowing for illumination of live *Drosophila* larvae with white and 488-nm wavelength light. Each larva that was tested was placed on a grape agar plate and left to adjust as in the thermal assay. Each larva was then exposed to alternating 5-s intervals of white light and blue light (488 nm) for 30 s. The resulting videos were analyzed, and behavior observed during the blue-light interval was recorded as the optogenetic response. The optogenetic responses were divided into four categories: roll, all rolls greater than or equal to 360°; turn, larvae that changed direction; look, larvae that stopped and looked on both sides; and no response, larvae that did not display any of these behaviors during optogenetic activation. The light intensities were measured using a Mastech Professional luxmeter and corresponded to 25.0 Klx with white light and 45.0 Klx with blue light. These light intensities are consistent with

those previously reported for inducing a photophobic response in larvae (Xiang *et al.*, 2010) and are at levels consistent with full daylight sun on a clear day as measured on a luxmeter. Statistical differences between experimental and control groups for each of the developmental time points were computed using the Mann-Whitney rank sum test.

Larval phototaxis assay

For the larval phototaxis assays, petri dishes (30 mm) with grape agar were divided in half. One half was exposed to blue light (488-nm wavelength) and the other half to white light. The light intensities measured were the same as described above and, to control for the photophobic response, are consistent with levels previously demonstrated to induce light avoidance by larvae (Xiang *et al.*, 2010). Yeast paste with 500 μmol l^{-1} all-*trans*-retinal (or without for control) was placed on the center of each half. *UAS-ChR2::eYFP* (×) *GAL4[477],UAS-mCD8::GFP* larvae were placed on a line separating the two halves of the dish. The dish was simultaneously illuminated by both the blue light and the white light on opposite halves of the petri dish for 10 min. The number of larvae on each side of the dish was quantified. Preference index was computed by subtracting the number of larvae on the white side from the number of larvae on the blue side and dividing by the total number of larvae as described previously (Bellmann *et al.*, 2010).

Results

Development of the larval thermal nocifensive response

Our analyses revealed that early *Drosophila* larvae do not perform the stereotypical "corkscrew-like" roll in response to noxious thermal stimuli as previously reported in third-instar larvae (Tracey *et al.*, 2003). This observation indicated the necessity to characterize the chronological progression of the nocifensive response throughout larval development to provide a baseline for further behavioral studies.

We began our study by testing wild-type (*Oregon-R*) larvae with a thermal response assay (Tracey *et al.*, 2003; Hwang *et al.*, 2007). To characterize the chronological progression of nocifensive response, we performed timed embryo collections on grape agar plates supplemented with wet yeast and aged them to different developmental stages at 25 °C. We then conducted the thermal response assay on larvae of specific ages after egg laying (AEL) in increments of 8 h and recorded the results (Fig. 1). We divided the responses into three categories: no response, which includes all rolling less than 180° (Supp. Video 1; http://www.biolbull.org/content/supplemental); ≥180°, which includes all rolling greater than or equal to 180° but less than 360° (Supp. Video 2; http://www.biolbull.org/content/supplemental); and ≥360°, which includes all rolling greater than or equal to 360° and constitutes the previously characterized "corkscrew-like" roll (Tracey *et al.*, 2003) (Supp. Video 3; http://www.biolbull.org/content/supplemental).

The earliest observed larval rolling behavior (≥180°), albeit at a very low frequency (<5%), occurred at 40-h AEL in response to noxious heat (Fig. 1). We observed more frequent rolling behavior at 56-h AEL. At 64-h AEL, about 50% of larvae were in either the no response or rolling categories; by 72-h AEL, the ≥180° rolling response became

predominant. At 80-h AEL, the ≥360° response became the most common, a trend that continued throughout the later developmental time points. Interestingly, for the most mature larvae, the ≥180° response was entirely absent.

Optogenetic activation of larval nociceptors reveals novel behavioral responses in early larvae

The inability of younger larvae to perform the corkscrew-like roll prompted us to search for different nocifensive responses that could be useful when studying first and second larval instar animals. We initiated these studies by optogenetically activating class IV da neurons, which have previously been shown to function as larval thermal nociceptors (Hwang *et al.*, 2007). Larvae expressing Channel-rhodopsin-2 (ChR2) in class IV neurons were fed with all-*trans* retinal, which is essential for the synthesis of ChR2 (Nagel *et al.*, 2003). Control larvae were not fed with all-*trans* retinal. To test the response of larvae to activation of nociceptive class IV da neurons, we isolated individual larvae and observed them through time-lapse microscopy. After a larva adjusted to the new plate, we subjected it to 488-nm blue light, which has the maximum absorption by ChR2 (Bamann *et al.*, 2008), for 5 s and recorded the results *via* time-lapse microscopy.

We did not notice any reproducible optogenetically induced responses from larvae younger than 64-h AEL (Fig. 2A). We attempted to quantify a variety of behaviors including stopping, crawling backward, and twitching. However, when compared with controls, none of these behaviors appeared to be a unique response to optogenetic activation of class IV da neurons. Two behaviors, in addition to rolling, do appear to represent novel and quantifiable nocifensive responses to optogenetic activation. In larvae aged for 64-h AEL, we observed a behavior we termed “look”, which entailed a stereotyped, left-to-right movement of the head (Supp. Video 4; http://www.biolbull.org/content/supplemental). Although this behavior seems to occur at low frequency (~20% at 64-h AEL) (Fig. 2A), we did not observe this response in any control animals (Fig. 2B), suggesting that it may represent a novel early response to nociceptive neuron activation. The other behavior that emerged was a change of direction in response to optogenetic activation, a behavior we termed “turn.” The turn response entails a complete change of direction immediately after optogenetic stimulation (Supp. Video 5; http://www.biolbull.org/content/supplemental). In experimental larvae aged for 80-h AEL, the turn behavior was the predominant response (Fig. 2A). Although the turn behavior was also observed in control animals between 72-h and 88-h AEL, the overall response was at a very low frequency (Fig. 2B), far below the frequency with which this behavior was observed in experimental animals at 80-h AEL (Fig. 2A). As most larvae respond by rolling at 88 h, we propose that this turning behavior could be a precursor behavior of the mature corkscrew-like roll response.

Perhaps the most striking observation from the optogenetic time-lapse experiments was the age at which the animals began the rolling behavior. Whereas larvae exposed to a noxious thermal stimulus began performing a full 360° roll at 56-h AEL (Fig. 1), rolling in response to optogenetic stimulation was not apparent until 72-h AEL (Fig. 2). To ensure that this difference did not arise from the different strains of flies used in the respective experiments, we performed the thermal nociception assay on larvae of the same genotype used in the

optogenetic experiments and found that they are also able to perform the 360° roll at 56 h (data not shown). The temporal discrepancy in the performance of the fully mature 360° nocifensive rolling behavior between the thermal assay and the optogenetic assay may suggest the presence of additional nociceptive neurons apart from the class IV da neurons; alternatively, it could indicate that ChR2-mediated activation of class IV neurons is less efficient than heat in stimulating the nocifensive response.

Optogenetic activation of class IV da neurons promotes an avoidance behavior from the onset of larval development

As we did not observe any quantifiable optogenetically induced nocifensive behavioral response in larvae aged less than 64-h AEL, we designed an experiment to test whether optogenetic activation of class IV da neurons promotes avoidance, a basic form of nocifensive response. To perform this experiment, we transferred groups of 10 larvae expressing ChR2 in class IV neurons and aged for a specific time onto a 35-mm grape agar plate divided into two sections. One section was illuminated with 488-nm light, which causes the light-gated channel to open, thereby activating class IV da neurons. The other half was illuminated with white light of a comparable intensity, to control for the photophobic behavior of larvae (Bellmann *et al.*, 2010; Xiang *et al.*, 2010). Using this assay (Fig. 3A), we found that animals as young as 24-h AEL, immediately after hatching from the egg case, actively avoid the optogenetic stimulus (Fig. 3B), exhibiting an aversive withdrawal behavior. Control animals, which were not fed all-*trans* retinal, did not display a reproducible avoidance of 488-nm light, but rather a stochastic distribution on both sides of the plate (Fig. 3B). These results suggest that activation of class IV da neurons is sufficient to elicit a specific avoidance behavior in newly hatched first-instar larvae, and that this behavior continues throughout early larval development. This behavior does not appear to represent a photophobic response to light-induced activation of class IV neurons, given that control animals fail to display any consistent aversive behavior in response to either white or blue light, whereas experimental animals specifically avoid the blue-light-illuminated side of the plate, a behavior that is more consistent with a nocifensive avoidance response to activation of these neurons.

Discussion

This study provides the first quantitative descriptions of the temporal acquisition of the nocifensive response in *Drosophila* larvae. As this model system has become increasingly important for addressing questions about the biological correlates and molecular bases of nociception, this information will prove a valuable resource for researchers, especially in experiments with younger animals. The absence of the mature “rolling” nocifensive response in younger animals (Figs. 1,2) may provide an entry point for further studies on the development of the ability to perceive and react to noxious stimulus. As an age-dependent change in nociception has previously been described in a mamma-lian model (Hiura *et al.*, 1992), studies in *Drosophila* may serve as a basis for subsequent, translational research into the nociceptive process.

The delay in the acquisition of the mature, “corkscrew roll” indicates that younger larvae either do not perceive the stimulus, do not transmit the signal to the central nervous system, or have not developed the neuromuscular connections to perform this behavioral response. The absence of the behavior may result from under-developed muscles, motor neurons, or neuromuscular junctions (NMJs). *Drosophila* NMJs are observed early in embryonic development, with axons from the ventral nerve chord reaching somatic muscles during stage 15 (~12 h after egg-lay) (Broadie and Bate, 1993a). However, coordinated movements develop more gradually, progressing from locally restricted twitches of the body wall to coordinated, sequential contractions of individual segments that define peristalsis. This progression has been found to occur primarily in the final 25% of embryonic development, during stages 16-17 (~15-22 h after egg-lay at 25 °C) (Pereanu *et al.*, 2007). The transverse muscles that presumably could function to perform the rolling motion are also present in first-instar larvae (Landgraf *et al.*, 1997).

As all of the gross physiological components to perform the corkscrew-roll appear to be in place in first- and second-instar animals, we speculate that maturation of the NMJ may be required for the corkscrew-roll nocifensive response in the third-instar larvae. The NMJ is a highly plastic structure, which continues to grow throughout the larval stages, in terms of both morphology and electrophysiology (Broadie and Bate, 1993b; Prokop *et al.* 1996). Another possibility is that the network of interneurons that connect and integrate the sensory and motor neurons do not become fully functional until later in development. Further experiments will be necessary to better understand the wiring of the neuronal circuits responsible for the nocifensive response.

We also observed a temporal discrepancy between the thermal and optogenetic nociception assays in the development of the mature nocifensive rolling behavior. That behavior manifests earlier in the thermal assay than in the optogenetic assay in which class IV da neurons are specifically activated by ChR2. This observation suggests that *Drosophila* larvae may possess nociceptive neurons in addition to the class IV da neurons that facilitate the nocifensive rolling response; alternatively, the ChR2-mediated activation of class IV neurons may be less efficient than heat in stimulating the nocifensive response.

Class IV da neurons have previously been demonstrated to function as thermal nociceptors in third-instar larvae (Hwang *et al.*, 2007). Moreover, another recent study has shown that these same neurons mediate light avoidance in third-instar larvae (Xiang *et al.*, 2010). We find that optogenetic activation of class IV da neurons promotes an avoidance behavior as early as first-instar larvae (Fig. 3); however, given that class IV neurons mediate both nociceptive and light-avoidance responses, there are two possible interpretations of our findings in early larvae. With respect to light avoidance, both white and blue light have been previously demonstrated to activate the firing of class IV neurons, thereby eliciting an avoidance behavior characterized by either a reverse in direction or turning of the head away from the light source (Xiang *et al.*, 2010). Moreover, this photophobic response is dependent upon the G-protein-coupled receptor Gr28b and the transient receptor potential channel TRPA1 for light transduction in class IV neurons (Xiang *et al.*, 2010). To control for photophobic responses in our assay (Fig. 3), we employed light intensities, for both white and blue light, equivalent to those previously demonstrated to activate the light transduction

pathway in class IV neurons (Xiang *et al.*, 2010). If the avoidance behavior we observed upon optogenetic activation of class IV neurons in first-instar larvae represented a photophobic response, then one would expect a stochastic distribution of larvae in both the control (ChR2 without all-*trans* retinal) and experimental (ChR2 with all-*trans* retinal) animals, given that white and blue light have previously been demonstrated to elicit a light-induced activation of class IV da neurons that manifests in a light-avoidance response. However, we observed an avoidance behavior only in the experimental animals that showed a specific aversion to the side of the assay plate illuminated with blue light; in contrast, in the control animals the distribution was stochastic with respect to the blue light and the white light sides of the plate. These data suggest that optogenetic activation of class IV neurons in early larvae elicits an avoidance behavior that is more consistent with a nocifensive response to a perceived painful stimulus than it is to a photophobic response. Also of note is that although negative phototaxis in larva has been widely documented in response to light (Busto *et al.*, 1999; Gong, 2009; Xiang *et al.*, 2010), these studies have been conducted using third-instar larvae rather than the younger larvae we used in aspects of the present study. Our analyses suggest that even if earlier-stage larvae do exhibit photophobia, their avoidance behavior is inconsistent with a photophobic response. Instead, their behavior favors interpretation as a nocifensive avoidance response that depends upon activation of class IV da neurons. The latter explanation suggests that these neurons function in nociception from the onset of larval development.

In addition to activity within the nervous system, interactions between sensory neurons and epidermal cells are central to nociception and the subsequent nocifensive response. Babcock *et al.* (2009) recently found that when *Drosophila* larvae were repeatedly exposed to UV-induced tissue damage, they exhibited both thermal hyperalgesia (an exaggerated response to noxious thermal stimuli) and thermal allodynia (a response to thermal stimuli that are normally below the response threshold). Using genetic methods, the authors showed that thermal allodynia depended on tumor necrosis factor signaling. It would be interesting to determine the developmental progression of epidermal/sensory neuron interactions and the allodynia and hyperalgesia properties.

Our study describes the development of the nocifensive response throughout the development of *Drosophila* larvae. We find that larvae are not capable of performing the stereotypical “corkscrew-like” roll until later developmental stages. Using optogenetic stimulation, we show that class IV da neurons appear to function as nociceptors throughout larval development. Moreover, our observation that thermal stimuli are able to elicit a nocifensive response earlier than optogenetic stimulation of class IV neurons suggests that *Drosophila* larvae may have additional nociceptors, although future studies would be required to demonstrate this point. This study can serve as a resource for use of the *Drosophila* larva as a model system in further studies of the molecular bases of nociception and the maturation of the nocifensive response.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

Acknowledgments

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Abbreviations

AEL	after egg laying
ChR2	Channelrhodopsin-2

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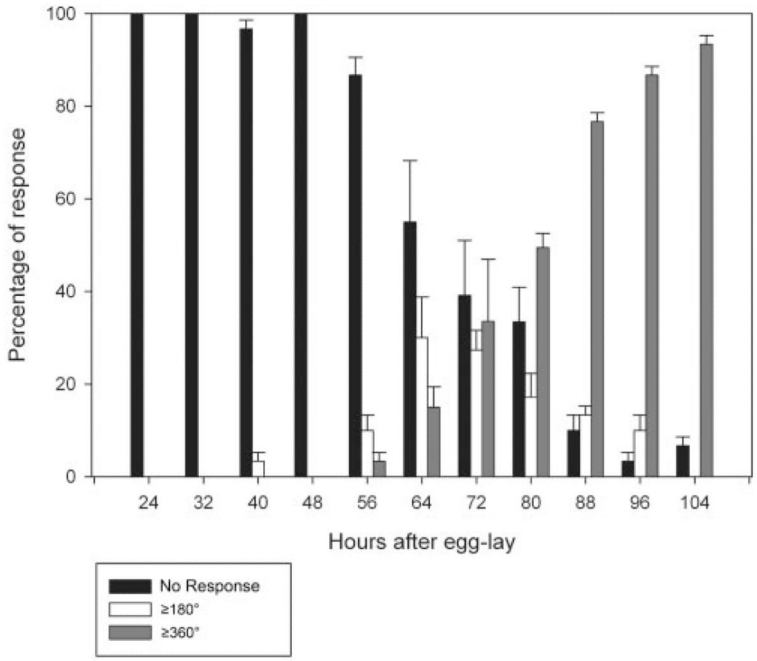

Figure 1.

Development of the larval nocifensive response. Wild-type larvae were isolated at specific time-points during normal development (hours after egg laying) and then subjected to a light touch on the lateral side between abdominal segments A4 and A6 with a probe set to a noxious thermal stimulus of 45 °C. Behavioral nocifensive responses were divided into three categories: no response, which includes all lateral movement less than 180°; ≥180°, which includes all movement between 180° and 360°; and ≥360°, which constitutes the fully developed “corkscrew-like” roll. Bars represent percentage of animals that perform the indicated behavior. Error bars indicate STDEV. Ten animals were tested for each time point (n = 10).

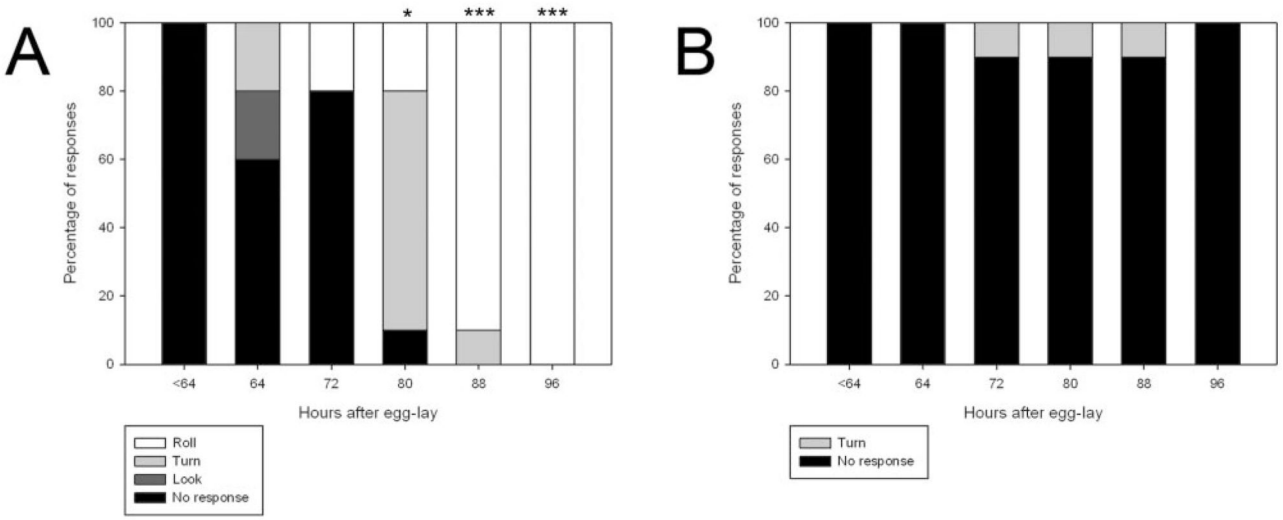

Figure 2.

Development of response to optogenetic stimulation of class IV neurons. *477ChR2::eYFP* larvae were isolated at specific developmental time-points (hours after egg laying) and fed yeast paste in the presence (A) or absence (B) of all-*trans* retinal for optogenetic assays and controls, respectively. (A) Quantified responses to optogenetic stimulation. Notice that optogenetically stimulated larvae do not perform the "corkscrew-like" roll until 72 h after egg-lay. (B) In contrast to experimental animals, control animals not fed all-*trans* retinal typically failed to respond to optogenetic stimulation. Ten animals were tested for both the experimental and control groups for all time points ($n = 10$). Statistically significant P values obtained by comparison of the experimental and control groups are reported on the graph as follows (* = $P < 0.05$; *** = $P < 0.001$).

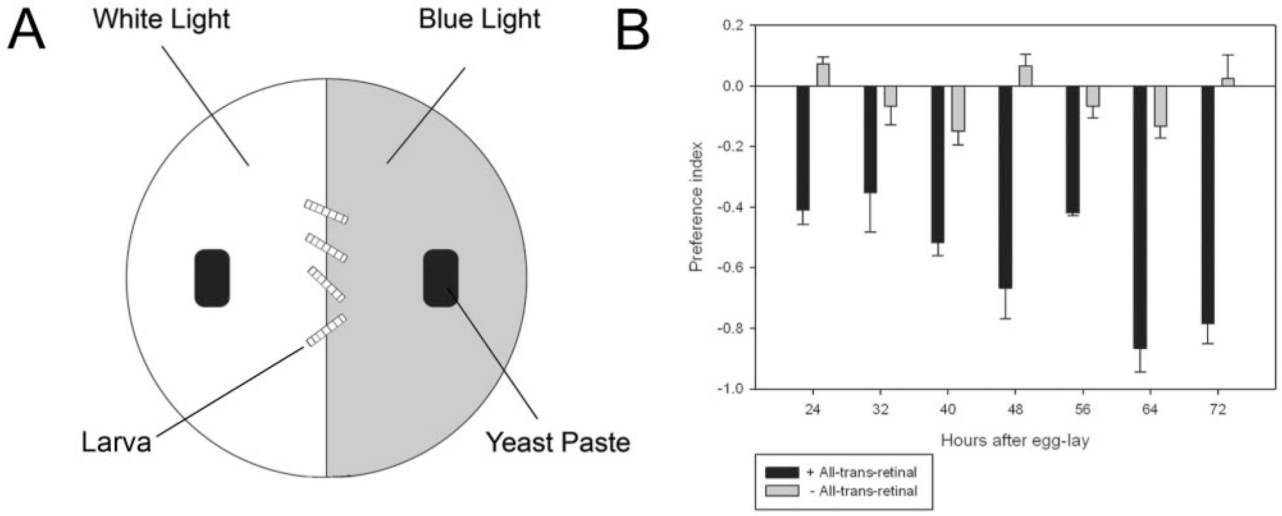

Figure 3.

Class IV neuron activation promotes avoidance behavior from the onset of larval development. *477ChR2::eYFP* larvae were isolated at specific developmental time-points (hours after egg laying) and fed yeast paste in the presence or absence of all-*trans* retinal for nocifensive avoidance assays. (A) Schematic of experimental design. Larvae of specified age were placed on a line in the center of a plate illuminated half by blue light (488 nm) and half by white light. The number of larvae in each half of the plate was counted after 10 min. (B) Preference index was computed for animals expressing functional ChR2 ((+) all-*trans* retinal) and control ((−) all-*trans* retinal) animals. A negative preference index score reflects an active aversion to the side illuminated by blue light (488 nm). Note that experimental animals expressing a functional ChR2 display consistent nocifensive avoidance behavior, whereas control animals display a more stochastic response. Each bar represents the average of three experiments with 10 larvae each ($n = 3$) and error bars represent STDEV.