Glypicans Dally and Dally-like control injury-induced allodynia in Drosophila

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Abstract
Over 100 million people are challenged by the effects of chronic pain in the United States alone. This burden also impacts the U.S. economy; 600 billion dollars annually is spent on medical care, medications, and lost productivity in the workplace. Current opioid treatments cause adverse effects including nausea, constipation, tolerance, and addiction liability. Nociceptive sensitization is thought to perpetuate chronic pain, but too little is known about its mechanisms. Components of the pathways that sensitize the nociceptors after injury are likely to be valuable targets for novel medications for the relief or prevention of chronic pain. Utilizing the Drosophila melanogaster cell targeting and RNA interference toolkit, we are investigating the bone morphogenetic protein pathway and its role in ultraviolet light injury-induced nociceptive sensitization. Bone morphogenetic proteins are well known as secreted developmental morphogens that control development, but other functions are known. We have previously identified bone morphogenetic protein signaling components used in nociceptors to modulate injury-induced allodynia, including Decapentaplegic (Dpp, orthologous to mammalian bone morphogenetic protein 2/4), and its downstream signaling components. The morphogen Hedgehog has also been shown to be necessary for allodynia following injury. Here, we show that two membrane-embedded regulators of the Dpp and Hedgehog pathways, Dally and Dally-like, are necessary for injury-induced thermal allodynia, as the formation of sensitization was reduced when either component was suppressed. These bone morphogenetic protein components are highly conserved and, because dysregulation of nociceptor sensitization underlies chronic pain, the homologs of Dally and Dally-like may represent novel therapeutic targets in humans challenged by chronic pain. Furthermore, because of their extracellular location, Dally and Dally-like represent attractive therapeutic drug targets because such drugs would not need to cross the plasma membrane.

Keywords
nociceptor, hypersensitivity, transforming growth factor beta, glypican, damage, ultraviolet

Introduction
Chronic pain is a dysregulated pain which, perpetuated by nociceptive sensitization, lasts longer than the original injury. Annually, the U.S. economy is impacted by over $600 billion spent on continued pain research, pain-related health care, and medications. The current standard of pain treatment is the utilization of opioid analgesics which, although useful in the short term, often induce side effects including constipation, nausea, tolerance, and addiction liability. Despite the critical need for effective drugs to treat chronic pain, we have an incomplete understanding of the underlying mechanisms that perpetuate sensitization. A deeper knowledge of the pathways that underlie neuronal sensitization may help identify novel targets for the more effective treatment of chronic pain. Because 77% of human disease genes have relatives in Drosophila melanogaster, the fly represents a model organism that can be powerfully genetically manipulated to study more complex mammalian processes.

In the Drosophila larva, there are four classes of sensory neurons, primarily distinguished by the complexity...
of their dendritic branching. Those with the most elaborate branching, class IV, underlie the epidermis and detect both noxious thermal and mechanical stimuli. These neurons, referred to here as nociceptors, express many channels, including the sodium ion channel, Pickpocket (Ppk). Pickpocket is expressed exclusively in class IV multidendritic neurons and detects noxious mechanical stimuli. In this study, we utilize the Gal4-UAS system to drive gene expression in only the Ppk-expressing nociceptors.

It is possible to utilize ultraviolet (UV) light to induce epidermal damage and sensitize the underlying nociceptors in Drosophila larvae. A controlled dose of UV irradiation can be delivered via a crosslinker to anesthetized third instar larvae. Twenty-four hours later, the wild-type animals become thermally allodynic; they demonstrate an increased sensitivity to the light touch of a thermal probe heated to a normally subthreshold temperature.

Interestingly, this sensitization pathway is dependent on the activity of bone morphogenetic proteins (BMPs), important ligands involved in developmental patterning. Previous research has shown that the UV injury induces apoptosis in the larval epidermal cells via the caspase Dronc. The morphogen, Hedgehog (Hh) is released and binds its receptor, Patched, on the underlying nociceptor. Following Hh binding, Dpp (orthologous to mammalian BMP2/4) is released and interacts with type I receptors, Saxophone and Thickveins, and type II receptor, Punt. This leads to activation of SMAD intracellular transducers via phosphorylation of Mothers Against Dpp (Mad) and subsequent complex formation with Medea. The signaling continues to the nucleus, where gene expression is controlled to induce sensitization of the nociceptor. Both allodynia and hyperalgesia can be detected after injury by assaying larvae with a thermal stimulus and observing the frequency of an innate avoidance behavior. Importantly, BMPs are highly conserved across species. For example, flies lacking Dpp (BMP2/4) can be provided the human BMPs are highly conserved across species. For example, flies lacking Dpp (BMP2/4) can be provided the human BMPs and detect noxious mechanical stimuli. In this study, we utilize the Gal4-UAS system to drive gene expression in only the Ppk-expressing nociceptors.

In mammals, glypicans are classified GP1 to GP6 and are further subdivided into two major families: glypicans 3/5 and glypicans 1/2/4/6. Dally and Dally-like serve as the Drosophila homologs of these two families, respectively. Although they are characteristically anchored into the membrane, glypicans can also be cleaved and released into the extracellular space. Because the glypicans are well conserved between the fly and mammals, Drosophila melanogaster represents a simplified model for studying the role of glypicans in relation to neuronal sensitization in more complex organisms, including humans.

Dally and Dally-like play critical roles in morphogen movement and maintaining gradients through the extracellular binding of these ligands, thereby controlling their distribution. Among other roles, Dally and Dally-like (see Figure 1) are involved in regulating the morphogens Dpp (in the case of Dally) and Hedgehog (in the case of Dally-like) during development. Dally-like is necessary for Hedgehog signal transduction, perhaps working as a coreceptor at (or just above) the level of the Hh receptor, Patched, on the responding cell.

One known class of regulators of BMP and other morphogen signaling are the heparan sulfate proteoglycans (HSPGs). HSPGs are glycoproteins located on the surface of cells and within the extracellular matrix that bear heparan sulfate (HS) glycosaminoglycan side chains. HSPGs play important roles in cell and organ growth through the binding of a variety of ligands, including chemokines, cytokines, various growth factors, and morphogens, including Dpp and Hedgehog, to manage their distribution and signaling.

HSPGs can be classified by their location: those found on the membrane (transmembrane syndecans and anchored glypicans), secreted extracellular matrix HSPGs (agrin, perlecan, type XVIII collagen), and on secretory vesicles (serglycin). In Drosophila, there exists one representative of the secreted extracellular HSPGs, Terribly Reduced Optic Lobes, and one representative of the elongated cell surface HSPGs, syndecan (Sdc). Division Abnormally Delayed (Dally) and Dally-like (Dlp) are the two Drosophila glypicans, characterized as such by their disulfide-bond stabilized globular domains and anchoring to the plasma membrane by a glycosylphosphatidylinositol linkage.

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Figure 1. Model of injury-induced nociceptor sensitization. Injury by ultraviolet light triggers release of Hedgehog, which then binds to its receptor Patched. Dally-like may help to facilitate this binding by securing Hedgehog and inhibiting its long-range travel. This eventually results in the release of Dpp which is also necessary for sensitization. Dally may sequester Dpp on the membrane, stabilizing it in its receptor complex of Thickveins, Punt, and Saxophone. As a result, effector genes are eventually regulated to sensitize the nociceptor.
acts as a coreceptor to the Dpp receptor Thickveins and likely helps to stabilize the binding of the Dpp ligand and thereby enhance Dpp signaling.32

Because the Dally/Dally-like–interacting morphogens (Dpp, Hedgehog) are necessary for neuronal sensitization following injury in larvae,4,5 it was hypothesized that the presence of these glypicans in the class IV multidendritic neurons, the primary nociceptors of the larval fly, also influences the fly nociceptors’ ability to sensitize following injury-inducing UV irradiation. In this report, we present evidence that these Drosophila glypicans are necessary for injury-induced nociceptive sensitization.

Materials and methods
Fly stocks and genetics
Experimental flies were purchased from the Bloomington Drosophila Stock Center in Bloomington, Indiana. Flies were maintained in 9 oz stock bottles containing sucrose-cornmeal-yeast medium at 50%–60% humidity and a temperature of 25°C. Bottles were stored in Percival Scientific Incubators with a 12-h light/12-h dark cycle, with an arbitrary dawn time set to 9:00 a.m. The GAL4/UAS system was utilized to drive expression of RNA interference of particular genes. To restrict GAL4 expression to the nociceptors, all experiments employed the driver ppk1.9-GAL4. The UAS-RNAi lines used were as follows: UAS-DallyIR-1 (BDSC#28747, TRiP JF03175, used previously by Ortmann et al.33), UAS-DallyIR-2 (BDSC#33952, TRiP HMS00905, used previously by Ferreira and Milán,34 Zhang et al.,35 and Ortmann et al.33), UAS-Dally (BDSC#5397), UAS-DlpIR-1 (BDSC#34091, TRiP HMS00903, used previously by Brann et al.,33), UAS-DlpIR-2 (BDSC#34089, TRiP HMS00875, used previously by Zhang et al.35). The Dally and Dally-like RNAi lines produced the predicted underexpression phenotypes in the studies referenced. UAS-SecDally was generously donated by Hiroshi Nakato.

UV injury
Injury by UV light was previously described by Babcock et al.11 Prior to UV exposure, three- to five-day-old early wandering larvae were selected and rinsed with tap water on a mesh filter; 15–30 larvae were put into a porous chamber and patted dry with a Kimwipe. Under a hood, 1.5 mL diethyl ether was added to a cotton ball and placed into a 5 mL beaker. The chamber containing the larvae was placed above the ether-wetted cotton ball, and the beaker was kept in a closed Coplin jar for ~2 min. After 2 min, the anesthetized larvae were removed from the apparatus. Larvae were rinsed into a small dish and then arranged dorsal-side up on a microscope slide. The slide was placed into a UV cross-linker, and the anesthetized larvae were exposed to a dosage of UV-C between 12.0 and 18.0 mJ, which was recorded with a UV meter. Uninjured control larvae were subjected to the same anesthesia and other manipulations but no exposure to UV irradiation. The larvae were rinsed from the slide and then transferred to a recovery vial. The recovery vial was blinded as to genotype and treatment and allowed to sit in the 25°C incubator for a 24-h recovery period.

Thermal nociception assay
In response to noxious heat applied by a thermal probe (ProDev Engineering, Missouri City, Texas), Drosophila melanogaster larvae perform an unmistakable 360° nociceptive roll.3 This roll is an innate avoidance response to noxious stimuli. In allodynia experiments, the thermal probe was set to 41°C, previously described as the highest innocuous temperature.4 In normal nociception experiments, the thermal probe was set to 45°C, a temperature in the middle of the responsive range.4 Only a full 360° roll was counted as a response, and it was only counted if it occurs within 20 s. A response between 6 and 20 s was recorded as a slow responder, and an animal that responded in 6 s or less was recorded as a fast responder. Animals that did not respond within 20 s were recorded as nonresponders. For a complete experiment, a sample size (n) of at least 90 per treatment was assessed, with and without UV injury. A mixed logistic regression (MLR) analysis was performed to check for statistical significance. The probe operator was blinded to the experimental treatment.

Quantification of dendritic morphology
Nociceptors were analyzed for total dendritic length and number of branches. Third instar larvae were paralyzed in 81°C water and arranged on a microscope slide in a 2:1 halocarbon–ether mixture. Using a Leica S5 confocal microscope, the nociceptors were imaged from abdominal segments 4–6. Z-stack was restricted to 0.8 μm and image resolution was 1024 × 1024. Utilizing Fiji Image J, images were skeletonized and analyzed for dendritic length and branching.

Statistical analysis. To estimate the predicted probability of reacting for the different treatment groups in sensitization experiments, the response variable (reaction time) was collapsed into a binary variable and a generalized linear mixed model (with a logistic link function) was fit to the data for each pathway component using the lme4 package (version 1.1.736) in R 3.1.3,37 where larvae batch was modeled as a random effect and the explanatory variable was genotype and injury (MLR analysis).
In bar graphs depicting allodynia and normal nociception experiments, black boxes denote fast responders (<6 s), gray boxes denote slow responders (6–20 s), and white boxes denote nonresponders (>20 s). Whiskers indicate the standard error of the mean of at least three groups of larvae. On graphs: * = p < 0.05, ** = p < 0.01, *** = p < 0.001.

Results

To investigate the role of Dally and Dally-like in the induction of nociceptive sensitization, we utilized the GAL4-UAS system to produce cell specific promotion or suppression (RNAi knockdown) of gene expression. Twenty-four hours after UV injury, larvae were tested for sensitivity to a normally innocuous 41°C heated probe stimulus. The probability of responding to the probe significantly increased in control animals following injury (Figure 2). Strikingly, in larvae where Dally was knocked down specifically in the nociceptors, there was no significant increase in response frequency following the injury (Figure 2(a)). This experiment was repeated utilizing a separate nonoverlapping inverted repeat construct and a similar phenotype was observed (Figure 2(b)). Uninjured Dally-knockdown larvae showed normal nociceptive behavior when challenged with a noxious 45°C probe (Figure 3). The morphology of the nociceptor was measured to assess if suppression of Dally reduced neuronal structure, which could contribute to the observed lack of nociceptive sensitivity seen in allodynia experiments. Instead, significant hypermorphy was measured in Dally knockdown nociceptors, in both branching and overall

Figure 2. Injury-induced allodynia requires Dally. Response latencies were characterized as follows: none (>20 s, white), slow (6–20 s, gray), or fast (<6 s, black). After 20 s, the assay was stopped. Error bars indicate the standard error of three groups of larvae. Twenty-four hours post-UV exposure (+) or no UV exposure (−), larvae were assayed with a thermal probe set to a normally innocuous 41°C. Knockdown of Dally was achieved using Ppk1.9-Gal4 and two nonoverlapping UAS-inverted repeat constructs. (a) DallyIR-1 (BDSC#28747, p < 0.05) and (b) DallyIR-2 (BDSC#33952, p < 0.01). Larvae with these RNAi genotypes failed to produce allodynia to the extent observed in control genotypes no UAS (Ppk1.9-Gal4>y¹y¹) and no Gal4 (w¹¹¹¹>UAS-DallyIR¹²); n = 90–120 for all experiments. Data were analyzed using MLR. UV: ultraviolet.
dendritic length (p < 0.001 for each comparison, Figure 4). Experimental overexpression of Dally specifically in the nociceptors caused a significant decrease in response compared to controls following UV injury (Figure 5(a)). Still, the uninjured larvae with overexpressed Dally showed normal nociception when challenged with a noxious 45°C probe (Figure 5(b)). A synthetic, secreted form of Dally, SecDally, was overexpressed specifically in the nociceptors, and a significant difference was observed compared to only one control following injury and assay at 41°C (Figure 6(a)). Uninjured larvae expressing SecDally showed normal nociceptive behavior when challenged with a noxious 45°C probe (Figure 6(b)).

The necessity of a second *Drosophila* glypican, Dally-like (Dlp), was also assessed for its involvement in injury-induced sensitization. In larvae where Dally-like was knocked down specifically in the nociceptors, there was no significant increase in response frequency following UV injury, whereas in control animals, this same injury led to allodynia (Figure 7(a)). This experiment was repeated utilizing a separate nonoverlapping inverted repeat construct and a similar phenotype was observed (Figure 7(b)). Uninjured Dally-like knockdown animals also displayed normal nociception and responded identically to controls at a noxious 45°C (Figure 7(c)). The morphology of Dlp knockdown nociceptors was assessed and no significant changes were observed (Figure 8).

**Discussion**

Chronic pain poses a difficult challenge because of the limitations of current treatments. Neuronal sensitization is thought to underlie and perpetuate chronic pain. Previous data have shown that the fly morphogens...
Hedgehog and Decapentaplegic (Dpp) are necessary for the development of thermal allodynia after injury.\textsuperscript{4,5} The data presented here provide supporting evidence for the involvement of Hedgehog and Dpp regulators, glypicans Dally and Dally-like (Dlp), presumably working at the extracellular level to regulate Hedgehog and Dpp availability to their receptors on the nociceptor.

Suppression of Dally in the nociceptors reduces injury-induced thermal allodynia 24 h after UV injury (Figure 2). This supports the hypothesis that the extracellular regulator Dally is necessary for nociceptive sensitization. Previous studies show that Dally promotes Dpp signaling by reducing Thickveins' ability to antagonize the Dpp ligand and that Dally also likely works to stabilize Dpp at the receptor complex.\textsuperscript{32} It is likely that the absence of Dally makes Dpp binding and signaling less efficient following a UV injury. Interestingly, overexpression of Dally by the nociceptors also suppresses nociceptive sensitization following injury (Figure 3). This may indicate that the excess Dally, in the absence of excess receptor to which Dpp may bind, seems to have a slight negative effect on Dpp signaling. It is possible the excess Dally sequesters Dpp and reduces its opportunity for receptor binding. Similarly, experimental expression of SecDally, which is not membrane bound, produces a nonsignificant hyposensitivity in injured larvae, $p = 0.03$ for SecDally versus the no UAS control, and $p = 0.30$ for SecDally versus the no Gal4 control. SecDally may also sequester extracellular Dpp, but far enough from the cell surface so that its effect is smaller than that seen in the overexpression of the normal membrane-embedded Dally.

Another \textit{Drosophila} glypican, Dally-like protein (Dlp), is also required in the nociceptor for the formation of allodynia (Figure 7) and may also act as a coreceptor. It is likely that these effects take place earlier on in the sensitization pathway, at the level of Hedgehog and its receptor, Patched.\textsuperscript{31}

To address the possibility that any observed reductions in sensitization resulting from genetic manipulation were accompanied by any reductions in the morphology of the nociceptors, we assessed the dendritic morphology

\textbf{Figure 5.} Overexpression of Dally in the nociceptors suppresses injury-induced allodynia 24 h post-UV exposure (+) or no UV exposure (−). larvae were assayed with a thermal probe set to an innocuous 41°C. (a) Overexpression of Dally in the nociceptors using Ppk1.9-Gal4>UAS-Dally (BDSC#5397) suppresses injury-induced allodynia compared to control genotypes no UAS (Ppk1.9-Gal4>w$^{1118}$) and no Gal4 (w$^{1118}$>UAS-Dally) ($p < 0.05$); $n = 90–121$. (b) Normal nociception was observed in UAS-Dally larvae compared to controls when challenged with 45°C probe ($p > 0.05$); $n = 90–94$. Response latencies were characterized as follows: none (> 20 s, white), slow (6–20 s, gray), or fast (<6 s, black). Data were analyzed using MLR.
of Dally-suppressed and Dally-like-suppressed nociceptors. Neither the suppression of Dally nor Dally-like reduced the overall dendritic length (Figure 4) or degree of branching (Figure 8). In fact, Dally-suppressed nociceptors were hypermorphic in both dendritic parameters assessed (Figure 4), whereas Dally-like-suppressed nociceptors were no different from controls (Figure 8). These results indicate a role for Dally in negatively regulating the development of the dendritic field of these neurons, but whether or not the loss of sensitization observed in Dally-suppressed animals is the result of the increased dendritic development remains to be determined. Similar evidence of BMP involvement in dendritic development was previously observed.4 Injury to the epidermis triggers a signaling cascade that results in the binding of extracellular morphogen, Hedgehog, to its receptor Patched.5 We hypothesize that Dally-like helps to facilitate this binding by securing Hedgehog and inhibiting its long-range travel (see Figure 1). This may result in a signaling cascade within the nociceptor itself that eventually results in the release of Dpp, possibly in an autocrine fashion.4 Dally also may sequester Dpp on the membrane (see Figure 1), similarly to the association between Dally-like and Hedgehog, stabilizing Dpp in its own receptor complex of Thickveins, Punt, and Saxophone. From there, the signaling cascade likely continues to the nucleus, where effector genes are regulated to sensitize the nociceptor. The observed reduction in sensitivity resulting from suppression of Dally and Dlp was specific to injury-induced sensitization, as demonstrated by the normal nociceptive response to a normally noxious temperature observed in uninjured Dally-knockdown and Dlp-knockdown larvae. This would be advantageous if BMP mediators were to be used for treating pain patients, because blocking BMP signaling via these components might alleviate abnormal pain while leaving the protective normal pain processes unperturbed.

The goal of this research is to better understand how neurons sensitize, because understanding those

Figure 6. Expression of SecDally, a secreted form of Dally, in the nociceptors does not significantly affect injury-induced allodynia. Twenty-four hours after UV exposure (+) or no UV exposure (−), larvae were assayed with a thermal probe set to an innocuous 41°C. (a) Expressing a synthetic secreted form of Dally, SecDally, in the nociceptors using Ppp1.9-Gal4>UAS-SecDally suppresses injury-induced allodynia compared to the no UAS control (Ppp1.9-Gal4> w 1111) (p = 0.03), but not the no Gal4 control (w 1118>UAS-Sec-Dally) (p = 0.30); n = 90–121. (b) Normal nociception was observed in uninjured Ppp1.9-Gal4> UAS-SecDally larvae compared to controls when challenged with 45°C probe (p > 0.05); n = 91–92. Data were analyzed using MLR. UV: ultraviolet.
mechanisms will reveal potential targets for the development of novel drugs to treat chronic pain. Due to their extracellular location, Dally and Dally-like represent particularly attractive potential drug targets for this pathway because of the challenge of delivering drugs across a cell membrane. The therapeutic potentials of Dally and Dally-like are not limited to pain. These also represent targets for the development of drugs to treat Fibrodysplasia Ossificans Progressiva (FOP), a disorder in which tissues are replaced by bone, caused by overactivated BMP signaling. A future direction of this work, in addition to developing targeted drugs for

Figure 7. The glypican Dally-like (Dlp) is required in the nociceptors for injury-induced allodynia. Twenty-four hours after UV exposure (+) or no UV exposure (−), larvae were assayed with a thermal probe set to an innocuous 41 °C. Suppression of Dlp using Ppk1.9-Gal4 > UAS-DlpIR (BDSC#34091). (a) Dlp^{IR-1} (BDSC#34091, p < 0.01) and (b) Dlp^{IR-2} (BDSC#34089, p < 0.05). Larvae with these RNAi genotypes failed to produce allodynia to the extent observed in control genotypes no UAS (Ppk1.9-Gal4>y^{v1}) and no Gal4 (w^{1118}>UAS-DlpIR^{IR-1}); n = 90–101 for all experiments. (c) Normal nociception was observed in uninjured Dlp^{IR-1} larvae compared to controls when challenged with 45 °C probe (p > 0.05); n = 91–92. Data were analyzed using mixed logistic regression analysis. UV: ultraviolet.
chronic pain, includes testing drugs developed for FOP for their effectiveness to ameliorate allodynia.

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Author Contributions
CLB, JKM and GKG designed the research. CLB and JKM performed the experiments and analyzed the results. CLB, JKM and GKG wrote the manuscript.

Declaration of Conflicting Interests
The author(s) declared no potential conflicts of interest with respect to the research, authorship, and/or publication of this article.

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