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Bone Morphogenetic Protein Glass Bottom Boat (BMP5/6/7/8) and its receptor Wishful Thinking (BMPRII) are required for injury-induced allodynia in *Drosophila*

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Abstract

Background: Chronic pain affects millions of people worldwide; however, its cellular and molecular mechanisms have not been completely elucidated. It is thought that chronic pain is triggered by nociceptive sensitization, which produces elevated nocifensive responses. A model has been developed in *Drosophila melanogaster* to investigate the underlying mechanisms of chronic pain using ultraviolet-induced tissue injury to trigger thermal allodynia, a nociceptive hypersensitivity to a normally innocuous stimulus. Larvae were assayed for their behavioral latencies to produce a distinct avoidance response under different thermal conditions. Previously, Decapentaplegic, a member of the Bone Morphogenetic Protein (BMP) family and orthologous to mammalian BMP2/4, was shown to be necessary for the induction of allodynia. Here, we further investigate the BMP pathway to identify other essential molecules necessary to activate the nociceptive sensitization pathway.

Results: Using the GAL4-UAS-RNAi system to induce a cell-specific knockdown of gene expression, we further explored BMP pathway components to identify other key players in the induction of nociceptive sensitization by comparing the responses of manipulated animals to those of controls. Here, we show that a second BMP, Glass Bottom Boat, and its receptor Wishful Thinking are both necessary for injury-induced thermal allodynia since the formation of sensitization was found to be severely attenuated when either of these components was suppressed. The effects on pain perception appear to be specific to the sensitization system, as the ability to respond to a normally noxious stimulus in the absence of injury was left intact, and no nociceptor morphological defects were observed.

Conclusion: These results provide further support of the hypothesis that the BMP pathway plays a crucial role in the development of nociceptive sensitization. Because of its strong conservation between invertebrates and mammals, the BMP pathway may be worthy of future investigation for the development of targeted treatments to alleviate chronic pain.

Keywords

Nociceptor, hypersensitivity, transforming growth factor beta, damage, ultraviolet, pain

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Introduction

Chronic pain can be defined as pain that persists beyond the duration of the injury. It is thought that chronic pain is perpetuated by nociceptive sensitization.[1–3] Among various manifestations of nociceptive sensitization is allodynia or pain resulting from a normally innocuous stimulus.[4] One mechanism of nociceptive sensitization is the prolonged activation of nociceptors, which are high-threshold sensory receptors of the peripheral somatosensory nervous system that transduce and encode noxious stimuli.[4] The mechanisms by which peripheral nociceptors undergo sensitization are important to understand,

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as their components represent targets for potential medications that may block sensitization and therefore alleviate chronic pain.

In *Drosophila*, there are four classes of peripheral sensory neurons (class I–IV). Those with most elaborately branched dendritic arbors are the Class IV neurons.[5] Six of these peripheral nociceptive neurons reside in each of the nonterminal segments of the larval body wall (Figure 1) and can detect noxious thermal and mechanical stimuli[6] and are directly or indirectly activated by ultraviolet (UV) light.[7] Unlike their mammalian counterparts, in which the somata of the primary afferent neurons are located proximal to the spinal cord in dorsal root ganglia, the somata of the multipolar *Drosophila* nociceptors are located in the centers of their multidendritic receptive fields, directly beneath the epidermis, and their axons project somatotopically to the ventral nerve cord. They express several ion channels, such as pickpocket (Ppk), which is a degenerin/epithelial sodium channel subunit[8] capable of mechanosensation[6] and painless, which is a transient receptor potential channel.[8] Ppk is expressed in all class IV neurons[9] and has been shown to be necessary for mechanical, but not thermal, nociception.[10] The class IV multidendritic neurons are necessary and sufficient to trigger reaction to noxious stimuli[11] and are referred to here as nociceptors.

The only existing model of nociceptive sensitization in *Drosophila* is that triggered by UV light injury.[12] In this model, larvae are subjected to UV irradiation, which induces epidermal injury while sparing the nociceptors. Apoptosis of the injured epidermal cells, specifically the activation of the initiator caspase Dronc,[13] causes release of TNF-α,[12] which together with Tachykinin[14] bind their receptors on the juxtaposed nociceptors. Subsequent Hedgehog release then activates its receptor Patched on the nociceptor.[15] Release of Decapentaplegic (Dpp), orthologous to mammalian BMP2/4, together with primary serine/threonine kinase BMP type II (BMPRII) receptor Punt (Put), and type I (BMPR) coreceptors Thick Veins (Tkv) and Saxophone (Sax), leads to the activation of Mad (Mothers Against Dpp) and Medea.[16] These intracellular transducers belong to the SMAD family, named for the *Caenorhabditis elegans* protein Small and the *Drosophila* protein Mad. The SMADs in turn are predicted to interact with other transcription factors to control gene expression. The resulting sensitization of the nociceptors by this pathway can be detected by assaying larval animals for a distinct avoidance behavior in response to a thermal stimulus. In this model, injured larvae develop sensitization that includes thermal allodynia and hyperalgesia.[12]

Bone morphogenetic proteins (BMPs) are secreted ligands belonging to the TGF-β superfamily. BMPs

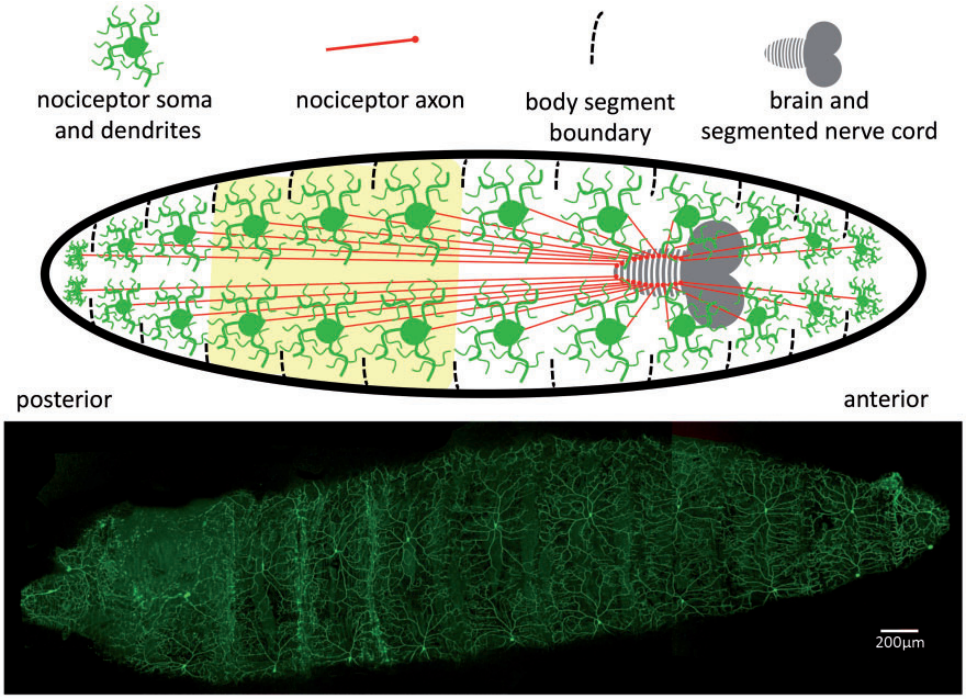

Figure 1. Arrangement of nociceptors in *Drosophila* larva. The somata and respective dendritic fields (green) of nociceptors are arranged in the segmented body wall of the animal as represented in the diagram (top) and visualized in a live animal (bottom). Axons (red) project to and insert somatotopically into the segmented ventral nerve cord. Abdominal segments 4–6 (yellow) were thermally stimulated for allodynia experiments.

were first described for their role in bone and cartilage formation, yet have been shown to play crucial roles in several processes by acting as gradient morphogens in various developmental contexts.[17] BMPs have been shown to be essential for cell fate decisions at the neural plate border[18] and gastrulation.[19] Furthermore, BMPs are essential for axon regeneration[20] and neurogenesis, neural tissue specification, induction of the nervous system, axon guidance, dendritic growth, and neuronal migration.[21]

BMPs are necessary for normal synaptic growth and stability in fruit flies and vertebrates and are strongly conserved across the animal kingdom.[22] As an example of this remarkable functional similarity, transfer of human BMP4 coding sequences to flies lacking the BMP2/4 ortholog Dpp resulted in rescue of the deficiency.[23] Similarly, bone formation in rats can be induced by *Drosophila* Dpp protein.[24] Mutations that hyperactivate the BMP receptor ALK2 have been connected to the human disorder fibrodysplasia ossificans progressiva (FOP) such that ALK2 hyperactivation dysregulates the BMP signaling pathway, leading to ectopic bone formation and allodynia.[25–27]

In addition to Dpp, *Drosophila melanogaster* has two other BMPs. They are Screw, which appears to be a distantly related BMP with similarity to the activins, and Glass Bottom Boat (Gbb), the *Drosophila* ortholog of the mammalian BMPs 5, 6, 7, and 8. BMPs are known to be able to function as heterodimers[28–30] and have been found to bind to their receptors,[22,31] forming a heterotetrameric receptor complex, and ultimately signaling through a hexameric BMP ligand-receptor complex.[17] It was hypothesized that in addition to Dpp, the Type II receptor Punt, and the Type I receptors Thick Veins and Saxophone identified previously as necessary to induce nociceptive sensitization,[16] a second BMP ligand and Type II receptor may also be necessary. In this report, we present evidence that the BMP ligand Gbb and the Type II BMP receptor Wishful Thinking (Wit) are necessary for injury-induced nociceptive sensitization (Figure 6).

Materials and methods

Fly stocks and genetics

Flies were maintained in 6 oz stock bottles on sucrose-cornmeal-yeast medium at a temperature of 25°C with a humidity of 50%–60%. Stock bottles were kept in Percival Scientific Incubators (Perry, IA) with a 12-h light, 12-h dark cycle. The GAL4/UAS system was used to drive expression of *UAS-RNAi* transgenes targeting specific genes of interest. The driver in all experiments was *Ppk1.9-Gal4*, which expresses GAL4 specifically in the class IV multidendritic nociceptors.[32] The *Ppk1.9-Gal4* line used also contained a *ppk-eGFP* element, allowing direct visualization of morphology of these neurons (generous gift from Daniel Cox). All other flies were obtained from the Bloomington *Drosophila* Stock Center (BDSC) in Bloomington, IN. The transgenic *UAS-RNAi* lines used, featuring Inverted Repeat (IR) construction for RNAi suppression, were as follows: *UAS-Gbb^{IR}* (BDSC#34898) and UAS-Gbb^{IR}/CyO (generous gift from Kristi Wharton; data not shown) and *UAS-Wit^{IR}* (BDSC#25949). A *UAS-Gbb* line was used to overexpress Gbb and the wing disc driver *A9-Gal4* was used to confirm its activity (generous gifts from Kristi Wharton). Animals were included without regard to sex.

UV injury

A method of UV-induced allodynia was applied.[12] Third instar larvae were collected at the late foraging stage, just prior to wandering, rinsed in water, and anesthetized with diethyl ether. Anesthetized larvae were gently adhered dorsal side up to double-sided adhesive tape on a microscope slide and were subjected to 12–15 mJ/cm^2 of UV light over a period of 2–5 s in a Spectronics Corporation Spectrolinker XL-1000 UV crosslinker. The larvae were then placed in a small vial containing 1 ml of sucrose-cornmeal-yeast media.

Thermal nociception assay

To test the nociceptive behavior, the larvae were assayed with a thermal probe (Pro-Dev Engineering, Houston, TX) 24 h after the UV exposure. The probe tip was gently applied to the dorsal side of the larvae and held on abdominal segments 4–6 for a maximum of 20 s. To test for allodynia, larvae were subjected to 41°C, the highest temperature that did not elicit a behavioral response in wild-type animals.[16] The larval response consisted of a nocifensive 360° lateral rolling that larvae only exhibit in response to a noxious stimulus.[6] The latency for response was recorded and categorized as follows: fast was 0–6 s (indicated by black regions on graphs), slow was from 6 to 20 s (gray), and no response if the 20-s cutoff was reached (white). To assay the normal nociceptive function, larvae were tested at 45°C, a temperature to which approximately 50% of uninjured wild-type larvae respond, and the latency of response was recorded. The probe operator was blinded to the treatment and genotype, and all groups contained a sample size of at least 90 animals.

Quantification of dendritic morphology

Class IV multidendritic neurons were analyzed for total dendritic length and number of dendritic branches.[33] Third instar larvae of control genotypes *Ppk1.9-*

Gal4>y^{1}*v*^{1}, or experimental genotypes *Ppk1.9-Gal4>gbb*^{IR} (BDSC#34898) and *Ppk1.9-Gal4>wit*^{IR} (BDSC#25949) were anesthetized with CO_2 and placed on a microscope slide with a halocarbon–ether mixture (2:1). Live larvae were imaged with a Leica SP5 confocal laser microscope using a 20× objective with 3× zoom. ddaC neurons, the most dorsal pair of nociceptors,[34] were imaged from abdominal segments 4–6 and z-stacks were taken with a 0.76-μm step size to capture the whole dendritic field of nociceptors of 18 to 30 animals. Images were taken with a resolution of 1024×1024. Images were collapsed into maximum projections, skeletonized, and analyzed for parameters of dendritic length and dendritic branching in the image processing package Fiji (https://imagej.net/Fiji). While consistent within this study, operator variation in the preparation of images for skeletonization produces differences in baselines, so care must be taken to compare these quantification results to those of other studies.

Immunohistochemistry and imaging

Third instar larvae bearing *ppk-eGFP* to specifically visualize the nociceptors were filleted longitudinally by incision along the ventral midline without anesthesia, and all tissues internal to the muscular body wall were removed. The body wall was pinned flat on a silicone (Sylgard) surface, fixed 20 min with fresh 4% paraformaldehyde in phosphate-buffered saline (PBS) at room temperature, washed in PBS containing 0.1% Triton X-100 (PBS-T), and blocked overnight at 4°C with 5% normal goat serum in PBS-T. The intensity of Gbb and Wit immunoreactivity were compared in control and *UAS-RNAi*-expressing animals. Monoclonal antibodies to Gbb (DSHB 3D6-24;[35] 1:500) and Wit (DSHB 23C7;[36] 1:300) were incubated for 1 h at room temperature, then overnight at 4°C, washed in PBS-T and visualized with fluorescently conjugated secondary antibodies (Alexafluor 568: Abcam) at 1:500, incubated for 2 h at room temperature followed by washing with PBS-T. The ddaC nociceptors in abdominal segments 4–6 were identified by eGFP fluorescence and studied for a total of 10–12 cells per component and analyzed by confocal microscopy. Images were collected using a Leica SP5 Scanning Confocal Microscope using a 40× oil objective. All images were 1024×1024, and a Z-stack was collected to ensure that the entire cell body was analyzed. The Z-stacks were collapsed into maximum projections, and the relative intensity of staining was analyzed using Fiji (https://imagej.net/Fiji). In immunohistochemistry results, mean integrated density was measured as the product of the area of the region of interest and the mean gray value in the desired channel.

Wing morphology

Wings were dehydrated in ethanol, mounted in Canada Balsam as previously described,[37] and imaged using phase contrast at 4× on a Keyence BZX-700 inverted digital widefield microscope.

Statistical analysis

In graphs depicting results of allodynia and normal nociception experiments, whiskers indicate standard error of the mean of at least three groups of larvae. For morphometric and immunohistochemical analysis, a Student's *t* test was performed. A mixed logistic regression (MLR) was performed to analyze the predicted probability of reacting for the different treatment groups according to the methods previously reported.[16] Briefly, the response variable (reaction time) was compared to the explanatory variable (genotype and treatment) by generating a linear model and running an MLR using R. Results were considered nonsignificant if $p > 0.05$. On graphs, $*p < 0.05$, $**p < 0.01$, $***p < 0.001$.

Results

Using a cell-specific RNAi approach, the expression of BMP pathway components was suppressed in the primary nociceptors. The successful suppression, or "knockdown," of Gbb expression in nociceptors was assessed using immunohistochemistry. In control larvae, nociceptors show reactivity to a monoclonal anti-Gbb antibody throughout the soma (Figure 2(a) and (c)). Gbb immunoreactivity was significantly reduced in knockdown larvae (Figure 2(a) and (c)). There was no significant difference in Gbb immunoreactivity outside the nociceptors between the control and *Gbb*^{IR} groups (data not shown). The degree of Wit knockdown in the nociceptors was also assessed using immunohistochemistry. In control animals, Wit immunoreactivity was observed in the nociceptors using a monoclonal anti-Wit antibody. In the nociceptors of animals in which Wit was knocked down by cell-specific RNA interference, a significantly lower amount of Wit immunoreactivity was measured compared to the controls (Figure 2(b) and (d)). There was no significant difference in Wit immunoreactivity outside the nociceptors between the control and *Wit*^{IR} groups (data not shown). To determine the efficacy of overexpressing *Gbb* in the nociceptors, the expression of Gbb was visualized via immunohistochemistry using the same anti-Gbb antibody as previously mentioned. Gbb immunoreactivity was significantly higher in *UAS-Gbb* nociceptors than in the control (Figure 2(a) and (c)). The ability of the *UAS-Gbb* tool to induce overexpression of biologically active Gbb was tested by activating it in the developing wing disc using the wing disc driver *A9-Gal4*. The

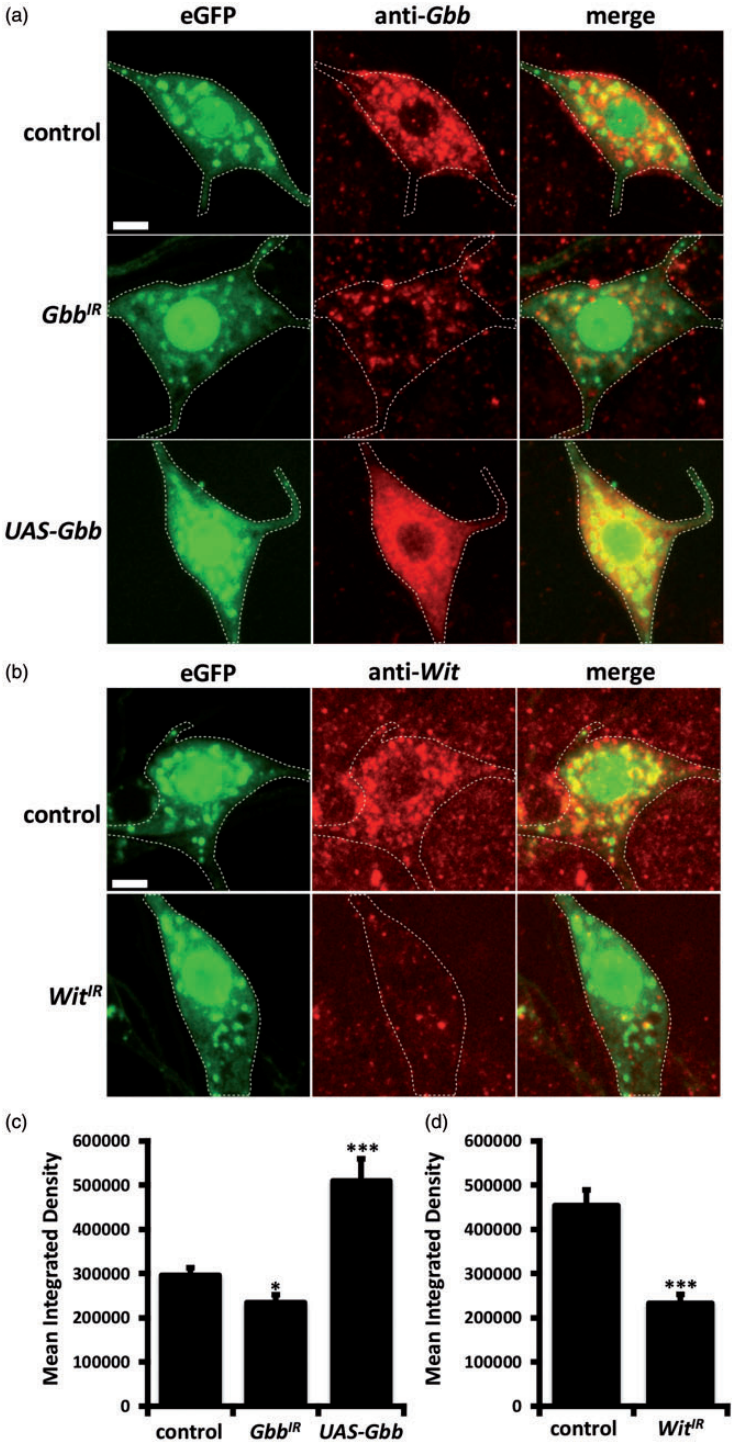

Figure 2. *Gbb* and *Wit* immunolocalization in nociceptors. Nociceptors were identified by GFP expression in larvae expressing eGFP under the control of the *Ppk1.9* nociceptor specific promoter (green). Fillets were incubated with primary antibodies recognizing Gbb or Wit and visualized by confocal microscopy (red). Gbb expression was observed in nociceptors (a). Compared to controls, the expression of Gbb within the soma was reduced in Gbb^{IR} neurons ($p = 0.012$) and increased in *UAS-Gbb* neurons ($p < 0.001$) (a and c). Wit expression was observed in nociceptors (b). Compared to controls, the expression of Wit within the soma was reduced in Wit^{IR} neurons ($p < 0.001$) (b and d). Dotted lines outline soma and major dendritic branches. Scale bars are 5 μm; n = 10–14 for each group. Data were analyzed by Student's *t* test, *$p < 0.05$, and ***$p < 0.001$.

resulting animals showed the severe wing deformation characteristic of perturbation of Gbb signaling in this tissue (Figure 3(d)).

We then proceeded to test our hypothesis that the BMP Gbb and its receptor Wit are required for the formation of allodynia. Twenty-four hours after UV injury, larvae were tested for responses to a normally innocuous 41°C stimulus. In control animals, injury led to a significantly greater frequency of avoidance behavior, characteristic of allodynia (Figure 3(a)). In contrast, larvae in which Gbb was knocked down in nociceptors exhibited no significant increase in response frequency after injury. Larvae in which Gbb expression was knocked down using a separate nonoverlapping IR construct showed similar results (data not shown). Gbb was overexpressed in the nociceptors and uninjured larvae were assayed for allodynia at 41°C. Compared to controls, Gbb overexpression caused no significant change in response frequency (Figure 3(c)).

Wit, the primary Gbb receptor, was knocked down specifically in the nociceptors. Control and Wit-knockdown larvae were tested for the formation of allodynia. In control animals, injury led to a significantly greater frequency of avoidance behavior, characteristic of allodynia (Figure 3(b)). However, larvae in which Wit was knocked down in nociceptors exhibited no significant increase in response after injury.

The normal nociceptive behavior of Gbb or Wit knockdown larvae was then tested to determine if they still had the ability to respond to normally noxious stimuli in the absence of injury. To that end, uninjured controls and experimental larvae were assayed at 45°C, a normally noxious temperature. The response frequencies of the Gbb^{IR} knockdown larvae were found to be lower than seen in no Gal4 controls ($p < 0.01$), but not different than that of no UAS controls ($p > 0.05$) as shown in Figure 4(a). The sensitivity of uninjured Wit knockdown animals to normally noxious temperatures was also assessed. When responses to 45°C were compared, no significant difference in Wit knockdown larvae and normal larvae was found ($p > 0.05$) as shown in Figure 4(b).

The morphology of the dendritic arbor of the dorsal nociceptors, $ddaC^5$ of larvae in which Gbb or Wit was knocked down specifically in these cells, was measured in live animals to assess whether there were any changes to neuron structure that could be associated with the observed nociceptive sensitization. The dendritic branching and overall dendritic length of the nociceptors were not significantly different than observed in control larvae for Gbb^{IR} knockdown larvae. The morphology of the dendritic arbors of the dorsal nociceptors of living larvae in which Wit was knocked down specifically in these cells was also measured. In this case, the dendritic branching and overall dendritic length of the Wit knockdown nociceptors were significantly higher than observed in control larvae (Figure 5(a) to (c)).

Discussion

Nociceptive sensitization is thought to perpetuate chronic pain.[1–3] The *Drosophila* BMP Dpp and its receptor Put have previously been shown to be necessary for the full development of thermal allodynia after injury.[15] In this report, evidence is presented that supports the hypothesis that another member of the BMP family, Gbb and its receptor, Wit, are also necessary components for injury-induced nociceptive sensitization (Figure 6).

The successful suppression of Gbb and Wit expression in nociceptors was assessed using immunohistochemistry. In normal larvae, nociceptors show reactivity to a monoclonal anti-Gbb antibody and anti-Wit antibody and both were significantly reduced in RNAi-suppressed larvae (Figure 2), providing confirmation that these genes were successfully suppressed or "knocked down" using the GAL4/UAS coupled to IR constructs triggering cell-specific RNA interference.

When either Gbb or Wit was knocked down specifically in the nociceptors, injury-induced thermal allodynia was abolished (Figure 3). This observation indicates that Gbb and Wit are both necessary for nociceptive sensitization in this paradigm. On the other hand, experimental targeted overexpression of the ligand Gbb revealed that Gbb is insufficient to induce the thermal allodynia sensitization pathway in the absence of injury (Figure 3(c)). The ability of the *UAS-Gbb* tool to cause overexpression of biologically active Gbb was tested by targeting its expression to the developing wing disc. Since a Gbb gradient controls morphogenesis in this tissue, a dramatically disturbed phenotype compared to the control was observed (Figure 3(d)) as expected.[38,39] Previously, overexpression of Dpp in the nociceptors was shown to be sufficient to produce genetic sensitization in the absence of any injury.[16] Given the established necessity of Dpp, Put, Sax, Tkv, and now Gbb and Wit, the observation that Gbb overexpression is not similarly sufficient might indicate that a Dpp/Gbb heterodimer might be required for assembly of a heterohexameric ligand/receptor signaling complex and that Dpp, not Gbb, may represent a limiting step in the control of this pathway.

The reduction in nociceptive sensitivity of Gbb knockdown animals and Wit knockdown animals was specific to the injury-induced sensitization system. This was evidenced by uninjured Wit knockdown animals that showed no significant decreases in observed behavioral sensitivity upon exposure to a normally noxious temperature and Gbb knockdown animals that showed a significant decrease in sensitivity compared to only one

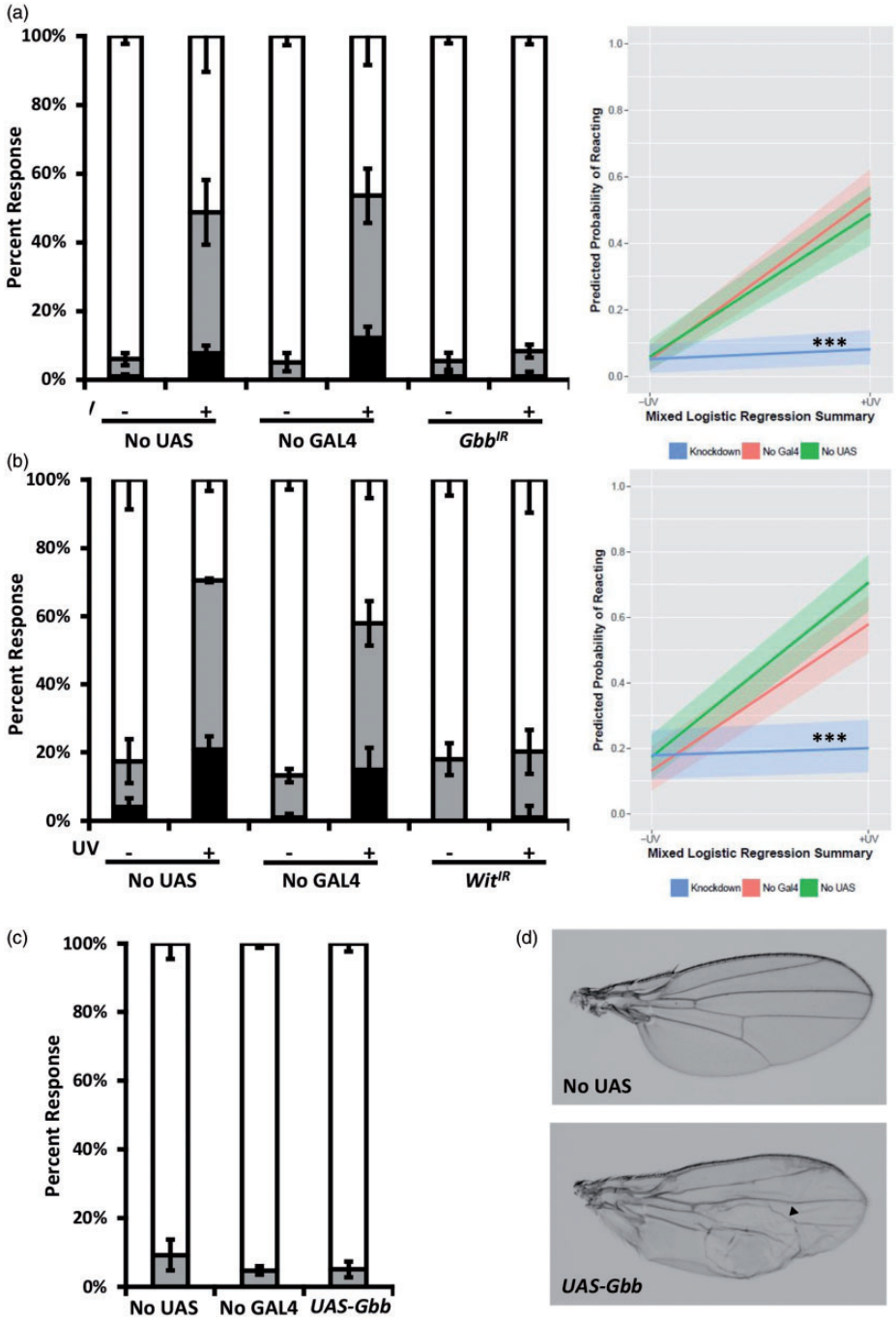

Figure 3. BMP ligand *Gbb* and Type II BMP receptor *Wit* are required in nociceptors for injury-induced allodynia. (a–c) Response latencies were recorded in seconds and categorized as follows: none (>20 s, white area), slow (6–20 s, gray area), or fast (<6 s, black area). Whiskers indicate SEM of at least three groups of larvae. Data were analyzed using mixed logistic regression analysis. Significant differences are indicated by asterisks, ***$p < 0.001$; $n = 92–148$. (a–c) Following UV exposure (+) or no UV exposure (−), larvae were assayed by gentle touch with a thermal probe set to the innocuous temperature, 41°C. (a) Suppression of Gbb using a *Ppk1.9-Gal4>UAS*-inverted repeat (IR) RNAi genotype resulted in a failure to produce allodynia, compared to control genotypes no UAS (*Ppk1.9-Gal4>y^1v^1*) and no Gal4 (*w^{1118}>UAS-Gbb^{IR}*) ($p < 0.01$). There was no difference among *Gbb*^{IR} UV treatments ($p \geq 0.05$). (b) Suppression of Wit using a *Ppk1.9-Gal4>UAS*-IR RNAi genotype resulted in a failure to produce allodynia, compared to control genotypes no UAS (*Ppk1.9-Gal4>y^1v^1*) and no Gal4 (*w^{1118}>UAS-Wit^{IR}*) ($p < 0.001$). There was no difference among *Wit*^{IR} UV treatments ($p > 0.05$). (c) Overexpression of Gbb (*UAS-Gbb*) in *Ppk1.9-Gal4>UAS-Gbb* larvae did not cause hypersensitivity in the absence of UV injury compared to the no UAS (*Ppk1.9-Gal4>y^1v^1*) and no GAL4 (*w^{1118}>UAS-Gbb*) controls ($p < 0.001$). (d) The effectiveness of *UAS-Gbb* to produce Gbb overexpression confirmed by its effect on wing development. An adult fly wing *A9-Gal4>UAS-Gbb* (bottom) shows expected dramatic wing phenotype, including blistering (arrowhead) compared to control *A9-Gal4>w^{1118}* (no UAS: top).

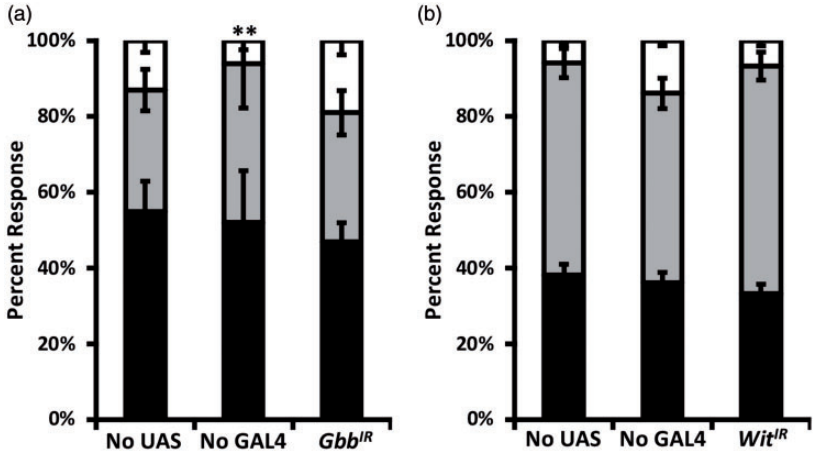

Figure 4. Suppression of *Gbb* or *Wit* in nociceptors does not affect normal nociception. (a) Uninjured animals bearing inverted repeat (IR) RNAi genotypes reducing expression of Gbb responded to normally noxious thermal stimuli (45°C) no differently than the no UAS control ($p > 0.05$) but was hyposensitive compared to the no Gal4 control ($p < 0.01$). (b) Uninjured animals bearing IR RNAi genotypes reducing expression of Wit responded to normally noxious thermal temperature (45°C) no differently than the controls ($p > 0.05$); $n = 96–102$ for each group. Data were analyzed by MLR.

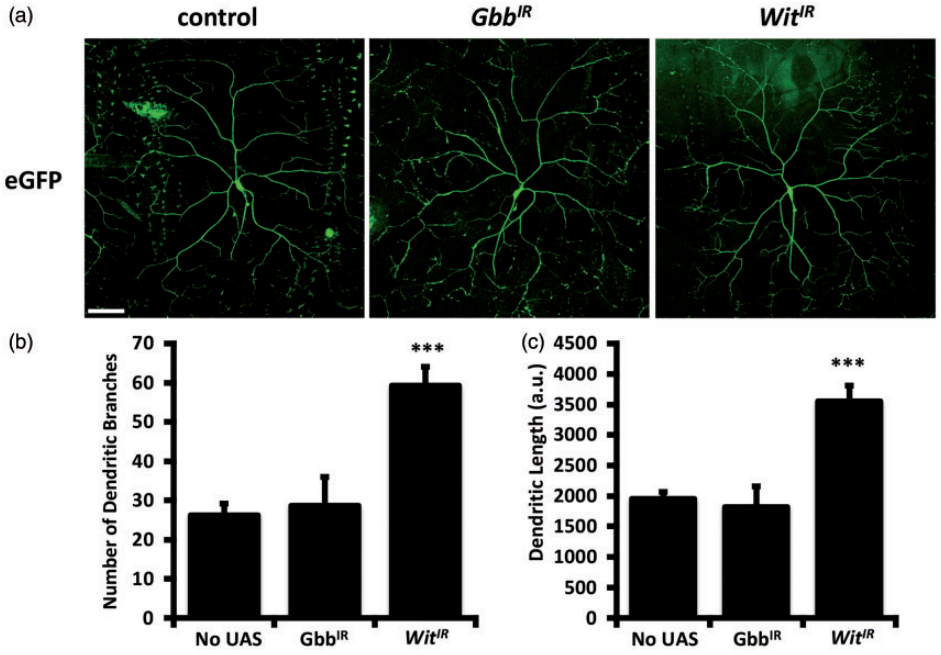

Figure 5. Morphology of nociceptor dendritic fields of larvae in which *Gbb* or *Wit* is suppressed. Images of live nociceptors were obtained by visualizing GFP expression in larvae expressing eGFP under the control of the *Ppk1.9* promoter (green) (a) to assess the morphological parameters of total number of dendritic branches (b) and total dendritic length (c). Larvae in which Gbb was suppressed in nociceptors by inverted repeat (IR) triggered RNAi exhibited neither significant differences in dendritic branching nor total dendritic length compared to control genotype *Ppk1.9-Gal4>y^{1}v^{1}* ($p > 0.05$). Larvae in which Wit was suppressed in nociceptors by IR triggered RNAi resulted in a significant increase in both dendritic branching and total dendritic length, compared to control ($p < 0.001$ and $p < 0.001$, respectively). Scale bar is 100 μm. Skeletons were constructed in Fiji, and total dendritic length and total number of branches were calculated. Data were analyzed using Student's *t* test; $n = 18–30$ for each condition, ***$p < 0.001$.

of its controls (Figure 4). This is promising for future treatment of chronic pain in that the blockade of BMP signaling might be able to suppress abnormal pain while leaving helpful normal pain sensitivity intact.

The necessity of Gbb or Wit in the regulation of the dendritic morphology of the nociceptors was assessed by confocal imaging of GFP-expressing neurons in living larvae. Imaging in live larvae provides better

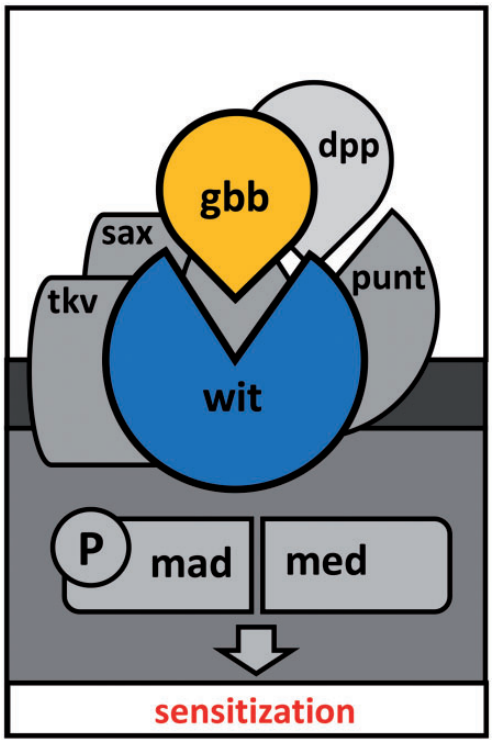

Figure 6. Proposed model of Bone Morphogenetic Protein (BMP) signaling components necessary for nociceptive pain sensitization in *Drosophila* larval nociceptors. Gbb (orthologous to BMP 5/6/7/8) ligand binds to Dpp (orthologous to BMP2/4) ligand and to its primary Type II receptor Wit (orthologous to Type II TGFβ receptor). Dpp binds to its primary Type II receptor, Punt (orthologous to the Type II TGFβ receptor). Subsequent activation of Type I receptors Tkv (orthologous to ALK3/6) and Sax (orthologous to ALK1/2) leads to phosphorylation of the SMAD transducer Mad (cofounder of SMAD family), which then binds Medea (orthologous to SMAD4), ultimately leading to sensitization. This signaling cascade via ligand/receptor multimerization appears to be necessary to induce pain sensitization, with Dpp possibly acting as the limiting factor. Suppression of any one of these components blocks pain sensitization but not normal pain-sensing abilities.

preservation of the fine structure of these cells than do methods requiring dissection and fixation. Gbb suppression was not observed to have any significant effect on either dendritic branching or overall dendritic length of the nociceptors. However, a significant increase in both branching and length was observed in Wit knockdown nociceptors (Figure 5). This observed hypertrophy may not be related to the decrease in sensitivity observed in injury-induced allodynia experiments but may suggest that Wit regulates nociceptor sensitivity by negatively regulating dendritic growth and agrees with the known neural development importance of BMP signaling.[40,41] Although the dendritic arbors of the manipulated neurons became larger and more complex, there was no detectable change in the thermal sensitivity of the animals.

In conclusion, the BMP ligand Gbb (BMP 5/6/7/8) and its type II receptor Wit (BMPRII) are necessary for injury-induced pain sensitization. It is known that Dpp (BMP 2/4) and its primary receptor Punt (BMPRII) as well as the type I TGFβ receptors Tkv and Sax are also necessary.[16] It appears that to successfully produce allodynia, several BMP components are needed to associate in a hexameric ligand–receptor complex. While noncanonical signaling was not examined in this study, given previous results,[16] it appears that the BMP receptors act through the SMAD pathway to bring about sensitization (Figure 6). When any one of these components is knocked down in the nociceptors, the hypersensitivity of injured larvae to non-noxious stimuli is alleviated. Taken together, these findings further implicate the BMP signaling pathway in pain sensitization. Novel therapeutics that will suppress the function of any one of these BMP targets may have the potential to alleviate chronic pain in humans.

Author Contributions

KJG, TLF, and GKG designed the research. KJG performed the experiments and analyzed the results. KJG, TLF, and GKG wrote the manuscript.

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Declaration of Conflicting Interests

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