Duox mediates ultraviolet injury-induced nociceptive sensitization in *Drosophila* larvae

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**Abstract**

**Background:** Nociceptive sensitization is an increase in pain perception in response to stimulus. Following brief irradiation of *Drosophila* larvae with UV, nociceptive sensitization occurs in class IV multiple dendritic (mdIV) neurons, which are polymodal sensory nociceptors. Diverse signaling pathways have been identified that mediate nociceptive sensitization in mdIV neurons, including TNF, Hedgehog, BMP, and Tachykinin, yet the underlying mechanisms are not completely understood.

**Results:** Here we report that duox heterozygous mutant larvae, which have normal basal nociception, exhibit an attenuated hypersensitivity response to heat and mechanical force following UV irradiation. Employing the ppk-Gal4 line, which is exclusively expressed in mdIV neurons, we further show that silencing duox in mdIV neurons attenuates UV-induced sensitization.

**Conclusions:** Our findings reveal a novel role for duox in nociceptive sensitization of *Drosophila* larvae, and will enhance our understanding of the mechanisms underlying this process in *Drosophila* sensory neurons.

**Keywords:** Duox, ROS, Nociception, *Drosophila*

**Background**

Animals perceive noxious stimuli as pain. Peripheral sensory nociceptive neurons are activated upon nociceptive stimuli and transmit electric signals to central pain pathways, giving rise to pain perception and inducing escape behavior [1]. Nociceptive sensory neurons are ‘sensitized’ when nearby tissues are damaged, giving rise to pain hypersensitivity, which is manifested as hyperalgesia (pain amplification by painful stimuli) and allodynia (pain creation by non-painful stimuli) [2]. This pain sensitization is beneficial to animal survival since it helps to avoid touching damaged tissues until they are healed. However, in certain pathological conditions, persistent nociceptive sensitization generates chronic pain [3]. The molecular mechanisms underlying nociceptive sensitization are not fully understood.

In *Drosophila* larvae, class IV multiple dendritic (mdIV) neurons are polymodal nociceptive sensory neurons that induce arborization of dendrites underneath the larval skin [1, 4]. mdIV neurons acutely respond to diverse noxious stimuli including heat, mechanical force, noxious chemicals and reactive oxygen species (ROS) [1, 5–7]. Diverse ion channels are expressed in mdIV neurons to evoke depolarization in response to corresponding noxious stimuli [1, 5, 8–10]. Next to their acute nociceptive response, mdIV neurons accomplish nociceptive sensitization in response to brief ultraviolet-induced tissue damage in the larval skin [11]. Sensitized mdIV neurons give rise to hyperalgesia and allodynia in *Drosophila* larvae [11]. Like in mammals, tumor necrosis factor (TNF) signaling was shown to operate in mdIV neurons for nociceptive sensitization [11]. Recently, additional signaling including Hedgehog (hh) signaling, Bone Morphogenetic Protein (BMP) signaling and Tachykinin-like signaling have been shown to mediate nociceptive sensitization in mdIV neurons [12–14]. However, the underlying mechanisms are incompletely understood. Here we report the genetic analysis of ROS-generating Dual Oxidase (Duox) enzymes and find that...
Duox is required to mediate nociceptive sensitization in mdIV neurons.

Results
To examine whether Duox is involved in pain processing, we undertook a genetic analysis of duox mutants in D. melanogaster. The MiMiC element line MI11825 features an insertion into the 2nd intron of the duox gene (Fig. 1a). Duox [MI11825] homozygotes die as embryos, and are thus not available in the 3rd-instar larval stage for nociception analysis. We therefore used duox [MI11825] heterozygous mutant larvae, in which the transcript level of duox is greatly reduced (Fig. 1b). Duox heterozygous mutant larvae are normal in appearance, larval locomotion, and gentle touch response [15].

Acute nociceptive response is not impaired in duox heterozygous mutant larvae
Wild-type larvae perceive heat and harsh mechanical force as nociceptive, and thus sensing these stimuli on the skin elicits a characteristic nociceptive response [1] (Additional files 1 and 2). Duox heterozygous mutant larvae exhibit a normal acute nociceptive response to heat and mechanical force, comparable to wild-type larvae (Fig. 1c-d), suggesting that their acute response to nociceptive stimuli is not impaired.

Nociceptive sensitization is impaired in duox heterozygous mutant larvae
Irradiating wild-type larvae briefly (5 s) with UV induces tissue damage that gives rise to nociceptive sensitization in mdIV neurons [11]. Accordingly, UV-irradiated wild-type larvae exhibited an increased nociceptive response to heat over time (Fig. 2a). Specifically, 20% of larvae demonstrated nociceptive response to a 40 °C heat probe, increasing to 40% of larvae at six hours post-irradiation; thus, this nociceptive sensitization is hyperalgesic (amplifies pain). In contrast, homozygous mutant larvae of the transient receptor potential ankyrin 1 (TrpA1) channel, a heat and chemical irritant sensor, exhibited no increased nociceptive response following UV irradiation (Fig. 2b), which is consistent with a published report [11]. Likewise, duox heterozygous mutant larvae exhibited no increased nociceptive response to heat (Fig. 2c), suggesting that duox is required for nociceptive sensitization, and hyperalgesia in particular.

To examine whether duox functions in mdIV neurons, we silenced duox in mdIV neurons employing the pickpocket (ppk)-Gal4 line, which directs expression of Gal4 to mdIV neurons ([16]), and two duox RNAi lines (38,907 and 32,903). When driving expression of duox RNAi with ppk-Gal4 (ppk-Gal4 > UAS-Duox RNAi 38,907, 32,903), Duox transcript levels were reduced to 64% for duox RNAi 38,907 and to 78% for duox RNAi 32,903 (Additional file 3: Figure S1A-B). Importantly, nociceptive sensitization following UV treatment was attenuated for both duox RNAi 38,907 and 32,903.
and duox RNAi 32,903 (Additional file 3: Figure S1C). Likewise, silencing of TrpA1 in mdIV neurons (ppk-Gal4 > UAS-TrpA1 RNAi) attenuated nociceptive sensitization following UV treatment (Fig. 2e), consistent with a previous report. However, silencing of ppk1, a mechanosensitive channel, in mdIV neurons (ppk-Gal4 > UAS-ppk1 RNAi) did not abrogate heat nociceptive sensitization following UV irradiation (Fig. 2f), suggesting that Ppk1 is not involved in nociceptive sensitization.

It is of note that basal nociception was similar between ppk-Gal4 > UIAS-duox (Fig. 2d) and duox RNAi 32,903 (Additional file 3: Figure S1C). Likewise, silencing of TrpA1 in mdIV neurons (ppk-Gal4 > UIAS-TrpA1 RNAi) attenuated nociceptive sensitization following UV treatment (Fig. 2e), consistent with a previous report. However, silencing of ppk1, a mechanosensitive channel, in mdIV neurons (ppk-Gal4 > UIAS-ppk1 RNAi) did not abrogate heat nociceptive sensitization following UV irradiation (Fig. 2f), suggesting that Ppk1 is not involved in nociceptive sensitization.

Discussion
We describe a novel role of duox in nociceptive sensitization in mdIV neurons. Firstly, our data show that duox heterozygous mutant larvae, which exhibit basal nociception, display defective hyperalgesia (pain amplification) to heat and mechanical force following UV irradiation. Secondly, duox silencing in mdIV neurons impairs induced hypersensitivity. Altogether, these genetic studies suggest that Duox is required in mdIV neurons to mediate UV irradiation-derived nociceptive sensitization.
It is of note that ~28% of larvae expressing either duox or ppk1 RNAi in mdIV neurons (ppk > Duox-RNAi and ppk > ppk1-RNAi) exhibited nociceptive response to 40 °C heat, as opposed to ~12% of larvae expressing TrpA1 RNAi. This suggests that silencing of duox or ppk1 in mdIV neurons does not affect basal nociception against 40 °C heat, while TrpA1 silencing reduces it. This makes sense in that Duox and Ppk1 are not heat sensors, while TrpA1 is [5, 17, 18]. Notably, duox silencing abrogated heat hypersensitivity while ppk1 silencing did not, highlighting the role of Duox in nociceptive sensitization.

We have shown that basal nociception against heat and harsh mechanical force is not affected by duox reduction, suggesting that mdIV neurons with reduced duox expression retain normal function in sensing nociceptive stimuli and in depolarization. To further confirm this notion, we determined whether structural defects were present in duox heterozygous mutant larvae. We examined the dendrites of duox heterozygotes using ppk-td-GFP lines that specifically expressed td-GFP in mdIV neurons [19]. Confocal images showed that the dendrites of mdIV neurons in duox heterozygous mutant larvae were not reduced in comparison to those of control larvae (Additional file 4: Figure S2); thus, the nociceptive sensitization defects in duox mutants are not due to a reduction of dendrites.

We propose that UV irradiation either directly or indirectly activates Duox expression and/or Duox activation in mdIV neurons. Diverse signaling pathways including TNF, Hedgehog, BMP, and Tachykinin have been shown to mediate UV irradiation-induced nociceptive sensitization in mdIV neurons [11–14]. These signaling pathways could induce the expression and/or activity of Duox [13, 20], and further research should be done to determine whether they do so in mdIV neurons.

The genetic knockdown of heat sensors painless and TrpA1 abolishes not only basal nociception but also UV-induced nociceptive sensitization [12]. This suggests that Painless and TrpA1 mediate nociceptive sensitization following UV irradiation. Duox is a member of the

Fig. 3 Mechanical nociceptive sensitization assay. Larvae were irradiated with UV at 2 mJ/cm². Larval nociceptive response to mechanical force (30 mN) was evaluated. a Wild-type (n = 120 per time section), TRPA1 homozygous mutant (n = 45 per time section), and duox heterozygous mutant larvae (n = 75 per time section). b Duox silencing in mdIV neurons. Ppk > duox-RNAi indicates ppp-Gal4 > UAS-duox RNAi (n = 60 per time section). c TrpA1 silencing in mdIV neurons. Ppk > TRPA1-RNAi indicates ppp-Gal4 > UAS-TRPA1 RNAi (n = 45 per time section). Error bars denote +/− SEM. One-way ANOVA with Tukey post-test was used to analyze the differences. *, ** and *** indicate p < 0.05, 0.01, and 0.001 respectively. n.s., non-significant.
NADPH oxidase family, which produces reactive oxygen species (ROS) in a regulated manner [21]. We speculate that ROS produced by Duox following UV irradiation increase the gating of Painless and TrpA1 through direct oxidation.

Conclusions
Duox has been shown to catalyze dityrosine cross-links in epithelial cuticles, hormone synthesis, and mucosal immunity in Caenorhabditis elegans, D. melanogaster, and mammals. However, the role of Duox in pain signaling has not been addressed in any animal models. Our data uncover a novel role for Duox in the nociceptive sensitization of sensory nociceptors in Drosophila. Intriguingly, mammalian nociceptors employ a different member of the NADPH oxidase family in nociceptive sensitization [22]. Thus, our findings support the notion that the underlying mechanisms of nociceptive sensitization are evolutionarily conserved from insects to mammals.

Methods

Drosophila strains
Flies were reared on standard yeast/cornmeal agar medium at 25 °C. The ppk-GAL4 (#32078, #32079), ppk1-RNAi (#29571), TrpA1-RNAi (#31504), duox-RNAi (#32903, #38907), duox mutant line (duoxΔ11825), #59037 and Ppk-td-GFP (35843) were from Bloomington Drosophila Stock Center.

RT-PCR
Larvae were collected under CO2 and frozen rapidly in liquid nitrogen. Total RNA from larvae was extracted using TRIzol (MRC) according to the manufacturer’s instructions. Reverse transcription (RT) was performed using AccuPower™ RT Premix (Bioneer K-2041) with 2 μg of total RNA in a 20-μl reaction. PCRs were performed on an AccuPower PCR Premix (Bioneer K-2016) with duox primers 5′-CTGCCCATCGCACAAGCATT-3′ and 5′-CTATCCAAAGTCTCGAAGT-3′ and Rp49 primers 5′-AGATCGTGAAAGCGCCACC-3′ and 5′-CACCAGGAACCTTCTTGAAATCCGG-3′.

UV treatment
Lightly ice-anesthetized early third-instar larvae were deposited on a 2% agarose plate and placed in a CL-1000 UV crosslinker (UVP). We used 0 mJ/cm2 (control) and 2 mJ/cm2 at a wavelength of 254 nm. After UV treatment, larvae were returned to the rearing medium at 25 °C before nociceptive sensitivity was assessed at various times after UV exposure.

Behavioral assays
Larval thermal nociception assays were performed as described previously [1]. Briefly, 3rd instar larvae were placed on 2% agarose medium in plastic petri dishes, and were laterally touched with a soldering iron with a 0.6-mm-wide chisel; its temperature was calibrated with a fine thermocouple. The behavioral responses of the larvae were recorded using a digital camera (Kenox, Samsung) and analyzed.

Larval mechanical nociception assays were performed as described earlier [5]. Briefly, 3rd instar larvae were stimulated at 45 mN with a calibrated Sulon monofilament fishing line (6-lb test, diameter 0.23 mm, length 18 mm) that was attached to a pipette. Noxious mechanical stimuli were delivered by rapidly depressing the larvae with the fiber on the dorsal side. Each larva was tested only once.

Additional files

Additional file 1: Video 1. Typical larval behavior upon exposure to non-nociceptive substance. Heat probe, 35 °C. (MP4 4170 kb)

Additional file 2: Video 2. Typical larval nociceptive rolling behavior upon heat exposure. Heat probe, 45 °C. (MP4 4658 kb)

Additional file 3: Figure S1. A. (left) RT-PCR of 3rd instar larvae from lines Ppk-Gal4+/ (1), Ppk-Gal4 > UAS-duox-RNAi (38907) (2), and Ppk-Gal4 > UAS-Duox-RNAi (32903) (3). The primers used for duox PCR are the same as in Fig. 1. Rp49 was used as a loading control. (Right) Quantification of RT-PCR band areas by Image-J. (NIH). B. The band intensity of duox normalized to that of Rp49, and set to one for Ppk-Gal4+. C. Larval thermal nociception assay. Rolling within 10 s of a 40 °C touch was counted as response (n = 30 per time section). Error bars denote +/- SEM. One-way ANOVA with Tukey post-test was used to analyze the differences. * and *** indicate p < 0.05 and 0.001 respectively. n.s., non-significant. (PPTX 148 kb)

Additional file 4: Figure S2. Confocal microscopy reveals dendrites of mdIV neurons for Ppk-td-GFP/+ (left) and Ppk-td-GFP/duox [MI11852] larvae (right). These larvae specifically express td-GFP in mdIV neurons. (PPTX 755 kb)

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Availability of data and materials
The datasets are included within the article.

Authors’ contributions
WJ conducted most of the genetic experiments, analyzed the results, and wrote most of the paper. MB conducted initial UV experiments. YSH provided materials. CK conceived the idea and wrote the paper with WJ. All authors read and approved the final manuscript.

Ethics approval and consent to participate
Not applicable

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Competing interests
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