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Leucokinin signaling regulates hunger–driven reduction of behavioral responses to noxious heat in *Drosophila*

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ABSTRACT

In the fruitfly *Drosophila melanogaster*, hunger has a significant impact on its sensory systems and brain functions, and consequently modifies related behaviors. However, it remains unclarified whether hunger affects nociceptive behavioral responses to heat stimuli. In this study, we show that food deprivation reduces responses to noxious heat in wild–type flies. We further identified that the neuropeptide Leucokinin (Lk) and its receptor (Lkr) are essential for the reduction of responses to noxious heat. Temporal silencing of Lk–expressing neurons and a knockout mutation of *Lkr* generated using the CRISPR/Cas9 system inhibited the reduction of responses to noxious heat. Thus, our results reveal that hunger induces reduction of responses to noxious heat through the Lk/Lkr signaling pathway in *Drosophila*.

1. Introduction

In the fruitfly *Drosophila melanogaster*, food deprivation induces active food–search behavior and gustatory responses to sugar [1,2]. In contrast, sensitivity to bitter taste is reduced in food–deprived flies [3]. Furthermore, food deprivation affects context–dependent CO_2 avoidance, sleep, and memory in *Drosophila* [4–6]. Thus, hunger has a significant impact on *Drosophila* sensory systems and brain functions.

Nociception, the neuronal process for reception and perception of acute pain, is essential for recognition of injury and survival in nature. Animals show a variety of escape responses to noxious stimuli, and several types of stimuli (e.g., noxious heat, noxious chemicals, and harsh mechanical stimulation) can activate the specific nociceptors [7]. In rats, acute food deprivation reduces formalin–induced nociceptive behavioral response [8], indicating that hunger also affects nociception in rats. However, it remains unknown whether hunger–driven reduction of responses to noxious stimuli is prevalent among other animal species. In this study, we examined whether *Drosophila* adults also show the hunger–driven reduction of responses to noxious stimuli and how the responses are controlled by the nervous system. *Drosophila* responds to noxious stimuli and has been used to identify genes involved in heat, mechanical, or chemical nociception [9–12]. The *painless* gene (*pain*), which encodes a transient receptor potential (TRP) channel, was identified as the first 'pain' gene in *Drosophila* [12]. Responses to noxious heat are inhibited in *pain* mutant larvae and adults [12,13]. After that, more genes associated with heat nociception in *Drosophila* have been reported [14–17]. Thus, *Drosophila* can be used to identify genes and molecular mechanisms regulating the hunger–driven reduction of responses to noxious heat.

In *Drosophila*, various neuropeptides contribute to the regulation of feeding behavior or food intake [18,19]. Because the hunger and satiety states are modulated by complex signaling networks using such neuropeptides, it is possible that neuropeptide signaling pathways also play a critical role in the regulation of the hunger–driven reduction of nociception. In this study, we found that flies show the reduction of their responses to noxious heat after food deprivation, and the silencing of Leucokinin (Lk)–expressing neurons inhibits the hunger–driven reduction of responses to noxious heat. Lk is a neuropeptide found in most invertebrate species and regulates meal size in *Drosophila* [20–22]. We generated flies with Lk and its receptor (Lkr) knocked out using the CRISPR/Cas9 system [23], and we examined whether knockout mutations of *Lk* and *Lkr* affect hunger–driven reduction of responses to noxious heat.

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2. Materials and methods

2.1. Fly strains

The fly stocks used for this study were as follows: wild−type *D. melanogaster* Canton−S (CS), *pain*^{1}, *pain*^{2}, *pain*^{3}, *pain*−genome (obtained from Dr. Toshihiro Kitamoto), UAS−*mCD8*::*GFP* (BL−5137), *Lk*^{KO} (see next section), *Lkr*^{KO} (see next section), *Lk*−GAL4 (obtained from Dr. Pilar Herrero), MB−LexA, LexAop−*mCD8*::*GFP* (BL−32203), UAS−*Kir2.1*::*GFP* (BL−6595), and *tub*−GAL80^{ts} (BL−7108). Flies were raised on glucose−yeast−cornmeal medium at 25.0 ± 0.5 °C in a 12−h light:12−h dark (LD) cycle. All lines except for UAS−*mCD8*::*GFP* and LexAop−*mCD8*::*GFP* were outcrossed for at least six generations to *white* flies with the CS genetic background.

2.2. Generation of Lk^{KO} and Lkr^{KO} flies

Lk^{KO} and *Lkr*^{KO} were generated by the CRISPR/Cas9 system [23]. Two complementary oligonucleotides corresponding to 20−b target sequences were annealed and cloned into *Bbs*I−digested pBFv−U6.2 and pBFv−U6.2B vectors. The 20−b target sequences were designed using Cas9 Target Finder (https://shigen.nig.ac.jp/fly/nigfly/cas9/cas9TargetFinder.jsp). The 20−b target sequences are as follows: *Lk* gRNA1, 5′−GGCAAAGATAGTCCTGTGTA−3'; *Lk* gRNA2, 5′−GTGGGGCGGCAAAAGGTCAC−3'; *Lkr* gRNA, 5′−GCAATGGACTTAATCGAGC−3'; *Lkr* gRNA2, 5′−GTGTCGCGAGTCCACCTGCC−3'. The pBFv−U6.2B−gRNA1−gRNA2 plasmid (200 μg/ml) was injected into *y*^{1} *v*^{1} *nos−phiC31*; attP40 (TBX−002) eggs. For the generation of knockout flies, U6.2B−gRNA transgenic flies were crossed with *vas*−Cas9 transgenic flies (BL−51323). Gene deletion was confirmed by PCR using genomic DNA. The primer sequences are described in Supplemental Materials and Methods.

2.3. Behavioral assay of responses to noxious heat

Behavioral assay of responses to noxious heat was performed as previously reported with substantial changes [13]. Newly emerged males were collected, and their wings removed with microscissors during 4 min cold anesthesia treatment (<3 °C) to prevent them from flying away during the assay of their noxious heat responses. Wingless males were kept at 25.0 ± 0.5 °C in an LD cycle until the experiments. A single wingless male (3−6 d old) was placed at the center of a peltier plate (15 cm × 22 cm) at a controlled predetermined temperature (25, 40, 44, 46, 48, or 52 °C) (SANSYO, Cool plate SA−800) using a manual aspirator and its movements were recorded for 30 s or until it goes outside the plate. In a group of 10 males, we determined the number of jumping flies (N_j). When decapitated flies were used in the experiments, we determined the number of tumbling flies (N_t). We calculated response index (RI) using RI (%) = (N_j or N_t)/10 × 100. When a fly went outside the plate without showing responses to noxious heat during recording, it was regarded to have no response. Student's *t*−test or the Mann−Whitney *U* test was used for statistical analysis.

2.4. Decapitation of flies

Decapitated flies can maintain their normal standing posture and respond to gentle mechanical signals [24]; however, it is unknown whether nociception remains intact in decapitated flies. Thus, we examined in this study whether decapitated flies also show responses to noxious heat. Decapitated wild−type and *pain* mutant flies were prepared by cutting the heads of cold−anesthetized flies with microscissors. Cold anesthesia treatment (<3 °C) during decapitation was minimized and limited to 4 min. The decapitated flies then placed in a temperature− regulated container (25 °C) for 0.5−1.5 h.

2.5. Food deprivation

Newly emerged males were collected and their wings removed with microscissors during 4 min cold anesthesia treatment (<3 °C). After wing removal, flies were kept in standard food vials until just before the start of food deprivation. For food deprivation, flies without wings were transferred into vials (about 20 flies/vial) with agar medium (water, 1 L; agar, 8 g) 6, 12, or 18 h before behavioral assay.

2.6. Immunohistochemistry

An anti−Lk antibody to the full−length amidated Lk peptide was generated by Sigma−Aldrich Japan. Immunohistochemistry was performed as previously described [25]. Brains were immunostained for Lk with a rabbit anti−Lk antibody (1:500−4000) followed by Alexa Fluor 488−conjugated anti−rabbit IgG (Invitrogen

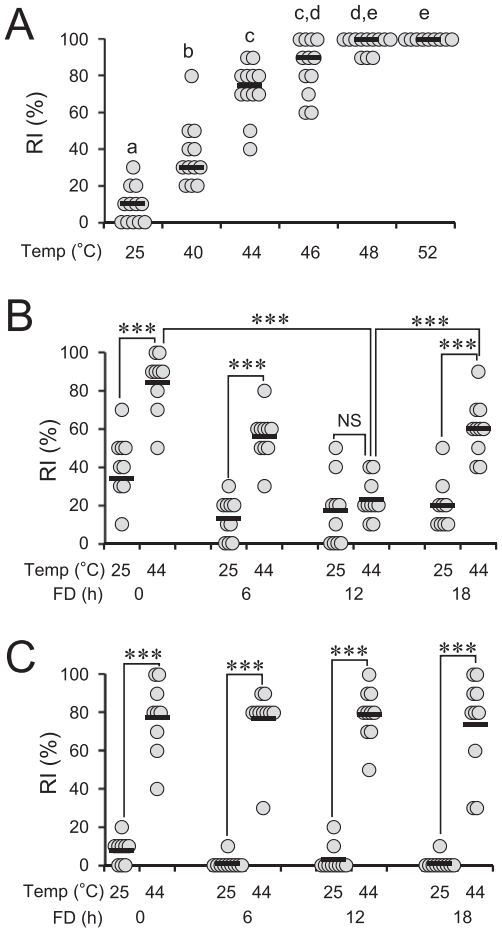

Fig. 1. Hunger−driven reduction of responses to noxious heat. NS, not significant; **, $P < 0.01$. ***, $P < 0.001$. **(A)** Dot plot graph of response index (RI) (%) at various temperatures (25, 40, 44, 46, 48, and 52 °C). Wild−type males were used. Each dot represents the data of each group, and means are shown as black lines. Using the computer software BellCurve for Excel (Social Survey Research Information Co., Ltd.), nonparametric ANOVA (Kruskal−Wallis test) followed by post−hoc analysis using the Steel−Dwass test was carried out for multiple pairwise comparisons. Values with the same letters indicate that they are not significantly different ($P > 0.05$). $N = 12$ in each group. **(B)** RIs (%) at 25 and 44 °C. Food−deprived wild−type flies were used. $N = 9$−10 in each group. **(C)** RIs (%) at 25 and 44 °C. Food−deprived and decapitated flies were used. $N = 9$−10 in each group.