Genetic studies of hunger-driven reduction of behavioral responses to noxious heat in *Drosophila*

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Contents

Summary

Feeding is essential for animal survival in nature and influences internal satiety-hunger state. The hunger can modify not only feeding behavior but also other behaviors or physiology. The powerful genetic tools in the fruitfly *Drosophila melanogaster* have been used to clarify the cellular and molecular mechanisms of modification of innate and learned behavior induced by hunger. Food deprivation promotes the activity of olfactory and sweet taste neurons and consequently induces active food-search behavior and gustatory responses to sugar. In contrast, sensitivity to bitter taste is reduced in food-deprived flies. In addition, food deprivation is known to influence context-dependent CO_2 avoidance, sleep, and memory in *Drosophila*. Thus, hunger has a significant impact on modification of sensory systems or brain functions in *Drosophila*.

Nociception, the neuronal process for reception and perception of acute pain, is essential for recognition of injury and survival in nature. In vertebrates and invertebrates, 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. Acute food deprivation reduces formalin-induced nociceptive behaviors, 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. *Drosophila* reacts to a variety of noxious stimulus and many studies focused on noxious heat. Many genes associated with heat nociception have been reported in *Drosophila*. Thus, *Drosophila* can be used to identify genes and molecular mechanisms regulating the hunger-driven reduction of responses to noxious heat. In this study, I examined whether adult flies also show the hunger-driven reduction of responses to noxious heat stimuli and how the responses are controlled by the nervous system.

When wild-type adults are placed on a hot plate (≧ 44°C), they show robust jumping behavior as a response to noxious heat. First, I examined whether food deprivation affects responses to noxious heat. When wild-type flies were food-deprived for 12 h (referred to as 12 h FD), responses to noxious heat were reduced. However, 12 h FD slightly increased locomotor activity and did not affect climbing activity. In contrast to 12 h FD, when wild-type flies were food-deprived for 6 h and 18 h, responses to

noxious heat were not reduced. In addition, I confirmed that food intake in food-deprived flies for 12 h increased in comparison with the control flies. These results indicate that 12 h FD induces hunger.

To investigate whether the brain regulates hunger-driven reduction of responses to noxious heat, I used decapitated flies in the experiments. Decapitated flies are known to retain a variety of functional sensory inputs, although it is unknown whether they can respond to noxious heat. Decapitated wild-type flies showed tumbling at 44 °C. In *Drosophila*, the Painless (Pain) Transient receptor potential channel is thought to be a heat nociceptor because *pain* mutations inhibit the responses to noxious heat. The frequency of tumbling was significantly reduced by *pain* mutations, indicating that the tumbling is a response to noxious heat in decapitated flies. Next, I examined whether food deprivation also affects responses to noxious heat in decapitated flies. When flies were decapitated after 12 h FD, the responses to noxious heat did not change, suggesting that hunger-driven reduction of responses to noxious heat results from the modification of physiological states of the brain.

In *Drosophila*, complex signaling networks using neuropeptides regulate satiety-hunger state. A neuropeptide, Leucokinin (Lk) and its receptor [Lk receptor (Lkr)] are known to regulate meal size. However, it remains unclear whether food deprivation modifies fly behaviors through Lk signaling. First, I confirmed that the temporal electrical silencing of adult *Lk*-expressing neurons (*Lk* neurons) have effect on the reduction of responses to noxious heat after food deprivation. Next, I generated Lkr^{KO} using CRISPR/Cas9 system. Heterozygous and homozygous flies for Lkr^{KO} also did not show the hunger-driven reduction of responses to noxious heat, indicating that Lk/Lkr signaling pathway induces the hunger-driven reduction of responses to noxious heat. Using anti-Lk antibody, I examined whether 12 h FD affects Lk levels in the brain neurons. Compared with the control, Lk signal in food-deprived flies significantly decreased. However, the qRT-PCR analysis revealed that no significant differences are detected in *Lk* mRNA level between food-deprived flies and the control flies. Taken these results together, it is possible that hunger induces Lk release in the brain neurons and consequently responses to noxious heat are inhibited.

In this study, I found that hunger induces reduction of responses to noxious heat through Lk/Lkr signaling in *Drosophila*. This system may improve a possibility for flies to find available feeding site under blazing heat in summer.

General introduction

Animals modify their behaviors in response to individual interactions and external environments. They receive and recognize various environmental signals to select proper behavior in nature. Thus, this behavioral decision in animals may be based on a series of sensory processing (Gold and Shadlen, 2007; Heekeren *et al*., 2008). Internal states also influence the behavioral decision. For example, hunger may induce food searching behavior, while animals will face danger during food searching in nature. Thus, based on a balance of risks and benefits, animals have to make a decision whether they seek food resources or refrain from food seeking. However, molecular and cellular underpinnings of behavioral decision remain elusive.

In the fruitfly *Drosophila melanogaster*, feeding depends on nutrient availability and metabolism as was observed in mammals (Moran, 2009; Itskov and Ribeiro, 2013; Jourjine, 2017). In *Drosophila*, various neuropeptides contribute to regulate feeding behavior or metabolic pathway as follows: (1) low amino-acid medium and 24 h food deprivation increase protein levels of Insulin-like peptide 2 (Ilp2) and Ilp5 in insulin-producing cells, suggesting that feeding of amino acid and sugar induces Ilps secretion (Géminard *et al*., 2009), (2) Allatostatin A (AstA) inhibits food intake and it regulates signaling of glucagon-related adipokinetic hormone (Akh) and Ilp (Hergarden *et al*., 2012; Hentze *et al*., 2015), (3) Neuropeptide F (NPF), which is a *Drosophila* homolog of mammalian Neuropeptide Y (NPY), regulates hunger-driven feeding behavior and overexpression of *NPF receptor* can mimic food-deprived phenotype (Wu *et al*., 2003; Wu *et al*., 2005; Lingo *et al*., 2007), (4) short Neuropeptide F (sNPF) controls food intake and regulates body size in larvae and adults (Lee *et al*., 2004), and (5) Leucokinin (Lk), Hugin (Hug), and myoinhibiting peptide precursor also contribute to feeding behavior (Al-anzi *et al*., 2010; Melcher and Pankratz, 2005; Min *et al*., 2016). Therefore, *Drosophila* can be used as a model organism for validating the neuronal mechanisms of various biological phenomena associated with hunger/satiety state.

Hunger/satiety state positively and negatively modifies sensory physiology in vertebrate and invertebrates. In *Caenorhabditis elegans*, hunger increases chemoreceptor expression and food

attractive behavior via dopaminergic neurons (Ryan *et al*., 2014; Hills *et al*., 2004). In *Drosophila*, sugar sensing and olfactory processing are enhanced by hunger (Meunier *et al*., 2007; Inagaki *et al*., 2012; Root *et al*., 2011; Beshel and Zhong, 2013). In zebrafish larvae, hunger promotes the approach to small moving objects ("prey") by modulating visual processing via neuroendocrine and serotonergic systems (Filosa *et al*., 2016). These results suggest that hunger modifies sensory processing in animal, although the mechanisms are poorly understood.

Nociception is one of the most important sensory functions for animal survival. Noxious stimuli, which are divided into several types of stimuli (noxious heat, noxious chemicals, noxious cold and noxious mechanical stimulation), induce acute pain (Dubin and Patapoutian, 2010). *Drosophila* also shows responses to noxious stimuli as was observed in mammals, and it has been used to identify genes involved in heat, mechanical, or chemical nociception (Tracey *et al*., 2003; Al-Anzi *et al*., 2006; Ohashi and Sakai, 2015). *Drosophila* is an excellent model organism to study nociception because (1) various genetic information and tools are available, (2) genetic studies using *Drosophila* have identified several molecular components associated with nociception, and (3) the molecules identified in *Drosophila* are mostly shared by mammals (Milinkeviciute *et al*., 2012).

In Part I, to clarify whether the fly brain regulates behavioral responses to noxious heat, I established novel behavioral assay for heat nociception using decapitated flies. Using this behavioral assay, I found that decapitated flies also show specific behavioral responses to noxious heat. In Part II, I examined whether hunger state influences behavioral responses to noxious heat in flies. I found that food deprivation reduces behavioral responses to noxious heat in intact flies but not in decapitated flies, although food deprivation has little impact on heat sensation in sensory neurons. Furthermore, I identified that neuropeptide signaling pathways regulate the hunger-driven reduction of responses to noxious heat in *Drosophila*.

Part I

Establishment of behavioral assay for nociception using intact and decapitated flies

Introduction

Nociception, the neuronal process for reception and perception of acute pain, is essential for recognition of injury and survival in nature. Noxious stimulus is detected by nociceptor, and this information is transmitted into the brain. *D. melanogaster* is especially useful for identification of genes and molecular mechanisms involved in reception and perception of acute pain because there are far fewer ethical issues with studying mutant flies than mammalian model systems. Mammals show a variety of escape responses to noxious stimuli (e.g., noxious heat, noxious chemicals, and noxious mechanical stimulation) (Dubin and Patapoutian, 2010). Similarly, flies also escape from noxious stimuli and they have been used to identify genes involved in heat, mechanical, or chemical nociception (Tracey *et al*., 2003; Al-Anzi *et al*., 2006; Milinkeviciute *et al*., 2012; Ohashi and Sakai, 2015).

The transient receptor potential (TRP) channels are six-transmembrane cation channel, and they form homo- or hetero-tetramers (Talavera *et al*., 2008). In 1989, *trp* was firstly isolated as a membrane protein required for phototransduction in *Drosophila* because an electrical potential of the retina in *trp* mutant was “transient” (Montell and Rubin, 1989). The TRP channel families are conserved from yeast to human and classified into seven subfamilies (TRPC, TRPV, TRPN, TRPM, TRPA, TRPP, and TRPML) whose functions are relevant to various sensory physiology (Vriens *et al*., 2004; Ramsey *et al*., 2006). TRPV, TRPM, and TRPA subfamilies are known to be thermosensors in mammalian species (Talavera *et al*., 2008; Uchida and Tominaga, 2011). TRPV1 and TRPV2 channels are identified as heat sensors, while TRPV (TRPV3 and TRPV4) and TRPM (TRPM2, TRPM4, and TRPM6) are warm sensors (Uchida and Tominaga, 2011). In addition, TRPM8 and TRPA1 are cool or cold sensors (Uchida and Tominaga, 2011). Like mammalian TRPs, *Drosophila* TRPs also contribute to sensing of a specific temperature. The *painless gene* (*pain*), which encodes a TRPA channel, was identified as the first ‘pain’ gene in *Drosophila* (Tracey *et al*., 2003). Responses to noxious heat are inhibited in *pain* mutant larvae and adults (Tracey *et al*., 2003; Xu *et al*., 2006). *Drosophila* TRPA1 (dTRPA1) is required for thermal nociception and works as a worm sensor in flies (Hamada *et al*., 2008; Neely *et al*., 2011), and TRPC (TRPL and TRP), TRPV (Inactive), TRPP (Brivido) are related to behavioral response to cool

temperatures in larvae or adults (Rosenzweig *et al*, 2008; Kwon *et al*., 2010; Gallio *et al*., 2011). Moreover, TRPP (Polycystic kidney disease 2, Pkd2*),* TRPN (No mechanoreceptor potential C, NOMPC), and TRPM (Trpm) contribute to noxious cold responses in larvae (Turner *et al*., 2016). Thus, in mammals and *Drosophila,* TRP channels are essential for thermosensation ranging from cold to heat.

Although flies escape from noxious heat stimuli by jumping (Xu *et al*., 2006), it still remains unclarified whether this jumping behavior is regulated by the brain or central nervous system other than the brain [e.g., ventral nerve cord (VNC)]. In this study, to estimate whether the brain regulates heat-inducible jumping, decapitated flies were used. Decapitated flies can maintain their normal standing posture and respond to gentle mechanical signals (Vandervorst and Ghysen, 1980); however, it is unknown whether nociception remains intact in decapitated flies. I identified that decapitated flies do not show jumping behavior toward noxious heat stimuli and that they show unique responses to noxious heat stimuli instead of jumping. Furthermore, I examined whether Pain TRP channel is also essential for behavioral responses to noxious heat in decapitated flies.

Materials and Methods

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}, and *pain-genome* (obtained from Dr. Toshihiro Kitamoto). 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 were outcrossed for at least six generations to *white*^{1118} flies with the CS genetic background.

Behavioral assay of responses to noxious heat with intact flies

Behavioral assay of responses to noxious heat was performed as previously reported with some modifications (Xu *et al*., 2006). Newly emerged males were collected, and their wings were 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 male flies, I determined the number of jumping flies (N_j). In a group of 10 decapitated male flies used in the experiments, the number of tumbling flies (N_t). I 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 pairwise comparison.

Decapitation of flies

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.

Results

Behavioral responses to noxious heat in intact adult flies

I measured behavioral responses to various temperatures using wild-type flies (Fig. 1A). When the temperature was at 44 °C or higher, RI was significantly higher than that at the control temperature (25 °C). Xu *et al*. (2006) has reported that flies jump quickly at 45 °C or higher. My results shown in Fig. 1A were almost consistent with their report (Xu *et al*., 2006). Next, I used *pain* mutant flies (Fig. 1B). At 46 °C, RIs in $pain^1$ *and* $pain^3$ mutant flies were significantly lower than that in wild-type flies, while no significant difference was detected between *pain* mutants and wild-type flies at 25 °C (Fig. 1B). Thus, I confirmed that Pain is required for behavioral responses to noxious heat in adult flies as previously reported (Xu *et al*., 2006).

Behavioral responses to noxious heat in decapitated flies

At room temperature, decapitated wild-type flies stood on the plate without moving until the end of the observation period (Fig.2, white bar). In contrast, they showed various behaviors as responses to noxious heat (Fig.2, light gray bar). Although they did not jump in response to noxious heat (46 °C), they frequently tumbled and then got up on a hotplate (tumbling). Thus, it is possible that tumbling is a response to noxious heat in decapitated flies. At 44 and 46 °C, RI of decapitated flies carrying $pain^1$, $pain^2$, and $pain^3$ was lower than that of decapitated wild-type flies (Fig. 3). Next, I used a genomic DNA fragment containing the *pain*-coding region and 2.0 kb of upstream genomic DNA (*pain*-genome) (Tracey *et al.*, 2003). The RI of *pain*-genome; $pain^1$ flies was significantly higher than that of $pain^1$ flies at 44 °C (Fig. 4), indicating that *pain* expression in $pain^1$ flies restores the $pain^1$ mutant phenotype. Thus, I concluded that tumbling is a response to noxious heat in decapitated flies.

Discussion

In this study, I established a novel behavioral assay for responses to noxious heat using decapitated flies (Figs. 2, 3, and 4). At 44 °C or higher, decapitated flies showed tumbling, but not jumping. *pain* mutations inhibited frequency of tumbling, indicating that *pain* TRP channel is also required for behavioral responses to noxious heat in decapitated flies (Figs. 3 and 4). Since Pain TRP channels, which act as a heat nociceptor in *Drosophila* (Sokabe *et al*., 2008), are expressed in sensory neurons in the legs (Al-Anzi *et al*., 2006; Xu *et al*., 2006), flies may sense noxious heat directly through their legs. Although intact adult flies showed jumping as the response to noxious heat (Fig. 1), decapitated flies did not (Fig. 2), indicating that the brain is essential for coordinating proper jumping in intact adult flies.

Oswald *et al*. (2011) demonstrated that water droplet (>29 °C) induced escape behavior in *Drosophila* larvae and $pain^{1}$ mutant respond to water droplet at about 34 °C. These results are inconsistent with previous reports using a heat probe, suggesting that water droplet can activate many neurons at the same time in comparison with a heat probe (Tracey *et al*., 2003). In a temperature-regulated peltier plate using my study, only legs containing heat nociceptors, but not all nociceptors in fly body, may be activated. Although Pain is a direct sensor of noxious heat (Sokabe *et al*., 2008), heat nociception at 44–46 °C was not completely inhibited by *pain* mutations, suggesting that there are other heat sensors in *Drosophila*. However, I cannot completely deny the possibility that Pain TRP channel in VNC may also be involved in response to noxious heat.

Figures

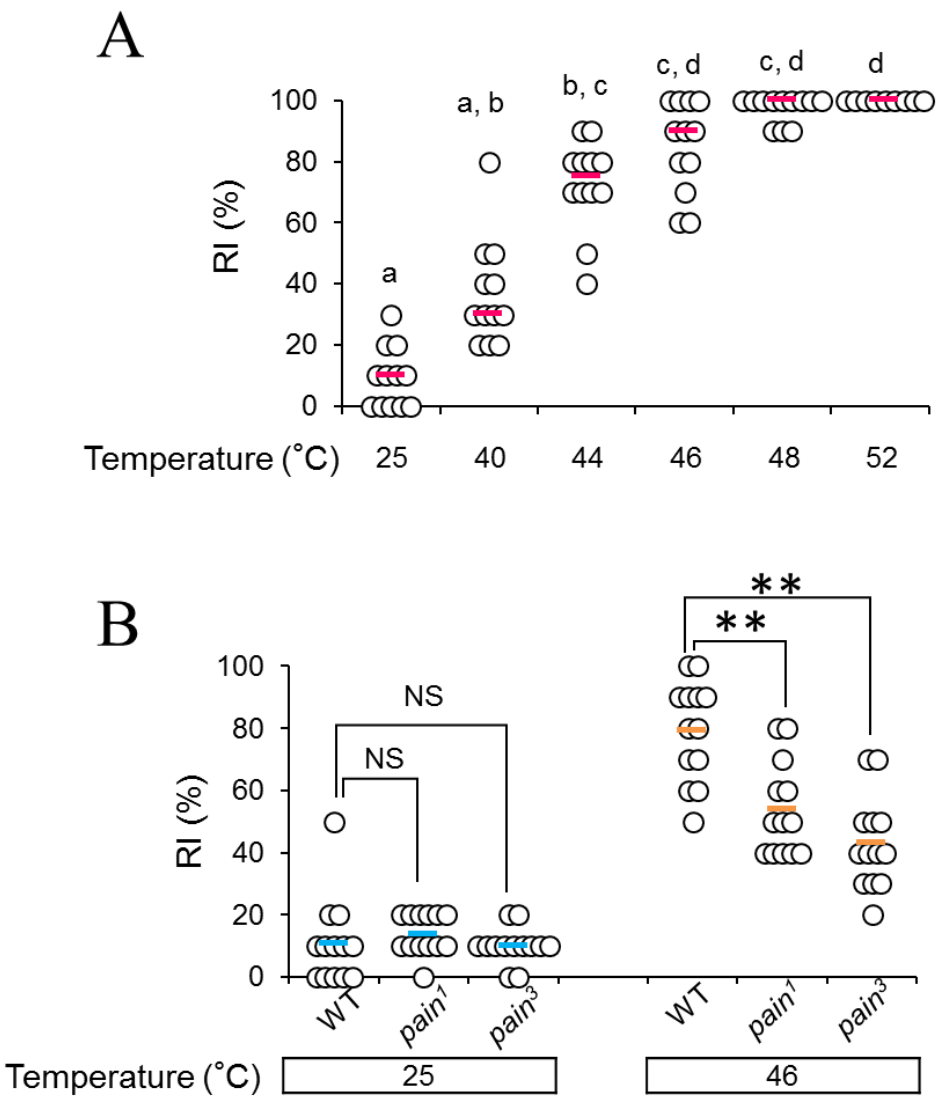

Fig. 1. Responses to noxious heat in wild-type and *pain* mutants.

In a group of 10 male flies, the number of jumping flies (N_j) was checked. Response index (RI) was calculated using RI (%) = N_j / 10 ×100. (A) Dot plot graph of RIs (%) at various temperatures (25, 40, 44, 46, 48, and 52 °C). Wild-type (WT) males were used. Each dot represents the data of each group, and means are shown as 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 46 °C. WT, *pain*^{1} and *pain*^{3} flies were used. $N = 13$ in each group. NS, not significant; **, $P < 0.01$.

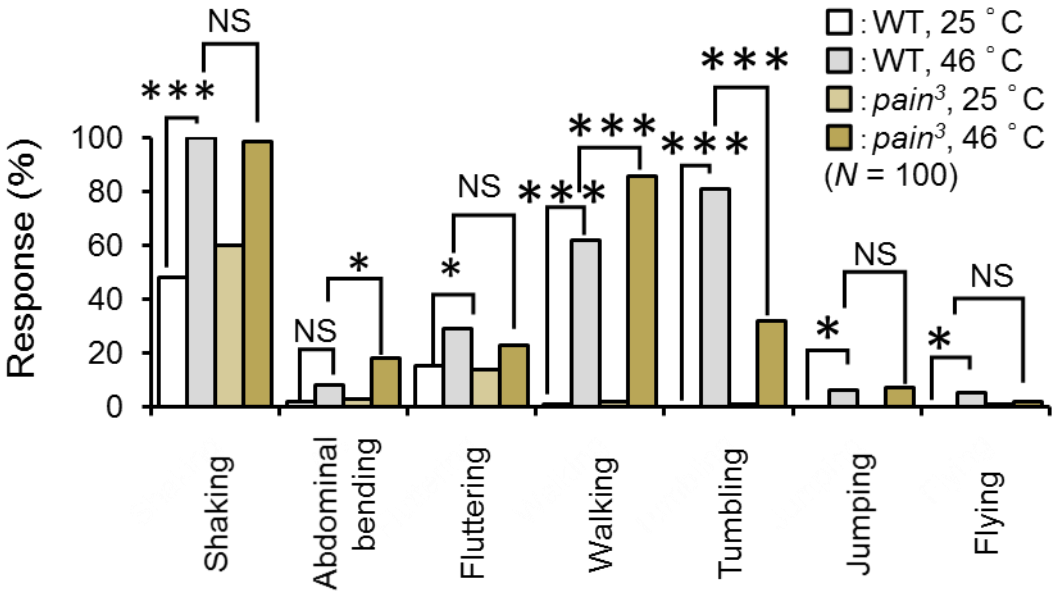

Fig. 2. Detailed observation of responses to noxious heat in decapitated flies.

Chi-square test was used for statistical analysis. Percent of each response (%) at 25 and 46 °C. Wild-type (WT) males were used. WT and *pain*^{3} flies were used. $N = 100$ in each group. NS, not significant; *, $P < 0.05$. ***, $P < 0.001$.

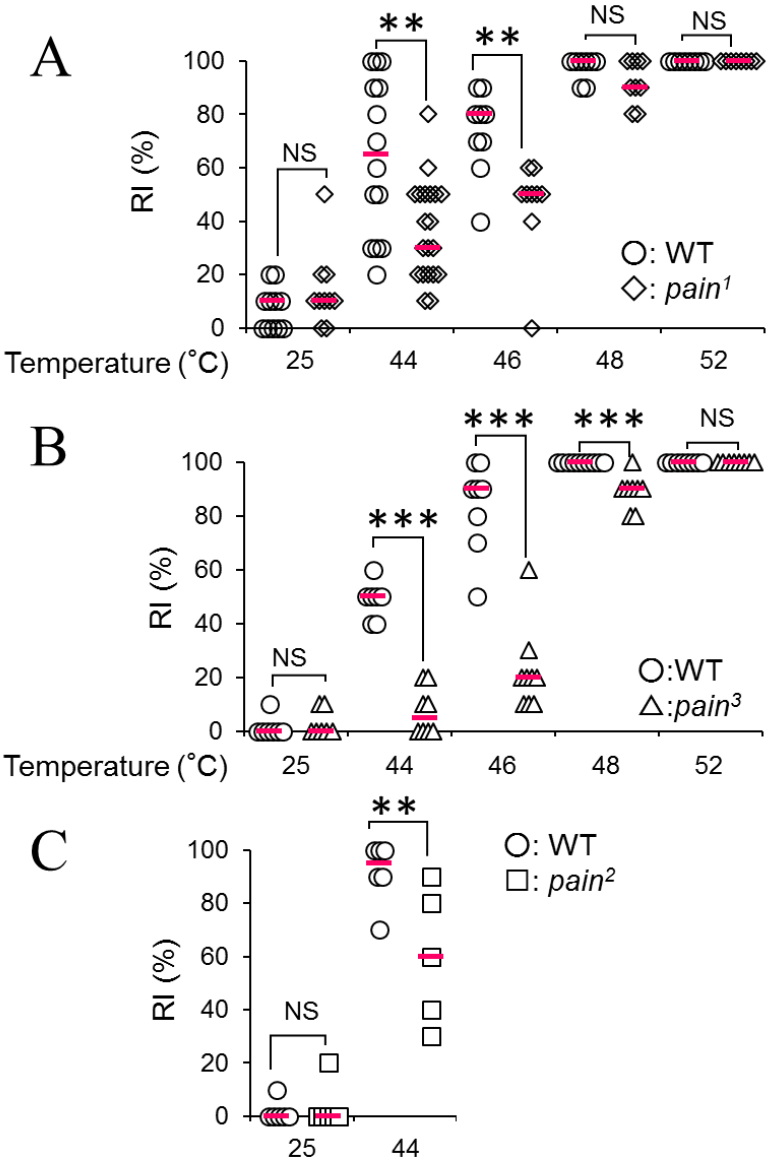

Fig. 3. Responses to noxious heat in decapitated flies at various temperatures.

In a group of 10 decapitated male flies used in the experiments, I determined the number of tumbling flies (N_t). Response index (RI) was calculated using RI (%) = N_t / 10 ×100. (A–C) Each dot represents the data of each group, and means are shown as lines. (A) Dot plot graph of RI (%) at various temperatures (25, 40, 44, 46, 48, and 52 °C). Wild-type (WT) and *pain*^{1} males were used. Each dot represents the data of individual group, and means are represented as horizontal lines. N = 9–20 in each group. (B) Dot plot graph of RI (%) at various temperatures (25, 40, 44, 46, 48, and 52 ˚C). WT and *pain*^{3} males were used. N = 7–10 in each group. (C) Dot plot graph of RI (%) at 25 and 44 °C. WT and *pain*^{2} males were used. N = 5–6 in each group. NS, not significant; **, $P < 0.01$. ***, $P < 0.001$. Student's t-test or the Mann-Whitney U test was used for pairwise comparison.

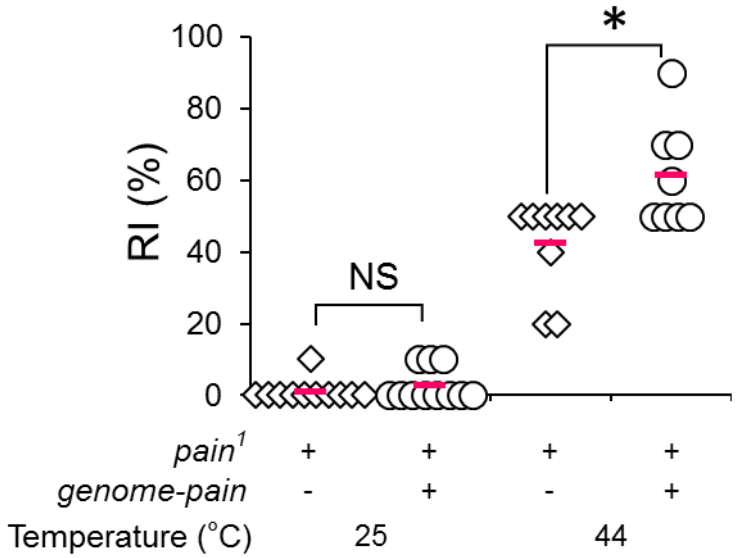

Fig. 4. Induction of a wild-type *pain* transgene in *pain* mutant background restores *pain* mutant phenotype in decapitated flies.

The Mann-Whitney *U* test was used for pairwise comparison. Dot plot graph of RIs (%) at 25 and 44 °C. Each dot represents the data of each group, and means are shown as lines. *pain*^{1} and *pain-genome*; *pain*^{1} males were used. N = 8–11 in each group. NS, not significant; *, $P < 0.05$.

Part II

Hunger-driven reduction of responses to noxious heat

Introduction

In the fruitfly *Drosophila melanogaster*, various neurotransmitters and neuropeptides contribute to the regulation of feeding behavior or food intake (Itskov and Ribeiro, 2013; Martelli *et al*., 2017). Food deprivation enhances olfactory responses to food source via increasing sNPF receptor (sNPFR) expression in olfactory sensory neurons (Root *et al.*, 2011). Food deprivation also increases gustatory responses to sugar (Meunier *et al*., 2007; Inagaki *et al*., 2012). However, it suppresses the sensitivity to bitter taste (Inagaki *et al*., 2014). Furthermore, food deprivation affects context-dependent CO_2 avoidance, sleep, and memory in *Drosophila* (Bräcker *et al*., 2013, Murakami *et al.*, 2016; Hirano *et al.*, 2013). Thus, hunger has a significant impact on *Drosophila* sensory systems and brain functions.

A neuropeptide, Leukokinin (Lk), is well conserved in invertebrates (Radford *et al*., 2002; Radford *et al*., 2004). Lk increases intracellular Ca^{2+} in stellate cells, which is one of cell types composing the malpighian tubules (MTs), induce fluid secretion of *Drosophila* MTs (Terhzaz *et al.*, 1999). *Drosophila* Lk receptor (Lkr, CG10626) encodes a G-protein coupled receptor (GPCR), and it is considered that Lkr activation induces IP_3-mediated elevation of the Ca^{2+} level via activation of phospholipase C β (PLCβ) (Radford *et al*., 2002; Pollock *et al*., 2003). Lk functions as a diuretic hormone (Terhzaz et al., 1999). In addition, it also affects various behavior. Al-Anzi *et al*. (2010) reported that Lk/Lkr signaling might control the termination of *Drosophila* feeding. In *Drosophila*, *Lk* is expressed in the brain (lateral horn and subesophageal ganglion) and VNC (abdominal neuromeres: A1-8) (Haro *et al*., 2010). Abdominal *Lk* (ABLK) neurons and serotonin neurons modulate tuning behavior in larvae (Okusawa *et al*., 2014). Lateral horn *Lk* (LHLK) neurons and *Lkr* neurons play an important role in circadian rhythms in adult flies (Cavey *et al*., 2016). A recent study reported that activation of *Lk* neurons decreases whereas *Lk* downregulation by RNAi increases postprandial sleep, suggesting that Lk release inhibits postprandial sleep in *Drosophila* (Murphy *et al*., 2016). However, it remains elusive how hunger state modifies Lk release in the fly brain.

In rats, acute food deprivation reduces formalin-induced nociceptive behavioral responses (Gheibi *et al*., 2013) 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, I 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. Intact wild-type flies showed the reduction of responses to noxious heat after food deprivation, but decapitated flies did not, suggesting that brain function contributes to the reduction of responses to noxious heat. Next I examined whether neuropeptides involved in the regulation of feeding affect the hunger-driven reduction of responses to noxious heat, and found that neurosecretion of *Lk* neurons is most effective among various neuropeptides. Thus, I generated knockout flies of *Lk* and *Lkr* using the CRISPR/Cas9 system (Kondo *et al*., 2013) and examined whether these knockout mutations affect the hunger-driven reduction of responses to noxious heat.

Materials and Methods

Fly strains

The fly stocks used for this study were as follows: wild-type *D. melanogaster* Canton-S (CS), UAS-*mCD8::GFP* (BL-5137), Lk^{KO} (see following section), Lkr^{KO} (see following section), *Lk*-GAL4 (obtained from Dr. Pilar Herrero), *sNPF*-GAL4 (DGRC113901), *AstA*-GAL4 (BL-51979), *Akh*-GAL4 (BL25684), *Ilp2*-GAL4 (obtained from Dr. Toshihiro Kitamoto), *NPF*-GAL4 (BL25681), *Hug*-GAL4 (BL58769), UAS-*Kir2.1::GFP* (BL-6595), *tub*-$GAL80^{ts}$ (BL-7108), MB-LexA, and LexAop-*mCD8::GFP* (BL-32203). 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.

Food deprivation

Newly emerged males were collected, and their wings were 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 (see also Material and method of Part I).

Behavioral analysis of locomotor activity

A male fly (5-6 d old) was introduced into a glass tube (3 mm in diameter × 65 mm in length). Glass tubes were placed under a video camera (Sony, Handycam HDR-CX590V) and recorded for more than 10 min. Using video tracking software (Move-tr/2D ver. 8.2; Library Co., Tokyo, Japan), traces were generated, and the total distance moved (mm) for 10 min was measured for each fly.

Analysis of climbing activity

The climbing activity of flies was determined as described previously (Quan *et al*., 2017). Twenty flies

(5–6 d old) were introduced into an acrylic vial (20 cm high and 2 cm in diameter) after 12h food deprivation, and bumped down to the bottom. Pictures were taken immediately after pushing them down. The distance that each fly climbed was measured by the pictures. In a group consisting of 20 flies, I repeated the measurement of the distance 5 times and the average was defined as the climbing activity of the group. Mean climbing activity was calculated from 6 groups.

Quantification of food consumption

Adult flies (5–6 d old) were transferred into food vials containing standard medium with blue dye (Brilliant Blue FCF, 027-12842, WAKO, 1.25 mg/ml). One hour later, 6 decapitated flies were homogenized in a 150 μl solution (1xPBS:100% EtOH = 1:1). After centrifugation, the absorbance of the supernatant was measured at 630 nm (A630) on a spectrometer (Eppendorf BioSpectrometer® basic). After determining the equivalent dye concentration of each fly homogenate using the linear fit of the standard curve ($R^2 > 0.99$), food consumption was calculated by multiplying equivalent concentration with homogenate volume (150 μl) and dividing by the number of flies per sample.

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

Lk^{KO} and Lkr^{KO} were generated by the CRISPR/Cas9 system (Kondo *et al*., 2013). Two complementary oligonucleotides corresponding to 20-bp target sequences were annealed and cloned into BbsI-digested pBFv-U6.2 and pBFv-U6.2B vectors. The 20-bp target sequences were designed using Cas9 Target Finder (https://shigen.nig.ac.jp/fly/nigfly/cas9/cas9TargetFinder.jsp). The 20-bp target sequences are as follows:

Lk gRNA1, 5′-GGCAAAGATAGTCCTGTGTA-3′;

Lk gRNA2, 5′-GTGGGGCGGCAAAAGGTCAC-3′;

Lkr gRNA1, 5′-GCAATGGACTTAATCGAGC-3′;

Lkr gRNA2, 5′-GTGTCGCGAGTCCACCTGCC-3′.

The pBFv-U6.2B-gRNA1-gRNA2 plasmid (200 μg/ml) was injected into *y^1v^1nos-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). The gene deletion was confirmed by PCR analysis using genomic DNA with the following primers.

For Lk^{KO}, forward, 5′-GTGTGGAAAACGTAGAGACG-3′;

reverse, 5′-TAGTTGTCGATAGCTTCAGC-3′.

For Lkr^{KO}, forward, 5′-GTTCCTTGAGGGCAAGACTG-3′;

reverse, 5′-CAGAGGCAAATGGTCATGTTCC-3′.

Real-time quantitative reverse transcription PCR (qRT-PCR)

qRT-PCR was performed as previously described (Shimada *et al*., 2016). Total RNA was collected from the heads of wild-type males (4–6 d old). The expression level of *Lk* mRNA was normalized by that of *rp49* mRNA. The primer sequences used for real-time PCR were as follows:

Lk-forward, 5′- AAAGGTGCCGCATCAAATCC-3′;

Lk-reverse, 5′-TTAGTAGTCCGGCAGGATCG-3′;

Lkr-forward, 5′-GCAATAGCTGCTACAATCCC-3′;

Lkr-reverse, 5′-TGCTCGTCTTGAACTTGCAG-3′;

rp49-forward, 5′-AAGATCGTGAAGAAGCGCAC-3′;

rp49-reverse, 5′-TGTGCACCAGGAACTTCTTG-3′.

Immunohistochemistry

An anti-Lk antibody to the full-length amidated Lk peptide was generated by Sigma-Aldrich Japan. Immunohistochemistry was performed as previously described (Shimada *et al*., 2016). Brains were immunostained for Lk with a rabbit anti-Lk antibody (1:500–4000) followed by Alexa Fluor 488-conjugated anti-rabbit IgG (Invitrogen A11008) used as the secondary antibody (1:1000). Fluorescence was observed under a confocal microscope (Nikon, C2).

Quantitative analysis of Lk signals in the adult brain

For the measurement of Lk signal intensity with or without food deprivation, Mushroom body (MB)-

LexA, LexAop-*mCD8::GFP* adult male brains (5–6 d old) were collected at ZT1.5–3. In these flies, GFP is expressed in the adult brain structure, MB. Brains were extracted and immunostained with a rabbit anti-Lk antibody as previously described (Shimada *et al*., 2016). An Lk-positive cell was Z-projected into 11–28 sequential sections (1 μm intervals). Lk level was measured by Alexa Flour 568-conjugated anti-rabbit IgG (Invitrogen A11011) or GFP fluorescence intensity determined using the NIS-Elements software (Nikon). Fluorescence intensity and area size were measured in a manually set ROI of the Lk-positive cell region. To compensate for differences in fluorescence intensity between different ROIs, Lk fluorescence intensity was normalized to the fluorescence intensity of GFP in a section of the anterior peduncle of the MB in each brain. For all samples, their image data were acquired under identical conditions. Using the computer software SPSS (IBM), one-way ANOVA followed by post-hoc analysis using Scheffe's test was carried out for multiple pairwise comparisons.

Results

Hunger state reduces responses to noxious heat in flies

To examine whether hunger affects responses to noxious stimulus in *Drosophila*, I measured heat nociception in wild-type flies after food deprivation. I mainly used 44 °C for noxious heat stimulation in the subsequent experiments, because I confirmed that wild-type flies show active responses to noxious heat at 44 °C or higher (Part I, Fig.1A). Flies with food-deprived for 6, 12, and 18 h (hereafter, referred to as 6 h, 12 h, and 18 h FD, respectively) were prepared and flies without food deprivation (0h FD) were used as a control (Fig. 5). In wild-type flies, only those with 12 h FD showed a significantly reduced RI compared with the control (Fig. 5). In contrast to responses to noxious heat, 12 h FD slightly increased general locomotor activity (Fig. 6A) and did not affect climbing activity (Fig. 6B). Thus, 12 h FD-induced reduction of responses to noxious heat does not simply result from immotility of food-deprived flies. Furthermore, 6 h, 12 h, and 18 h FD increased food intake in comparison 0 h FD (Fig. 7A), indicating that the FD induces hunger.

Decapitated flies do not show the hunger-driven reduction of responses to noxious heat

It is possible that the hunger-driven reduction of responses to noxious heat results from the attenuation of heat sensation. Thus, I determined whether food deprivation induces the reduction of responses to noxious heat in decapitated flies. In contrast to intact flies, responses to noxious heat did not change in decapitated wild-type flies regardless of the presence or absence of food deprivation (Fig. 8). Thus, unlike normal intact flies, food deprivation did not affect the responses to noxious heat in decapitated flies, indicating that food deprivation has little impact on heat sensation in sensory neurons. These findings support the idea that the hunger-driven reduction of responses to noxious heat in intact flies is not caused by the attenuation of heat sensation.

Neuropeptide-expressing neurons involved in the hunger-driven reduction of responses to noxious heat.

Because the hunger/satiety states are modulated by complex signaling networks using various neuropeptides (Itskov and Ribeiro, 2013; Martelli *et al*., 2017), 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, I focused on seven neuropeptides involved in the regulation of feeding as follows: Lk, sNPF, AstA, Akh, Ilp, NPF, and Hug (Al-Anzi *et al*., 2010;Hergarden *et al*., 2012; Hentze *et al*., 2015; Lee *et al*., 2004; Lingo *et al*., 2007; Melcher and Pankratz, 2005). The activity of neurons expressing each neuropeptide was electrically silenced by induction of an inward rectifier K^+ channel, Kir2.1 (Hodge, 2009). As was observed in wild-type flies, silencing of *sNPF*-, *Ilp2*-, and *NPF*-expressing neurons also did not affect the hunger-driven reduction of responses to noxious heat (Fig. 9A). However, silencing of *Lk*-, *AstA*-, *Akh*-, and *Hug*-expressing neurons inhibited the hunger-driven reduction of responses to noxious heat (Fig. 9A).

Temporal silencing of adult Lk neurons affects the hunger-driven reduction of responses to noxious heat.

In this study, I focused on a neuropeptide Lk. Since Lk signaling appears to mediate feeding termination (Al-anzi *et al*., 2010), it is possible that food deprivation disturbs Lk signaling and subsequently induces the reduction of responses to noxious heat. I examined whether temporal electrical silencing of adult *Lk* neurons also inhibits the hunger-driven reduction of responses to noxious heat. Kir2.1 was used in combination with the TARGET system (McGuire *et al.*, 2003). In *Lk*-GAL4 / *tub*-GAL80^{ts}; +/ UAS-*Kir2.1::GFP* flies, electrical activity should be suppressed in adult *Lk* neurons by placing them at the restrictive temperature. In these flies which were kept at the restrictive temperature, 12 h FD did not reduce RI at 44 °C , while control flies (+/ *tub*-GAL80^{ts}; +/ UAS-*Kir2.1::GFP*) showed the reduced RI at 44 °C after 12 h FD (Fig. 10B, RT). Furthermore, in both genotypes which were kept at the permissive temperature, their RI was reduced at 44 °C (Fig. 10B, PT), indicating that temporal electrical

silencing of adult *Lk* neurons inhibits the reduction of responses to noxious heat. These results indicate that *Lk* neurons play an important role in the hunger-driven reduction of responses to noxious heat.

Knockout of Lk receptor does not show the hunger-driven reduction of responses to noxious heat.

To examine whether the Lk/Lkr signaling pathway induces the hunger-driven reduction of responses to noxious heat, I generated null mutations in *Lk* and *Lkr*, respectively, using the CRISPR/Cas9 system (Kondo *et al*., 2013). To delete almost the entire protein-coding sequence (CDS), a pair of guide RNAs were targeted to two regions (flanking regions of start, and stop codons, respectively) of each gene (Figs. 11A and 11D). PCR and the subsequent sequencing of the genomic region revealed a 471-bp deletion of *Lk* (Figs. 11B and 11C), and those revealed a 5321-bp deletion of *Lkr* (Figs. 11E and 11F). Since almost all CDS of *Lk* and *Lkr* are lacking in these mutants, I named these alleles Lk^{KO} and Lkr^{KO}.

Next, I examined whether Lk^{KO} and Lkr^{KO} flies also do not show the hunger-driven reduction of responses to noxious heat. In heterozygous Lk^{KO} flies, as was observed in the wild-type, 12 h FD induced the reduction of RI at 44 °C (Figs. 12A and 12B). In homozygous Lk^{KO} flies, 12 h FD slightly but significantly reduces RI at 44 °C. In contrast, heterozygous and homozygous Lkr^{KO} flies did not show the hunger-driven reduction of responses to noxious heat (Figs. 12C and 12D). These results indicate that Lkr plays an important role in the hunger-driven reduction of responses to noxious heat. Although the reason why a knockout mutation of *Lk* does not completely inhibit the hunger-driven reduction of responses to noxious heat is largely unknown, it seems likely that efficiency of Lk is partial and other ligands of Lkr are also involved in the hunger-driven reduction of responses to noxious heat.

Pan-neural knockdown of *Lk* and *Lkr* induces arrhythmic locomotor activity (Cavey *et al*., 2016). Thus, the behavioral arrhythmicity may contribute to the hunger-driven reduction of responses to noxious heat. Under constant light conditions (LL), the *Drosophila* circadian clock does not normally work, resulting in arrhythmic locomotor activity (Qiu *et al*., 1996). In my study, wild-type flies were kept under LL for at least 4 days, and then they were food-deprived for 12 h. However, they also

showed the hunger-driven reduction of responses to noxious heat (Fig. 13). Thus, it is most unlikely that the impairment of the hunger-driven reduction of responses in Lkr^{KO} flies results from the behavioral arrhythmicity or the dysfunction of their circadian clock.

Food deprivation decreases Lk expression levels in Lk neurons.

Previous studies showed that there are several *Lk* neurons in the adult brain (Herrero *et al*., 2003; Al-anzi *et al*., 2010; Haro *et al*., 2010; Fig. 14A). Two of them are located in the lateral horn (LH) and are denoted as LHLK neurons, and the other neurons are located in the subesophageal (SE) zone and are denoted as SELK neurons (Fig. 14A). In this study, I generated the anti-Lk antibody to the full-length amidated Lk peptide. First, I confirmed that Lk signals by an anti-Lk antibody are detected in LHLK and SELK neurons using *Lk*-GAL4 / UAS-*mCD8::GFP* flies (Figs. 14B and 14C). Secondly, I also confirmed that Lk immunoreactivity in the adult brain was evident in the wild-type flies, but not in Lk^{KO} flies, when this antibody was used (Fig. 15). Finally, I examined whether food deprivation affects Lk expression levels in *Lk* neurons with this anti-Lk antibody. Signals of internal standard (GFP signal in MB) did not change by food deprivation (Figs. 16A and 16B). Compared with a control (0 h FD), 12 h FD decreased Lk signal intensity, whereas 6 h or 18 h FD did not (Figs. 16A, 16C, and 16D). Furthermore, the qRT-PCR analysis revealed that no significant differences are detected in both *Lk* and *Lkr* mRNA expression level between the food-deprived flies and a control flies (Fig. 17). Thus, it is most likely that food deprivation for 12 h promotes Lk secretion in *Lk* neurons.

Discussion

In this study, I identified for the first time that food deprivation induces the reduction of responses to noxious heat in *Drosophila* (Fig. 5). Since 12 h FD did not inhibit locomotion and climbing ability (Fig. 6), the reduction of responses to noxious heat was not simply caused by decreased locomotor activity. The hunger-driven reduction of responses to noxious heat was not observed in decapitated wild-type flies (Fig. 8), suggesting that hunger state does not affect reception of heat nociception. In addition, my study showed the novel function of Lk. Temporal silencing of *Lk* neurons and a knockout mutation of *Lkr* inhibited the hunger-driven reduction of responses to noxious heat (Fig. 10), indicating that the Lk/Lkr signaling predominantly contributes to the hunger-driven reduction of responses to noxious heat.

Neural and molecular mechanism of hunger-driven reduction of responses to noxious heat

Hunger reduces the responses to noxious heat in wild-type flies (Figs. 5 and 7A). Temporal silencing of *Lk* neurons impaired the reduction of responses to noxious heat after 12 h FD (Fig. 10). These results indicated that silencing of *Lk* neurons eliminates the effect of food deprivation. Similarly, Lkr^{KO} flies also did not show the reduction of responses to noxious heat after 12 h FD (Figs. 12C and 12D), indicating that transmitter release from *Lk* neurons and Lkr activation are required for the reduction of responses to noxious heat. In contrast to *Lkr*, a knockout mutation of *Lk* did not completely inhibit the hunger-driven reduction of responses to noxious heat (Figs. 12A and 12B), suggesting that there are other ligands of Lkr in *Drosophila*. The mechanisms of the hunger-driven reduction of responses to noxious heat may be explained as follows (Fig. 18). 12 h FD induces the release of Lk or other ligands of Lkr, and subsequently activates Lkr in the fly brain. Furthermore, the activation of Lkr signaling inhibits the neuronal activity in the brain, which is associated with the regulation of jumping in response to noxious heat. Although the neural pathway of responses to noxious heat in *Drosophila* adults is poorly understood, the Lk/Lkr signaling may affect the activity of giant fiber (GF) neurons during hunger because it is considered that GF, which projects from the brain to VNC, regulates fly jumping (Lima and Miesenböck, 2005).

Why is the reduction of responses to noxious heat unaffected by 18h FD?

Although previous studies reported that Lk/Lkr signaling regulates meal size and postprandial sleep (Al-anzi *et al*., 2010; Murphy *et al*., 2016), they did not reveal how hunger influences *Lk* expression and release. Although the mRNA expression of *Lk* and *Lkr* was not affected by food deprivation (Fig. 17), Lk level in LHLK neurons reduces after 12 h FD (Fig. 16). Therefore, I concluded that hunger state induces Lk secretion in the fly brain. It was previously reported that activation of *Lk* neurons decreases Lk signals in SELK neurons (Liu *et al*., 2015). This report also supports my idea that hunger induces Lk secretion.

In contrast to 12 h FD, 18 h FD did not affect the responses to noxious heat and Lk signal intensity in brain neurons (Fig. 18). Thus, the hunger-driven reduction of responses to noxious heat is a 12 h FD-specific phenomenon, although it remains unclarified how Lk level recovers after 18 h FD. Other neuropeptides may be released after 18 h FD, and the activation of these signaling may inhibit Lk secretion.

12h FD modifies physiological properties in Drosophila

In this study, I found that 12 h FD decreased responses to noxious heat. However, locomotor activity was enhanced by 12 h FD (Figs. 5 and 6A). Although it remains unclear how 12h FD enhances locomotor activity, my results suggest that 12 h FD drastically modify physiological properties in adult flies. In *Drosophila*, previous studies reported that 12 h FD also modifies olfactory and gustatory sensitivities, sleep, or long-term memory formation (Fig. 19) (Meunier *et al*., 2007; Inagaki *et al*., 2012; Root *et al*., 2011; Beshel and Zhong, 2013; Yang *et al*., 2015; Murakami *et al.*, 2016; Hirano *et al.*, 2013). These reports also support my idea that 12 h FD drastically modify physiological properties such as sensory processing and higher brain function.

Figures

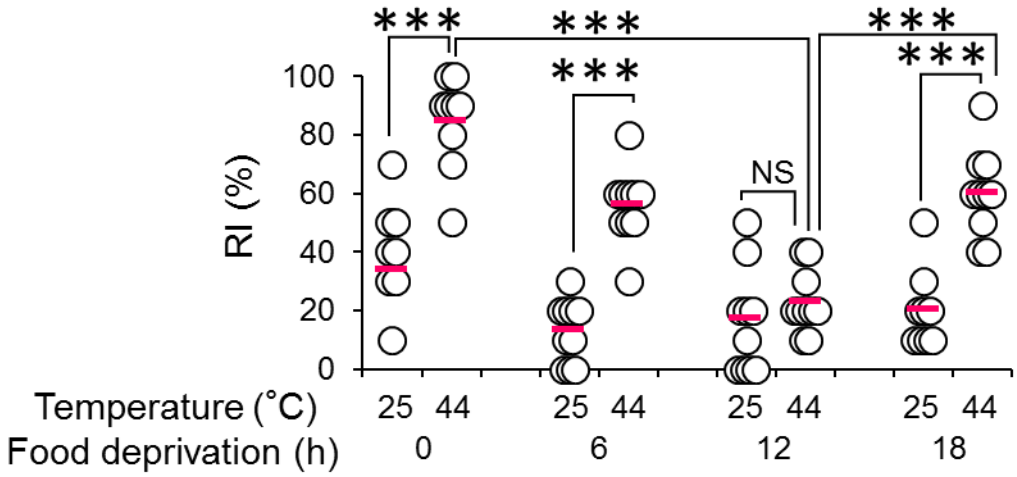

Fig. 5. Responses to noxious heat after 6 h, 12 h, and 18h FD in intact flies.

In a group of 10 male flies, the number of jumping flies (N_j) was checked. Response index (RI) was calculated using RI (%) = N_j / 10 ×100. Dot plot of RIs (%) at 25 and 44 °C. Each dot represents the data of each group, and means are shown as lines. Food-deprived wild-type flies were used. $N = 9–10$ in each group. The Mann-Whitney *U* test was used for statistical analysis. NS, not significant. ***, $P < 0.001$.

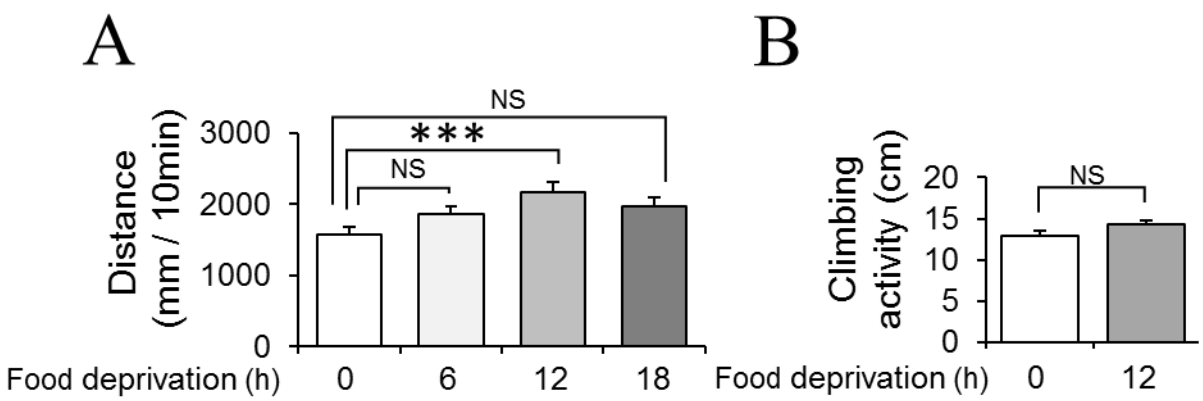

Fig. 6. Motility after 6 h, 12 h, and 18h FD in wild-type.

(A) Total walking distance (mm) of food-deprived wild-type flies. Flies without food deprivation were used as the control. Student's *t*-test was used. Error bars show SEM. *N* = 29–30 in each bar. NS, not significant; ***, *P* < 0.001. (B) Climbing activity (cm) in food-deprived flies. Flies without food deprivation were used as a control. *N* = 6 in each bar. Error bars show SEM. Student's *t*-test was used. NS, not significant.

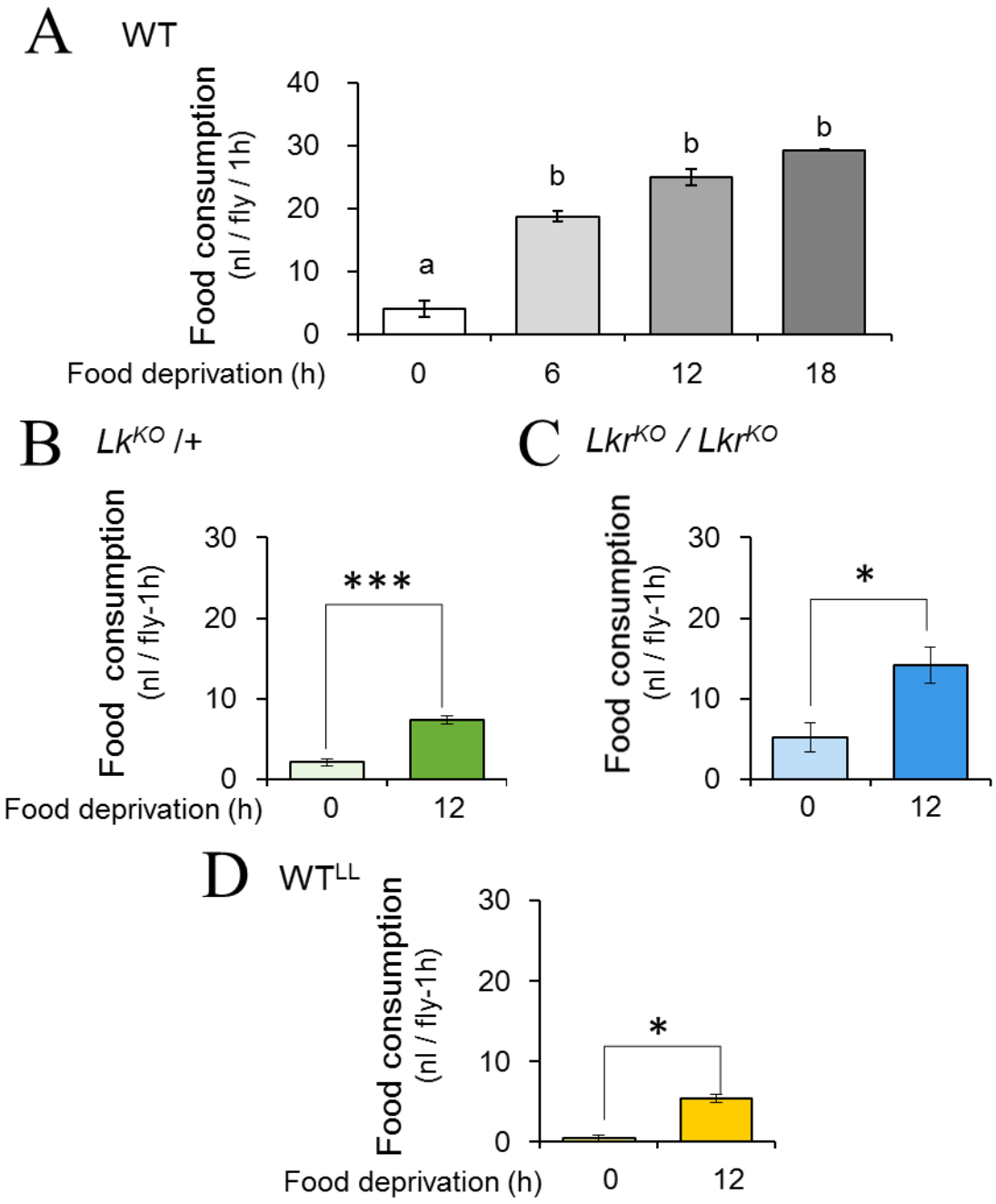

Fig. 7. Food intake after 6 h, 12 h, and 18h FD.

(A) Amount of food intake of food-deprived wild-type (WT). Flies without food deprivation were used as a control. Student's *t*-test was used. Nonparametric ANOVA (Kruskal-Wallis test) followed by post-hoc analysis using the Steel-Dwass test was carried out for multiple pairwise comparisons. Value with the same letters indicates that they are not significantly different ($P > 0.05$). Error bars show SEM. $N = 4–8$ in each bar. (B and C) Student's *t*-test was used. Amount of food intake of Lk^{KO} and Lkr^{KO} after food

Figure 7 continued on next page

deprivation. Lk^{KO} / + (B) and Lkr^{KO} / Lkr^{KO} (C) flies were used. Flies without food deprivation were used as a control. Error bars show SEM. $N = 4$ in each bar. *, $P < 0.05$. ***, $P < 0.001$. (D) Food intake after 12 h food deprivation in WT flies with the dysfunctional circadian clock. (WT^{LL}). The Mann-Whitney *U* test was used for statistical analysis. Amount of food intake of WT flies with dysfunctional circadian clock after 12 h food deprivation. WT flies were kept under constant light conditions for at least 4 days. In addition, they were food-deprived for 12 h and subsequently subjected to behavioral analysis. Flies without food deprivation were used as a control. Error bars show SEM. $N = 4$ in each bar. *, $P < 0.05$.

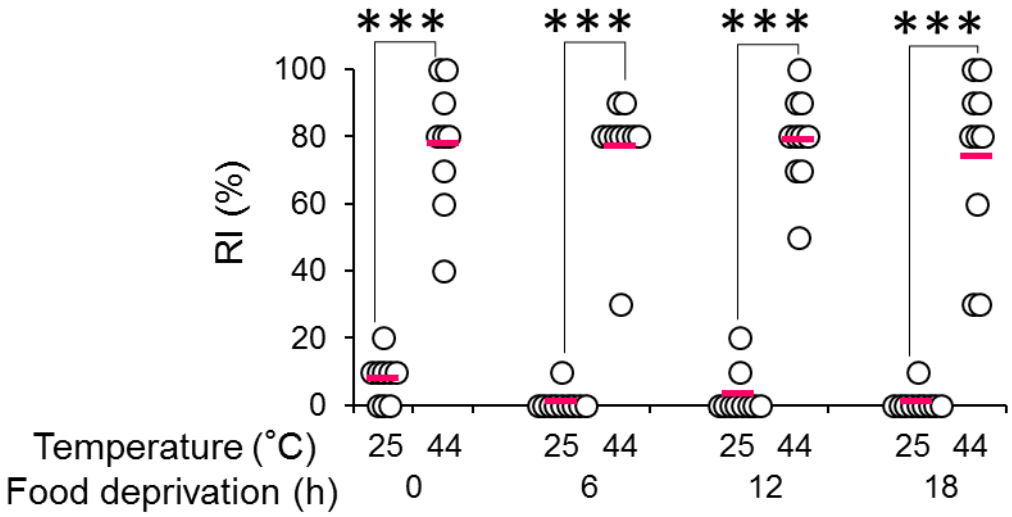

Fig. 8. Responses to noxious heat after 6 h, 12 h, and 18 h food deprivation in decapitated flies.

Dot plot of RIs (%) at 25 and 44 °C. Each dot represents the data of each group, and means are shown as lines. Food-deprived and decapitated flies were used. $N = 9–10$ in each group. The Mann-Whitney *U* test was used for statistical analysis. NS, not significant. ***, $P < 0.001$.

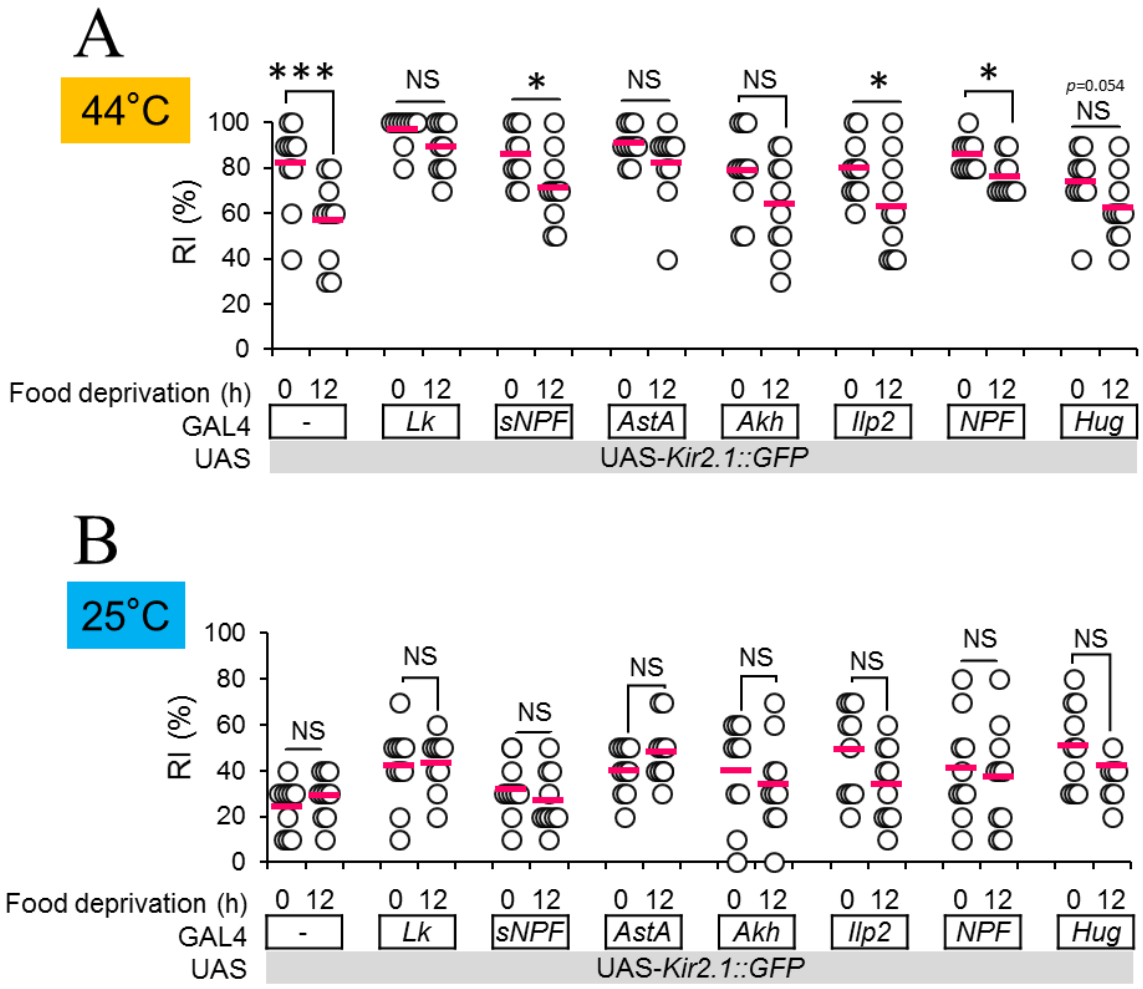

Fig. 9. Screening of neuropeptide expressing neuron involved in hunger-driven reduction of responses to noxious heat.

Flies with chronic inhibition in neuropeptide expressing neurons and control flies (+ / UAS-*Kir2.1::GFP*) were used. (A) Dot plot graph of RIs (%) at 44 °C. $N = 10$ in each group. (B) Dot plot graph of RIs (%) at 25 °C. Student's t-test or the Mann-Whitney *U* test was used for pairwise comparison. $N = 10$ in each group. NS, not significant; *, $P < 0.05$. ***, $P < 0.001$.

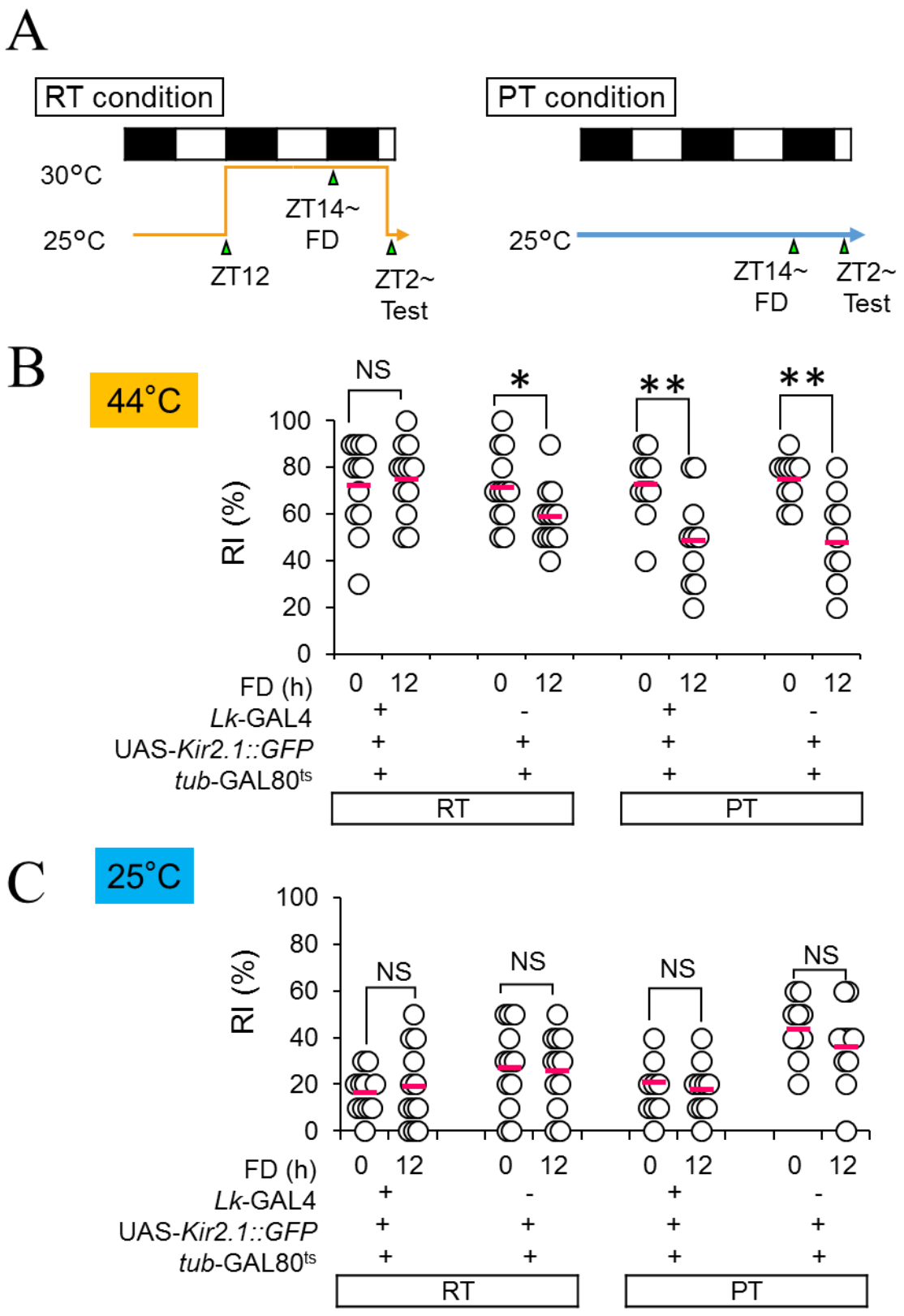

Figure 10 continued on next page

Fig. 10. Conditional silencing of *Lk* neurons suppressed hunger-driven reduction of responses to noxious heat.

(A) Schematic image of the experimental procedure. RT, restrictive temperature (30 °C); PT, permissive temperature (25 °C). The Flies were kept at 25.0 ± 0.5 °C during the embryonic, larval and pupal stages. RT condition: flies were collected and kept at the PT until 14 h before experiments, then they were kept at the RT until just before the start of the experiments. PT condition: flies were collected and kept at the PT until just before the start of experiments; (B and C) Temporal electrical silencing of *Lk*–positive neurons. Dot plot graph of RIs (%) at 25 °C (B) and 44 °C (C). *Lk*-GAL4 / *tub*-FAL80^{ts}; +/ UAS-*Kir2.1::GFP* flies were used. +/ *tub*-GAL80^{ts}; +/ UAS-*Kir2.1::GFP* flies were used as a control. $N = 10–12$ in each group. Student's *t*-test or the Mann-Whitney *U* test was used. NS, not significant; *, $P < 0.05$. **, $P < 0.01$.

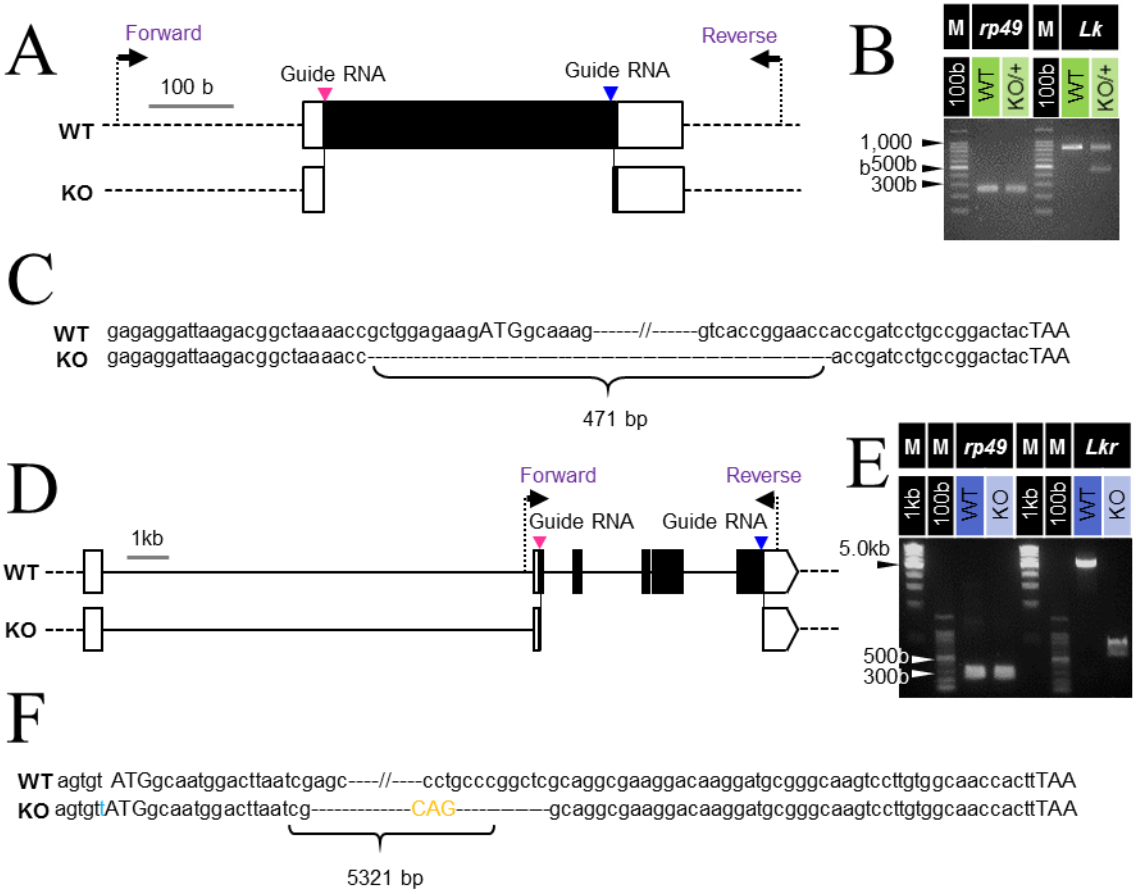

Fig. 11. Generation of *Lk*^{KO} and *Lkr*^{KO} flies.

The CRISPR/Cas9 system was used to generate knockout flies. (A) Schematic representation of the *Lk* locus. White and black boxes are the protein-coding and untranslated regions, respectively. (B) Genomic DNA PCR of *Lk*. A 975-bp (wild-type, WT) and a 504-bp (*Lk*^{KO}) fragments were detected. M, DNA ladder maker. The primer sequences used in the experiments are mentioned in Materials and Methods. (C) Nucleotide sequences of *Lk*-coding region in WT and *Lk*^{KO} flies. (D) Schematic representation of the *Lkr* locus. (E) Genomic DNA PCR of *Lkr*. A 5994-bp (WT) and a 677-bp (*Lkr*^{KO}) fragments were detected. M, DNA ladder marker. (F) Nucleotide sequences of *Lkr*-coding region in WT and *Lkr*^{KO} flies.

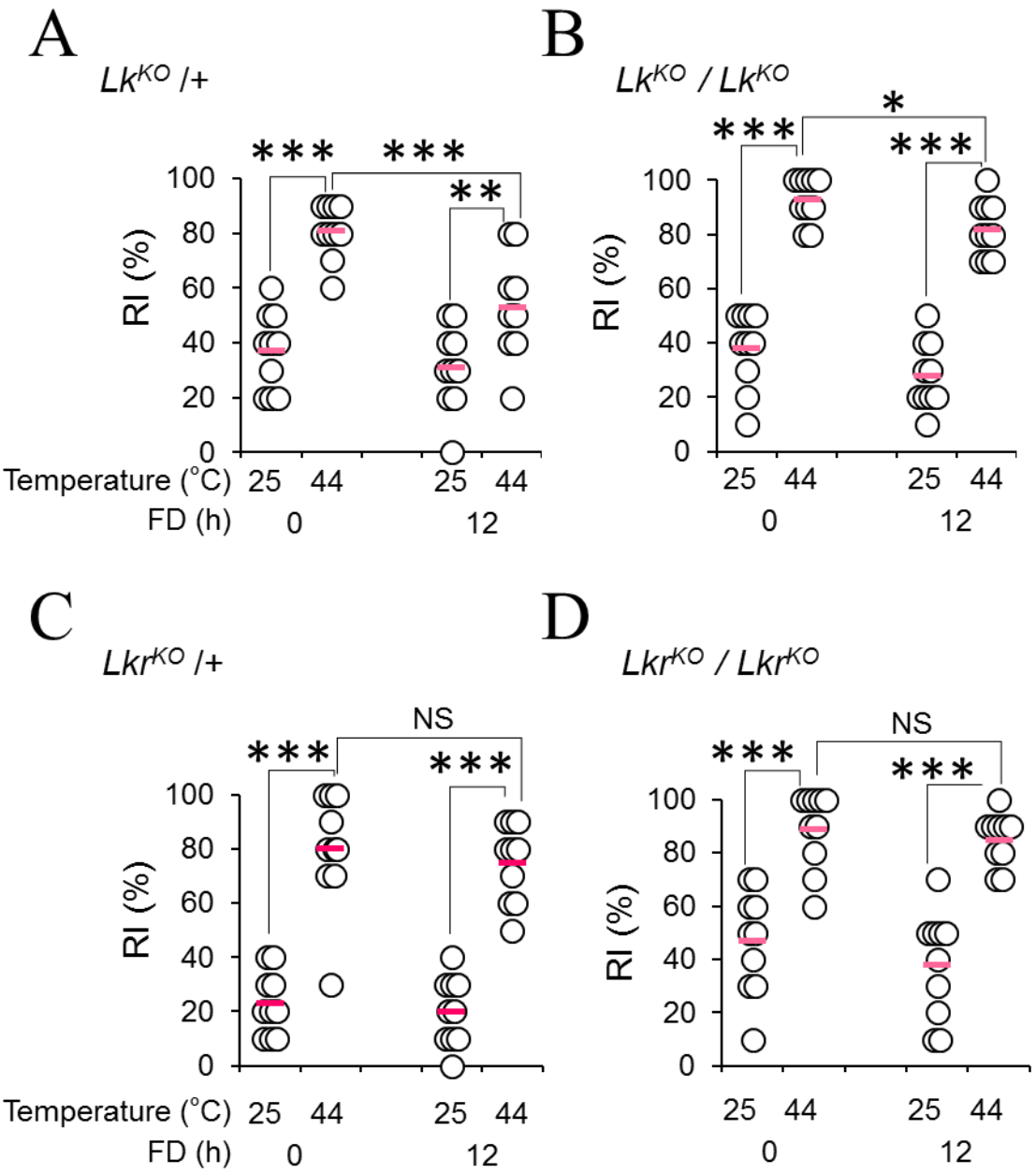

Fig. 12. Responses to noxious heat after 12 h FD in *Lk*^{KO} and *Lkr*^{KO} flies.

Dot plot of RIs (%) at 25 and 44 °C in *Lk*^{KO} /+ (A), *Lk*^{KO} / *Lk*^{KO} (B), *Lkr*^{KO} /+ (C), and *Lkr*^{KO} / *Lk*^{KO} (D) flies. Food-deprived flies (12 h FD) were used. Flies without food deprivation were used as a control. $N = 10$ in each group. Student's *t*-test or the Mann-Whitney *U* test was used for statistical analysis. NS, not significant; **, $P < 0.01$. ***, $P < 0.001$.

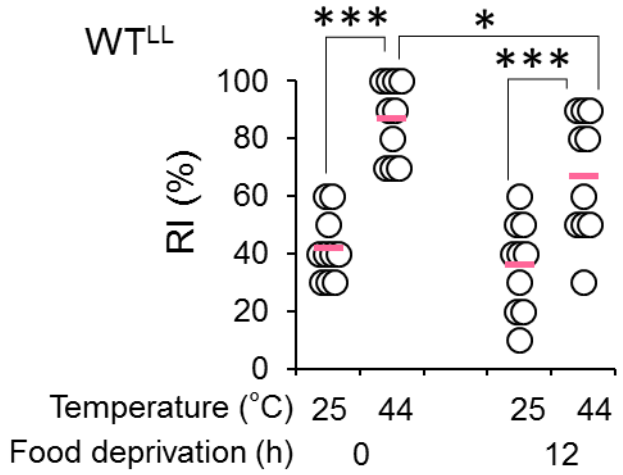

Fig. 13. Responses to noxious heat after 12 h food deprivation in wild-type flies with the dysfunctional circadian clock.

Dot plot of RIs (%) at 25 and 44 °C. Wild-type flies with dysfunctional circadian clock (WT^{LL}) were used in the experiments. WT flies were kept under constant light conditions for at least 4 days. They were food-deprived for 12 h and subsequently subjected to behavioral analysis. $N = 10$. Student's *t*-test or the Mann-Whitney *U* test was used for statistical analysis. NS, not significant; *, $P < 0.05$. ***, $P < 0.001$.

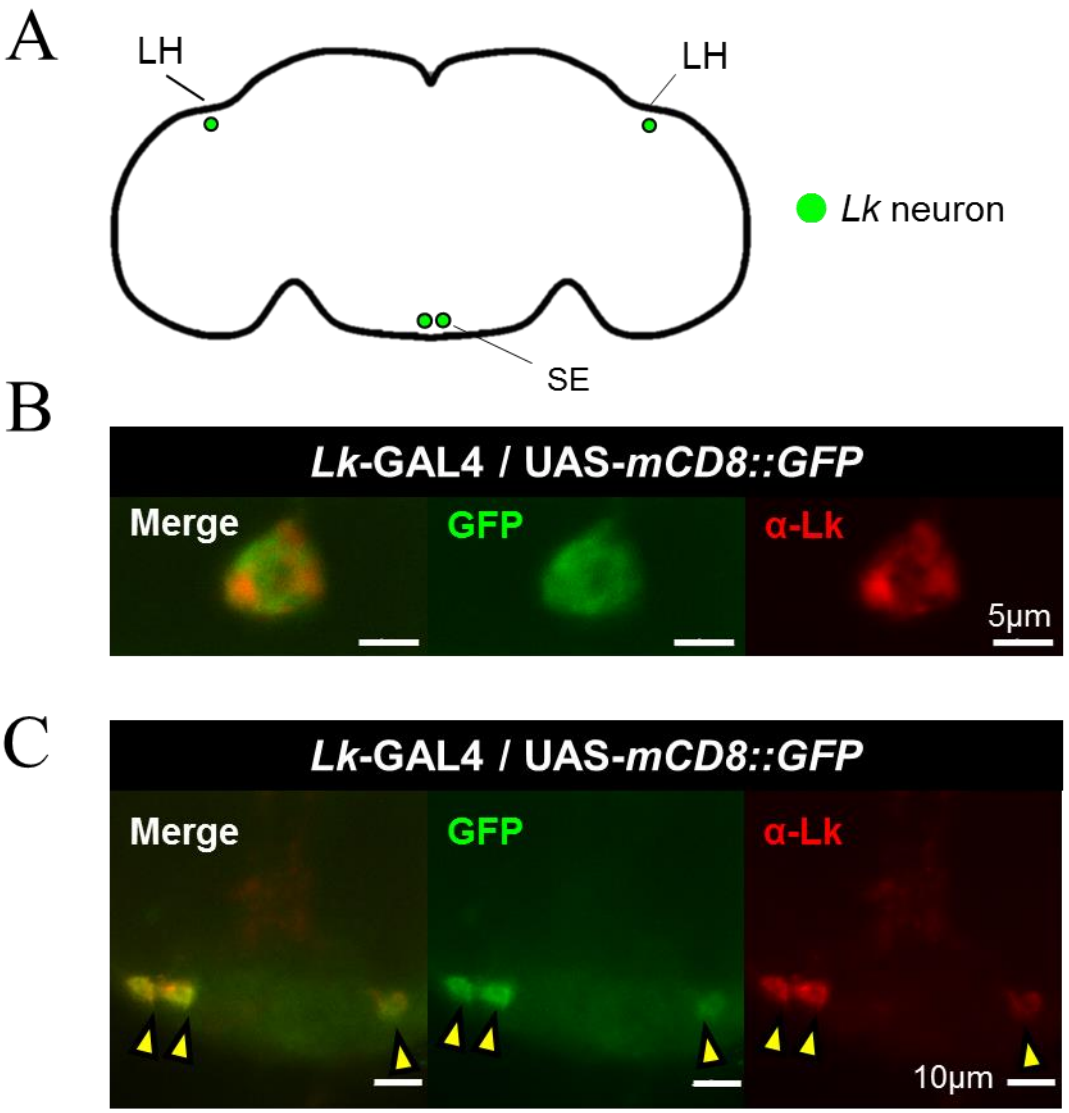

Fig. 14. Lk immunostaining signals are colocalized with GFP driven by *Lk*-GAL4.

(A) *Lk* neurons in *Drosophila* adult brain. Anterior view of the brain. LH, lateral horn; SE, subesophageal ganglion. Green circles, *Lk* neurons. (B and C) Confocal section image at the level of Lk neurons of the adult brain. *Lk* neurons in the adult brain. *Lk*-GAL4 / UAS-*mCD8::GFP* flies were used. GFP is shown in green, Lk immunolabeling is shown in red, and the overlap is shown in yellow. The brain *Lk* neurons (SELK and LHLK) are revealed by *Lk*-GAL4-driven GFP. (B) A single section images indicating an LHLK neuron. Scale bars represent 5 μm. (C) Stacked confocal images showing SELKs. Triangles, SELKs. Scale bars represent 10 μm.

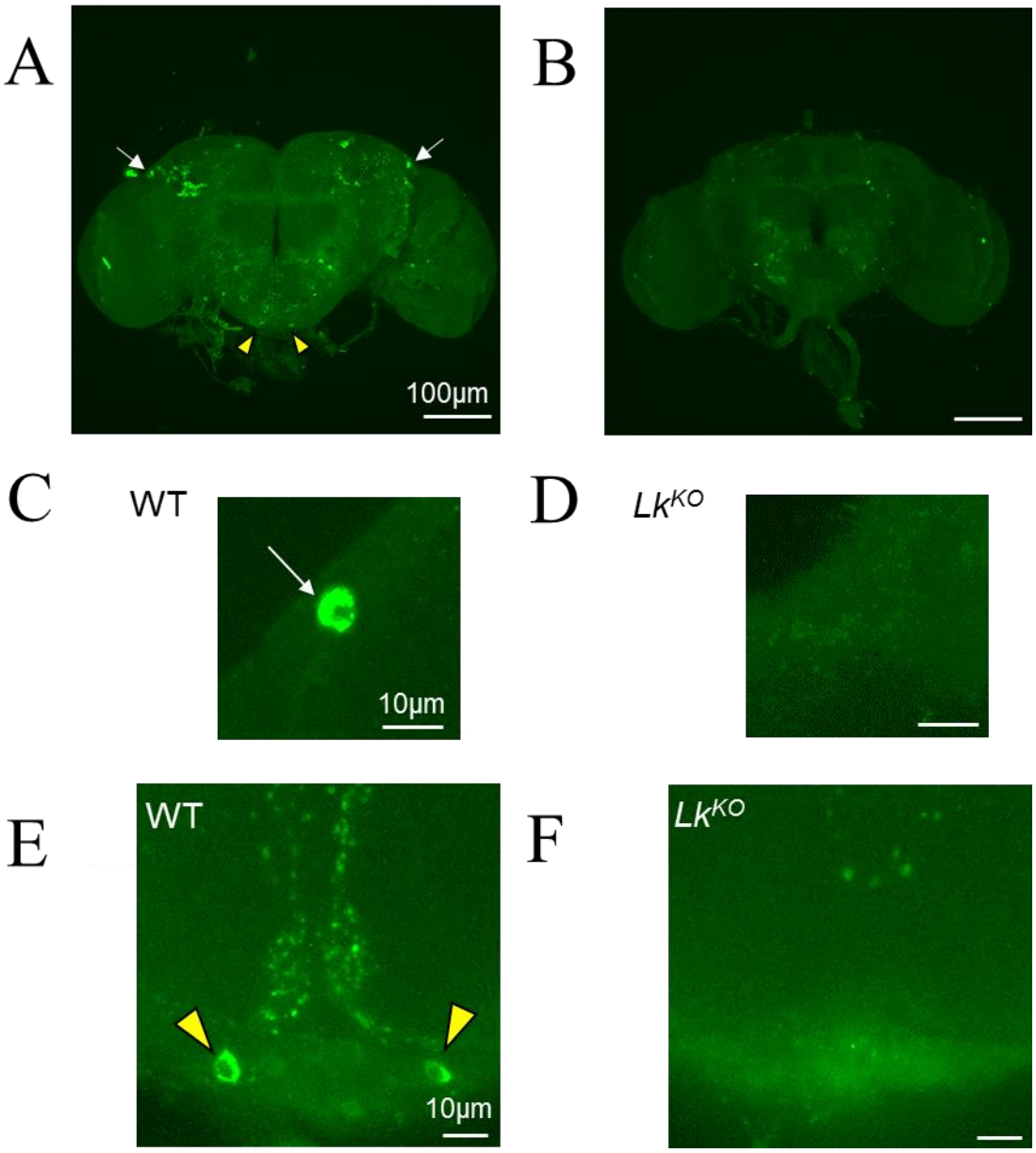

Fig. 15. Lk immunostaining in WT and *Lk*^{KO} adult brains.

Lk immunolabeling is shown in green. Wild-type (WT) flies (A, C, and E) and *Lk*^{KO} / *Lk*^{KO} (B, D, and F). (A and B) Stacked confocal images showing a front view of the adult brain. One LHLK neuron (C and D) and two SELK neurons (E and F) of the adult brain. Arrows, LHLK neurons; Triangles, SELK neurons. (A and B) Scale bars represent 100 μm. (C–F) Scale bars represent 10 μm.

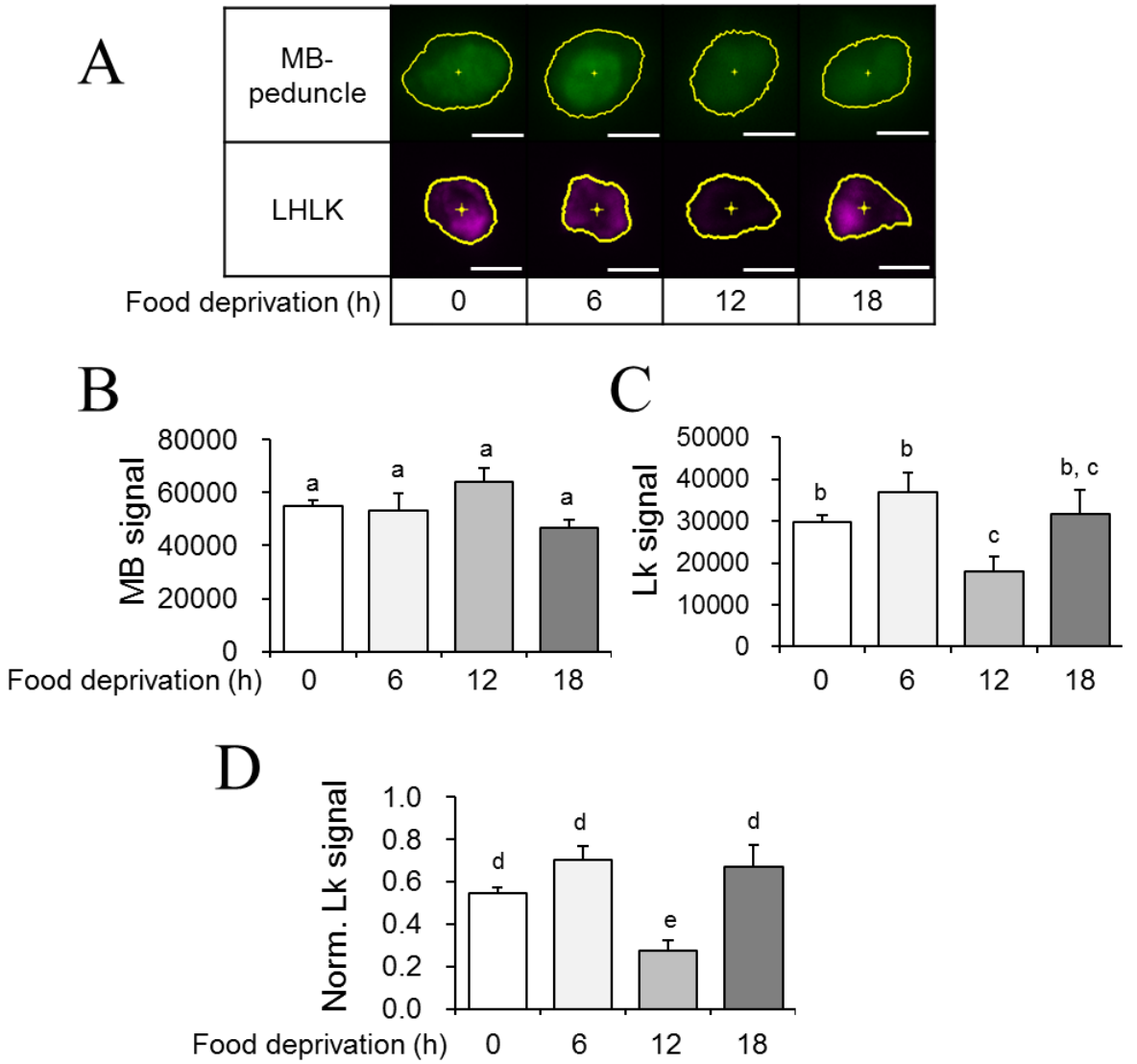

Fig. 16. Food deprivation for 12 h inhibits Lk signals in the brain.

MB-LexA, LexAop-*mCD8::GFP* flies were used. Brains were dissected at ZT 1.5–3.0 after 6, 12, and 18 h food deprivation. Flies without food deprivation were used as a control. (A) The MB neurons (MB-peduncle) are revealed by MB-LexAop-driven GFP. Scale bars represent 10 μm. Lk immunolabeling in an LHLK neuron is shown in magenta. Scale bars represent 5 μm. (B–D) Four to five brains were used in food-deprived flies. 14 brains were used in control fed flies. (B) GFP level in MB peduncle. (C) Lk levels in an LHLK neuron. (B and C) 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 ($P > 0.05$). (D) Relative Lk levels in an LHLK neuron. One-Way ANOVA followed by post-hoc analysis using Scheffe's test was carried out for multiple pairwise comparisons. Values with the same letters indicate that they are not significantly different ($P > 0.05$).

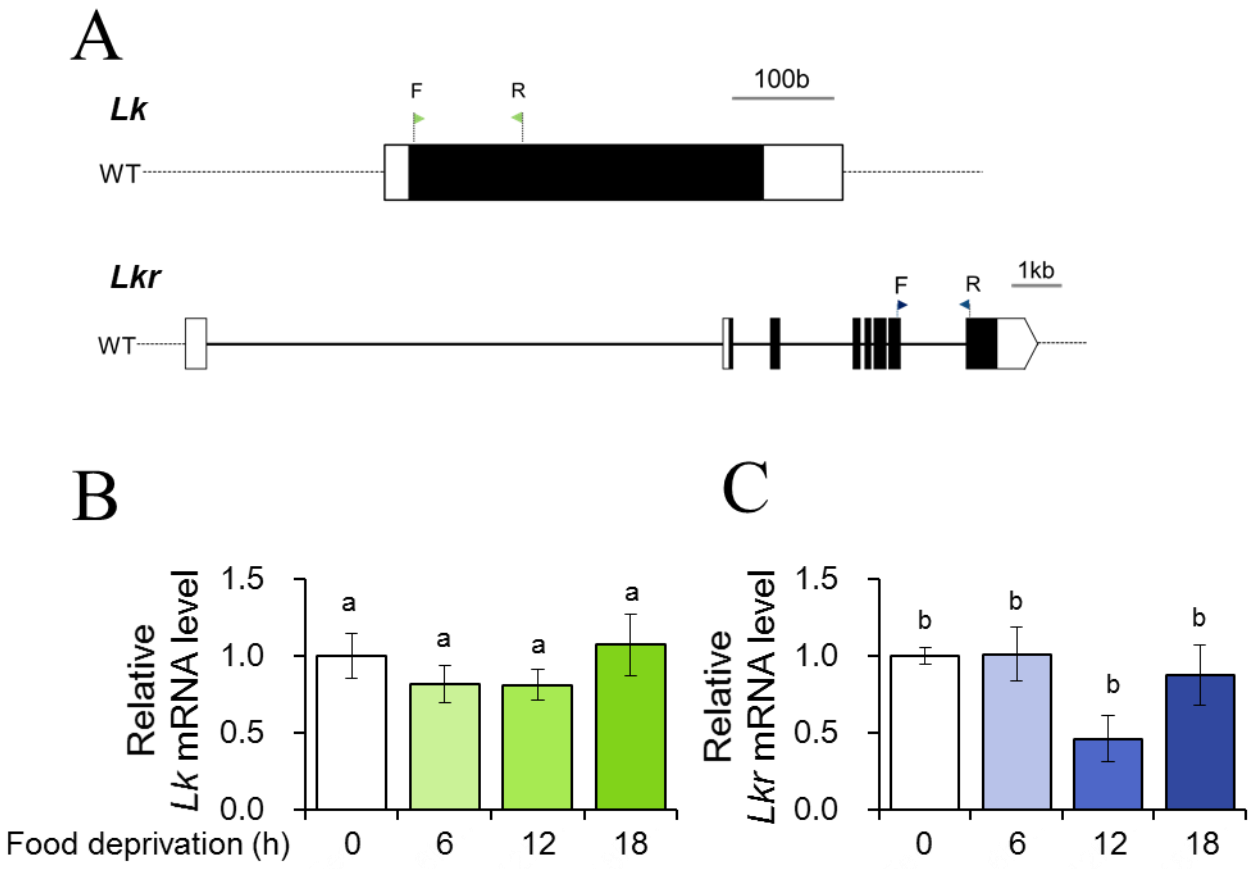

Fig. 17. Food deprivation does not affect *Lk* and *Lkr* mRNA expression in adult brains.

(A) Schematic representation of the *Lk* gene, *Lkr* gene, and primers for Real-time qRT-PCR. White and black boxes are the untranslated and protein-coding regions, respectively. Primer locations were indicated as flags. The primer sequences used in the experiments are mentioned in Materials and Methods. (B and C) One-Way ANOVA followed by post-hoc analysis using Scheffe's test was carried out for multiple pairwise comparisons. Values with the same letters indicate that they are not significantly different ($P > 0.05$). (B) Real-time qRT-PCR analysis of *Lk* mRNA expression level. Mean ± SEM values were calculated from five to six replicates. (C) Real-time qRT-PCR analysis of *Lkr* mRNA expression level. Mean ± SEM values were calculated from four to five replicates.

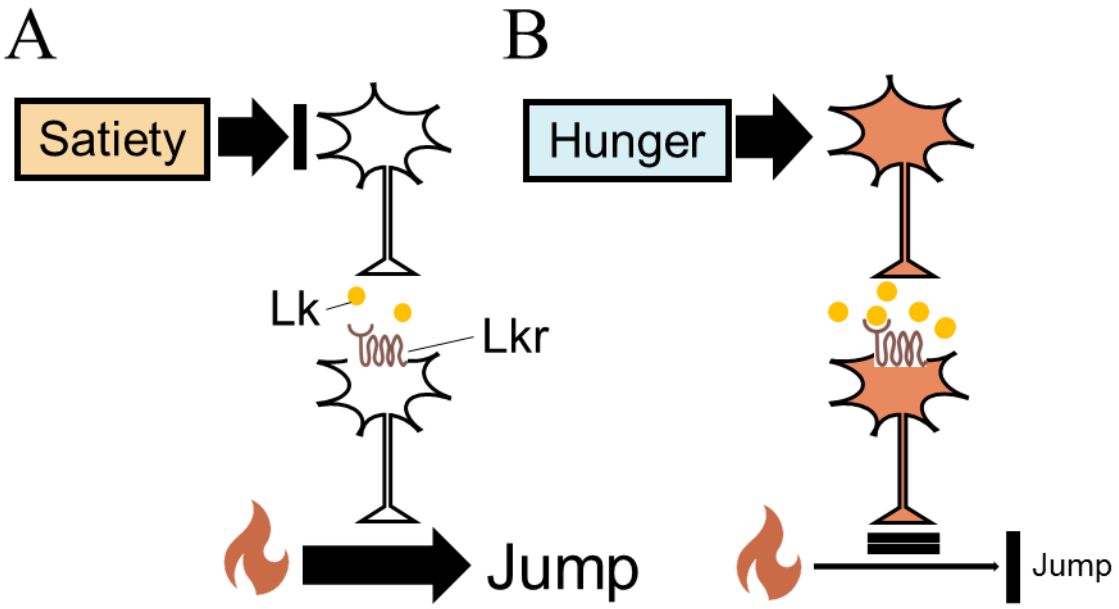

Fig. 18. A model for the neural mechanics that hunger signal modulates response to noxious heat via Lk/Lkr signaling.

(A) Satiety signal inhibits the neural activity of *Lk* neurons. Lk/Lkr signaling does not affect response to noxious heat. Thus, flies show a high frequency of jumping behavior as a response to noxious heat. (B) Hunger signal activates the neural activity of *Lk* neurons and promotes Lk secretion from LHLK neurons. The increase of Lk secretion induces the neural activity of *Lkr* neurons. This Lk/Lkr signaling attenuates behavioral responses to noxious heat.

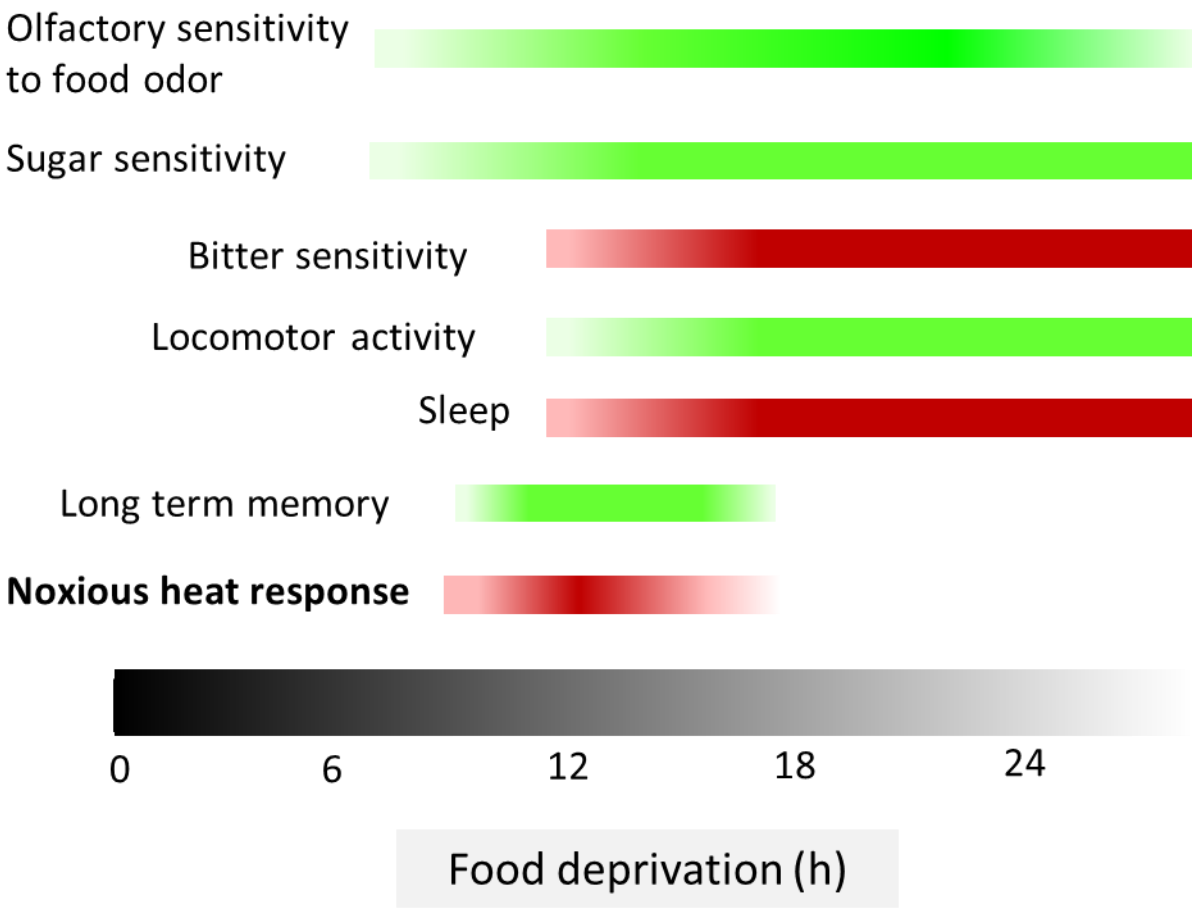

Fig. 19. Hunger state modifies sensory processing and various behaviors in *Drosophila*.

Food deprivation enhances olfactory sensitivity to food odor, sugar sensitivity, locomotor activity, and long-term memory (red bars), while it inhibits bitter sensitivity, sleep, and responses to noxious heat (green bars). A horizontal axis shows a duration of food deprivation (h).

General discussion

In this thesis, I established the novel behavioral assay of nociception using decapitated flies (Part I). Decapitated flies showed the behavioral responses to noxious heat. However they showed tumbling instead of jumping as was observed in intact wild-type flies (Part I, Fig. 2). I also demonstrated that frequency of tumbling in decapitated flies is inhibited by *pain* mutations (Part I, Figs. 2, 3, and 4), suggesting that flies sense noxious heat directly through their legs with *pain*-expressing sensory neurons. Furthermore, I found that intact wild-type flies show the reduction of responses to noxious heat after 12 h FD (Part II, Fig. 5). However, decapitated flies did not, indicating that food deprivation has little impact on heat sensation in sensory neurons (Part II, Fig. 8). I also demonstrated that Lk/Lkr signaling is involved in the hunger-driven reduction of responses to noxious heat (Part II, Figs. 9, 10, 12, and 16).

I found the hunger-driven reduction of responses to noxious heat in *Drosophila* (Part II, Fig. 5). What is the advantage of this physiological property in nature? Previous studies showed that food deprivation enhances food odor-sensing and increases response to sugar (Root *et al*., 2011; Beshel *et al*., 2013; Meunier *et al*., 2007; Inagaki *et al*., 2012), suggesting that food searching is driven by hunger in flies. The hunger-driven reduction of responses to noxious heat may help to expand food searching area because flies can move in hot places which are exposed to direct sunlight. Lkr is known as mammalian NPY receptor homolog (Radford *et al*., 2002), and NPY peptides and NPY/AgRP neurons promote eating in mammals (Sobrino Crespo *et al*., 2014; Jourjine *et al*., 2017). Interestingly, Alhadeff *et al*. (2018) demonstrated that hunger reduces behavioral response to inflammatory pain. They also revealed that NPY/AgRP neurons activate the hindbrain parabrachial nucleus (PBN) through NPY signaling and PBN neurons decrease responses to inflammatory pain during hunger (Alhadeff *et al*., 2018). Although it remains unknown whether hunger also reduces the responses to noxious heat in mammals, it will be interesting whether mammalian species has similar physiological property as was observed in *Drosophila*. My study may provide new insight into research on animal survival strategy in nature.

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