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Mapping of Courtship Behavior-Induced Neural Activity in the Thoracic Ganglia of Silkmoth *Bombyx mori* by an Immediate Early Gene, *Hr38*

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In the central nervous system of insects, motor patterns are generated in the thoracic ganglia under the control of brain, where sensory information is integrated and behavioral decisions are made. Previously, we established neural activity-mapping methods using an immediate early gene, *BmHr38*, as a neural activity marker in the brain of male silkmoth *Bombyx mori*. In the present study, to gain insights into neural mechanisms of motor-pattern generation in the thoracic ganglia, we investigated expression of *BmHr38* in response to sex pheromone-induced courtship behavior. Levels of *BmHr38* expression were strongly correlated between the brain and thoracic ganglia, suggesting that neural activity in the thoracic ganglia is tightly controlled by the brain. In situ hybridization of *BmHr38* revealed that 20–30% of thoracic neurons are activated by courtship behavior. Using serial sections, we constructed a comprehensive map of courtship behavior-induced activity in the thoracic ganglia. These results provide important clues into how complex courtship behavior is generated in the neural circuits of thoracic ganglia.

Key words: silkmoth, pheromone, immediate early gene, courtship, thoracic ganglion

INTRODUCTION

Information received in the sensory organs is processed and integrated in the insect brain. Behavioral decisions are made in response to such information, and the behavioral program is transmitted to the thoracic ganglia, the equivalent of the vertebrate spinal cord, through command neurons in the brain. Thoracic ganglia of insects consist of three segmental ganglia (prothoracic, mesothoracic, and metathoracic ganglia), and each segmental ganglion regulates the legs in each segment. Mesothoracic and metathoracic ganglia control fore- and hind-wings, respectively (Kondoh and Obara, 1982). Thoracic ganglia are interconnected with each other, and the local neural circuits control motor programs, such as locomotor and wing-flapping patterns, that coordinately generate insects' behavior (von Philipsborn et al., 2011). Furthermore, even after decapitation, insects can exhibit stereotypic behavior in response to mechanical stimulation, indicating that thoracic neural circuits can function as independent regulators of behavior.

Male silkmoth *Bombyx mori* is an important model insect for use in neuroethological studies (Blomquist and Vogt, 2003; Sakurai et al., 2014). Since adult silkmoths are specialized for reproduction, they do not exhibit any significant behavior other than courtship behavior. Also, courtship behavior of male silkmoth is completely dependent on information on a single sex pheromone, bombykol (Sakurai et al., 2011; Sakurai et al., 2015; Hara et al., 2017). Bombykol is a major sex pheromone component emitted from female moths and elicits all sequence of courtship behavior of the male silkmoth, including orientation, zig-zag turns, circling, and abdominal bending (Kaissling, 1978; Obara, 1979). This simple relationship between sensory input and behavior makes male silkmoth an ideal model for investigating the neural circuits that regulate stereotypic courtship behaviors.

Although many studies have been conducted to reveal neural mechanisms of courtship behavior of male silkmoths, most have focused on the brain, since it plays essential roles in sex pheromone recognition, information processing, and behavioral decision (Fujita et al., 2013; Namiki et al., 2014; Sakurai et al., 2014). Thoracic neural circuits that regulate sex pheromone-induced courtship behavior remain elusive, despite the strong interest in behavior generation. Thus, in the present study, we investigated neural activity pattern of thoracic ganglia in response to courtship behavior, focusing specifically on the sequential behaviors from pheromone stimulation to mating behavior.

Previously, we established novel methods to comprehensively map neural activity in the brain of male silkmoths, using an immediate early gene (IEG), *BmHr38* (Fujita et al., 2013). IEGs are a group of genes whose expression is regulated by neural activity and can be used as neural activity markers. In vertebrates, a variety of IEGs, including *c-fos* and *Arc*, are known and used for mapping active neurons during behavior, since their expression is well correlated with neural activity (Flavell and Greenberg, 2008). In insects, to date, only three genes (*kakusei*, *Hr38*, and *Egr*) have been identified as IEGs which can reliably be used as neural activity markers (Kiya et al., 2007; Fujita et al., 2013; Ugajin

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et al., 2013). *BmHr38* belongs to the NR4A1 family of genes, is an evolutionally conserved IEG (Fahrbach et al., 2012), and is useful as a neural activity marker in insect brains. In the present study, we show that *BmHr38* can also be used as a neural activity marker in the thoracic ganglia and, for the first time, reveal comprehensive neural activity pattern of sex pheromone-induced courtship behavior.

MATERIALS AND METHODS

Insects and pheromone stimulation

Eggs of a racial hybrid of *B. mori*, Kinshu × Showa, were purchased from a local dealer (Ueda Sanshu, Nagano, Japan). Larvae were reared on an artificial diet (Silkmate 2M, Nihon Nosan Kogyo, Yokohama, Japan) at 25°C under a 12-h light/12-h dark photoperiodic cycle. Adult moths 1–4 days after eclosion were used for experiments.

Male silkmoths were stimulated with 100 ng bombykol [(E,Z)-10,12-hexadecadien-1-ol] (synthesized by local dealer; Sumika Techno Service, Kobe, Japan) for 30 min, as previously reported, and rested in clean air for 30 min, to maximize the level of *BmHr38* expression (Fujita et al., 2013). One hundred ng bombykol dissolved in water (100 ng/μl) was put on a filter paper that was laid in the bottom of a closed plastic cup (ϕ = 70 mm) housing a male silkmoth. After 30 min, the plastic cup was opened and the filter paper was replaced to a new filter paper. After another 30 min, silkmoth was sacrificed for experiment. Samples without pheromone stimulation were used as control. Moths were dipped in 100% ethanol, anesthetized in ice-cold water, and kept on ice until dissection. Brains, thoracic ganglia, and antennae were dissected in phosphate-buffered saline (PBS; Takara Bio, Japan) and immediately frozen in plastic tube put on dry-ice. Samples were kept −80°C until use.

Quantitative RT-polymerase chain reaction (QRT-PCR)

QRT-PCR was conducted as described previously (Fujita et al., 2013). Tissues from three male silkmoths were used for each sample. Expression levels of *BmHr38* were divided by that of *rpl3*, and relative values to control are indicated. Data are presented as mean ± standard error.

In situ hybridization

In situ hybridization was performed as described previously (Fujita et al., 2013). Thoracic ganglia dissected in PBS were embedded and frozen in OTC compound (Sakura Finetek Japan, Tokyo, Japan). Serial frozen sections of 10 μm thickness were made using a cryostat. Number of *BmHr38*-positive cells was counted as described previously. Total number of cells were counted utilizing background staining. Data are presented as mean ± standard error.

RESULTS

Levels of BmHr38 expression in the thoracic ganglia correlate with those in the brain

To investigate whether *BmHr38* can be used as a neural activity marker in the thoracic ganglia in male silkmoths, we determined levels of *BmHr38* expression in the thoracic ganglia with or without bombykol stimulation by QRT-PCR analyses. In addition, to compare the relationship of *BmHr38*

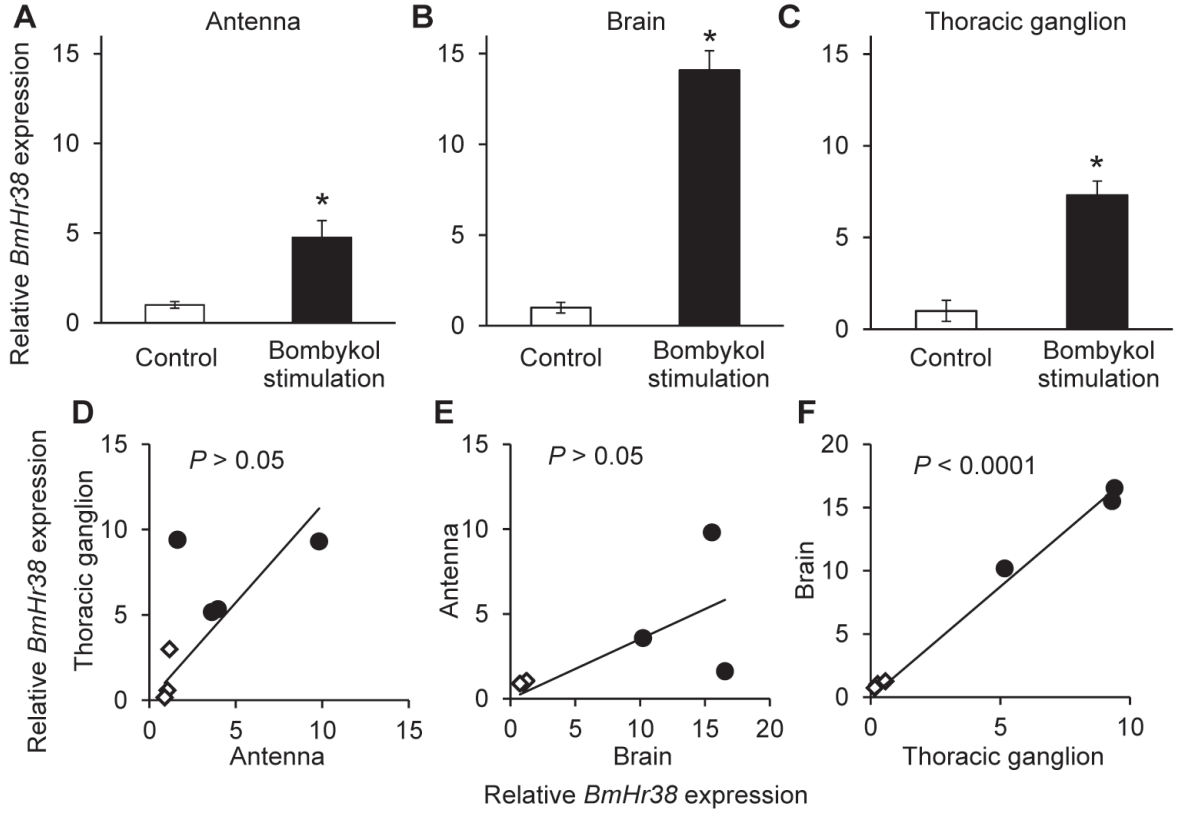

Fig. 1. QRT-PCR analysis of *BmHr38* expression in response to sex pheromone stimulation in the antennae, brains, and thoracic ganglia. **(A–C)** Relative expression levels of *BmHr38* in the antennae **(A)**, brains **(B)**, and thoracic ganglia **(C)** of control (no stimulation) or bombykol-stimulated male silkmoths. $n = 3$ (brains) or 4 (antennae and thoracic ganglia), each. *: $P < 0.05$, *U*-test. **(D–F)** Regression analysis of *BmHr38* expression levels between tissues. Open squares and filled circles indicate control and bombykol-stimulated samples, respectively.

expression levels among tissues, we collected the antennae and brains from the same silkmoths. As reported previously (Fujita et al., 2013), levels of *BmHr38* expression significantly increased in response to bombykol stimulation in the antennae and brains (Fig. 1A and B). A significant increase in *BmHr38* expression was also observed in the thoracic ganglia (Fig. 1C). These results indicate that *BmHr38* is expressed in a stimulus-induced manner in the thoracic ganglia, as well as in the antennae and brain. Levels of *BmHr38* expression in the brains or thoracic ganglia did not correlate with that in the antennae, due to highly variable levels of up-regulation in *BmHr38* expression in the antennae (Fig. 1D and E). In contrast, expression levels in the brains and thoracic ganglia showed significant correlation (Fig. 1F). Since amount of neural activity is reflected in the levels of *BmHr38* expression (Fujita et al., 2013), the levels of neural activity of these tissues are tightly correlated.

Localization of BmHr38 expression in the thoracic ganglia by in situ hybridization

To visualize the expression pattern of *BmHr38*, we performed in situ hybridization using serial sections of thoracic ganglia prepared from bombykol-stimulated or control male silkmoths. As expected from the results of QRT-PCR analyses, *BmHr38*-expressing cells were detected in a bombykol stimulation-dependent manner (Fig. 2A–F). We counted the number of *BmHr38*-expressing cells and found that 20–30% of thoracic ganglion cells are *BmHr38*-positive in response to stimulation (Fig. 2G–I). The percentage of *BmHr38*-expressing cells was slightly higher in mesothoracic and metathoracic ganglia than in prothoracic ganglion, probably due to additional neural activity by wing-flapping in these ganglia.

To gain insights into the neural mechanisms that generate courtship behavior, we comprehensively visualized *BmHr38* expression pattern in the thoracic ganglia (Fig. 3). *BmHr38*-expressing cells were detected across the entire

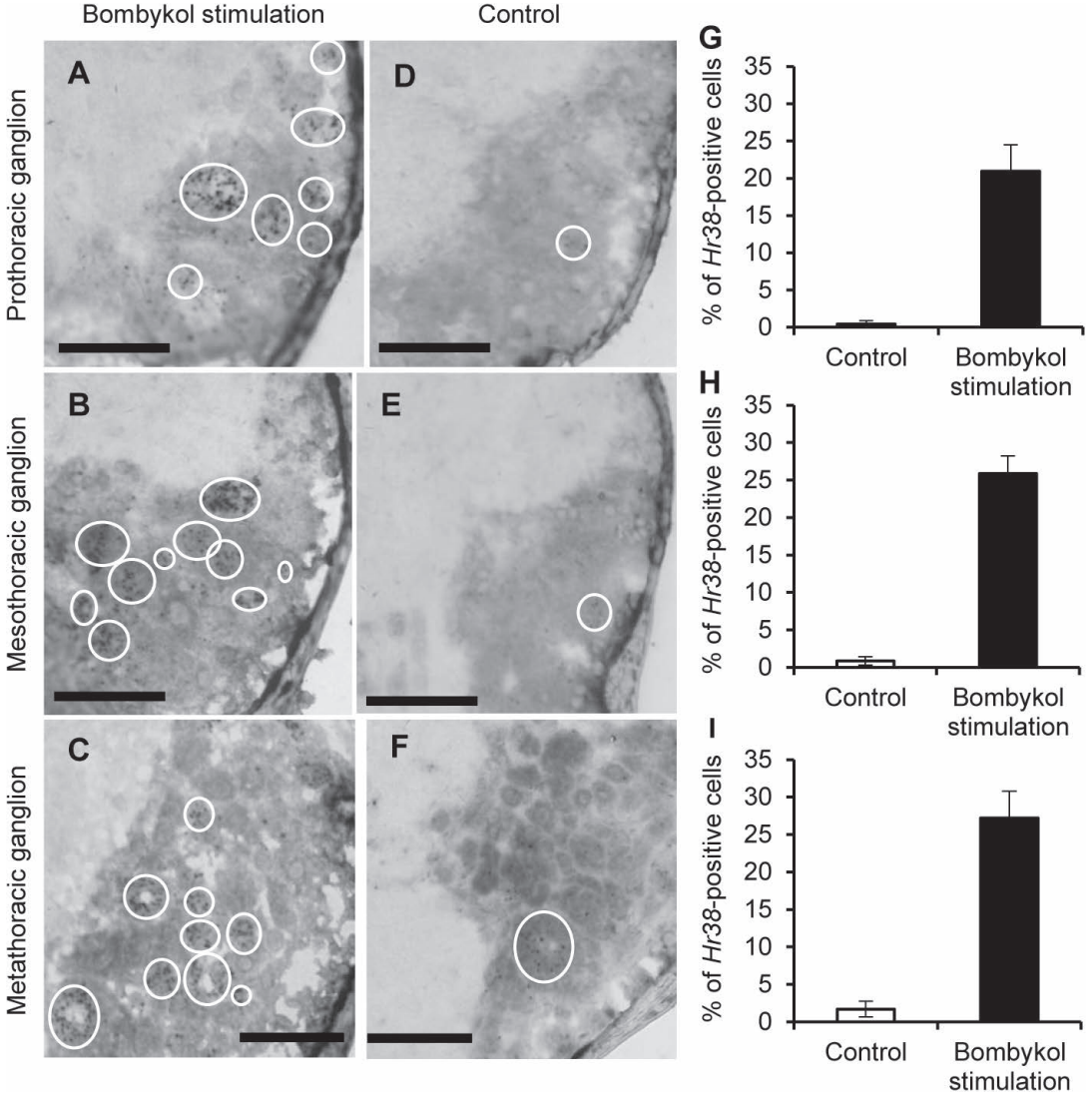

Fig. 2. Expression pattern of *BmHr38* in the thoracic ganglia revealed by in situ hybridization. **(A–F)** Expression pattern of *BmHr38* in the prothoracic **(A, D)**, mesothoracic **(B, E)**, and metathoracic **(C, F)** ganglia with **(A–C)** or without **(D–F)** bombykol-stimulation. White circles indicate *BmHr38*-expressiong cells. Scale bars, 50 μm. **(G–I)** Proportion of *BmHr38*-positive cells in each ganglion. $n = 4–7$, each.

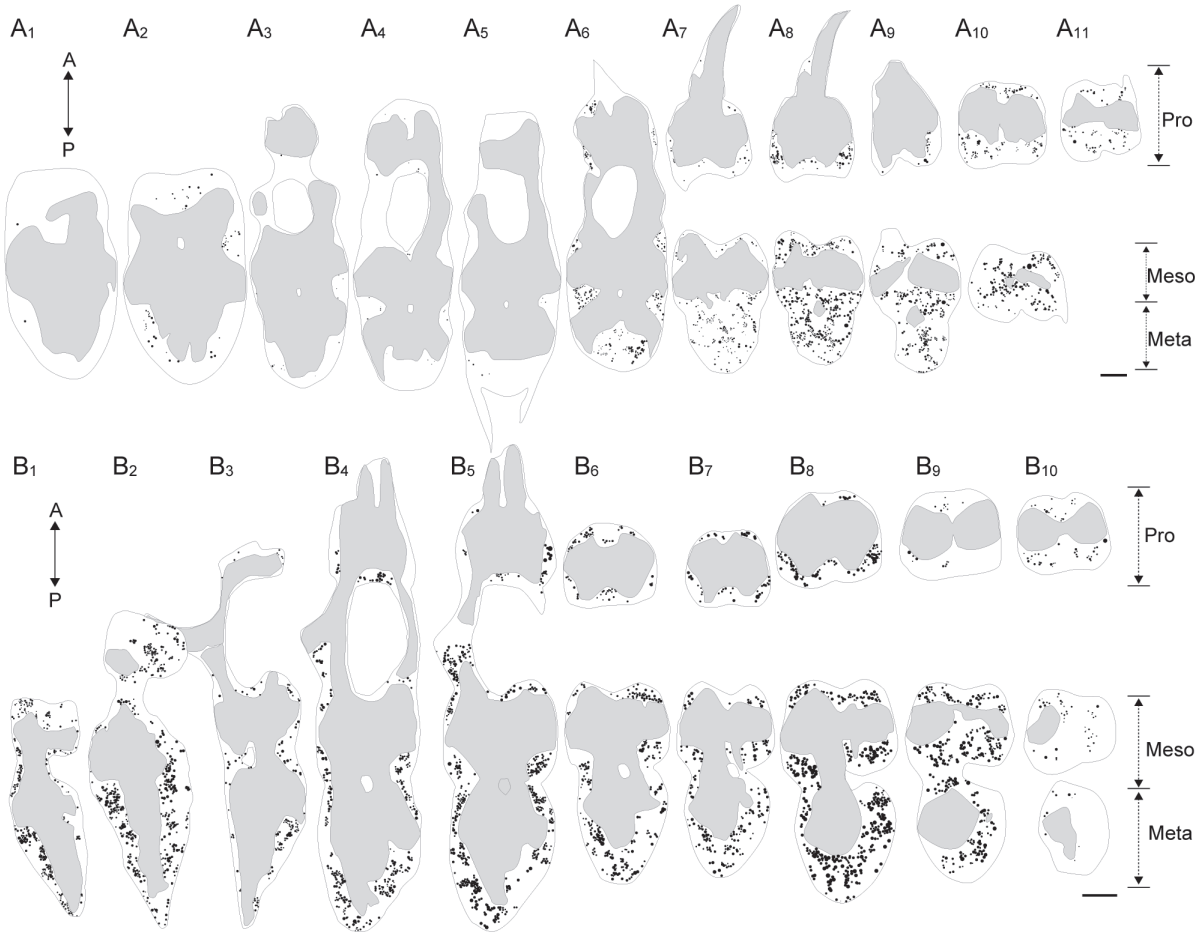

Fig. 3. Schematic drawings of *BmHr38* expression pattern in the thoracic ganglia of male moths stimulated with bombykol. Schematic summaries of expression pattern of *BmHr38* in two representative thoracic ganglia (A and B) of bombykol-stimulated silkmoths. The sections ($A_{1–11}$ and $B_{1–10}$) are numbered and placed from dorsal to ventral. Signals and neuropilar area are shown in black and gray, respectively.

area of thoracic ganglia, rather than specific areas, suggesting that a large population of thoracic ganglion neurons is active during courtship behavior in male silkmoths.

DISCUSSION

In the present study, we showed that *BmHr38* can be used as a neural activity marker in the thoracic ganglia of male silkmoths, and mapped neural activity during courtship behavior in the entire thoracic ganglia. To our knowledge, this is the first comprehensive visualization of neural activity in the thoracic ganglia of a free-moving insect. We revealed that neurons across the entire range of thoracic ganglia, not specific neural circuits, are active during courtship behavior. Since a variety of neurons are expected to be active during courtship behavior, these neurons may include not only motor neurons that directly control courtship behavior, but also sensory neurons and interneurons that transmit sensory information perceived during behavior.

IEGs take time to be expressed in the cells (> 30 min) but behavior occurs immediately upon sensory inputs (Guzowski et al., 2001; Fujita et al., 2013). This difference in time course sometimes makes it difficult to interpret the results of IEG expression analysis. Advantageously, male silkmoths do not show any other behavior than courtship behavior, and thus we can conclude that *BmHr38* expression observed in sex pheromone-stimulated male is related to courtship behavior. Since the thoracic ganglia reside in the ventral side of thorax, it is impossible to record neural activity from free-moving and intact insects using conventional techniques, such as electrophysiology and Ca^{2+} imaging. From IEG expression analysis, the present study provides important insights into the neural mechanisms of how thoracic ganglia regulate stereotypic behavior.

In the vinegar fly *Drosophila melanogaster*, thermogenetic and optogenetic studies have revealed that neural circuits in the thoracic ganglia expressing sex-determining genes *fruitless* and *doublesex* regulate wing extension, a motor pattern specific to courtship behavior (von Philipsborn et al., 2011; Shirangi et al., 2016). In the present study, we could not isolate active neurons to specific neural population with specific functional characters, as many cells were labeled by *BmHr38* in response to courtship behavior. Our methods can detect active cells during behavior, but are not capable of elucidating the specific functional importance of neurons. Thus, further analyses to reveal functions of labeled neurons on courtship behavior is needed in the future studies. In addition, neural activity mapping using IEG expression is compatible to double labeling with other

marker genes by double in situ hybridization or immunostaining. Co-localization analyses to sex-determining genes and/or neurotransmitters in the future studies will be promising to characterize functions of these neurons. We expect that these analyses will collectively clarify neural mechanisms that generate complex behavior.

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COMPETING FINANCIAL INTERESTS

The authors declare no competing financial interests.

AUTHOR CONTRIBUTIONS

TK conceived the project; KM conducted every experiment; MI contributed reagents/analytic tools; KM and TK analyzed the data. TK wrote the paper.

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