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Severe developmental timing defects in the prothoracicotropic hormone (PTTH)-deficient silkworm, *Bombyx mori*

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ABSTRACT

The insect neuropeptide prothoracicotropic hormone (PTTH) triggers the biosynthesis and release of the molting hormone ecdysone in the prothoracic gland (PG), thereby controlling the timing of molting and metamorphosis. Despite the well-documented physiological role of PTTH and its signaling pathway in the PG, it is not clear whether PTTH is an essential hormone for ecdysone biosynthesis and development. To address this question, we established and characterized a *PTTH* knockout line in the silkworm, *Bombyx mori*. We found that *PTTH* knockouts showed a severe developmental delay in both the larval and pupal stages. Larval phenotypes of *PTTH* knockouts can be classified into three major classes: (i) developmental arrest during the second larval instar, (ii) precocious metamorphosis after the fourth larval instar (one instar earlier in comparison to the control strain), and (iii) metamorphosis to normal-sized pupae after completing the five larval instar stages. In *PTTH* knockout larvae, peak levels of ecdysone titers in the hemolymph were dramatically reduced and the timing of peaks was delayed, suggesting that protracted larval development is a result of the reduced and delayed synthesis of ecdysone in the PG. Despite these defects, low basal levels of ecdysone were maintained in *PTTH* knockout larvae, suggesting that the primary role of PTTH is to upregulate ecdysone biosynthesis in the PG during molting stages, and low basal levels of ecdysone can be maintained in the absence of PTTH. We also found that mRNA levels of genes involved in ecdysone biosynthesis and ecdysteroid signaling pathways were significantly reduced in *PTTH* knockouts. Our results provide genetic evidence that PTTH is not essential for development, but is required to coordinate growth and developmental timing.

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1. Introduction

In insects, molting and metamorphosis are intricately regulated by two major hormones, 20-hydroxyecdysone (20E) and juvenile hormone (JH) (Jindra et al., 2013, 2015; Nijhout, 1981, 1998; Riddiford, 2012). The precursor of 20E, ecdysone, is synthesized in the endocrine organ prothoracic gland (PG), secreted into the hemolymph, and then converted to 20E at peripheral tissues (Gilbert et al., 2002; Rewitz et al., 2013). The synthesis and release of ecdysone is induced at specific times during development to trigger molting and metamorphosis (Gilbert et al., 2002; Nijhout, 1998; Rewitz et al., 2013). It has been shown that the production and release of ecdysone is primarily controlled by a neuropeptide hormone, prothoracicotropic hormone (PTTH) (Gilbert et al., 2002; Ishizaki and Suzuki, 1994; Nijhout, 1981, 1998; Ou et al., 2016; Rewitz et al., 2013; Yamanaka et al., 2015).

PTTH was originally identified in the silkworm, *Bombyx mori*. PTTH is a homodimeric protein consist of subunits composed of 109 amino acid residues (Kataoka et al., 1991; Kawakami et al., 1990; Noguti et al., 1995). PTTH is synthesized in two pairs of dorsolateral neurosecretory cells in the brain and transported to the corpora allata (CA), an endocrine organ that produces JH, by axons running through the contralateral hemisphere of the brain (Mizoguchi et al., 1990). PTTH is secreted into the hemolymph from arborized axon endings in the CA (Dai et al., 1995). In PG cells, PTTH binds to its receptor, Torso (a receptor tyrosine kinase), and activates ecdysone synthesis via the MAPK/ERK (mitogen-activated protein kinase/extracellular signal-regulated kinase) pathway (Lin and Gu, 2007;

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Rewitz et al., 2009). Then, dietary cholesterol is sequentially converted to ecdysone in PG cells by ecdysteroidogenic enzymes encoded by the "Halloween genes", which include *neverland* (*nvd*), *noppera-bo* (*nobo*), *shroud* (*sro*), *spook* (*spo*), *spookier* (*spok*), *phantom* (*phm*), *shadow* (*sad*), and *disembodied* (*dib*) (Enya et al., 2014, 2015; Gilbert, 2004; Niwa and Niwa, 2014; Rewitz et al., 2013). Synthesized ecdysone is released from the PG into the hemolymph and converted to the more active form, 20E, in the peripheral tissues by *shade* (*shd*), another member of the Halloween genes (Petryk et al., 2003). In the cultured PG of *Bombyx*, PTTH stimulates the transcription of *spo*, *phm*, and *dib*, but not *sad* (Namiki et al., 2005; Niwa et al., 2005; Yamanaka et al., 2007), indicating that PTTH selectively upregulates Halloween genes at the transcriptional level.

Physiological roles for PTTH during larval-pupal metamorphosis have been extensively studied in classic model lepidopteran insects such as the tobacco hornworm *Manduca sexta* and the silkworm *Bombyx* (Nijhout, 1998). In a proposed model for the endocrine control of pupal metamorphosis, the activity of the PG is directly or indirectly suppressed by JH during the early stages of the last larval instar (Gu et al., 1997; Nijhout and Williams, 1974; Sakurai, 1984; Truman and Riddiford, 1974). When larvae attain the critical size for metamorphosis, the synthesis of JH in the CA is shut-off and the JH titer declines to a very low level, allowing for the release of PTTH at the next photoperiodic gate (Muramatsu et al., 2008; Nijhout, 1998). The release of PTTH induces the synthesis of ecdysone in the PG, thereby initiating the cessation of feeding and the onset of wandering behavior (Nijhout, 1998; Rewitz et al., 2009). Thus, the regulation of the release of PTTH during the last larval instar is considered to be the key physiological event that determines the timing of metamorphosis and final body size (Mizoguchi et al., 2015; Nijhout, 1998).

Despite this knowledge, a fundamental question has not yet been answered: is PTTH an essential hormone for ecdysteroidogenesis? In the fruit fly, *Drosophila melanogaster,* the ablation of the PTTH-producing neurons resulted in a severe developmental delay and an increase in body weight and size (McBrayer et al., 2007). However, PTTH mutants have not been characterized yet in any insects, including *Drosophila*.

Here, we report the establishment and characterization of *PTTH* knockout *Bombyx*. To knockout the *PTTH* gene, we used transcription activator-like effector nucleases (TALENs), which have been shown to efficiently introduce mutations in target genes in *Bombyx* (Daimon et al., 2014, 2015; Takasu et al., 2013), and successfully established a *PTTH*-null mutant line. The present study provides strong evidence that PTTH is required to upregulate ecdysone biosynthesis in the PG during molting stages, thereby regulating developmental timing in *Bombyx*.

2. Materials and methods

2.1. Silkworm strains

Silkworms were reared on an artificial diet (Silk Mate PM, Nosan Corporation, Kanagawa, Japan) at 24–26 °C under a 12:12 L:D photoperiod as previously described (Daimon et al., 2003). Knockout *Bombyx* were generated using TALENs as previously described (Daimon et al., 2015). Briefly, TALEN mRNAs (400 ng; 200 ng + 200 ng for left and right TALEN mRNA, respectively) were injected into preblastoderm embryos of *pnd w-1* strain, a standard strain for transgenesis (Tamura et al., 2000). *PTTH* knockout lines used in this study were not outcrossed to other strains so that we could compare the phenotypes and gene expressions in the same genetic background (i.e., *pnd w-1*). The parental *pnd w-1* strain was used as a control strain throughout this study. Nucleotide sequences for the generated knockout alleles and TALEN target sites are shown in Fig. 1.

2.2. Genotyping

For genotyping, the heads of moths, the whole body of young instar larvae (first–second instar larvae, L1–L2 larvae), or small parts of larval bodies (L3 and thereafter) were crushed in alkaline buffer (50 mM NaOH) and then heated at 95 °C for 10–15 min. After neutralization by adding the same volume of 0.2 M Tris-HCl (pH 8.0), the supernatants were used as templates for PCR. PCR was performed using ExTaq (TaKaRa, Shiga, Japan) or KOD FX Neo DNA polymerase (TOYOBO, Tokyo, Japan). Primers used for genotyping were CelI-F (5′-ATTACTCGACCGATTATATTAGTC-3′) and CelI-R (5′-CGCGTTCTCGTTTCAACGGA-3′). The PCR products were directly sequenced using BigDye terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, CA) and the CelI-R primer.

2.3. Phenotypic analysis

To investigate development of *PTTH* knockouts, larvae were individually reared in a Petri dish (90 mm diameter) and their development was recorded daily until adult emergence or death. The body weight of larvae was measured every 24 h or after each larval molt or pupation. The head capsule width of larvae was measured using a digital micrometer caliper (Digimatic Caliper CD67-S15PS; Mitsutoyo, Ibaraki, Japan).

2.4. Whole-mount immunohistochemistry

Whole-mount immunohistochemistry (IHC) was performed as previously described (Yamanaka et al., 2005). Brain-corpora cardiaca-corpora allata (Br-CC-CA) complexes were dissected in PBS (137 mM NaCl, 2.68 mM KCl, 8.10 mM Na_2HPO_4, 1.47 mM KH_2PO_4; pH 7.4) and fixed with 4% (w/v) paraformaldehyde in PBS at 4 °C. After washing with PBS, the Br-CC-CA complexes were treated with 1 mg/ml collagenase (Sigma-Aldrich Japan, Tokyo, Japan) in PBS for 1 h. Tissues were blocked with 5% (w/v) bovine albumin fraction V (Nacalai Tesque, Japan) in PBST [PBS with 0.5% (v/v) Triton X-100] for 1 h, and incubated with anti-PTTH mouse monoclonal antibody (Mizoguchi et al., 1990), diluted 1:1000 with PBST, at 4 °C for 48 h. After washing 3 times with PBST for 10 min, the tissues were incubated with Alexa Fluor 488-labeled goat anti-mouse IgG (Life Technologies, Canada), diluted 1:500 with PBST, at 4 °C for 24 h. After washing 3 times with PBST for 10 min, samples were cleared with 70% (v/v) glycerol, and observed using a confocal laser-scanning microscope LSM 700 (Carl Zeiss, Germany).

2.5. Feeding rescue experiments

The artificial diet containing 20E (Kyoritsu Seiyaku, Tokyo, Japan) was prepared as previously described (Kamimura et al., 2003). For rescue experiments, newly hatched larvae were first reared on a standard diet. On day 4 of the second instar (L2), they were transferred to the diet containing selected doses of 20E (10–400 ppm on a dry-weight basis) for 24 h, and then transferred to the normal diet.

2.6. Ecdysteroid titer measurements

Ecdysteroids were extracted from whole bodies of L1 and L2 larvae, and from the hemolymph of L3–L5 larvae. To prepare whole body samples of L1 and L2 larvae, three individuals were pooled in a single 1.5 ml tube and homogenized in 100% methanol. The hemolymph of L3–L5 larvae was collected individually in a 1.5 ml

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