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Glue protein production can be triggered by steroid hormone signaling independent of the developmental program in *Drosophila melanogaster*

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ABSTRACT

Steroid hormones regulate life stage transitions, allowing animals to appropriately follow a developmental timeline. During insect development, the steroid hormone ecdysone is synthesized and released in a regulated manner by the prothoracic gland (PG) and then hydroxylated to the active molting hormone, 20-hydroxyecdysone (20E), in peripheral tissues. We manipulated ecdysteroid titers, through temporally controlled overexpression of the ecdysteroid-inactivating enzyme, CYP18A1, in the PG using the GeneSwitch-GAL4 system in the fruit fly *Drosophila melanogaster*. We monitored expression of a 20E-inducible glue protein gene, *Salivary gland secretion 3 (Sgs3)*, using a Sgs3:GFP fusion transgene. In wild type larvae, Sgs3-GFP expression is activated at the midpoint of the third larval instar stage in response to the rising endogenous level of 20E. By first knocking down endogenous 20E levels during larval development and then feeding 20E to these larvae at various stages, we found that Sgs3-GFP expression could be triggered at an inappropriate developmental stage after a certain time lag. This stage-precocious activation of Sgs3 required expression of the Broad-complex, similar to normal Sgs3 developmental regulation, and a small level of nutritional input. We suggest that these studies provide evidence for a tissue-autonomic regulatory system for a metamorphic event independent from the primary 20E driven developmental progression.

1. Introduction

Animals usually follow a programmed timeline for proper development. To this end, developmental programs contain checkpoints to ensure that a precise sequence of events occurs prior to allowing development to proceed further. Steroid hormones are crucial regulators of physiological and many morphological changes in most higher organisms (Wollam and Antebi, 2011). Holometabolous insects are excellent models for deciphering how developmental programs are coordinated, because their developmental transitions, including molting and metamorphosis, are primarily controlled by the insect steroid hormone, 20-hydroxyecdysone (20E). During steroid hormone biosynthesis in insects, ecdysone (E) is synthesized in the prothoracic gland (PG) and after secretion into the hemolymph is hydroxylated to 20E in peripheral tissues (Gilbert and Warren, 2005; Petryk et al., 2003). Distinct peaks of 20E are observed just prior to the various larval molts and at the end of the third larval instar (L3) stage just before pupariation (Warren et al., 2006). While elevation of the 20E titer is critical for physiological events, decline of the 20E titer by enzymatic inactivation is also important to drive developmental progression (Riddiford et al., 2003). A cytochrome P450, CYP18A1, is a key enzyme for E and 20E inactivation via 26-hydroxylation in the many target tissues of ecdysteroids (Guittard et al., 2011). The decline in the 20E titer by CYP18A1 is essential to promote metamorphosis, as the loss-of-CYP18A1 function results in pupal lethality in *Drosophila melanogaster* (Guittard et al., 2011; Rewitz et al., 2010).

The onset of metamorphosis in holometabolous insects is intimately linked to nutritional cues which likely regulate secretion of the brain neuropeptide, prothoracicotropic hormone (PTTH), which triggers the production and release of E from the PG (McBrayer et al., 2007; Nijhout and Williams, 1974a). The secretion of E commits larvae to pupariation and cessation of growth and controls the final body size. There are at least three check points during the *Drosophila* L3 stage which larvae must pass through to ensure that they have sufficient nutrient stores and intact imaginal tissue to survive

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metamorphosis (Mirth and Riddiford, 2007; Nijhout, 1975; Stieper et al., 2008). The first is minimal viable weight (MVW) which is the minimum weight needed to successfully survive metamorphosis. The second checkpoint is termed critical weight (CW) which is the minimum weight after which starvation can no longer delay metamorphosis. A third recently characterized checkpoint involving *dilp8* signaling ensures that the imaginal tissue is not damaged before metamorphosis commences (Garelli, 2012; Colombani, 2012; Hackney et al., 2012). The timing of E synthesis induction in the PG at the early L3 stage corresponds to the attainment of the CW (Koyama et al., 2014; Mirth and Riddiford, 2007). The duration of the larval growth period prior to CW is regulated by multiple nutrition-dependent signaling pathways including Insulin/insulin-like (IIS) and Target of Rapamycin (TOR) signaling (Caldwell et al., 2005; Colombani, 2005; Koyama et al., 2014; Layalle et al., 2008; Mirth et al., 2005; Walkiewicz and Stern, 2009). This regulatory network, composed of multiple signaling pathways likely endows the larva with the flexibility to adapt to environmental conditions, notably nutrition, before a commitment to metamorphosis via monoaminergic signaling (Ohhara et al., 2015; Shimada-Niwa and Niwa, 2014).

In *D. melanogaster*, the 20E titer rises in a stepwise manner during the feeding L3 stage, culminating in a large peak around the time of pupariation (Warren et al., 2006; Lavrynenko et al., 2015). The first threshold at 8 h after L2-L3 ecdysis (AL3E) on standard growth media is thought to be a commitment peak since it just precedes the attainment of CW (Koyama et al., 2014; Mirth and Riddiford, 2007). The second and third thresholds at 20 h and 28 h AL3E are correlated with the onset of glue protein synthesis in the salivary gland (SG) and the behavioral switch from the feeding to the wandering stage, respectively (Mirth and Riddiford, 2007; Warren et al., 2006). The glue proteins allow the larva to adhere to a solid surface when it becomes a pupa. One of the glue proteins, Salivary gland secretion 3 (Sgs3), is a mucoprotein composed largely of tandem repeats of the five amino acids PTTTK (Garfinkel et al., 1983). The process of glue protein secretion and SG histolysis have been well studied (Biyasheva et al., 2001; Jiang et al., 1997). In these processes, traditional 20E-regulated gene cascades are activated via a nuclear receptor heterodimer consisting of ecdysone receptor (EcR) and ultraspiracle (USP) (Hill et al., 2013). In contrast, expression of the glue gene *Sgs3* is induced by an atypical 20E signaling pathway which requires EcR, but not USP and the complete underlying mechanism remains unclear (Biyasheva et al., 2001; Costantino et al., 2008). However *Sgs3* induction also requires activation of the 20E-inducible Broad Complex (Br-C), which is a large transcription unit that produces four transcription factor protein isoforms (Costantino et al., 2008). The Br-C has been well-characterized as an early gene required for expression of the glue genes, including *Sgs3,* through activation of promoter/enhancer elements in the gene cluster (Crowley et al., 1984; DiBello et al., 1991; Guay and Guild, 1991). Indeed, overexpression of any isoform of Br-C in SG cells could induce Sgs3 synthesis, even in loss-of-EcR function mutants (Costantino et al., 2008).

In this study, we temporally over-expressed the ecdysteroid-inactivating enzyme, CYP18A1, in the PG using the GeneSwitch-GAL4 system in order to manipulate ecdysteroid titers. We especially focused on the expression of Sgs3 triggered by 20E at the midpoint of the L3 stage, because it was easy to monitor the initiation of its expression using transgenic flies expressing a Sgs3-GFP fusion protein (Biyasheva et al., 2001). We found that Sgs3-GFP expression could be triggered by administration of 20E even in an aberrant developmental timeline. This stage-aberrant induction of Sgs3 in the SG was observed only after the larval developmental schedule was disrupted, and required a certain level of nutrition, a time lag after 20E feeding, and the products of the Br-C. We propose here a tissue-autonomic regulatory system for a metamorphic event independent from the coordinated developmental system in whole body.

2. Materials and methods

2.1. Drosophila strains

phm-GS-GAL4/Tm6B, Tb^{1} was generated as described below. *UAS-CYP18A1* (Rewitz et al., 2010) and *UAS-Grim* (McBrayer et al., 2007) were obtained from M.B. O'Connor. *UAS-spok-IR*; *UAS-spok-IR* was described in Ono et al. (2006). *UAS-GFP* (#107–870) and *Sgs3-GFP* (#5885) were obtained from KYOTO Stock Center (DGRC) in Kyoto Institute of Technology and Bloomington Drosophila Stock Center, respectively. Flies were cultured on a standard cornmeal/yeast extract/dextrose medium.

2.2. Vector construction and transformation

The phmGeneSwitch vector harboring a *phantom* (*phm*) promoter positioned upstream of the GeneSwitch sequence containing GAL4 DNA binding domain and progesterone receptor ligand binding domain was generated as shown in Fig. S1-S2. The primers used for construction are listed in Table S1. Briefly, the GeneSwitch component was first inserted in pUAST vector. Next, a region of *phm* promoter of *D. melanogaster* was PCR amplified from genomic DNA. This fragment was inserted into pGeneSwitch-UAST. Germline transformant was obtained using standard protocol. For experiments, strain carrying the transgene on the third chromosome was used.

2.3. Chemicals

Mifepristone (RU486) and ecdysone were purchased from Sigma-Aldrich (St. Louis, MO, USA). 20-Hydroxyecdysone was purchased from SciTech (Prague, Czech Republic). Methoprene was purchased from AccuStandard (New Haven, CT, USA).

2.4. Developmental analyses

Eggs were collected and hatched larvae were reared on instant food with or without steroid(s) as described previously (Ono, 2014). For steroid feeding experiments, RU486, E, 20E or both RU486 and ecdysteroid dissolved in ethanol were added to instant food at 500 μM final concentration unless otherwise noted. Methoprene dissolved in acetone was suspended in water, and then the suspension was applied to instant food at 500 μM final concentration. When ectopic expression of a transgene was induced using GAL4 or GeneSwitch-GAL4 driver, animals were reared at 29 °C.

2.5. Nutritional deficient experiments

Individual larva carrying a transgene *Sgs3:GFP* was transferred to 250 μl of 1.8% agar medium in a half-cut collection tube (2.0 ml), and then plugged with a sponge. For 20E supplemental experiments, 20E was added to the agar medium to a final concentration of 500 μM. L2 larvae were used at 0, 2, 4, 6 h after L1-L2 molting. L2 or L3 larvae within 2 h after ecdysis were used for the 12, 18 h-L2 or L3 samples. Dead animals within three days after transfer were excluded from data collection. Animals were reared at 25 °C during these experiments.

2.6. RT-PCR and quantitative RT-PCR (qPCR)

Individual larvae to measure expression of GFP or glue genes were collected and homogenized in TRIzol Reagent (GIBCO-BRL, NY, USA). Otherwise, SGs collected from ten larvae were treated in the same fashion to measure expression levels of EcR and Br-C. Total RNA purification, reverse transcription and qPCR were performed as described (Ono, 2014). Alternatively, the generated cDNAs were subjected to PCR amplification with gene-specific primers using GoTaq Green Master mix. (Promega, Madison, WI). PCR conditions