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Prothoracicotropic hormone regulates developmental timing and body size in Drosophila

Zofeyah McBrayer[1,*], **Hajime Ono**[1,2,*,#], **MaryJane Shimell**[1,*], **Jean-Philippe Parvy**[3], **Robert B. Beckstead**[4], **James T. Warren**[5], **Carl S. Thummel**[4], **Chantal Dauphin-Villemant**[3], **Lawrence I. Gilbert**[5], and **Michael B. O'Connor**[1,2,#]

1 *The Department of Genetics, Cell Biology and Development, University of Minnesota, Minneapolis, MN, 55455*

2 *Howard Hughes Medical Institute, University of Minnesota, Minneapolis, MN, 55455*

3 *FRE2852 Protéines Biochimie structurale et fonctionnelle, CNRS- Université P. et M. Curie, Bat A, 5ème ét., Case 29, 7 Quai St Bernard, 75252 Paris CEDEX 05 France*

4 *Department of Human Genetics, University of Utah School of Medicine, Salt Lake City, UT 84112*

5 *Department of Biology, University of North Carolina, Chapel Hill, NC 27599-3280*

Summary

In insects, control of body size is intimately linked to nutritional quality as well as environmental and genetic cues that regulate the timing of developmental transitions. Prothoracicotropic hormone (PTTH) has been proposed to play an essential role in regulating the production and/or release of ecdysone, a steroid hormone that stimulates molting and metamorphosis. In this report we examine the consequences on Drosophila development of ablating the PTTH-producing neurons. Surprisingly, PTTH production is not essential for molting or metamorphosis. Instead, loss of PTTH results in delayed larval development and eclosion of larger flies with more cells. Prolonged feeding, without changing the rate of growth, causes the developmental delay and is a consequence of low ecdysteroid titers. These results indicate that final body size in insects is determined by a balance between growth rate regulators such as insulin and developmental timing cues such as PTTH that set the duration of the feeding interval.

Introduction

Proper development of all multicellular organisms requires not only correct spatial control of cellular interactions, but also accurate timing of specific developmental programs of gene expression. These timed programs involve systemic signaling systems that respond to key nutritional and environmental cues to direct coordinated developmental responses throughout the animal. In humans, for example, passage from adolescence to adulthood is accompanied by rapid changes in growth and acquisition of sexual maturity (reviewed in Navarro et al., 2007). Likewise, in frogs a dramatic hormone stimulated transformation remodels the

[#]To whom correspondence should be addressed. Michael B. O'Connor, Depart. of Genetics, Cell Biology and Develop., HHMI/ University of Minnesota, 6-160 Jackson Hall, Minneapolis, MN. 55455, Tel. 612-626-0642, E-mail: E-mail: moconnor@umn.edu.
[*]These authors contributed equally to this work
[#]Present address. Graduate School of Agriculture, Kyoto University, Sakyo-ku, Kyoto, 606-8502, Japan

immature swimming tadpole into a sexually active, air-breathing adult (Furlow and Neff, 2006). Perhaps the most dramatic examples, however, can be found in insects where developmental transitions occur at regularly defined intervals. These transitions include molting, a process whereby the rigid exoskeleton is shed and re-synthesized to accommodate increasing larval body size as a result of cell growth, and metamorphosis, a transformation in which the immature larva changes into a reproductively mature adult.

In most arthropods, the timing of molts and metamorphosis is coordinated by a rise in the titer of the steroid hormone 20-hydroxyecdysone (20E) (Henrich et al., 1999; Warren et al., 2006). In insects, the production and release of ecdysone in response to developmental cues is thought to be primarily regulated by a small, secreted peptide known as prothoracicotropic hormone (PTTH) (reviewed in Rybczynski, 2005). PTTH was originally purified from *Bombyx mori* brain extracts as a substance that could stimulate ecdysone production in the prothoracic glands (Kataoka et al., 1991). Active *Bombyx* PTTH is a ~25 kDa disulfide linked homodimer that is processed from a larger precursor protein. In Lepidoptera, it is produced primarily in a pair of bilateral neurosecretory neurons whose axons terminate in specialized neurohemal varicosities on the corpus allatum, a secretory gland of the neuroendocrine system (Agui et al., 1979; Dai et al., 1994; Mizoguchi et al., 1990). Once released from the corpus allatum into the hemolymph, PTTH targets the prothoracic gland where it binds to an unknown receptor and triggers production and release of ecdysone via one or more second messenger pathways that include Ca^{2+}, cAMP and a MAP kinase cascade (Rybczynski et al., 2001; Rybczynski and Gilbert, 2003; Smith and Gilbert, 1989).

Understanding the regulation of PTTH production and release is key to deciphering the mechanisms that regulate developmental timing in insects. Studies in Lepidoptera and several other insect groups suggest that PTTH release is controlled by at least two components, weight gain and photoperiod. In some blood sucking hemipteran species such as *Rhodnius prolixus* or the milkweed bug *Oncopeltus fasciatus*, weight gain triggers PTTH release and molting (Nijhout, 1979; 1984). Since injection of air into the gut can also trigger a molt, distention of abdominal stretch receptors is thought to be the key event that signals for PTTH release and the molting response (Nijhout, 1979). In Lepidoptera and Drosophila however, artificial inflation of larvae with air does not induce a molt, suggesting that similar stretching of the larval abdomen cannot, by itself, stimulate PTTH release in these insects. Instead, a more complex nutritional assessment is made in which larvae must pass through several checkpoints to ensure that they have achieved an appropriate size and amassed sufficient nutritional storage, primarily in the fat body, to survive the prolonged period of non-feeding during metamorphosis (reviewed in Edgar, 2006; Nijhout, 2003).

Attainment of “minimal viable weight” is the first checkpoint that must be attained to survive metamorphosis. Starvation before this time results in a prolonged larval stage and eventual death without pupation or a partial attempt at pupation. A second checkpoint, referred to as “critical weight” is reached when starvation no longer affects the time to pupation (Nijhout, 2003). Attainment of minimal viable weight ensures that there is enough nutrient storage to undergo metamorphosis, while reaching critical weight is thought to initiate the metamorphic process.

In Lepidoptera, achieving critical weight is thought to be a key factor in stimulating PTTH release (Nijhout, 1981). Superimposed on this control is a photoperiodic gating mechanism in which PTTH can only be released during a specific eight-hour window each day (Truman, 1972; Truman and Riddiford, 1974). If critical weight is achieved outside this time-frame, then the larvae continue to feed until the next photoperiodic gate is reached the following day.

Although PTTH is considered to play a key role in regulating ecdysone production and release, and therefore in the timing of insect development, this hypothesis has never been rigorously tested by genetic loss-of-function studies. In this report, we describe the identification and characterization of a Drosophila PTTH-related gene. Similar to its lepidopteran homologs, Drosophila PTTH is a secreted factor that is produced by a pair of bilateral neurosecretory cells in the brain. In Drosophila, however, these neurons directly innervate the prothoracic gland, instead of the corpus allatum, to regulate ecdysone production. Using the Gal4/UAS system (Brand and Perrimon, 1993), we specifically ablated the PTTH-producing neurons and examined the developmental consequences. Surprisingly, ablation does not completely halt development. Rather, loss of PTTH substantially increases the time required to pass through the larval period, especially the third instar stage. This prolonged developmental period results in a longer duration of larval feeding and eclosion of larger adult flies with more cells. Feeding 20E to PTTH producing neuron-ablated larvae can reverse these phenotypes. These results indicate that PTTH directs proper temporal progression through larval stages and contributes to the determination of final body size in insects by regulating the duration of growth through control of ecdysteroid production.

Results

Identification of a Drosophila PTTH family member

Previous biochemical attempts to purify a factor from Drosophila with PTTH-like ecdysteroidogenic activity led to the identification of two partial peptide amino acid sequences, neither of which showed significant similarity to lepidopteran PTTH (Kim et al., 1997). Searching the Drosophila database using NCBI Blast and several different lepidopteran amino acid sequences as templates revealed no gene with a P value score lower than 0.01. However, the predicted gene CG13687 was similar in length to the moth sequences and showed high conservation in the spacing pattern of 7 cysteine residues (Fig. 1A). In lepidopteran PTTH, six of these cysteines form intra-molecular disulfide bridges creating a cysteine knot-type structure similar to those found in NGF, PDGF and TGF-β-type factors (Noguti et al., 1995), while the seventh cysteine links two monomers together to form a homodimer. Phylogenetic comparisons using a neighbor-joining method indicate that the Drosophila sequence as well as related sequences found in mosquitoes, are distant relatives of the lepidopteran sequences, (Fig. 1B). The similarity of this sequence to PTTH has been noted previously (Rybczynski 2005: Riehle et al. 2002), and we propose to call it Drosophila PTTH.

The predicted Drosophila PTTH contains a hydrophobic stretch of amino acids at its N-terminal end that likely serves as a signal peptide or a type II transmembrane segment suggesting that it is secreted. It also contains a dibasic (KR) sequence just prior to the first of the conserved cysteines. Proteolytic cleavage at this dibasic sequence would release a C-terminal fragment from the precursor protein, as found for lepidopteran PTTH.

This PTTH-like sequence is conserved in other Drosophilidae species. However, the methionine identified by Flybase as the start codon for the *D. melanogaster* sequence was not conserved (Supplement Fig. 1). Instead, a valine is substituted at this position with an open reading frame continuing upstream for an additional 24 amino acids to a second methionine codon that is preceded by an in-frame stop codon. In *D. melanogaster*, the reading frame also remains open upstream of the predicted start codon suggesting that the upstream Met may indeed represent the true N-terminus (Fig. 1C).

To determine if Drosophila *ptth* is transcribed and to determine its molecular structure, we isolated several cDNAs. This analysis revealed three isoforms that only differ in the region upstream of the probable signal peptide suggesting that all transcripts will likely produce the same mature secreted protein (Fig 1C).

Drosophila PTTH is produced in the PG neurons that innervate the prothoracic gland

To examine the expression of Drosophila *ptth*, we carried out *in situ* hybridization to embryos and dissected larvae. We first detect *ptth* expression in stage 17 embryos in a pair of bilaterally symmetric central brain neurons (Fig. 2A). This expression continues through all larval stages and is prominent in wandering third instar larvae (Fig. 2B) but not in sense probe controls (Fig. 2C).

Since it is difficult to survey all developmental times and stages by *in situ* hybridization, we also created a promoter/enhancer Gal4 fusion using approximately 1 kb of intergenic DNA that spans the region upstream of CG13687 to the next identified gene *Pph13* (CG2819). When crossed to a strain containing a UAS-cd8 membrane bound GFP reporter, expression is observed in two central brain neurons starting in late embryos, similar in position to those identified by *in situ* hybridization. We also occasionally see transient expression during the first and second instar stages in several additional central brain neurons of unknown identity (Supplement Fig. 2). During the third instar stage we see the same two prominent neurons and their dendritic arbors (Fig. 2D). To trace the axon projections of these neurons we generated a genomic PTTH construct tagged with a hemagglutinin (HA) epitope. In this case, PTTH-HA localization is seen prominently in the axons and in terminal varicosities on the prothoracic gland (Fig. 2F). Since the dendritic arbors of these neurons extend in the same direction as the axons, the neurons are unipolar and appear to correspond to the PG neurons identified in a Gal4 enhancer trap screen for neurons that innervate the prothoracic gland (Siegmund and Korge, 2001). Consistent with this assignment, we also find that axons projecting from the pigment dispersion factor (PDF)-producing neurons terminate in close proximity to the dendritic arbors of the PTTH-producing neurons (Fig. 2G) similar to what has been described previously for the PDF axons. To confirm that these are the PG neurons, we localized *ptth* transcripts to the GFP positive cells of the Feb211-Gal4 line described by Siegmund and Korge (2001) (Supplement Fig. 2).

Since in Lepidoptera the prothoracic gland undergoes apoptosis during pupal stages (Dai and Gilbert, 1997), we were curious whether Drosophila PTTH is expressed in the adult, particularly since *Bombyx* PTTH was originally extracted from adult heads (Nagasawa et al., 1980). We found no expression in any tissue other than the brain, where we see several neurons per hemisphere that continue to express PTTH (Supplement Fig. 3) and some axons from these neurons appear to innervate the ellipsoid body, a circular structure with roles in regulating walking and flight behavior (Ilius et al., 1994; Martin et al., 2001).

PTTH transcription shows an unusual cyclic profile during third instar stage

To more closely examine the transcriptional profile of *ptth*, we carried out semi-quantitative RT-PCR on RNA isolated from carefully staged third instar larval brains. We find that *ptth* is expressed throughout the third instar stage (Fig. 3A). Its expression is not uniform, but instead shows an unusual cyclic pattern with an approximate 8-hour periodicity. In addition, expression shows a dramatic up-regulation roughly 12 hrs before pupariation (Fig. 3A,C). We also attempted to examine protein levels on Western blots using the genomic *ptth-HA* tagged lines, but were unable to detect the HA epitope in brain/ring gland extracts, probably because of the low expression level.

Since the PDF-producing neurons synapse with the PG neuron dendritic arbors, we sought to determine if PDF influenced the expression of *ptth*. RT-PCR on RNA extracted from pdf^{01} mutants displayed an altered periodicity, and the overall levels of *ptth* transcripts were significantly enhanced (Fig. 3B, C), suggesting that PDF signaling may contribute to the transcriptional periodicity and acts as a general negative regulator of *ptth* transcription or message stability.

Ablation of PG neurons produces developmental delay and a prolonged duration of feeding

To gain insight into the potential function of Drosophila *ptth*, we sought to examine the consequence of its loss on larval/pupal development. At present, no loss-of-function mutants are available for this gene. We tried expressing several different RNAi constructs in PG neurons, but none showed significant knockdown of PTTH-HA protein expression. As an alternative, we specifically ablated the PTTH producing neurons using the Gal4/UAS system. Such a method has been used successfully to examine the function of eclosion hormone-producing neurons (McNabb et al., 1997) as well as the functions of several other neuropeptide-producing neurons (Kim et al., 2006; Park et al., 2003; Terhzaz et al., 2007).

To achieve cell-specific ablation, UAS-Grim was expressed in the PG neurons using the *ptth* > Gal4 driver. A cd8-GFP reporter or a PTTH-HA genomic transgene was included in the background enabling us to monitor the timing and extent of ablation. We found that larvae containing two copies of UAS-Grim and two copies of *ptth* > Gal4 showed no detectable GFP or PTTH-HA at any stage (Fig. 4A vs B for third instar larvae) suggesting effective killing of PG neurons at an early age. Despite the complete loss of the PG neurons and PTTH production, some viable adults emerged from the ablation crosses. The females showed reduced fecundity, and the males exhibited male-male courtship behaviors similar to that seen in *fruitless* mutants (Gailey and Hall, 1989). We also observed that these adults, as well as the pupae and wandering third instar larvae, were larger than wildtype (Fig. 4 C,D,E,J). Female ablated pupae were on average 32% and 21% longer, than UAS-Grim and *ptth* > Gal4 pupae, respectively; ablated male pupae were 26% and 15% longer on average than UAS-Grim and *ptth* > Gal4 controls, respectively (Fig. 4J). The adult females and males ranged from 50 to 70 % heavier than the controls (Fig. 4J).

Not only were the bodies of adults larger and heavier, their wings were also larger (Fig. 4G). To determine if this enhanced size was due to an increase in cell number or to an increase in cell size, we counted wing hair numbers within a defined $100\mu^2$ area on each wing. Since each wing epidermal cell produces only one wing hair, the density of hairs is a useful indicator of cell size. This analysis revealed that there is no change in cell size (Fig. 4F) leading us to conclude that the larger wings are produced by an increase in cell number.

To determine if the enhanced body size is the result of an alteration in the rate of mass accumulation during feeding or the duration of feeding, we measured the time period spent in each larval instar stage, as well as the rate of weight gain during the third larval instar as a function of time. We found that the duration of each larval instar was lengthened in ablated animals compared to controls (Fig. 5). The average time to ecdysis from the first to the second instar stage for larvae in which the PG neurons were ablated increased by approximately 8 hours (Fig. 5A). Interestingly, during the second to third instar stage, the delay in larvae with ablated PG neurons did not become any more pronounced and stayed approximately 8 hours behind the controls (Fig. 5B). During the third instar stage, however, there is an additional dramatic developmental delay of 5 days. The average total time from egg deposition to pupariation increased from 5.5 days for controls to approximately 10.5 days for larvae in which the PTTH-producing neurons were ablated (Fig. 5C). After pupation, the time to eclosion for ablated animals is not different from the controls and averages about 5 days (data not shown). It should be noted that not all ablated animals were able to complete development. The death rate of ablated larvae varied between trials. Approximately 5% of ablated larvae died between the 1^{st} and 2^{nd} instar. By the 3^{rd} instar, 20% of the larvae had died and by puparium formation 50% of the larvae had died. Lastly, not all animals that succeeded in puparium formation were able to complete metamorphosis, and up to 50% of puparia died before ecloson. These dead puparia included elongated prepupae, ones with head eversion defects and other phenotypes characteristic of reduced ecdysone titers.

Since an increase in body size can be caused by either an increase in the duration of larval feeding, an enhanced rate of growth, or some combination thereof, we measured the rate of weight increase during the third instar stage of both controls and larvae with ablated PG neurons. We found that both control and PG neuron-ablated larvae gain mass at approximately the same rate (Fig. 6A), but that the ablated larvae continue to feed and gain mass for much longer times. This observation suggests that ablation of PG neurons primarily affects the duration of feeding and not the rate of weight gain.

Ablation of the PG neurons enhances critical weight

As described earlier, a key size assessment checkpoint for insect development during the third instar stage is attainment of critical weight. Once critical weight is reached, then starvation no longer delays the time to pupariation. We determined the critical weight threshold (Fig. 6B) for metamorphosis by starving L3 larvae of known weight and developmental age and then measured the time to pupariation (Mirth et al. 2005). Both the UAS-Grim and *ptth* > Gal4 control lines exhibited a 50% threshold for pupariation after starvation at about 0.75 mg/larva, slightly less than the 0.86 mg critical weight determined for the *phm* > Gal4 strain described by Mirth et al. (2005). In contrast, larvae in which the PTTH-producing neurons were ablated showed an approximate 4-fold increase (2.5 mg/larva) in the critical weight threshold. These larvae displayed no difference in the size of the prothoracic gland (Supplement Fig. 4), supporting the conclusion that prothoracic gland size alone does not determine the critical weight threshold (Mirth et al. 2005). In our experiments, approximately 50% of the animals that pupariated eclosed as adults. Taken together, these results demonstrate that PG neuron ablation disrupts the proper assessment of critical weight, leading to an increase in the duration of larval feeding and, as a result, enlarged adult body size.

The alteration in body size is likely due to loss of PTTH

The ablation experiments described above do not distinguish between whether the effects on body size and developmental time are a direct result of PTTH loss or an indirect effect caused by loss of some other factor that is produced by the PG neurons. While this cannot be determined directly without loss-of-function mutations, we sought to determine what the consequences on development might be as a result of PTTH overexpression. If loss of PTTH-producing neurons results in prolonged development, then perhaps a continuous high-level supply of PTTH might shorten developmental time resulting in small flies. Ubiquitous overexpression of PTTH using a *da* > Gal4 driver resulted in eclosion of adults that were approximately 20% (males) – 30% (females) smaller by weight than the control balancer-containing flies (Fig. 4H, J) and produced smaller wings (Fig. 4I). Wing hair density counts revealed that the small size of the adult wings is the result of reduced cell numbers and not a change in cell size (Fig. 4F). Since overexpression of PTTH itself results in small flies, we conclude that the larger flies produced by ablating the PTTH neurons is most likely caused by loss of PTTH and not some other factor produced by these neurons.

Low basal 20-hydroxyecdysone titers are responsible for the prolonged third instar larval stage

Since the primary function of PTTH in Lepidoptera is thought to be modulation of 20-hydroxyecdysone (20E) production and release, we compared 20E titers in control versus PG neuron-ablated larvae during the third instar stage. We find that larvae with ablated PG neurons show very low ecdysteroid titers throughout the prolonged third instar stage (Fig. 7A). As in wild type, however, an ecdysteroid peak is evident at the white prepupal stage suggesting that PTTH is not the only means by which the ecdysone titer can be increased to trigger metamorphosis.

The prolonged L3 stage and delayed pupariation of *ptth* > Gal4; UAS-Grim animals suggest that they are able to eventually mount an appropriate 20E response to initiate metamorphosis. If this is true, then these animals should express the proper temporal progression of 20E-regulated gene expression that directs entry into metamorphosis. To determine if this is the case, Northern blot hybridizations were used to detect the transcription of 20E-regulated genes in staged animals (Fig. 7B). As newly formed prepupae, UAS-Grim and *ptth* > Gal4 controls as well as the *ptth* > Gal4;UAS-Grim animals display very similar changes in gene expression, indicative of normal responses to a high titer late larval ecdysone pulse (Fig. 7B, final time point for each genotype). These include efficient repression of *EcR* and *E74B* as well as induction of the *E74A* and *BR-C* early regulatory genes. The imaginal disc-specific *IMP-L1* and fat body-specific *Fbp-1* genes are induced normally, and the *Sgs-4* glue gene is repressed. Looking at earlier stages reveals that both control genotypes proceed on a similar time course through the mid-L3 transition, as demonstrated by the repression of *ng-1* and induction of *Sgs-4* in the larval salivary glands at 36 hours. The *ptth* > Gal4 animals, however, show a delay between the mid-L3 transition and pupariation (36–72 hrs). Analysis of the *ptth* > Gal4;UAS-Grim ablated animals reveals an overall prolonged pattern of gene expression that is similar to that of the controls. During the second half of this prolonged L3, however, distinct asynchrony becomes apparent. The 120 and 144 hour time points in *ptth* > Gal4;*UAS*-Grim larvae appear to be at relatively late stages, with high levels of *EcR, E74B,* and *BR-C* mRNA and low levels of *Fbp-1*. In contrast, the subsequent 168 hr time point appears to be at a much earlier stage in development, with lower levels of the early mRNAs and prominent *ng-1* transcription. Taken together, these data suggest that animals lacking PTTH mount a proper transcriptional response to 20E, but increasing asynchrony occurs as these animals progress through their highly prolonged L3 stage.

To determine if low basal levels of ecdysteroid are responsible for the developmental delay and increased size, we fed 20E to larvae with ablated PG neurons just after ecdysis from the second to the third instar stage. As illustrated in Figure 7C, 20E-fed PG-ablated larvae were able to achieve puparium formation 2.5 days after ecdysis to the third instar, while controls lacking 20E did not pupariate until 5–6 days. 20E feeding also restored adult fertility and reduced larval, pupal, and adult size to that of wildtype (data not shown).

Since the developmental gene expression program is protracted and the rise in ecdysone titer is delayed in PG neuron-ablated animals, we examined whether ablation affected the transcription of the known ecdysone biosynthetic enzymes (see Supplemental Figure 4 for where each enzyme acts in the biosynthetic pathway). We and others have previously shown that transcription of *neverland (nvd), spookier (spok), phantom (phm), shadow (sad)* and *disembodied (dib)* all exhibit similar expression patterns during the third instar stage where transcription is initially quite low just after ecdysis and then rises dramatically just prior to wandering (Chavez et al., 2000; Namiki et al., 2005; Niwa et al., 2004; Ono et al., 2006; Parvy et al., 2005; Warren et al., 2006; Yoshiyama et al., 2006). Interestingly, the time at which this occurs, about 12 hr before pupariation, closely coincides with the time at which the PTTH transcription sharply rises (Fig. 3). To determine if ablation of the PG neurons affects transcription of the known biosynthetic enzymes, we carried out quantitative real time PCR (Q-PCR) on RNA samples prepared from well-timed control and ablated larvae after ecdysis to the third larval instar. The mRNA levels of *nvd, phm, spok,* and *dib* all remained quite low in ablated animals throughout the prolonged larval instar and into the white puparium stage (Fig 7D–G). In contrast, *sad* and *shd* expression, although delayed, rise substantially at late times (Fig. 7H,I). *shd* is the only larval biosynthetic enzyme so far identified that is not expressed in the ring gland (Petryk et al. 2003). As a result, it is not likely to be under the direct control by PTTH. We conclude that elimination of the PTTH-producing neurons results in prolonged developmental delay during the third instar stage as a consequence of low basal

ecdysteroid titers, that may be caused, at least in part, by reduced expression of several biosynthetic enzymes.

Discussion

Is Drosophila PTTH the functional equivalent of lepidopteran PTTH?

Although phylogenetic analysis suggests that we have identified a Drosophila homolog of lepidopteran PTTH, we were not able to directly stimulate ecdysone production and/or release in isolated prothoracic glands. Purified Manduca PTTH is able to stimulate ecdysone production/release approximately 4–10 fold when added to isolated glands (Gilbert et al. 2000). We have tried similar studies using Drosophila glands and recombinant Drosophila PTTH produced in S2 cells with variable success. The inconsistent stimulation might result from several factors. First, active lepidopteran PTTH is derived from the C-terminal portion of the protein by proteolytic processing (Kataoka et al., 1991; Kawakami et al., 1990). We do not know if endogenous Drosophila PTTH is similarly processed since we have been unable to detect it on a Western blot from brain extracts. In S2 cells, we see no evidence for PTTH processing, but these cells might not express the appropriate maturation enzyme.

While the inability to produce active PTTH in S2 cells might be caused by lack of proper processing, an alternative explanation for its lack of activity in a ring gland assay is that Drosophila PTTH may require a specific route of delivery that precludes it from working effectively when added exogenously to glands. Unlike lepidopteran PTTH, which is released into the hemolymph from specialized nerve endings in the corpus allatum (Agui et al., 1979; Dai et al., 1994; Mizoguchi et al., 1990), Drosophila PTTH is expressed in neurons that directly innervate the prothoracic gland itself where it may function more like a neurotransmitter rather than a circulating hormone. If PTTH receptors are primarily clustered in specialize regions around the terminal varicosities, then exogenously added PTTH may not have effective access to them.

Are there innervation differences for PTTH-producing neurons between species?

As described above, the axons of PTTH-positive neurons in Lepidoptera terminate on the corpus allatum (Dai et al., 1994; Mizoguchi et al., 1990), while in Drosophila the PG neurons send out processes that terminate on the prothoracic gland (Fig. 2). Although this might represent differences between the two species in the wiring of an equivalent set of neurons, it seems more likely that it reflects a difference in the identity of the neurons that express PTTH. We note in this regard, that like Lepidoptera, Drosophila also has a pair of bilaterally symmetric brain neurons, referred to as the CAs, that specifically innervate the corpus allutum (Siegmund and Korge, 2001). Likewise, Lepidoptera have a pair of bilateral neurons that innervate the prothoracic gland, and intriguingly these neurons express several prothoracicostatic peptides (Yamanaka et al., 2005; Yamanaka et al., 2006). Furthermore, some Drosophila Gal4 enhancer trap lines show expression in neurons that innervate both the PGs and CAs (Siegmund and Korge, 2001) suggesting that these neurons may be functionally or developmentally related.

PTTH transcriptional periodicity correlates with ecdysteroid titer

To fully understand the developmental timing mechanism, it is essential to identify and characterize the signals that regulate PTTH production and release. We examined the *ptth* transcriptional profile during the third instar stage and found that it shows an unusual periodicity and a dramatic up-regulation approximately 12 hr prior to metamorphosis. Interestingly, this periodicity is similar to that seen in the 20E titers of carefully staged third instar larvae using a highly sensitive RP-HPLC/RIA assay (Warren et al., 2006). In this study, several small ecdysone peaks were observed at 8, 20 and 28 hours after ecdysis to the third instar, roughly corresponding to the temporal periodicity of *ptth* transcriptional fluctuations

that we report here. Similar small increases of molting hormone have been described in the last larval stage of Lepidoptera and have been termed “commitment” peaks (Bollenbacher et al., 1975; Riddiford, 1976). Commitment peaks have long been thought to initiate re-programming of the larva in preparation for the subsequent larval-pupal transition (Riddiford, 1995). Consistent with this view is the observation that in Drosophila, these small ecdysteroid peaks temporally correlate with large-scale transcriptional profile changes that take place during the third instar stage (Fig. 7, and Andres et al., 1993; Sullivan and Thummel, 2003; Parvy et al. 2005; Warren et al., 2006). We speculate that the observed periodic fluctuations in PTTH transcriptional levels precede an increased burst of PTTH release at the terminal varicosities on the prothoracic gland that then determine the temporal progression of transcriptional responses during the third larval instar stage by stimulating small, periodic increases in the basal ecdysteroid titer.

How the periodic PTTH transcriptional profile is generated remains unclear. In some species, including Drosophila, photoperiod gating of PTTH release has been inferred (Mirth et al., 2005; Roberts et al., 1987; Truman, 1972; Truman and Riddiford, 1974; Vafopoulou and Steel, 1996) as one type of regulatory input, but no studies on the role of circadian cycles in regulating transcription of *ptth* have been documented. Pigment dispersing factor (PDF) is thought to play a role in coupling circadian outputs to downstream neurons to control rhythmic outputs (reviewed in Taghert and Shafer, 2006). The close apposition of the PDF-expressing axon terminals within the PG neuron dendritic field (Fig. 2) prompted us to examine whether PDF influenced the periodicity of PTTH transcription. We found that in *pdf* null mutant larvae, the cycle changed in complicated ways. The approximate 8 hr transcriptional periodicity was lost and replaced with a modulated cycle that varied in length from 12 to 16 hr. In addition, the sharp up-regulation in transcription prior to metamorphosis was attenuated, and the rise was spread out over a period of time beginning at approximately 20 hours instead of 12 hours prior to metamorphosis. These results suggest that not only is there a complex interaction between PTTH transcription and the circadian cycle, but also that other unknown processes likely influence PTTH production to account for its unique transcriptional periodicity.

PTTH controls the assessment of critical weight in Drosophila

Although photogating is one mechanism that regulates PTTH release, it is not the primary means by which larval developmental timing is regulated. One current model, based primarily on data from moths, suggests that an important factor in this regulation is attainment of critical weight (Mirth and Riddiford, 2007; Nijhout, 2003). Critical weight is operationally defined by the way larval size dictates response to starvation (Nijhout, 2003). Prior to attainment of critical weight, starvation prevents metamorphosis, while after reaching critical weight, metamorphosis takes place in the majority of animals. The final adult size and the length of the metamorphic process can vary depending on the manipulations and species of insect involved (Mirth and Riddiford, 2007). Critical weight is assumed to reflect a neuroendocrine timing switch that signals the readiness of the larva to begin the metamorphic process. Although the means by which critical weight is assessed is not clear, correlative timing studies have suggested a model for how critical weight initiates the metamorphic developmental program in moths and other insects (Mirth and Riddiford, 2007). Juvenile hormone (JH) levels need to drop below a threshold for the process to begin in lepidopteran insects (Dominick and Truman, 1985; Nijhout and Williams, 1974). In addition, it has been observed in Manduca that if JH levels are artificially raised by injection of hormone, PTTH secretion is delayed and a prolonged larval feeding phase ensues producing larger adults (Nijhout and Williams, 1974; Rountree and Bollenbacher, 1986). Whether reaching critical weight triggers the JH drop, or the JH drop is simply the operative signal indicating that critical weight has been achieved, is not clear. Once JH levels dip below this critical threshold, PTTH is then released, triggering the rise in ecdysteroid titers that initiate metamorphosis.

The role of JH in regulating Drosophila PTTH release and metamorphosis is not as clearly defined as in Lepidoptera. If an equivalent scenario applies to Drosophila, then PTTH release should be downstream of critical weight and respond to it. However, our data show that loss of Drosophila PTTH results in a dramatic increase in critical weight and a prolonged developmental delay. Therefore, rather than responding to critical weight, Drosophila PTTH appears to act upstream to set the critical weight threshold. In this scenario, critical weight is not an active developmental timing switch. Instead it is an indication that the developmental program has progressed past a certain point. We propose that the actual timing switches are the minor pulses of PTTH and subsequent small ecdysteroid peaks that occur prior to the major rise in ecdysteroid titer that initiates metamorphosis. The idea that critical weight responds to small changes in ecdysteroid titers is also consistent with recent observations that slightly enhancing basal ecdysteroid levels by manipulating insulin signaling in the prothoracic gland shifts critical weight to a smaller size and leads to precocious metamorphosis (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). An alternative view, however, is that critical weight does act as the operative timing signal, but since in ablated animals there is no PTTH, they cannot respond properly and continue to feed resulting in an apparent shift in critical weight.

Drosophila PTTH regulates developmental timing but is not essential for viability

Our *in vivo* data suggest that the primary function of Drosophila PTTH is to regulate the ecdysteroid level, especially during the third instar stage, to properly time metamorphosis. A surprise from our studies is the observation that loss of PTTH does not result in a complete block to development. While up to 60% of the progeny in which the PG neurons are ablated die during larval and pupal stages, the remainder are able to eclose after a prolonged developmental period. Although viable, the flies that do eclose have reduced fecundity and likely cannot compete well with wildtype flies for limited resources. The non-viable animals likely die due to the asynchronous expression of 20E-regulated genes (Fig. 7B).

During the extended developmental delay in ablated larvae, the ecdysteroid titer remains very low. However, it eventually rises in white prepupae suggesting that an alternative mechanism for triggering metamorphosis is in place. Consistent with this idea is the observation that extracts prepared from Drosophila ventral ganglia, which should not contain the PG neurons, possess an ecdysteroidogenic activity (Henrich, 1995; Henrich et al., 1987). One likely candidate is an insulin-like peptide. In Lepidoptera, the insulin-like peptide bombyxin was originally identified as a small molecule with PTTH-like activity (Ishizaki and Suzuki, 1994). While the role of bombyxin-like peptides in regulating ecdysteroid levels in Lepidoptera remains unclear, recent evidence from Drosophila points to a role for insulin in regulating ecdysteroid signaling (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005) and developmental timing. These investigators found that increased insulin signaling in the prothoracic gland results in small flies, while reduced insulin signaling produces large flies. Effects on size are likely caused by changes in basal levels of ecdysteroids (Colombani et al. 2005 and Cadwell et al. 2005). Similar to our findings, higher ecdysteroid titers decrease body size by reducing cell number while lower ecdysteroid levels leads to more cells and larger flies.

How insulin-like factors modulate the ecdysteroid level is not entirely clear. However, of note is the observation that the levels of both *dib* and *phm* transcripts were shown to moderately increase in response to activation of insulin signaling (Colombani et al. 2005). Curiously, we do not see an increase in the transcription of *dib, phm nvl*, or *spok* in white prepupae of ablated animals, despite the fact that ecdysteroid titer does rise. Therefore, it does not appear that transcription of these enzymes is the rate-limiting step in ecdysteroid production. It is possible, however, that the transcriptional level of *sad*, which does rise, or some other ecdysteroidogenic

enzyme is rate-limiting. Another possibility is that rate-limiting control is exerted at the level of enzymatic activity and not transcription.

The suggestion that both PTTH and insulin may control ecdysone production via separate pathways is consistent with the finding that manipulating the Ras/Raf pathway in the PGs also affects developmental timing and size. Increased Ras or Raf activity enhanced ecdysteroid levels and resulted in small flies, while expression of dominant negative Ras or Raf lowered ecdysteroid levels, prolonged larval development, and produced large flies (Caldwell et al. 2005). While the mechanism of PTTH signal transduction remains elusive, in part because the receptor has not been identified, it is interesting to note that addition of exogenous PTTH to Manduca PGs leads not only to enhanced Ca^{+} and cAMP signaling, but also to the rapid phosphorylation of ERK (Rybczynski et al., 2001; Rybczynski and Gilbert, 2003). The common phenotypes produced in Drosophila by manipulation of PTTH levels and Ras/Raf suggest that in this organism a major component of the PTTH signal in regulating ecdysone production/release may be accomplished by activation of a MAP kinase cascade. It is likely that the PTTH signal is subsequently integrated with nutritional signals via the insulin pathway (Colombani et al., 2005; Mirth and Riddiford, 2005)) and modulated by prothoracicostatic signals (Yamanaka et al., 2006) to determine developmental timing and final body size. In this respect, insect metamorphosis shows remarkable similarity to mammalian reproductive development in which production and release of the neuropeptide kisspeptin gates the timing of puberty in conjunction with input from nutritional and metabolic sensors (Fernandez-Fernandez et al., 2006; Navarro et al., 2007; Smith and Clarke, 2007).

Other developmental roles for PTTH?

In addition to the role for PG neurons in controlling developmental timing, we also note that PTTH expression continues in a limited number of neurons in the adult brain. Similarly, Manduca PTTH expression is also expressed in the adult brain (Westbrook et al. 1993) but it appears to be the same l-NSCs that innervate the CA. Since the prothoracic gland degenerates during the pupal stage (Dai and Gilbert, 1991), the Drosophila PTTH-positive neurons would have to undergo developmental pruning and rewiring if they are the direct descendants of the larval PG neurons. We do not see any projections from the adult PTTH-positive neurons to the ovaries, suggesting that PTTH does not directly regulate ecdysteroidogenesis in the ovary although it may still act indirectly via the hemolymph. We do note however, that adult males in which PTTH-expressing neurons are ablated exhibit male-on-male courtship behavior. Since ecdysteroid signaling is needed to remodel many neuronal connections during metamorphosis (Lee et al., 2000; Schubiger et al., 1998; Zheng et al., 2003), it is possible that the developmental delay and/or generally low ecdysteroid titers that result from ablation of the PG neurons might alter the axon guidance and connectivity during metamorphosis. Alternatively, the PTTH positive neurons in the adult male brain might directly affect the expression or activity of genes involved in determining courtship rituals. Other functions for PTTH in the adult brain are also possible.

Materials and Methods

Drosophila strains

The transgenic lines carrying UAS- PTTH-HA, *ptth* > Gal4 and genomic *ptth-HA* were generated in a y,w^{1118} background by the CBRC fly core facility (Massachusetts General Hospital) using standard methods. For *ptth*-Gal4, two strong expression lines (45 and 117b) on the third chromosome were recombined to make line *ptth*-Gal4-45, 117b that was used for all experiments. The genomic PTTH-HA-50 line was used for protein localization studies. The UAS-PTTH-HA-92 line was used for ectopic expression studies. UAS-Grim, was a kind gift from J R. Nambu (Wing et al., 1999). The cd8GFP stock was obtained from the Bloomington

Drosophila Stock Center. The *pdf*^{01} allele has been previously described (Renn et al., 1999). For ablation experiments, non-GFP containing first instar larvae were picked from the progeny of a UAS-Grim/Cy^{O}-actGFP; *ptth* > Gal4/TM3, Ser, *actin*-GFP stock.

Molecular and immunologic methods

All molecular methods including cDNA isolation, epitope tagging, Northern blot preparation, RT-PCR and Q-PCR involved the use of standard protocols. Details are provided in the supplementary materials.

Developmental timing analysis

Fertilized eggs were collected on apple juice agar plates. Before actual collections, adults were allowed to lay for 1 hr in order to remove held eggs. Collections were done in two-hour intervals for 6 hours, beginning one hour before lights on. First instar larvae were collected from these plates and put into small 35mm × 10mm petri dishes (Falcon) filled with approximately 2.5ml of standard cornmeal food. Larvae were raised in groups of 10 to prevent crowding. Larvae for all experiments were raised inside an insulated moist chamber at 25°C (lights out) to 28° C (lights on) under a 12hr light/dark cycle. For developmental progression, larvae were scored in two-hour intervals. Stages were determined by floating the larvae in 20% sucrose, and observing spiracle and mouth hook morphology. Pupariation was scored at 1 hr intervals for controls and 12 hr intervals for ablated larvae.

Preparation of larvae for Northern blot analysis and ecdysteroid titers

For Northern blot and ecdysteroid titers, freshly ecdysed 3rd instar larvae were collected every hour, placed in groups of 1–5 animals into 1.5ml Epppendorf tubes punctured by 23-guage needles for aeration, and filled with approximately 400μl of mashed cornmeal food. The time of ecdysis was noted, larvae were aged to the desired time, removed from the food, briefly rinsed and dried, placed in clean tubes, flash frozen in liquid nitrogen, and stored at −70°C.

Statistical analysis

Statistics were done using Statistical Analysis Software® (SAS). Means and standard errors were calculated for each time point. Two-sample t-tests were used to evaluate the results of 20E feeding. For larval developmental progression, ANOVA followed by pairwise comparisons were performed. P-values for each time point were chosen based on whether the variances were equal or unequal. Significance level (0.5/3 = 0.0167) was chosen using Bonferonni's adjustment to correct for multiple hypothesis testing.

20E feeding

To make 20E food, dried baker's yeast was added to 450 μl of sterile water and mixed to the consistency of creamy frosting. 50 μl of a 20E stock (10mg/ml 20E (Sigma) in 95% ethanol) solution was added, along with 1000 μl of mashed cornmeal foodand mixed throughly. This gave a final concentration of 0.33mg/ml of 20E and approximately 3% ethanol. For control food, 50 μl of 95% ethanol was used rather than 20E stock. Third instar larvae that had ecdysed within 6 hr of each other were added to the freshly made plates in groups of 10 animals, and allowed to feed at will.

Whole body larval ecdysteroid titers

Every 12 or 24 hr during the 3rd instar, selected groups of larvae (10) were collected in 1.5 ml plastic tubes and kept at −80°C prior to processing. They were homogenized in ethanol, sonicated at medium power and centrifuged at maximum speed. The solids were then repeatedly extracted with methanol, the solvents pooled, evaporated under low pressure and

the residues subjected to radioimmunoassay employing the H22 antisera (Warren et al., 2006).

Determination of critical weight

Critical weights were determined as described in Mirth et al. (2005) with minor modifications. Larvae were cultured at 25°C under a constant-light regime. Newly ecdysed third instar larvae were collected every hour. Collected larvae were placed on a 35 × 10 mm plate filled with standard cornmeal/yeast extract/dextrose medium for a designed time, then were weighed and transferred to same-sized plate filled with a medium of 2% agar in water. The larvae were then starved and pupariation was scored.

Supplementary Material

Refer to Web version on PubMed Central for supplementary material.

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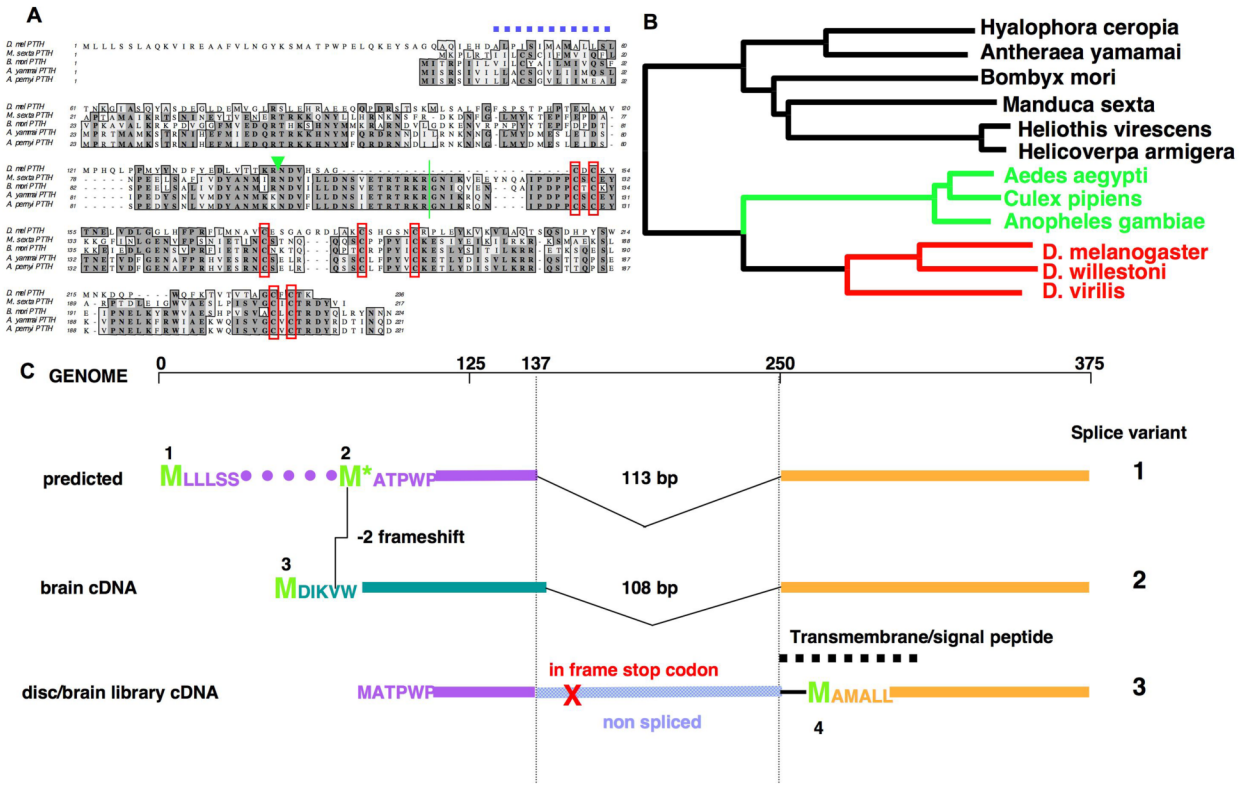


Figure 1.

Drosophila PTTH sequence, phylogeny and gene structure (A) Alignment of the *D. melanogaster* PTTH amino acid sequence to the indicated Lepidoptera species using the Clustal W alignment tool in MacVector ™. The dashed blue over line indicates a hydrophobic segment that likely serves as a signal peptide. The green arrowhead is positioned after a dibasic sequence that might serve as a maturation site for Drosophila PTTH. A green line is positioned at the maturation site of the Lepidoptera PTTH sequences. The red boxes indicated conserved cysteine residues. (B) Dendogram generated using the Clustal W tool of MacVector for several insect PTTH genes. Black lines Lepidoptera, green mosquito, and red Drosophilae. (C) The 5′ end gene structure of of various predicted and isolated Drosophila PTTH cDNAs. The numbers on top are base pairs starting at the first predicted methionine residue. The Flybase annotation of CG13687 starts at methionine 2 of the predicted sequence but the frame is open upstream for another 24 AA to a second potential start codon (M1). Line two shows the structure of a cDNA isolated by RT-PCR from third instar brain. It uses a different splice donor site such that the upstream sequence is in the -2 frame relative to the Flybase sequence and would use methionine 3 as a start. The third line indicates the structure of a cDNA isolated from a disc/brain library (Brown et al. 1988). In this case, the first intron is retained and the first in frame methionine (M4) is positioned downstream of the first intron splice acceptor site.

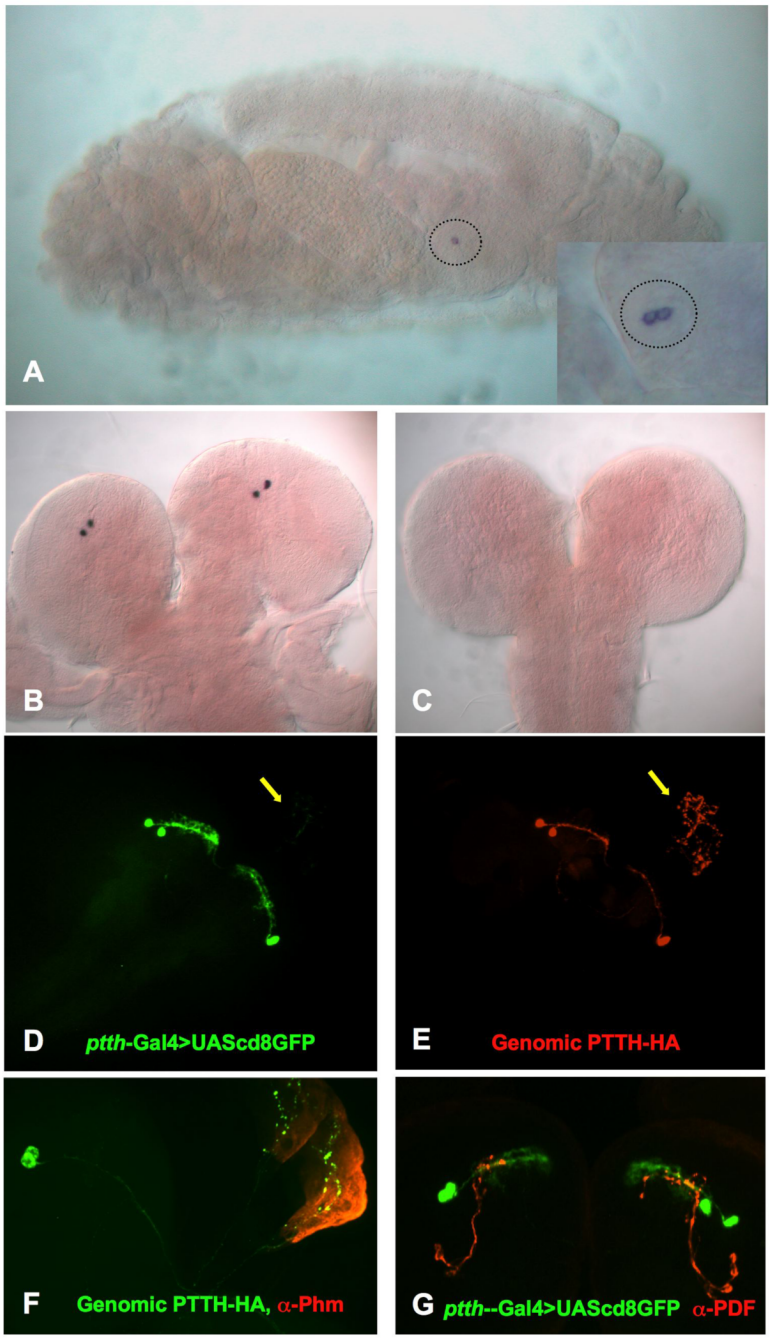


Figure 2.

In situ hybridization of an antisense *ptth* RNA probe to a stage 17 embryo (A) and wandering third instar brain (B). The black circle in A indicates the position of two positive neurons (enlarged in the inset) that correspond to one of the neuron pairs shown in B. (C) No staining is seen in a third instar brain hybridized with a sense control probe. (D) The dendritic arbors and axon terminals (yellow arrow) of the PG neurons are highlighted using *ptth*-Gal4 to drive expression of a membrane-bound UAS- cd8GFP reporter. (E) Immunolocalization of PTTH using an HA-tagged genomic transgene. The yellow arrow indicates synaptic connections on the prothoracic gland. (F) Immunolocalization of PTTH-HA (green) and the ecdysone

biosynthetic enzyme Phm (red) in the prothoracic gland. (G) Axons from the PDF-producing neurons (red) contact the dendritic arbors of PTTH-producing neurons.

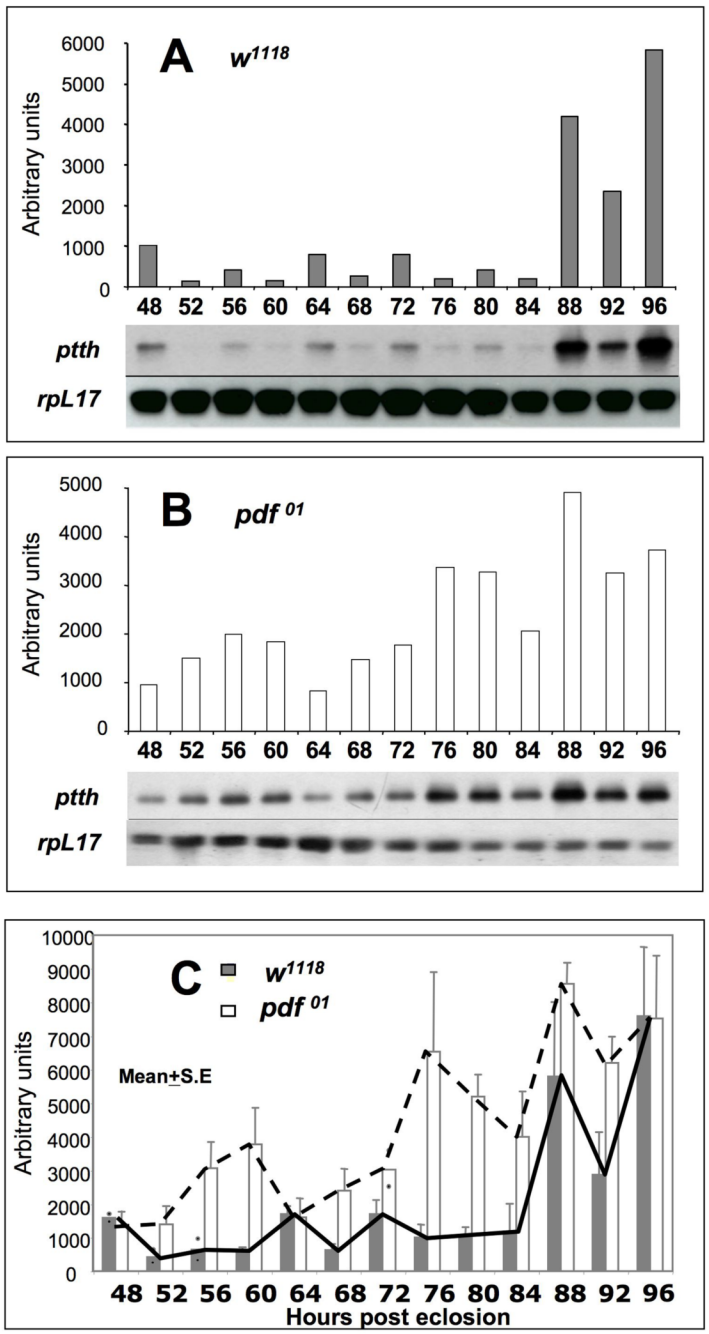
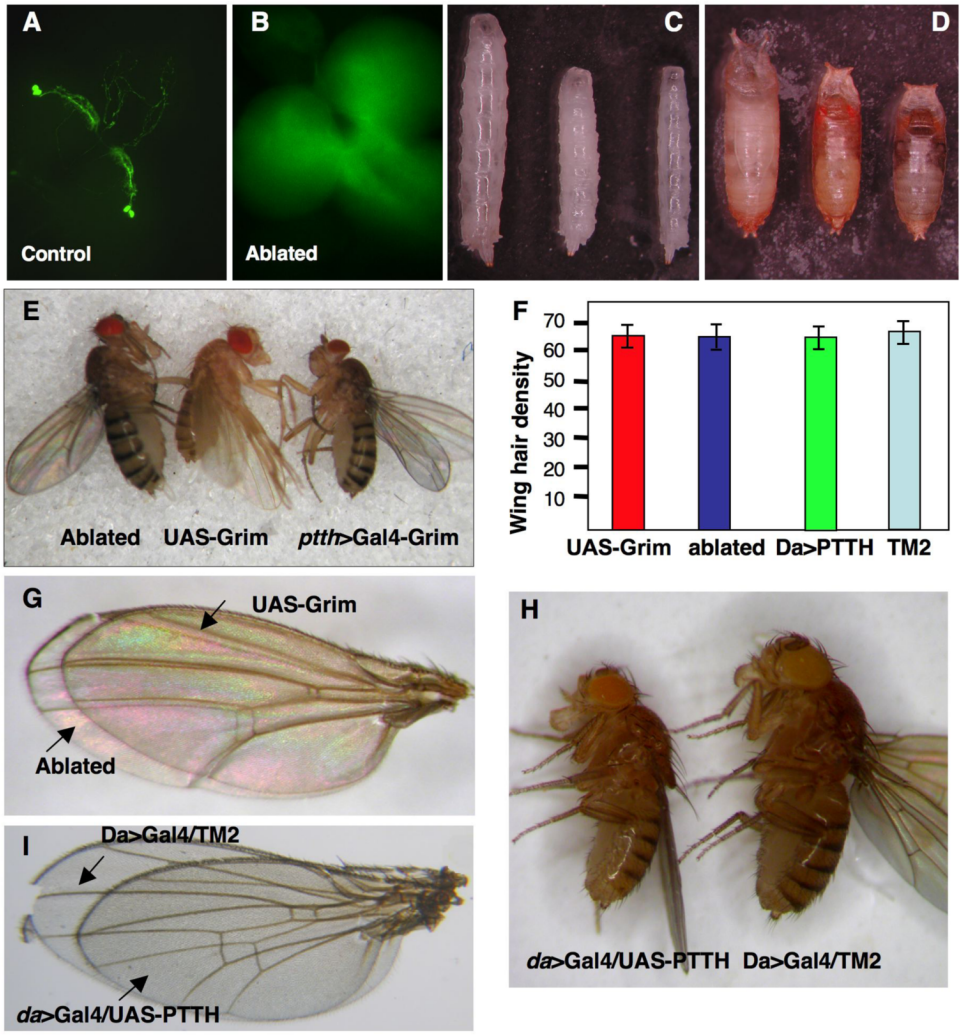


Figure 3.

Transcriptional profile of Drosophila *ptth* in wildtype y,w^{1118} and in pdf^{01} mutant as determined by semi-quantitative RT-PCR (A) Representative profile of *ptth* transcription in wild type animals. (B) Representative profile of *ptth* expression in pdf^{01} mutant. Top panels show the quantitative evaluation of *ptth* transcript levels using Scion Image software and the bottom panels the chemiluminescent detection... RPL17 transcription serves as an internal control. (C) Variations of *ptth* expression in wild type flies and pdf^{01} mutants. Mean values over three independent series of larvae were calculated. For each graph, the time of development is expressed in hours post ecolsion and intensity is given in arbitrary units. In C the dashed line and solid lines indicate the means of the pdf and wt measurements respectively.



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	Pupal Length (mm)		Adult Weight (mg)	
	Females	Males	Females	Males
Ablated	4.27 ± 0.13 (n=11)	3.81 ± 0.19 (n=6)	1.77 (n=35)	1.31 (n=35)
ptth>Gal4	3.51 ± 0.19 (n=10)	3.29 ± 0.10 (n=4)	1.17 (n=63)	0.80 (n=89)
UAS-grim	3.23 ± 0.11 (n=9)	3.02 ± 0.10 (n=6)	1.11 (n=92)	0.76 (n=75)
		da>Gal4/TM2	1.10 (n=64)	0.67 (n=48)
		da>Gal4/UAS-PTTH	0.88 (n=57))	0.54 (n=49)

Figure 4.

Loss of PG neurons generates large flies while ectopic PTTH leads to small flies. (A) Expression of *ptth* > Gal4/UAS-cd8GFP in the PG neurons of a wandering third instar larva (B) Coexpression of UAS-Grim (2X) with *ptth*-Gal4 (2X) results in loss of the PG neurons (no UAS-cd8GFP staining in a brain from a wandering third instar). (C–E) Crawling third instar larva, pupa and adult females, respectively, produced by ablation of PG neurons (left animal) or from control lines containing UAS-*grim* alone (middle animal) or *ptth*-Gal4 alone (right animal). (F) Wing hair density in a 100 μ^2 square section (n = 9 wings) located on the dorsal surface adjacent to the posterior crossvein (error bars SEM). (G) Overlay of wings produced from a PG neuron-ablated fly or a fly containing the UAS-Grim construct alone with no driver.

(H) Over-expression of PTTH produces small flies compared to expression of Gal4 alone. (I) Overlay of a wing produced by overexpression of PTTH compared to a wing produced by the driver alone. (J) Pupal lengths and adult weights of animals with the indicated genotypes. Photographing individual pupa and measuring pixel number compared to a calibrated standard determined pupal lengths. Adult flies were weighed in batches of 10–30 flies and the average weight per fly determined (error is SEM).

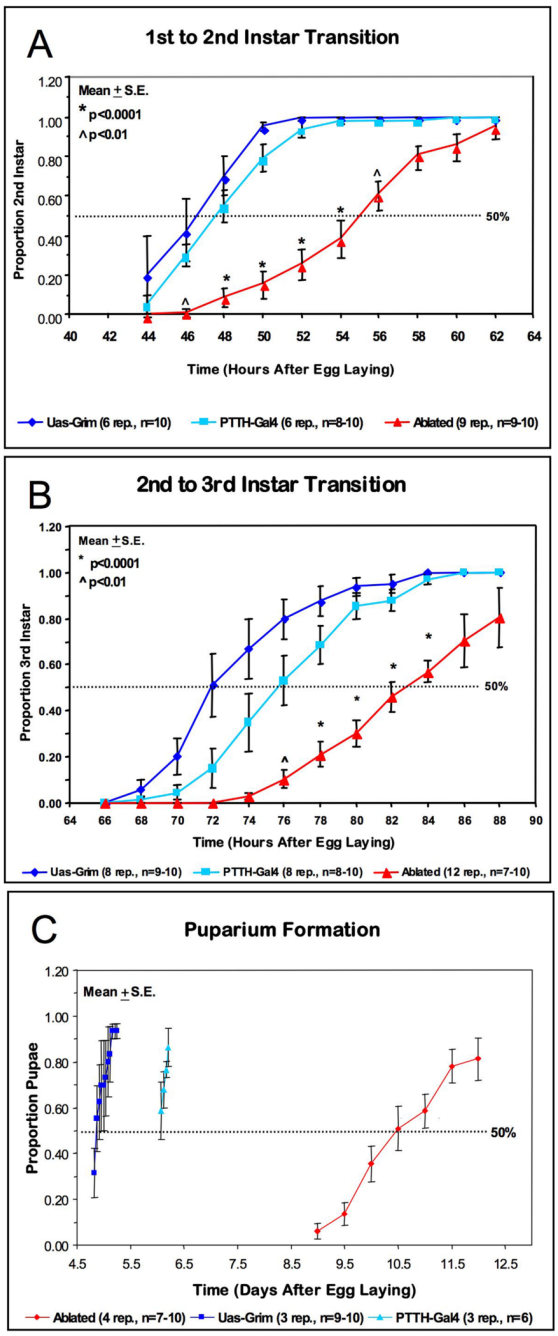

Figure 5.

Ablation of PG neurons produces developmental delay. (A) The percentage of larvae of the indicated genotype that had ecdysed to the 2nd instar stage (A) third instar stage (B) or undergone pupariation (C) are plotted relative to the time in hours after egg laying (AEL). Dark blue is UAS-Grim, turquoise is *ptth*-gal4 and red is ablated larvae. N = number of repetitions containing 8–10 larvae per sample. Results are expressed as mean ±-standard error of the mean.

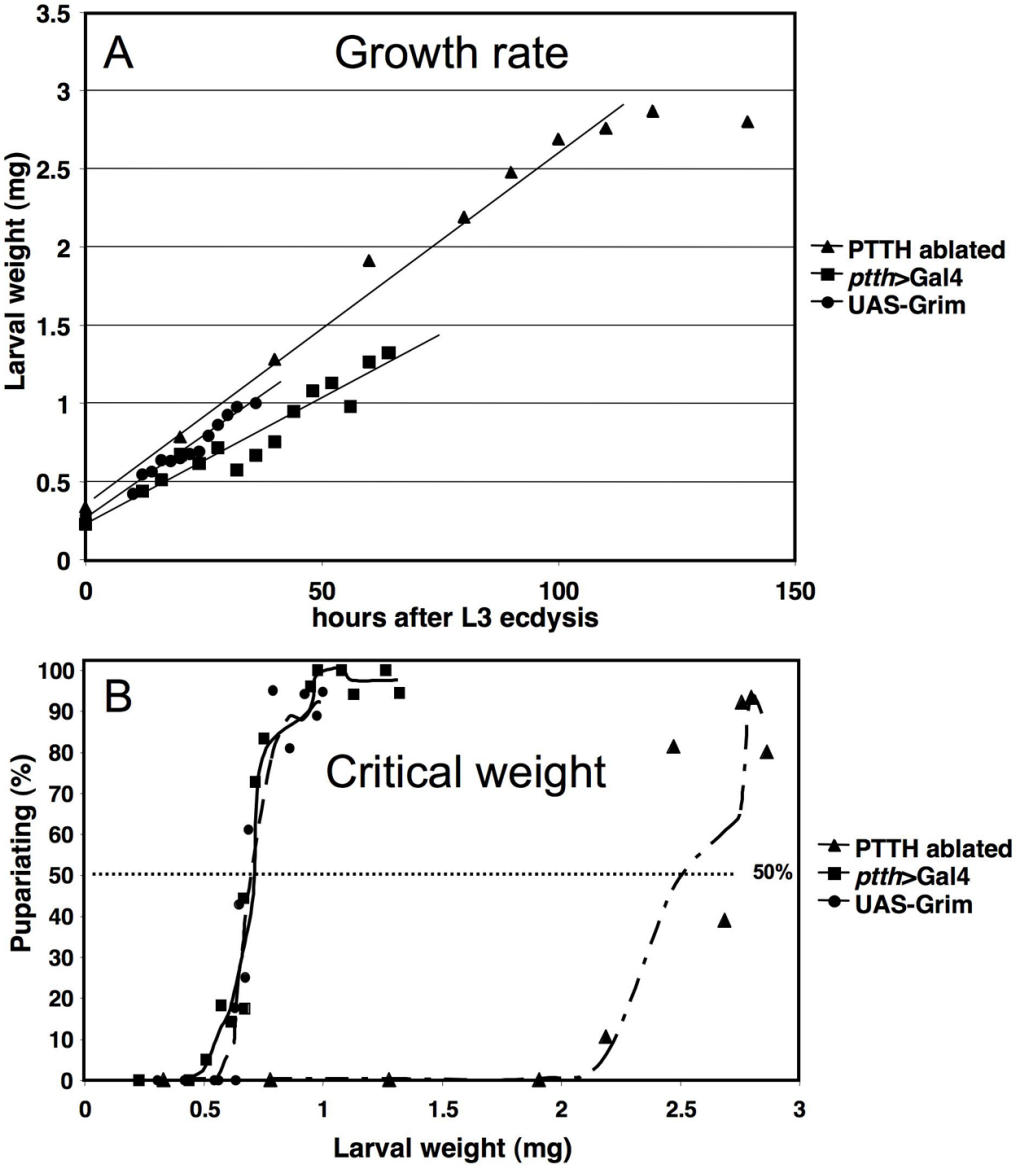

Figure 6.

Ablation of PG neurons results in a greater critical weight but no change in growth rates. (A) Percent of larvae that underwent pupariation after starvation at a given size after ecdysis to third instar (L3) for PTTH ablated larvae (*ptth* > Grim), *ptth* > Gal4 larvae and UAS-Grim larvae (*N* = 15–36 individuals at each data point). The critical weights that correspond to 50% threshold for pupariation were 2.5 mg, 0.72 and 0.71 for PTTH ablated larvae, *ptth* > Gal4 larvae and UAS-Grim larvae, respectively. (B) Plot of weight at a given time after L3 ecdysis for PTTH ablated larvae (*ptth* > Grim), *ptth* > Gal4 larvae and UAS-Grim larvae (*N* = 15–36 individuals at each data point).

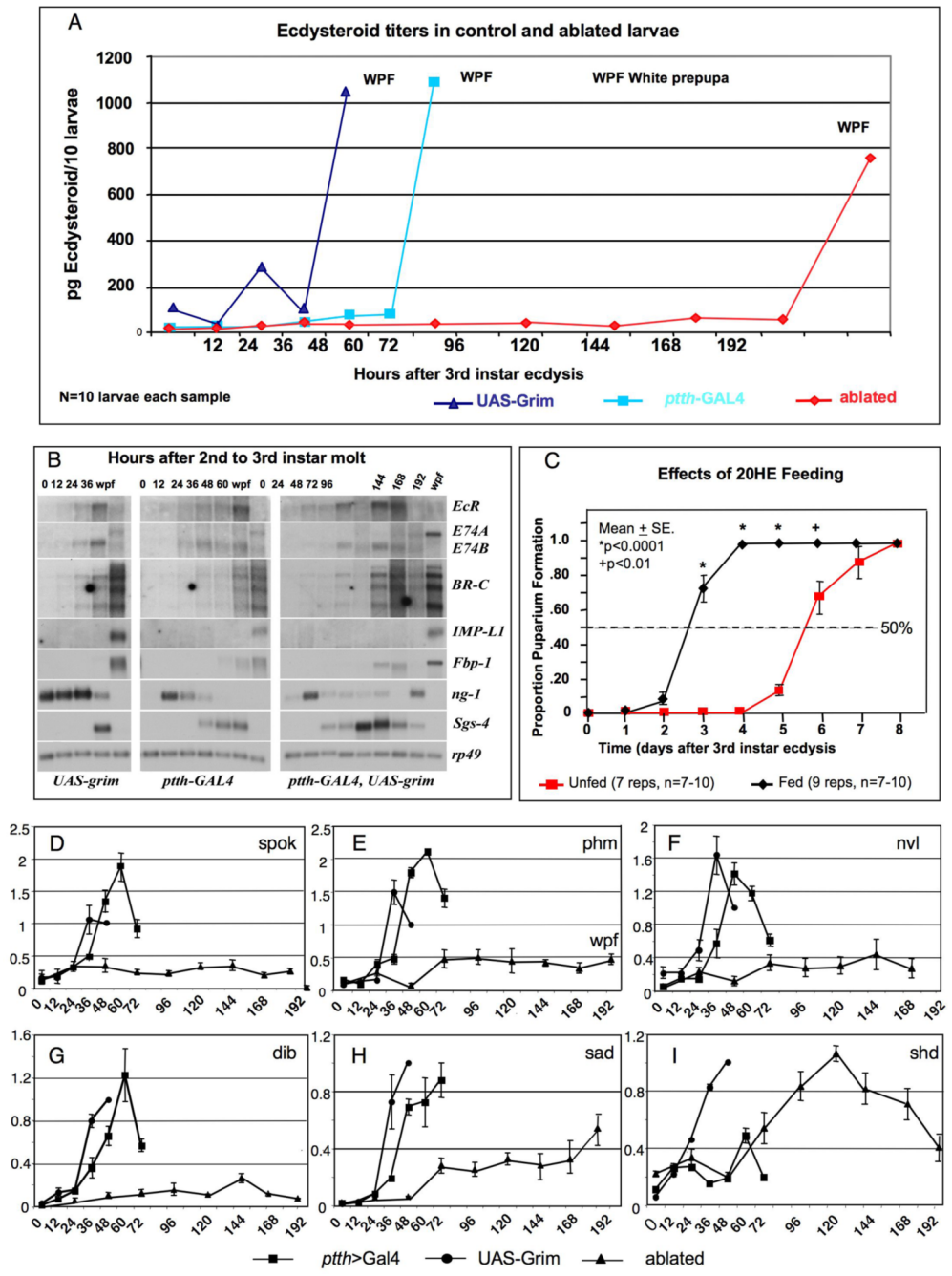

Figure 7.

Ablation of PG neurons results in a delayed rise in ecdysteroid titers (A) that is rescued by feeding larvae 20E (B), and produces an asynchronous developmental transcriptional profile (C) and low the transcription of several ecdysone biosynthetic enzymes prior to metamorphosis (D,E). (A) Ecdysteroid titer in pg/10 larvae plotted against time after ecdysis to third instar. (B) Larvae in which the PG neurons were ablated were fed food containing 0.33mg/ml 20E or control food and the time to pupariation measured. (C) Temporal profiles of 20E-regulated transcription in *UAS-grim*, *ptth-GAL4*, and *ptth-GAL4;UAS-grim* animals. Total RNA was isolated from animals staged in hours after the L2 to L3 molt. *UAS-grim* 48 hour, *ptth-GAL4* 96 hour, and *ptth-GAL4; UAS-grim* 192 hour animals were isolated as newly formed prepupae.

Hybridizations were performed to detect the *EcR*, *E74*, and *BR-C* early 20E-inducible mRNAs, as well as tissue-specific genes, *IMP-L1*, *Fbp-1*, *ng-1*, and *Sgs-4*. *rp49* was included as a control for loading and transfer. (D-I) Q-PCR analysis of the transcriptional levels of the indicated genes involved in ecdysteroid biosynthesis for PTTH ablated larvae (*ptth* > Grim), *ptth-Gal4* larvae and UAS-Grim larvae (mean ± SEM, $n = 3$). The levels were normalized to ribosomal protein rpL23 transcriptional levels in the same samples.