Ecdysone-dependent feedback regulation of prothoracicotropic hormone controls the timing of developmental maturation

Christensen, Christian F.; Koyama, Takashi; Nagy, Stanislav; Danielsen, E. Thomas; Texada, Michael J.; Halberg, Kenneth A.; Rewitz, Kim

Published in:
Development

DOI:
10.1242/dev.188110

Publication date:
2020

Document version
Publisher's PDF, also known as Version of record

Document license:
CC BY

Citation for published version (APA):
RESEARCH ARTICLE

Ecdysone-dependent feedback regulation of prothoracicotropic hormone controls the timing of developmental maturation

Christian F. Christensen, Takashi Koyama, Stanislav Nagy, E. Thomas Danielsen, Michael J. Texada, Kenneth A. Halberg and Kim Rewitz*

ABSTRACT

The activation of a neuroendocrine system that induces a surge in steroid production is a conserved initiating event of the juvenile-to-adult transition in many animals. The trigger for maturation is the secretion of brain-derived neuropeptides, yet the mechanisms controlling the timely onset of this event remain ill-defined. Here, we show that a regulatory feedback circuit controlling the Drosophila neuropeptide Prothoracicotropic hormone (PTTH) triggers maturation onset. We identify the Ecdysone Receptor (EcR) in the PTTH-expressing neurons (PTTHn) as a regulator of developmental maturation onset. Loss of EcR in these PTTHn impairs PTTH signaling, which delays maturation. We find that the steroid ecdysone dose-dependently affects Pth transcription, promoting its expression at lower concentrations and inhibiting it at higher concentrations. Our findings indicate the existence of a feedback circuit in which rising ecdysone levels trigger, via EcR activity in the PTTHn, the PTTH surge that generates the maturation-inducing ecdysone peak toward the end of larval development. Because steroid feedback is also known to control the vertebrate maturation-inducing hypothalamic-pituitary-gonadal axis, our findings suggest an overall conservation of the feedback-regulatory neuroendocrine circuitry that controls the timing of maturation initiation.

KEY WORDS: Drosophila, Ecdysone, Maturation, Prothoracicotropic, Ptth, Steroid

INTRODUCTION

The activation of a neuroendocrine axis leading to the production of steroid hormones is a conserved trigger of maturation onset in animals (Rewitz et al., 2013; Sisk and Foster, 2004; Tennessen and Thummel, 2011). In vertebrates, neuronal gonadotropin-releasing hormone (GnRH) secretion awakens the hypothalamic-pituitary-thyroid axis, our findings suggest an overall conservation of the feedback-regulatory neuroendocrine circuitry that controls the timing of maturation initiation.

© 2020. Published by The Company of Biologists Ltd| Development (2020) 147, dev188110. doi:10.1242/dev.188110

Handling Editor: Cassandra Ettavour
Received 10 January 2020; Accepted 26 June 2020

This is an Open Access article distributed under the terms of the Creative Commons Attribution License (https://creativecommons.org/licenses/by/4.0/), which permits unrestricted use, distribution and reproduction in any medium provided that the original work is properly attributed.
genetic programs underlying the juvenile-to-adult transition (Moeller et al., 2013). However, whether feedback also regulates the neurocircuitry that triggers these steroid pulses in animals, and thus the overall timing of the juvenile-to-adult transition, is presently unknown.

We report here the identification of EcR and usp in an RNAi-based screen for regulators of PTTH production or release. We find that ecdysone-mediated feedback via EcR in the PTTHn drives the pupariation-triggering PTTH surge, thereby determining the timing of maturation. Under rising ecdysone levels, EcR mediates Pth transcriptional upregulation, leading to the steep rise in PTTH prior to metamorphosis. This generates a PTTH surge that induces a high-level ecdysone peak that initiates maturation; high ecdysone levels subsequently feed back negatively to suppress PTTH production. Mammalian gonadal steroids act in positive and negative feedback loops to modulate the HPG axis (Acevedo-Rodriguez et al., 2018). Our results show that the developmental transition to adulthood in Drosophila is similarly controlled by positive and negative feedback mechanisms that modulate the PTTHn, suggesting that neuroendocrine feedback control of developmental maturation is evolutionarily ancient.

RESULTS

EcR induces maturation onset through positive regulation of the PTTHn

PTTH release is thought to be the main trigger of the juvenile-adult transition in insects (Rewitz et al., 2013). Loss of PTTH extends the larval period of feeding and growth, leading to increased pupal and adult size. To identify signals controlling maturation onset, upstream of PTTH, we performed an RNAi-based pupal-size screen targeting 608 membrane-associated proteins (known or potential receptors) and transcription factors in the PTTHn (Fig. 1A, Table S1). In this screen, expression of RNAi constructs (along with the RNA-processing enzyme Dicer-2) was driven in the PTTHn by NP423-GAL4 (NP432>) (Deveci et al., 2019; Gong et al., 2010; Yamanaka et al., 2013). Among the strongest hits was EcR: knockdown of this gene, or of usp, which encodes a transcriptional co-factor of EcR, in the PTTHn led to larval overgrowth and thus to increased pupal size (Fig. 1B). To verify these findings, we reduced EcR expression in the larval PTTHn and measured developmental timing and final body size. Knockdown using two independent RNAi lines (#1 and #2) confirmed that loss of EcR in the PTTHn causes developmental delay and overgrowth, like reduced expression of Pth itself (Fig. 1C-E). This suggested that EcR might act in the PTTHn as a positive regulator of PTTH production and release.

Next, we used the Pth-GAL4 (Pth>) driver, a weaker but PTTHn-specific line, to confirm that these effects were caused by knockdown of EcR in the PTTHn. Because of Pth-GAL4’s relative weakness, we recombined EcR-RNAi constructs #1 and #2 to increase the strength of RNAi-mediated knockdown. Animals expressing Pth>EcR-RNAi-1 +2 exhibited delayed pupariation and increased pupal size comparable to knockdown of Pth itself, and we re-confirmed these effects using a third independent EcR-RNAi line, #3 (Fig. 2A,B). All further EcR-RNAi experiments used RNAi lines #1 and #2 combined. To further attribute these defects to PTTHn-specific EcR deficiency, we disrupted the EcR gene in only these cells using tissue-specific somatic CRISPR/Cas9 gene editing. We generated a transgenic UAS-regulated construct that expresses a pair of guide RNAs (gRNAs) targeting exon 3 of the EcR gene, which encodes the DNA-binding domain of EcR and is shared between all its isoforms. By expressing this construct along with UAS-Cas9 under Pth> control (at 29°C to boost the activity of the GAL4 transcription factor and the Cas9 enzyme), we disrupted the EcR locus specifically in the PTTHn. To assess the efficacy of this setup, we immunostained brains from larvae at 96 h AEL (after egg laying) using antibodies against the PTTH neuropeptide, EcR, and the ecdysone-biosynthetic enzyme Phantom (Phm) to label the PG (Rewitz et al., 2006; Warren et al., 2004). No anti-EcR signal was visible in the PTTHn of knockout animals, whereas clear
anti-EcR signal was present in the PTTHn nuclei of controls, and in neighboring cells in both controls and EcR knockouts (Fig. 2C,D). Although the CRISPR/Cas9 system has induced cytotoxicity in some reports, we did not observe any morphological abnormalities in the PTTHn or their projections to the PG. Next, to investigate how this manipulation affected PTTHn activity, we measured developmental timing and final body size. PTTHn-specific EcR disruption significantly delayed pupariation and resulted in larger pupae, compared with controls (Fig. 2E,F). Furthermore, PTTH levels (anti-PTTH staining intensity in the PTTHn cell body) were significantly reduced in EcR knockouts (Fig. 2G). Together, these data support a role for EcR as an inducer of PTTH expression. We therefore next overexpressed EcR.A or EcR.B1 variants resulted in advanced pupariation onset, thereby shortening the larval growth period and reducing pupal size (Fig. 2B,H), consistent with a positive effect of EcR on PTTHn activity.

**EcR induces the PTTH surge prior to the juvenile-adult transition**

Because EcR induces pupariation through its actions in the PTTHn, we investigated EcR levels in these cells during the juvenile-adult transition. We found that EcR is present in PTTHn nuclei from early L3 (80 h AEL) throughout the L3 stage, increasing in abundance at the onset of the wandering stage (112 h AEL), when ecdysone levels rise prior to pupariation (Fig. 3A). Knockdown of EcR in the PTTHn attenuated this EcR rise, confirming that EcR expression is reduced by the RNAi, albeit not eliminated as with CRISPR-induced deletion (Fig. 2C,D).
As Pth is transcriptionally upregulated to induce the ecdysone pulse that triggers maturation (McBrayer et al., 2007), we analyzed whether EcR is required in the PTTHn for this upregulation. Anti-PTTH immunostaining intensity in cell bodies of the PTTHn displayed a dynamic profile in control animals, with a strong increase at 112 h AEL that coincided with the increase in EcR abundance (Fig. 3B), consistent with EcR being responsible for PTTH upregulation at this time. PTTHn-specific EcR knockdown markedly changed this pattern, eliminating the increase in PTTH intensity at 112 and 116 h AEL observed in control animals. Although a reduction in PTTH staining could potentially be explained by increased secretion, this would be inconsistent with these animals’ delayed development. Thus, this altered profile suggests that EcR positively regulates PTTH production, consistent with the results showing that EcR expression in the PTTHn correlates with pupariation advance. Because EcR is a transcriptional regulator, we asked whether EcR is required for transcriptional upregulation of Pth during the late L3 stage by performing a temporal gene-expression analysis of larvae expressing EcR-RNAi in the PTTHn throughout the second half of L3. Consistent with previous findings (McBrayer et al., 2007), in control animals, Pth expression underwent a dramatic upregulation from about 12 h prior to pupariation (112-124 h AEL) until the onset of metamorphosis (Fig. 3C). EcR knockdown significantly reduced Pth expression across the time series and prevented its pre-wandering enhancement, indicating that EcR is required for the late-larval transcriptional upregulation of Pth.

Loss of EcR in the PTTHn impairs the steroid increase that triggers maturation

PTTH signaling stimulates the production of ecdysone in the PG by inducing transcriptional upregulation of the ecdysone-biosynthetic
Halloween genes (McBrayer et al., 2007; Shimell et al., 2018). To examine the effects of EcR in the PTTHn on ecdysone production, we first analyzed the expression of these genes in the PG. Expression of spookier, phantom, disembodied and shadow are all upregulated in the PG in response to PTTH/Torso signaling. The enzyme Shade mediates the conversion of ecdysone to 20E in peripheral tissues, and its expression is therefore not regulated by PTTH signaling (McBrayer et al., 2007; Ou et al., 2016). Consistent with the requirement of EcR for inducing PTTH production, we found that the ecdysone-synthesis genes were not properly upregulated in Ptth>EcR-RNAi animals, whereas levels of shade were unaffected (Fig. S1A-E). This is consistent with a model in which the delay observed in EcR-knockdown animals is caused by a failure to upregulate PTTH towards the end of larval development, thus impairing the ecdysone biosynthetic pathway. To determine whether this is the case, we analyzed ecdysone levels directly by enzyme-linked immunosorbent assay (ELISA) and found reduced levels of ecdysone in animals with EcR knockdown in the PTTH towards the end of larval development (Fig. 3D), indicating that they do not produce a proper maturation-promoting ecdysone pulse. As a result, expression of the ecdysone-inducible genes E75A and E75B, which serve as proxies for the ecdysone titer, show a significantly reduced and delayed rise in Ptth>EcR-RNAi animals (Fig. 3E, Fig. S2). These data suggest that EcR stimulates Ptth transcription to generate the PTTH surge that initiates metamorphosis. We therefore examined whether the developmental-delay phenotype caused by knockdown of EcR in the PTTHN could be rescued by simultaneous overexpression of Ptth. Indeed, Ptth overexpression in the PTTHNs of animals with CRISPR/Cas9-mediated knockdown of EcR in these same neurons partially rescued the delayed pupariation and completely rescued their overgrowth, showing that the phenotype is caused by lack of PTTH (Fig. 3F,G). Together, our results suggest that EcR acts as a positive regulator of Ptth expression, just as steroids modulate the mammalian HPG axis, and that feedback through the steroid ecdysone is a key trigger of the neuroendocrine cascade that drives maturation onset in Drosophila.

**Initiation of maturation is triggered by feedback that activates PTTH**

EcR and 20E sit atop a large network of transcriptional regulators (King-Jones and Thummel, 2005). Our results show that EcR is required in the PTTHn for Ptth upregulation, suggesting a model in which PTTH or other factors induce a small ecdysone rise that acts via EcR in the PTTHN to increase PTTH production, leading to the activation of the metamorphosis-initiating neuroendocrine cascade. In this model, *in vivo* ecdysone manipulations should feed back to affect PTTH levels. Thus, we reduced ecdysone synthesis by silencing torso, which encodes the PTTH receptor, in the PG [PG-specific phm-GAL4 (phm>) driving UAS-torso-RNAi (torso-RNAi): phm>torso-RNAi]. As ecdysone synthesis is downregulated in these animals, their larval feeding stage was prolonged, resulting in overgrowth and increased pupal size (Fig. S3A). Next, we measured Ptth levels during L3 prior to the onset of pupariation. In controls, Ptth expression increased as expected from 96 h AEL to 120 h AEL (Fig. 4A). By contrast, when ecdysone synthesis was reduced via phm>torso-RNAi, Ptth expression remained uninduced, even when these animals eventually pupariated (at 189 h AEL). Expression of the PG-specific ecdysone-biosynthetic gene disembodied and the ecdysone-induced gene E75B remained low in phm>torso-RNAi animals, confirming reduced ecdysone synthesis (Fig. S3B,C). Thus, blocking ecdysone synthesis in the PG prevents the pre-metamorphic upregulation of Ptth.

**DISCUSSION**

EcR-mediated feedback induces developmental maturation by triggering PTTH neuronal activity

The activation of a neuroendocrine signaling cascade triggers maturation onset in most animals. This activation is associated with body-size gating to ensure the fitness of the reproductive adult. In insects, attainment of ‘critical weight’ during the last larval instar is the main such checkpoint gating the transition to adulthood (Mirth and Riddiford, 2007). After this checkpoint, a larva becomes committed to maturing on a fixed schedule irrespective of further nutrition. Thus, critical weight likely reflects energy stores sufficient to survive the non-feeding maturation process (metamorphosis) and obtain a final adult body size that maximizes fitness (Rewitz et al., 2013). Nutritional status is likewise a main factor permitting the entry into maturation in mammals (Mirth and Riddiford, 2007; Navarro et al., 2007). In humans, body weight correlates with the timing of menarche, which led to the use of the term ‘critical weight’ for the onset of reproductive cycles in humans (Frish and Revelle, 1971). Obese children enter puberty earlier than height-matched non-obese children, and malnutrition and lack of body fat can lead to delayed puberty (Kaplowitz, 2008; Soliman et al., 2014). These observations suggest that the maturation gate reflects not body size per se but rather the amount of body fat, and thus that the neuroendocrine system controlling the timing of this process somehow assesses nutritional and energetic stores. Interestingly, we next dissected larval brains, preserving PTTHN projections to the ring gland, which contains the PG, and cultured them *ex vivo* for 6 h in media containing 20E. Consistent with our model, 20E at 5 ng/ml (in the low physiological concentration range occurring during L3, prior to the large pre-metamorphosis pulse) increased Ptth expression (Fig. 4B). This suggests that pre-pulse ecdysone levels suffice to awaken the neuroendocrine system through Ptth upregulation, which then induces the steroid pulse initiating maturation. Interestingly, higher concentrations of 20E inhibited Ptth transcription, inconsistent with a solely positive role for 20E/ EcR. Ptth-inhibitory 20E concentrations (500 ng/ml) correspond to high physiological levels that occur during the large pupariation-associated ecdysone pulse. Consistent with this, these concentrations induced E75B upregulation (Fig. 4C), which occurs during this maturation-inducing pulse. We further tested the potential of negative feedback downregulation of PTTH following pupariation when the ecdysone titer falls rapidly. To do this, we induced RNAi-mediated knockdown of EcR 10 h before pupariation, at a time when Ptth expression has already been upregulated, to prevent interference with the positive effect of EcR on the pupariation-triggering rise in Ptth expression. This manipulation led to increased Ptth expression 3 h following pupariation (Fig. 4D), suggesting that EcR is required following pupariation to downregulate Ptth transcription to suppress ecdysone production. Consistent with this, these animals exhibited ecdysone levels similar to (or perhaps lower than) those of controls at pupariation, as reflected by E75A transcription, but they displayed increased E75A expression 3 h later, potentially because of a failure to downregulate ecdysone production (Fig. 4E; note that in Fig. S2, E75A expression was delayed when EcR was knocked down constitutively, suggesting that at pupariation in the temperature-shift experiment, EcR-RNAi had not had time to affect EcR levels). These data are consistent with a model in which an initial small 20E rise during L3 triggers a positive feedback circuit that generates the metamorphic PTTH/ecdysone surge. Surge-level 20E then suppresses PTTH production following pupariation (Fig. 4F).
the adipokine leptin regulates pubertal maturation in mammals (Shalitin and Phillip, 2003). Circulating leptin levels correlate with adiposity, and leptin-deficient humans and mice fail to undergo puberty. Leptin may therefore communicate body-fat levels to the neuroendocrine system controlling puberty, which could explain the link between childhood obesity and early onset of puberty. In insect larvae, the fat body is the main nutrient-storage and -sensing organ, releasing numerous nutrient-dependent insulin-regulating hormones (Boulan et al., 2015). Insulin is a stimulator of ecdysone production, thus coupling adipose-tissue nutrient sensing to the neuroendocrine maturation axis in Drosophila (Colombani et al., 2005; Mirth et al., 2005). Among the insulinotropic adipokines is Unpaired 2 (Upd2), which is structurally and functionally similar to human leptin. Upd2 acts through the JAK/STAT receptor Domeless (Dome) in GABAergic neurons that regulate insulin secretion from the insulin-producing cells (IPCs) in the brain, which are the primary source of circulating insulin (Rajan and Perrimon, 2012). Thus, related adiposity hormones that signal nutrition and energy storage influence the neuroendocrine events that lead to the onset of maturation in divergent systems.

Mammalian GnRH-producing neurons regulate the timing of puberty onset, and these cells are activated by the neuropeptide KISS1. The PTTHn, activated by the KISS1 ortholog AstA and its receptor AstA-R1 (Deveci et al., 2019), serve this function in Drosophila. This suggests conservation of the overall neuroendocrine architecture of the maturation-initiation system. AstA is regulated by nutritional intake, providing another potential link between energy status and maturation onset (Hentze et al., 2015). Furthermore, PTTHn-specific knockdown of Insulin receptor (InR) or Dome, encoding the Upd2 receptor, produced size phenotypes in our screen (Table S1), suggesting that the PTTHn integrate systemic nutrition-regulated signals and may also receive input via insulin from the IPCs themselves. Because PTTH controls developmental timing, and insulin is the main growth-regulatory factor, these results suggest that Upd2 may link growth and maturation by coordinating the activity of both the IPCs and the PTTHn. Knockdown of the amino-acid transporters Polyphemus and Minidiscs also induced strong growth effects in our screen, suggesting that the PTTHn may also sense nutrient status autonomously; in the IPCs, Minidiscs is required for inducing insulin secretion after intake of the amino acid leucine (Manière et al., 2016).

This raises the key question of how these nutritional cues lead to the surge mode of GnRH/PTTH release that initiates maturation. Our findings suggest that ecdysone feedback, via EcR in the PTTHn, is the mechanism that induces the PTTH and ecdysone surge towards the end of larval development. This is further

---

Fig. 4. Ptth is upregulated by ecdysone-mediated feedback at the onset of maturation. (A) The larval peak of Ptth expression observed in the control genotype does not appear in phm>torso-RNAi animals, indicating feedback from ecdysone produced by the PG to the PTTHn. (B,C) Expression of (B) Ptth and (C) the EcR-regulated proxy gene E75B increases in ex vivo cultured brains with increasing concentration of 20E in the medium, indicating biphasicity of ecdysone response at the Ptth locus. (D) RNAi-induced knockdown of EcR beginning 10 h before pupariation leads to increased Ptth transcription 3 h post-pupariation, consistent with a lack of EcR-mediated inhibition. (E) Levels of the ecdysone-induced transcript E75A are higher at the time of pupariation in temperature-induced EcR-knockdown animals than in controls, but after 3 h, E75A levels have fallen to a lower level in these animals than in controls, suggesting increased or prolonged ecdysone levels, consistent with loss of EcR-mediated Ptth inhibition. Colors in E are the same as in D. Statistics: one-way ANOVA with Dunnett’s multiple comparisons or an unpaired two-tailed t-test for pairwise comparison; *P<0.05; **P<0.01; ***P<0.001. (F) Graphical summary of the model presented here. A small rise in ecdysone production by the PG feeds back in an EcR-dependent manner in the PTTHn to drive the metamorphosis-inducing surge of PTTH release and ecdysone production; high ecdysone levels at the peak of the surge in turn inhibit further PTTH expression.

---

[Image of a graph showing the regulation of Ptth expression by ecdysone]
reinforced by EcR-mediated positive feedback on ecdysone production in the PG (Moeller et al., 2013). We propose that the triggering event that begins the feedback cycle is a small nutrient-dependent ecdysone peak early in the L3 stage. Nutritional signaling via insulin acts directly on the PG and is required for ecdysone production pre-critical weight but not post-critical weight (Koyama et al., 2014; Singleton et al., 2005). Furthermore, PTTH secretion is also controlled by nutrition and is required for normal attainment of critical weight (Galagovsky et al., 2018; Shimell et al., 2018), suggesting that PTTH acts together with insulin before attainment of critical weight to generate a small nutrient-dependent rise in ecdysone production at the beginning of L3. This small ecdysone peak up regulates Ptih via EcR and, under this scenario, corresponds to critical weight, which occurs ~10 h after the L2-L3 transition. Thus, when ecdysone reaches the threshold corresponding to critical-weight attainment, it generates an irreversible, self-sustaining feedback activation of the neuroendocrine system by promoting the PTTH surge that triggers the maturation-inducing ecdysone pulse towards the end of L3 (Fig. 4F). This model is supported by findings showing that a small nutrient-sensitive ecdysone peak early in L3 does indeed signal critical weight (Koyama et al., 2014).

The main feature of this model is ecdysone feedback onto the PTTHn via a mechanism requiring EcR in these cells. EcR-Usp may regulate Ptih expression by direct binding to the Ptih enhancer or through downstream target transcription factors regulated by this complex. Many transcription factors are known to be targets of EcR (King-Jones and Thummel, 2005), and EcR may indirectly regulate Ptih expression by altering the expression of one or more of these. Indeed, RNAi against certain known EcR-induced transcription factors, such as Hr39, Hr3 and ftz-f1, produced phenotypes in our screen, consistent with a possible role in Ptih regulation. Hr3 and Ftz-F1 are also known to participate in ecdysone regulation in the PG (Parvy et al., 2005, 2014), as is another nuclear receptor, Knirps (Danielsen et al., 2014), which was also identified in our screen as a potential regulator of PTTH. Clarifying the precise mechanism by which EcR controls Ptih expression will be an interesting topic for future investigation.

Conserved neuroendocrine circuitry triggers maturation onset

Early maturation is associated with smaller adult size in both flies and humans, as this event limits the juvenile growth period (Carel et al., 2004; Rewitz et al., 2009). The prevalence of precocious puberty has been linked with the increasing rates of childhood obesity; however, the mechanisms that gate GnRH secretion at the time of puberty are poorly understood (Tena-Sempere, 2012). The mammalian HPG axis controlling the onset of puberty is regulated by a complex of multiple factors, including sex steroids, GnRH, and other hormones. The interaction between these factors is critical for the proper timing of puberty; however, the mechanisms that gate GnRH secretion at the time of puberty are poorly understood (Tena-Sempere, 2012). The prevalence of precocious puberty is increasing worldwide, and this trend is likely driven by the rise in obesity; however, the mechanisms that gate GnRH secretion at the time of puberty are poorly understood (Tena-Sempere, 2012). The prevalence of precocious puberty is increasing worldwide, and this trend is likely driven by the rise in obesity; however, the mechanisms that gate GnRH secretion at the time of puberty are poorly understood (Tena-Sempere, 2012).

MATERIALS AND METHODS

Fly husbandry

All animals were reared on a standard cornmeal diet (Nutri-Fly Bloomington formulation) at 25°C under 12-h light/dark cycle conditions, with 60% relative humidity, unless otherwise stated. Larvae of mixed sex were used in all experiments. The following fly lines were used: UAS-EcR,gRNA was generated in this study; Pth-GAL4, UAS-Dicer-2 and NP423-GAL4, UAS-Dicer-2 were generous gifts from Pierre Léopold (Institut Curie, Paris, France); Pth-GAL4 and UAS-Ptih::HA (McBryar et al., 2007) and phm-GAL4 (Ono et al., 2006) were kindly provided by Michael O’Connor (University of Minnesota, Minneapolis, MN, USA); w1118 (#60000), UAS-EcR-RNAi #1 (#37058), UAS-EcR-RNAi #2 (#37059), UAS-Ptih-RNAi #102043 and UAS-torso-RNAi #101154 were obtained from the Vienna Drosophila Resource Center (VDRC); UAS-EcR.B1 (#64649), UAS-EcR.A (#6470), UAS-EcR-RNAi #3 (#50712) and UAS-Cas9.P (#54594) were obtained from the Bloomington Drosophila Stock Center (BDSC).

Fly genetics

To achieve CRISPR-Cas9-mediated disruption of EcR under GALA/UAS control, we generated a UAS construct expressing two EcR-targeted gRNAs (below) in the backbone of vector pCFD6 (Port and Bullock, 2016), which was obtained from AddGene (#73915). The gRNA sequences were designed and checked for specificity and efficiency using online tools at http://www.flyrnai.org/crispr/ and http://targetfinder.flycrispr.neuro.brown.edu. Two sequences with high predicted efficiency and no off-target binding sites were chosen 232 base pairs apart within exon 3 of EcR, an exon shared between all EcR transcripts that encodes the protein’s DNA-binding domain. Efficient induction of double-strand breaks should thus delete the DNA-binding domain and likely introduce frame-shift mutations as well, rendering the locus nonfunctional. Oligonucleotides containing the gRNA sequences were synthesized and inserted into pCFD6. The correct pCFD6-UAS-EcR,gRNA product was verified by sequencing, and transgenic animals were generated in-house and by Bestgene (Chino Hills, CA, USA). Fly stocks were constructed using standard techniques. gRNA sequence #1: TTATCGCGACATTTGTTCTC; gRNA sequence #2: GCAAGAGGGACCTGCGCCA.

Synchronization of development

To synchronize development for timed experiments, parental flies were allowed to lay eggs for 2-4 h on an apple-juice agar plate coated with a thin layer of yeast paste; hours AEL was measured from the midpoint of this time. After 24 h, newly hatched L1 larvae were collected and transferred to vials containing standard food at a density of 30 larvae per vial.

Ex vivo incubation with 20E

Four biological replicates of ten brains with an intact ring gland were dissected from synchronized L3 larvae at 110 h AEL in Schneider’s insect medium (Sigma-Aldrich, S0146). The tissue was transferred to Schneider’s medium containing 20E (Sigma-Aldrich, H5142) at 0, 5, 50, 500 or
5000 ng/ml and incubated for 6 h at room temperature (roughly 24°C). RNA was then extracted from tissue as described below.

**Real-time quantitative PCR (qPCR) analysis**

Four to six biological replicates of five whole larvae or ten dissected brains of each genotype were collected at the indicated times after egg laying (AEL) or puparium formation. Samples were flash-frozen in dry ice and stored at −80°C. The samples were thoroughly homogenized in 350 µl ice-cold lysis buffer containing 1% β-mercaptoethanol, and RNA was extracted using RNeasy mini kit (Qiagen) with DNase treatment according to the manufacturer’s instructions. RNA concentrations from whole-larval samples were measured using a NanoDrop spectrophotometer (Thermo Fisher) and adjusted to 300 ng/µl. RNA from dissected brains was transferred and split into two Eppendorf tubes, each containing approximately 150 µl supernatant. Methanol from both tubes was removed by evaporation in a vacuum centrifuge for 60 min, and pellets were re-suspended in 70% ethanol. RNA was then dissolved in 20 µl RNase free water. RNA from both tubes was transferred and split into two Eppendorf tubes, each containing 20 µl of RNA and 1% formaldehyde. 1% formaldehyde in PBS for 30 min, washed in PBS+0.1% Triton X-100 (PBST; one quick rinse followed by three 15 min washes with slow rocking motion), and blocked in PBST+5% normal goat serum (NGS) for at least 1 h. Blocking buffer was exchanged with PBST+5% NGS containing primary antibodies, and tissues were incubated overnight at 4°C. Samples were washed as before and incubated at 4°C overnight with secondary antibodies in PBST. Samples were washed again as above, washed in PBS to remove Triton X-100, and incubated in PBS at 4°C. Brains were mounted on poly-lysine-coated glass slides in ProLong Gold anti-fade reagent (Invitrogen). Fluorescence images were captured using a Zeiss LSM 800 confocal laser scanning microscope coupled with AiryScan technology and were then analyzed using the Fiji software package (https://imagej.net/Fiji). All samples for time-course data were imaged with identical settings. Quantifications of fluorescence intensity were performed by creating summed projections of each individual PTTHn followed by measurements of the anti-PTTH signal in the cell body and the anti-EcR signal in the nuclei using the following formula: integrated density−(area×mean background fluorescence). Mean fluorescence of brain tissue without positive PTTH and EcR signal in each individual projection was subtracted as background for each channel. Guinea pig anti-PTTH was purified using the Melon Gel IgG Spin Purification Kit (Thermo Scientific) from anti-PTTH serum generously provided by Pierre Léopold (Yamanaka et al., 2013); the purified IgG was isolated at 1.500. Rabbit anti-Phm (1:400) (Ono et al., 2006) was a generous gift of Michael O’Connor. Mouse monoclonal anti-EcR (clone Ag10.2) was obtained from Developmental Studies Hybridoma Bank and was used at 1 µg/ml. Secondary antibodies used were Alexa Fluor 555-conjugated goat anti-mouse, Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 647 goat anti-guinea-pig (Thermo Fisher, A21422, A1108 and A21450), all used at 1:200.

**Ecdysteroid measurements by ELISA**

Ecdysteroid levels were measured using a competitive 20-hydroxyecdysone ELISA kit (Bertin Bioreagent Cayman, 501390). Four biological replicates of ten larvae from each genotype were collected at the indicated times AEL. Larvae were washed in water and dissected in cold PBS, and tissues were fixed in 4% formaldehyde in PBS for 30 min, washed in PBS+0.1% Triton X-100 (PBST; one quick rinse followed by three 15 min washes with slow rocking motion), and blocked in PBST+5% normal goat serum (NGS) for at least 1 h. Blocking buffer was exchanged with PBST+5% NGS containing primary antibodies, and tissues were incubated overnight at 4°C. Samples were washed as before and incubated at 4°C overnight with secondary antibodies in PBST. Samples were washed again as above, washed in PBS to remove Triton X-100, and incubated in PBS at 4°C. Brains were mounted on poly-lysine-coated glass slides in ProLong Gold anti-fade reagent (Invitrogen). Fluorescence images were captured using a Zeiss LSM 800 confocal laser scanning microscope coupled with AiryScan technology and were then analyzed using the Fiji software package (https://imagej.net/Fiji). All samples for time-course data were imaged with identical settings. Quantifications of fluorescence intensity were performed by creating summed projections of each individual PTTHn followed by measurements of the anti-PTTH signal in the cell body and the anti-EcR signal in the nuclei using the following formula: integrated density−(area×mean background fluorescence). Mean fluorescence of brain tissue without positive PTTH and EcR signal in each individual projection was subtracted as background for each channel. Guinea pig anti-PTTH was purified using the Melon Gel IgG Spin Purification Kit (Thermo Scientific) from anti-PTTH serum generously provided by Pierre Léopold (Yamanaka et al., 2013); the purified IgG was isolated at 1.500. Rabbit anti-Phm (1:400) (Ono et al., 2006) was a generous gift of Michael O’Connor. Mouse monoclonal anti-EcR (clone Ag10.2) was obtained from Developmental Studies Hybridoma Bank and was used at 1 µg/ml. Secondary antibodies used were Alexa Fluor 555-conjugated goat anti-mouse, Alexa Fluor 488 goat anti-rabbit and Alexa Fluor 647 goat anti-guinea-pig (Thermo Fisher, A21422, A1108 and A21450), all used at 1:200.


Development (2020) 147, dev188110. doi:10.1242/dev.188110

RESEARCH ARTICLE


