Ligand-dependent de-repression via EcR/USP acts as a gate to coordinate the differentiation of sensory neurons in the *Drosophila* wing

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Summary

Loss of function of either the ecdysone receptor (EcR) or Ultraspiracle (USP), the two components of the ecdysone receptor, causes precocious differentiation of the sensory neurons on the wing of *Drosophila*. We propose that the unliganded receptor complex is repressive and that this repression is relieved as the hormone titers increase at the onset of metamorphosis. The point in development where the receptor complex exerts this repression varies for different groups of sensilla. For the chemosensory organ precursors along the wing margin, the block is at the level of *senseless* expression and is indirect, via the repressive control of *broad* expression. Misexpressing *broad* or *senseless* can circumvent the repression by the unliganded receptor and leads to precocious differentiation of the sensory neurons. This precocious differentiation results in the misguidance of their axons.

The sensory precursors of some of the campaniform sensilla on the third longitudinal vein are born prior to the rise in ecdysone. Their differentiation is also repressed by the unliganded EcR/USP complex but the block occurs after *senseless* expression but before the precursors undertake their first division. We suggest that in imaginal discs the unliganded EcR/USP complex acts as a ligand-sensitive 'gate' that can be imposed at various points in a developmental pathway, depending on the nature of the cells involved. In this way, the ecdysone signal can function as a developmental timer coordinating development within the imaginal disc.

Key word: Ultraspiracle, Ecdysone receptor, Broad-complex, Timing of differentiation, Imaginal discs

Introduction

Metamorphosis in *Drosophila* is induced by the steroid hormone 20-hydroxyecdysone (20E) (Riddiford, 1993). With the release of the hormone at the end of the third instar, larvae begin to wander and then pupariate. During this period proliferation of the imaginal discs slows and the differentiation of the adult sensilla begins. The response to 20E is mediated by the ecdysone receptor, a heterodimer of two nuclear receptors, Ecdysone receptor (EcR) and Ultraspiracle (USP) (Thomas et al., 1993; Yao et al., 1993). Based on studies of salivary gland puffing patterns, Ashburner et al. (Ashburner et al., 1974) proposed that a number of genes are direct targets of ecdysone. The gene products of these so called 'early' genes activate a large number of late or secondary response genes that in turn orchestrate the varied responses of cells and tissues to the hormone (Thummel, 1996). Different usage of EcR isoforms, the presence of specific co-regulators and co-factors and variation in structure and function of ecdysone response elements are thought to underlie the tissue-specific responses (e.g. Henrich et al., 1999).

Although critically important in the steroid response of larval tissues, the genes of the ecdysone cascade appear to have less impact in some imaginal tissues (Kozlova and Thummel, 2002). When the ecdysone receptor is non functional, as for example in loss-of-function USP clones in the wing imaginal disc, activation of early genes fails (Schubiger and Truman, 2000). But, surprisingly, the sensory neurons in the margin actually differentiate precociously, and can even now differentiate in the absence of 20E. Thus, in normal development USP represses sensory neuron development and 20E acts to remove this repression. Based on our results we proposed that there are two types of ecdysone response elements: inductive ones, in which the liganded receptor strongly activates transcription of target genes, and permissive ones, in which the unliganded ecdysone receptor represses transcription until binding of 20E relieves the repression (Cherbas and Cherbas, 1996), thereby allowing other factors at the promoter to regulate transcription. The important difference between the inductive and permissive response elements is that in the absence of a functional receptor there will be no activation of the gene controlled by the inductive ecdysone response element, whereas in the case of the permissive ecdysone response element, the repression is absent and the gene is activated precociously and independent of the

hormone. Its activation is now dependent on the presence of other transcription factors bound to regulatory regions of the gene.

The precocious sensory neuron differentiation found in loss-of-function USP clones reveals a key node where the ecdysone pathway intersects the neurogenic pathway in the imaginal discs. During the third instar, proneural genes are expressed in clusters of cells that are competent to form sensory organ precursors (SOPs) (Skeath and Carroll, 1991; Cubas et al., 1991). As development progresses one or a few cell(s) of the cluster are singled out to become the SOP. The selection of the SOP is under the control of the Notch signaling pathway (Kimble and Simpson, 1997; Artavanis-Tsakonas et al., 1999; Lai, 2004). Through the process of lateral inhibition, cells receiving the Notch ligand Delta activate *Enhancer of split* [*E(spl)*] genes and are inhibited from differentiating into a SOP. In the cell destined to form the SOP the proneural genes accumulate and allow the cell to take on the neural fate. Recently Nolo et al. (Nolo et al., 2000) and Jafar-Nejad et al. (Jafar-Nejad et al., 2003) showed that *senseless* (*sens;* also known as *Ly*) is also involved in SOP formation. The proneural genes are required to activate *sens* that in turn activates the proneural genes and promotes the accumulation of their gene products to high levels in the SOPs (Nolo et al., 2000). Nolo et al. (Nolo et al., 2000) also demonstrated that *sens* is required and sufficient for sensory organ differentiation. High levels of SENS activate the proneural genes but low levels serve to repress these genes (Jafar-Najad et al., 2003), leading these cells down the epidermal pathway. Thus both Notch-signaling and *sens* function as switches to determine neural versus epidermal fate.

We have used loss of function of USP or EcR to determine that both components of the ecdysone receptor complex are needed to repress differentiation of the sensory organs in the wing disc. This repression is indirect in the margin and is a consequence of de-repression of one of the ecdysone target genes, *broad* [*br*, formerly called *broad-complex* (*Br-C*)]. We show that BR is required for *sens* expression in the wing margin. Sensilla born during other developmental windows, such as the campaniform sensilla on the third vein, are also repressed by EcR/USP, but in this case the blockade is *br* independent and occurs after SOP maturation, blocking its divisions to form the cells of the sensory organ. We propose that 20E acts as a timer for the development of sensory neurons and that the correct timing is necessary for normal pathfinding of their axons.

Materials and methods

Fly strains and generation of clones

We used the following *usp* alleles: usp^2, which is a null protein (Oro et al., 1990) and usp^3, which is a point mutation in the DNA binding domain (Henrich et al., 1994). Both alleles fail to repress during low hormone titers, but usp^3 can activate some of the early genes whereas usp^2 cannot (Ghbeish et al., 2001). To remove *br*, we used npr^3 (Kiss et al., 1988), which is a null mutation of *broad* that abolishes all *br* function. Clones were made with the FRT/Flp system (Xu and Rubin, 1993), either in a *Minute* background or with the MARCM system (Lee and Luo, 1999).

Clones were induced with a 60-minute heat shock at 37°C in the progeny of the following crosses: (1) w^{1118} *Ub-GFP* $RpS5^2$ *FRT18A/FM7a* × usp^2 *(or* usp^3*) hs-N-myc FRT18A/Y; P[*usp^+*] Tb/MKRS, hs-FLP*; (2) w^{1118} *Ub-GFP* $RpS5^2$ *FRT18A/FM7a; A101/MKRS* × usp^2 *hs-N-myc FRT18A/Y; P[*usp^+*] Tb/MKRS, hs-FLP*; (3) w^{1118} *Ub-GFP* $RpS5^2$ *FRT18A/FM7a; ac-lacZ* ry^{506} × usp^2 *hs-N-myc FRT18A/Y; P[*usp^+*] Tb/MKRS, hs-FLP*; (4) w^{1118} *Ub-GFP* $RpS5^2$ *FRT18A/FM7a; Tb/MKRS, hs-FLP* × *y* npr^3 *FRT18A/*y^2*Y67g*; (5) *y* npr^3 *FRT19A/FM7 GG* × *Tub-GAL80, hsFLP, FRT19A/Y; Tub-GAL4, UAS-mCD8::GFP/MKRS.*

To misexpress genes we used the GAL4/UAS system (Brand and Perrimon, 1993) with *C96-GAL4* (from B. Edgar), *en-GAL4, dpp-GAL4* and *MS1096-GAL4* drivers and *UAS-GFP, UAS-br-Z* isoforms (from X. Zhou), *UAS-IR-EcRcore* (see below), *UAS-EcR-B1*^{W650A} (from L. Cherbas), *UAS-sc* (*scute:* from H. Bellen), and *UAS-sens* (from H. Bellen) responder lines. *A101* (*P[lArB]A101.1F3* ry^{503}; from the Bloomington Stock Center) and *ac-lacZ* (from P. Simpson) were used to mark SOPs and proneural expression.

UAS-IR-EcR-core constructs

A cDNA fragment was PCR amplified using the pCA1 (Antoniewski et al., 1996) plasmid as a template and primers AAGAATTCGGTACCAGGATGGCTATGAG and TTAGATCTCCTCGAGGAACTTG. The resulting PCR product corresponding to a 663 bp fragment between positions 2354 and 3017 relative to the EcR-B1 cDNA sequence (GenBank M74048) was cloned in the pUAST vector (Brand and Perrimon, 1993) in two consecutive steps, first in a reverse orientation between *Bgl*II and *Kpn*I, then in a forward orientation between *Eco*RI and *Bgl*II. Recombinant *UAS-IR* constructs were transformed at 30°C in Sure (Stratagene)-competent bacteria to minimize DNA recombination and screened using appropriate restriction enzyme digestions. Transgenic flies for the *UAS-IR-EcR-core* constructs were generated as previously described using a w^{1118} strain as a recipient stock (Rubin and Spradling, 1982).

In vitro cultures

In vitro cultures were set up as described by Schubiger and Truman (Schubiger and Truman, 2000) using Shield and Sang 3M medium (Sigma). The hormone 20-hydroxyecdysone (Sigma) was added to the cultures at a concentration of 1 μg/ml. To ensure that the correct stage of larvae were used, early wandering larvae were staged by (1) collecting larvae 1-3 hours after the onset of wandering, (2) their full gut and (3) the morphology of the wing disc at dissection; mid-wandering larvae were selected by their half full guts.

Antibodies, immunohistochemistry and imaging

We used the following antibodies: guinea pig anti-SENS (1:1000, a gift from H. Bellen), rabbit anti-β-galactosidase (1:1000), mouse anti-EcR common and mouse anti-EcR-B1 ascites (1:10000, a gift from C. Thummel), rabbit or mouse anti-BR-Z1 (1:3000 and 1:100; gifts from J.-A. Lepesant and G. Guild, respectively), mouse anti-Achaete (1:10; a gift from T. Orenic) and 22C10 (1:100, Developmental Studies Hybridoma Bank, Iowa City, IA, USA). We also used Alexa (Molecular Probes) secondary antibodies against mouse and rabbit, as well as Texas-Red- and FITC-conjugated antibodies from Jackson ImmunoResearch. Images were collected on a BioRad 600 confocal microscope and processed with Adobe Photoshop software.

Results

The unliganded ecdysone receptor has repressive function

We previously presented data demonstrating that loss of function of USP leads to precocious differentiation of the sensory neurons in the wing margin, as well as early expression of the Z1 isoform of BR (Schubiger and Truman, 2000). We proposed that in normal development the unliganded EcR/USP receptor complex represses the expression of BR-Z1 and

sensory neuron development, but since USP can form heterodimers with other nuclear receptors (Sutherland et al., 1995) we could not exclude the possibility that the repression we observed was due to a function of USP not associated with EcR. If the repression is caused by the unliganded ecdysone receptor complex, we would predict that the loss of EcR would produce the same effects as loss of USP function, namely precocious BR-Z1 expression and neuronal differentiation. Since *EcR* is located at 42A on the right arm of the second chromosome (Koelle et al., 1992), just proximal to the FRT site at 42D, the FLP/FRT system cannot be used to make loss-of-function clones. We therefore used silencing RNA to knockdown the levels of EcR by expressing *UAS-IR-EcR* in the wing disc. The construct we used contains a sequence common to all *EcR* transcripts. Using the *C96-GAL4* driver (Gustafson and Boulliane, 1996) to express *UAS-IR-EcR* along the dorsal-ventral boundary resulted in very low levels of EcR (Fig. 1A-A′′) in the margin of the wing disc. To test the effect of EcR knockdown on BR-Z1 expression, we expressed *UAS-IR-EcR* in the posterior compartment of the wing disc using the *en-GAL4* driver (Fig. 1B-B′′). To circumvent the lethality in the first instars, we raised early instars at 18°C, which leads to lower *GAL4* expression. Early third instar larvae were then brought to 25°C. Expressing *UAS-IR-EcR* in the posterior compartment made it possible to easily compare BR-Z1 expression between two large areas. The area of reduced EcR expression coincided with precocious BR-Z1 induction (Fig. 1B′′), suggesting that EcR levels were not sufficient to repress BR-Z1. This is in contrast to the effect of expressing *EcR-B1*^{W650A,} a dominant negative form of the receptor that fails to bind ecdysone (Cherbas et al., 2003). Using *MS1096-GAL4* to drive the dominant negative receptor in the dorsal wing compartment, BR-Z1 was not activated (Fig. 1C-C′′), indicating that the dominant negative receptor constitutively repressed BR-Z1.

The reduction of EcR levels also induced precocious differentiation of sensory neurons in the margin (in 27/27 discs, 0-4 hours APF; compare Fig. 1D′ and E′) as well as the sensilla on the third vein (compare Fig. 1F and G)

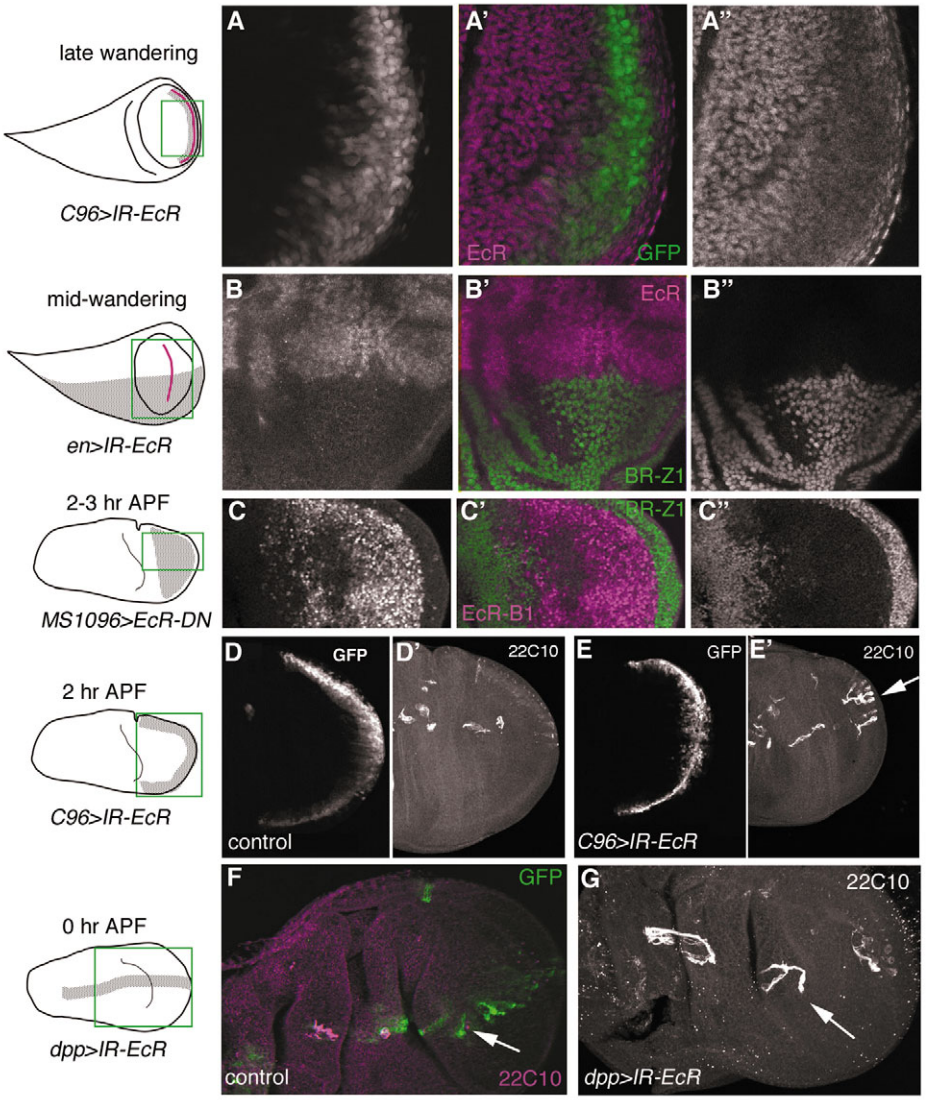

Fig. 1. Knockdown of EcR leads to precocious BR-Z1 expression and sensory neuron differentiation in contrast to the effects of dominant negative EcR. (A-A′′) *C96-GAL4* driving *UAS-GFP* and *UAS-IR-EcR* in the margin of a wing disc from a late wandering larva. In A GFP identifies areas of *C96-GAL4* expression; in A′′ low levels of EcR are seen in the region of GAL4 expression. A′ is the merged image. (B-B′′) *en-GAL4* driving *UAS-IR-EcR* reduces the levels of EcR in the posterior compartment of a wing disc from a wandering larva (B) and leads to precocious up-regulation of BR-Z1 (B′′). B′ is the merged image. (C-C′′) Wing disc 2-3 hours APF with *MS1096-GAL4* driving the dominant negative ecdysone receptor *EcR-B1*^{W650A}. The EcR-B1 antibody also recognizes the dominant negative isoform. At 2-3 hours APF EcR-B1 is normally low, thus the label in C reflects the domain of expression of the dominant negative ecdysone receptor which blocks the up-regulation of BR-Z1 (C′′). C′ is the merged image. (D,D′) Control disc 2 hours APF with *C96-GAL4* driving *UAS-GFP* (D). Sensory neuron differentiation has begun in the margin as seen with 22C10 (D′). (E,E′) *C96-GAL4* driving *UAS-GFP* and *UAS-IR-EcR*. At 2 hours APF 22C10 labeling (E′) shows advanced sensory neuron differentiation in the margin (arrows). (F,G) Sensilla on the third vein are also affected by loss of EcR function. 22C10 label shows the first axons (arrow) elongating in a 0 hour APF control (*dpp-GAL4* driving *UAS-GFP*) disc (F). The GFP reporter shows the domain of *dpp* expression. (G) Axons differentiate prematurely (arrow) in a 0 hour APF disc with *dpp-GAL4* driving *UAS-IR-EcR*. The schematic drawings indicate the morphology of the discs and the expression domain of the drivers used (stippled area). The dorsal/ventral boundary (margin) is indicated in magenta in discs from wandering larvae. In later stages the margin moves to the periphery as the disc elongates. All discs are oriented with anterior to the top and wing anlage to the right.

when *UAS-IR-EcR* was driven either by *C96-GAL4* or *dpp-GAL4*. The EcR loss-of-function phenotypes were similar to those observed in loss of function USP clones (Schubiger and Truman, 2000). This similarity suggests that in normal development the repression is indeed due to the unliganded EcR/USP dimer.

EcR/USP blocks differentiation of the sensory organ precursor cell (SOP) on the wing margin

The precocious neural differentiation observed in the margin raised the question: at which step was EcR/USP blocking the progression of sensory neuron development? To address this we induced USP loss-of-function clones in the second instar and analyzed the appearance of different gene products involved in neurogenesis in the presumptive wing margin. Gene products that appear before the EcR/USP block are predicted to be unaffected by loss-of-function USP, but those that arise at or after the block should appear precociously in loss-of-function USP clones. The proneural genes of the *achaete-scute* complex are required for the differentiation of the chemosensory neurons on the wing (Giangrande, 1995), and we used either an *ac-lacZ* construct or an antibody against Achaete (AC) (Skeath and Carroll, 1991) as a marker for proneural gene expression. As indicators for the formation of the SOPs we used Neuralized (NEUR) and Senseless (SENS) (Nolo et al., 2000) expression, the former followed by the *lacZ* reporter line A101 (Bellen et al., 1989). We fixed discs from late feeding and early wandering larvae when the markers normally begin to be expressed. Fig. 2 shows β-galactosidase (β-gal) expression of the *ac-lacZ* line in an usp^2 clone. There was no difference in the expression pattern between the mutant tissue and the surrounding control tissue (Fig. 2A,A′), suggesting that the unliganded receptor was not repressing proneural gene expression during this early stage. The anti-AC antibody gave the same result (data not shown). In discs from early wandering larvae, however, both NEUR (A101) and SENS were prematurely expressed in *usp* null clones. A101-*lacZ* was strongly expressed in the mutant clone but β-galactosidase had not yet appeared in wild-type cells (Fig. 2B,B′). We found a similar result for SENS expression. In normal development SENS protein initially appears in two bands along the dorsal-ventral boundary in both the anterior and posterior compartment. As development progresses SENS accumulates in the SOPs in the anterior compartment (Nolo et al., 2000). In the usp^2 clone SENS protein had already accumulated in the SOPs in the mutant patch (Fig. 2C,C′), but was only present at low levels in wild-type cells in the anterior margin. These results show that during normal development EcR/USP must be repressing the differentiation of the SOPs in the wing margin.

To further demonstrate that SOP formation is blocked by the unliganded receptor we used the GAL4 system to drive either *sc* or *sens* in the margin to test if they can by-pass the repression. Fig. 3 shows wing discs from animals at 0 hours APF where *sc* (Fig. 3A,A′) or *sens* (Fig. 3B,B′) was expressed using the *C96-GAL4* driver. When *sens* was expressed we observed precocious neuronal differentiation (11 of 11 discs). Expressing *sc* in the margin however, only led to a slight advancement in differentiation (n=20 discs). These results indicate that *sens* but not *sc* can override the block.

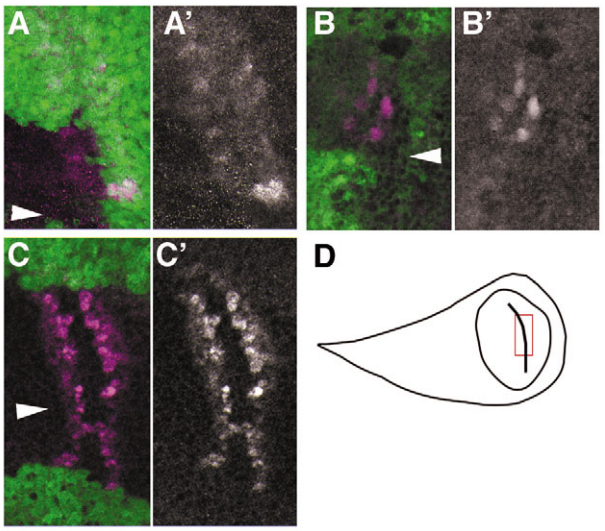

Fig. 2. Effect of loss-of-function USP on the expression of proneural and neurogenic genes in the wing margin [usp^2 clones in a Minute ($RpS5^2$) background]. Absence of GFP (green) marks usp^2 tissue. (A,A′) Wing disc from a late feeding larva. Expression of the *ac-lacZ* reporter is not affected by loss of USP function. (B,B′) The *neur* reporter A101 in a disc from an early wandering larva is precociously expressed in the absence of USP function. (C,C′) Senseless (SENS) is expressed precociously in the mutant clone in a disc from an early wandering larva. Note the accumulation of SENS in the mutant SOPs. In wild-type tissue low levels are detected anteriorly. (D) Diagram gives the orientation of the disc with the area depicted in A-C. Arrowheads indicate estimated intersection of the A/P boundary with the margin. A′-C′ show the expression of the different markers.

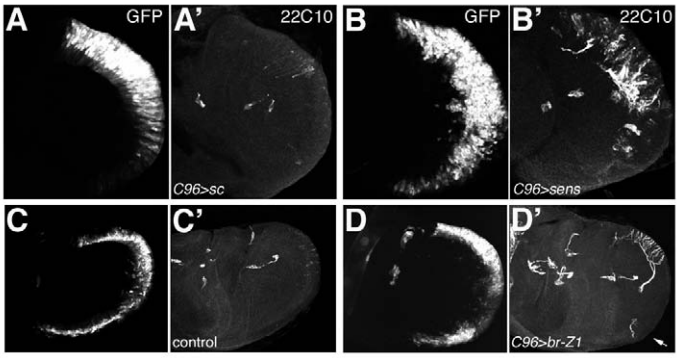

Fig. 3. *sens* and *br-Z1,* but not *sc* expression can by-pass repression of SOP formation in the wing margin by the unliganded EcR/USP complex. (A,A′) Wing discs 0 hours APF with *C96-GAL4* driving *UAS-sc* and *UAS-GFP* (A). *sc* misexpression does not by-pass EcR/USP repression. 22C10 expression (A′) reveals only a slight advancement in sensory neuron differentiation. (B,B′) Wing disc 0 hours APF with *C96-GAL4* driving *UAS-sens* and *UAS-GFP* (B). 22C10 expression shows precocious differentiation of the sensory neurons (B′). (C,C′) Control wing disc 2.5 hours APF with *C96-GAL4* driving *UAS-GFP*. 22C10 is beginning to be expressed in the margin (C′). (D,D′) Wing disc 2.5 hours APF with *C96-GAL4* driving *UAS-br-Z1* and *UAS-GFP* (D). Labeling with 22C10 shows the precociously differentiating neurons in the margin (D′). Note that their axons are projecting abnormally instead of following along the anterior margin. A differentiating sensory neuron is also seen in the posterior margin (arrow).

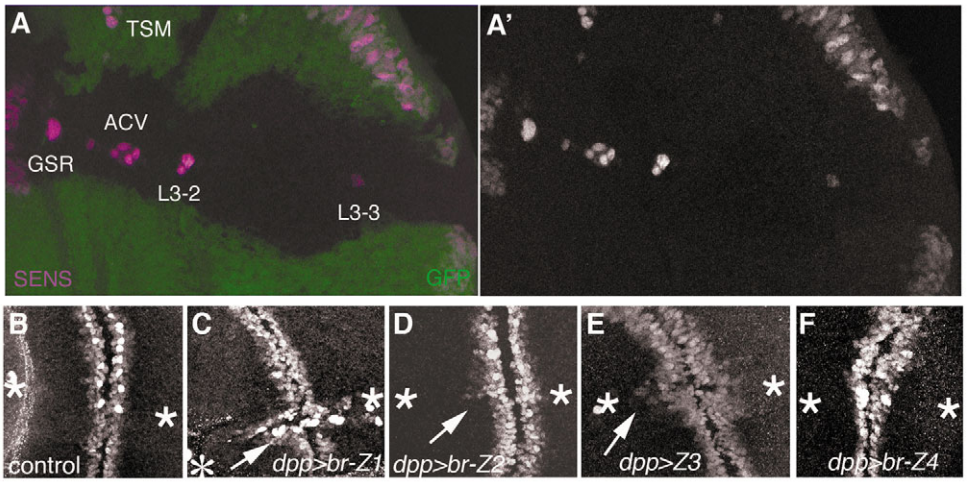

Fig. 4. BR function is required for *sens* expression in the wing margin. (A,A′) A large *npr*^{3} clone in a Minute (*Rp5S*) background. SENS protein (A′) is not detected in the *npr*^{3} clone (GFP negative area, A) in the wing margin, but is expressed normally in the region along the third vein [see Murray et al. (Murray et al., 1984) for nomenclature of these sensilla]. Image is a composite of projections of the dorsal epithelium only. (B-F) Misexpression of *br* isoforms and their effect on SENS expression in the wing margin. (B) Normal pattern of SENS expression in control disc. (C) *dpp-GAL4* driving *UAS-br-Z1* leads to ectopic SENS expression and early SOP differentiation (arrow). (D) *ddp-GAL4* driving *UAS-br-Z2* leads to ectopic SENS expression (arrow). However SENS has not accumulated in the SOPs in the area of ectopic SENS expression. (E) Ectopic SENS is expressed in a wing disc where *dpp-GAL4* drives *UAS-br-Z3* but does not lead to precocious accumulation of SENS in the SOPs. (F) Misexpressing *br-Z4* using the *dpp-GAL4* driver does not induce ectopic SENS expression. Asterisks in all panels indicate the approximate axis of *dpp-GAL4* expression.

The role of *br* in controlling sensory neuron differentiation in the wing margin

As we have shown previously, BR-Z1 is repressed by USP (Schubiger and Truman, 2000). Since *br* is a direct target of ecdysone we investigated whether BR isoforms are involved in SOP development. We expressed all four *br* isoforms (Zhou et al., 2004) in the presumptive margin of the wing disc using *C96-GAL4* and analyzed neuronal differentiation. Precocious sensory neuron development was only observed when BR-Z1 was expressed, as seen in a wing disc from a prepupa 2 hours APF (Fig. 3D,D′). The precocious differentiation of the margin neurons was similar to that observed when USP function was lost or EcR levels were knocked down. This result was seen in 39 of 40 discs analyzed from late wandering larvae to 2 hours APF. In about 15% of the discs one or two neurons also differentiated in the posterior margin, normally devoid of sensory neurons (Fig. 3D′).

From these results we predicted that loss of function of BR-Z1 should repress *sens* expression. To test this we made *npr*^{3} clones in the wild type or in a *Minute* background using the FRT/FLP system. *npr*^{3} clones eliminate the function of the entire *br* complex (Kiss et al., 1988). Since Bayer et al. (Bayer et al., 1997) showed that some BR isoforms can compensate for the function of other BR isoforms, we chose to use the *br* null allele *npr*^{3} *to* avoid functional redundancy and any interactions between the BR isoforms. We observed that in clones lacking *br* function both the high accumulation of SENS in the SOPs and the low levels of SENS expression in the posterior margin failed to occur (Fig. 4A,A′; 16/16 clones). As a consequence the marginal sensory neurons failed to differentiate. The loss of the marginal neurons was permanent since we never observed chemosensory bristles in adult wings with *npr*^{3} clones encompassing the wing margin (data not shown). Other SOPs situated on the third vein however, were insensitive to loss of *br* and expressed SENS (Fig. 4A; see below).

sens expression occurs in two steps with a low level seen prior to BR-Z1 expression followed by the high accumulation of SENS in the SOPs. Thus we asked if BR-Z2, -Z3 or -Z4 could induce low levels of *sens* expression. We used *dpp-GAL4* to drive the different *br* isoforms along the presumptive third vein and tested for SENS protein at the intersection of the *dpp* expression domain with the wing margin (Fig. 4B-F). As expected, BR-Z1 expression led to ectopic SENS expression with several cells showing high accumulation typical for SOPs. This ectopic accumulation happened before SENS accumulation in the SOPs along the anterior margin (Fig. 4C). BR-Z2 expression resulted in early larval lethality so we raised the animals at 18°C until the beginning of the third instar and then transferred them to room temperature. In such animals the misexpression of BR-Z2 induced ectopic SENS at the intersection of the *dpp* domain with the margin in about 50% of the discs analyzed, but we never observed precocious high accumulation in the SOPs (Fig. 4D). Misexpression of BR-Z3 also led to a widening of the SENS expression domain, but also without precocious high accumulation (Fig. 4E). Expressing BR-Z4 did not induce ectopic SENS expression (Fig. 4F).

These results from loss and gain of function of *br* show that it is required to activate *sens* in the margin, but is not required for *sens* activation in the early born SOPs on the third vein.

Differentiation of early born neurons is also controlled by EcR/USP

From our data on loss-of-function *npr*^{3} clones, it was obvious that *sens* activation that is required for the sensilla campaniformia along the third vein was not dependent on BR function (Fig. 4A,A′) and raised the question of how ecdysone controls the differentiation of these sensilla. We expressed the *UAS-IR-EcR* construct along the third vein by using the *dpp-GAL4* driver to knockdown EcR levels. Fig. 1F and G show wing discs at 0 hours APF. Precocious differentiation of the third vein sensilla was induced by the loss of EcR function. Similarly in loss-of-function USP clones the third vein sensilla differentiated early (data not shown). In contrast to the later birth of the chemosensory precursors, the SOPs of some of the third vein sensilla are born 20-30 hours before pupariation

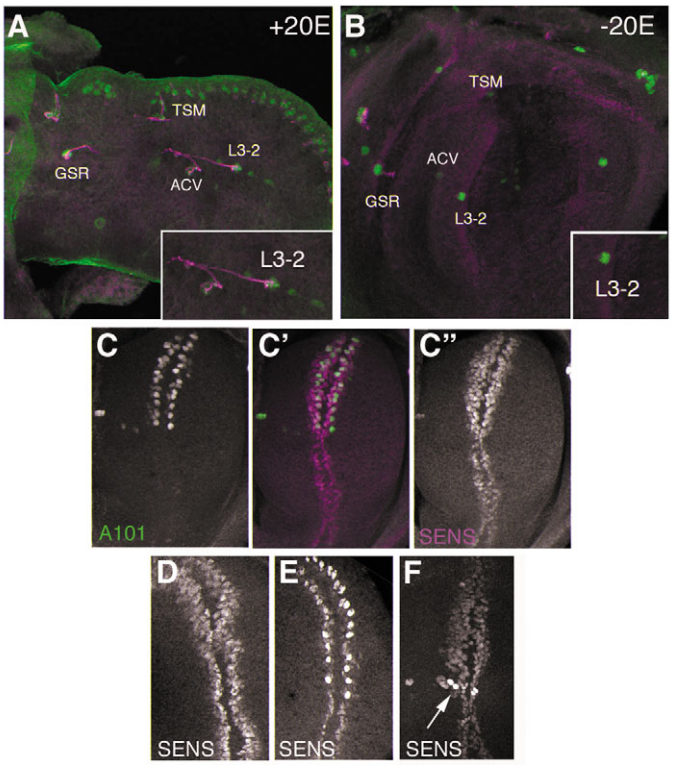

Fig. 5. In vitro culture in the absence of ecdysone reveals the step at which neurogenesis is blocked. (A) Disc from an early wandering A101 larva cultured in vitro for 24 hours in the presence of 20E shows the differentiation of the normal complement of neurons and outgrowth of their axons (22C10). (B) Disc from an early wandering larva cultured in vitro for 24 hours without ecdysone. Only the early born SOPs are visible with the A101 reporter line. Insets in A and B show higher magnification of the L3-2 region. (C-C′′) Disc from an early wandering A101 larva cultured in vitro without ecdysone for 24 hours. The SOPs are recognizable with A101-β-gal (C) but SENS fails to accumulate in them (C′′). (C′) merged image of A101 and SENS expression. (D-E) Changing pattern of SENS expression in the margin from two broad bands in the early wandering stage (D) to the accumulation in the SOPs by the mid-late wandering stage (E). (F) Block of SENS accumulation requires a functional ecdysone receptor: wing disc from an early wandering larva expressing *UAS-IR-EcR* under the control of *dpp-GAL4* cultured in vitro in the absence of ecdysone for 24 hours. SENS only accumulates in the margin (arrow) where it is intersected by the *dpp*-domain (compare with control in C′′). (C-F) Area shown is the same as in Fig. 2D. In all panels, anterior is to the top.

(Huang et al., 1991) at a time when the ecdysone levels are very low. Thus it is unlikely that the unliganded receptor blocks the same step in neurogenesis as we saw for the margin SOPs.

To test when repression might occur we cultured early wandering stage *A101* wing discs in vitro without 20E for 22-24 hours. The *A101 lacZ* reporter appears early in the formation of the SOP and can be followed through the subsequent divisions in the cells constituting the sensillum. To show that our in vitro cultures could support differentiation of the sensilla we first cultured wing discs from early wandering larvae in the presence of 20E. After 24 hours in culture we saw the normal complement of sensilla, with some sensory neurons showing extended axons (Fig. 5A). Differentiation was similar to a wing disc at about 5 hours APF, and agreed well with previously published developmental progress in culture, which was estimated to about half as fast as in vivo (Gibbs and Truman, 1998). However, when early wandering stage discs were cultured in the absence of 20E, differentiation was stalled (Fig. 5B).

The timing of production of the cells that make up the giant sensillum on the radius (GSR) and the anterior cross vein (ACV) sensillum were examined (Fig. 6). For the GSR, in discs of early wandering larvae, we observed either one or two A101-positive nuclei, indicating that at this stage the SOP of the GSR is about to or has just divided for the first time. After 24 hours in culture without 20E about half of the GSRs remained at the SOP stage, whereas the other half had progressed through the subsequent divisions to produce the four cells of the sensory organ (Fig. 6A). When the in vitro cultures were started with discs from mid-wandering larvae, at which stage the GSR had consistently gone through the first division, we observed that in the absence of 20E the second division occurred in the majority of cases (Fig. 6B). The few cases that did not undergo the second division were presumably placed in culture prior to the first division of the GSR. For the SOP of the ACV sensillum that develops slightly later than the GSR-SOP we found a similar pattern (Fig. 6C,D). From these data, we suggest that the early born SOPs have an ecdysone-dependent ‘gate’ just prior to their first division, and that after that time ecdysone is no longer required for the subsequent divisions.

Our in vitro cultures also gave us further insight into the differentiation of the chemosensory neurons on the margin.

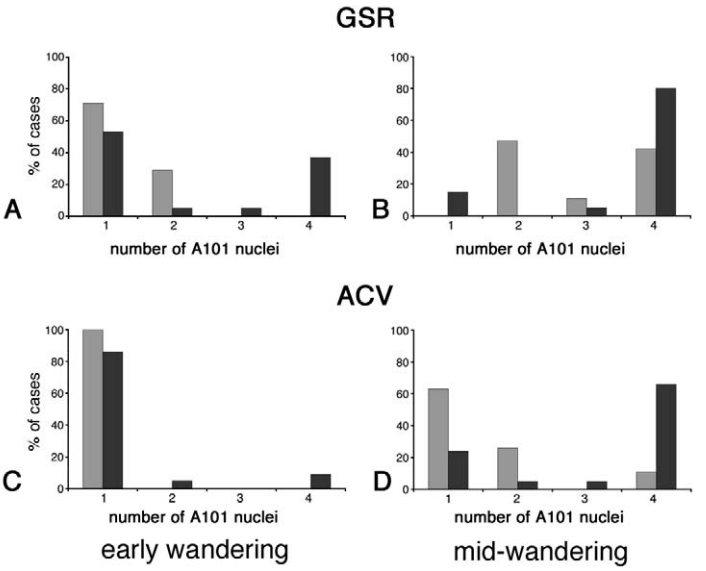

Fig. 6. Frequency of wings cultured in vitro without 20E that have 1, 2, 3, 4 or more A101-positive nuclei in two early born sensilla (GSR and ACV). Grey bars show the data from discs at the onset of culture, black bars show the data from discs cultured for 22-24 hours in vitro without 20E. A and C show the results from discs of early wandering larvae; B and D show discs from mid-wandering larvae. Frequencies are based on analysis of 19-24 discs.

SENS is normally expressed in two broad bands along the margin (Fig. 5D). In late third instar discs the SOPs in the anterior margin have accumulated SENS to high levels, whereas non-SOP cells expressed SENS weakly (Fig. 5E). When we initiated the in vitro cultures with *A101* discs, β-gal was expressed in the SOPs, whereas SENS was still only expressed at its early low level. Interestingly, after in vitro culture without ecdysone (Fig. 5C-C′′) A101 expression remained but SENS did not accumulate in the SOPs, indicating that the SOPs had formed but were not fully mature. This is in agreement with Nolo et al. (Nolo et al., 2000) who found that in *sens* mutant clones a SOP is nevertheless selected and initially accumulates AC/SC. Here, we furthermore demonstrate that the failure to accumulate SENS in the SOPs is dependent on a functional ecdysone receptor (Fig. 5F). When a wing disc expressing *UAS-IR-EcR* under the control of *dpp-GAL4* was cultured in vitro without ecdysone, SENS accumulated in the margin only at the intersection with the *dpp* domain.

Discussion

Both EcR and USP have repressive function

The ecdysone signal is transmitted via the ecdysone receptor to activate a number of direct target genes. It has generally been assume that the hormone and its receptor activate a hierarchy by activating early genes that then activate the many late genes (Ashburner et al., 1974). A large body of work based primarily on larval tissues has supported this model. Thus when the ecdysone receptor is non functional, the first step in the cascade fails, the early genes are not activated, and the tissues are unable to undergo a metamorphic response. In imaginal discs we previously showed that loss of function of USP (Schubiger and Truman, 2000) leads to the inability to activate early genes, such as *DHR3, EcR* and *E75B*, but also results in precocious differentiation, rather than in a failure to initiate a particular metamorphic response. We have now demonstrated that loss of EcR function in the wing discs gives similar results (precocious BR-Z1 expression and sensory neuron differentiation, Fig. 1B′,E′,G′) to the ones we reported for loss of USP function and we conclude that the unliganded EcR/USP heterodimer is the functional repressor. Thus at least some processes at the onset of metamorphosis are not controlled by the ecdysone-induced hierarchy, but rather through the relief of the repressive function of the unliganded EcR/USP complex once the ecdysone titers rise. The importance of this repressive function of the unliganded receptor is further demonstrated by our experiments using a dominant negative EcR, which does not bind the hormone, and as a consequence repression cannot be relieved and target genes are not expressed. The repressive role we propose for the unliganded ecdysone receptor complex would also explain why loss-of-function USP clones, in general, result in the differentiation of normal adult bristle organs (Oro et al., 1992) since loss of receptor function would only control the timing of differentiation. Such an interpretation is supported by early pigmentation of abdominal bristles in usp^3 clones that we have observed on occasions (M.S., unpublished observations). The in vivo studies of activation by EcR/USP by Kozlova and Thummel (Kozlova and Thummel, 2002) suggest that activation plays a major role in the metamorphic response of larval tissues but has only a minor role in the development of the imaginal discs. It remains to be seen which processes are activated by the ecdysone hierarchy and which by loss of the repressive actions of the unliganded receptor.

Ecdysone control of sensory organ differentiation in the margin

In our previous work (Schubiger and Truman, 2000) we found that adult chemosensory neurons on the wing margin underwent precocious differentiation in loss-of-USP clones. To understand which step is repressed by the EcR/USP complex we analyzed the early expression patterns of a set of genes involved in neuron differentiation in such mutant clones. In the absence of USP function the early pattern of Achaete (AC) expression in the margin is unaffected. In contrast, both NEUR (as visualized by A101) and SENS are expressed in *usp* mutant cells before they are detected in the surrounding wild-type tissue (Fig. 2). Our in vitro experiments revealed that A101 expression that is already on at the time we set up the cultures, remains on through the culture period, but that there is a block by EcR/USP at the level of *sens* expression that prevents the maturation of the SOPs of the chemosensory neurons in the wing margin. The block is released once the hormone titers rise.

The repressive function of the unliganded receptor does not act directly on the genes we tested. We have shown that the block of SOP differentiation is controlled through BR-Z1, and that *br* function is required for the activation of *sens*, a gene necessary and sufficient for sensory organ differentiation (Nolo et al., 2000). Thus expressing BR-Z1 or SENS early in the margin allows the inhibition from the unliganded ecdysone receptor to be by-passed and the sensory neurons in the margin to differentiate precociously. When SENS is misexpressed we found that clusters of extra neurons differentiate in the region of high driver expression (Fig. 3B′). This is in agreement with reports from Nolo et al. (Nolo et al., 2000) and Jafar-Nejad et al. (Jafar-Nejad et al., 2003) who showed that high levels of SENS activate the proneural genes and promote the formation of SOPs. By contrast, when BR-Z1 is misexpressed in the margin we observed a more normal pattern of sensory neuron arrangement (Fig. 3D′) that was very similar to what we observed in loss-of-function USP or EcR cells (Fig. 1E). This indicates that BR-Z1 does not induce the formation of SOPs but rather causes the up-regulation of SENS in cells that have already committed to the SOP fate. Occasionally expressing BR-Z1 in the margin led to the differentiation of a sensory neuron in the posterior margin, normally devoid of neurons. It is possible that in such a situation BR-Z1 misexpression can at times lead to sufficiently high expression of SENS to cause SOP differentiation.

Loss-of-function of BR demonstrated the requirement for BR to activate the high levels of SENS in the SOPs, as well as the low levels in the posterior margin, but without molecular data we do not know if *br* is directly activating *sens*. Since BR-Z1 normally appears later than the initial low expression of SENS, we propose (Fig. 7) that early SENS expression is most probably controlled by BR-Z2. It is expressed shortly after the molt to the third instar (J.W.T., unpublished) and we have shown that ectopic BR-Z2 expression induces low levels of SENS (Fig. 4D). BR-Z3 that also induces SENS when ectopically expressed (Fig. 4E) may induce low levels of SENS

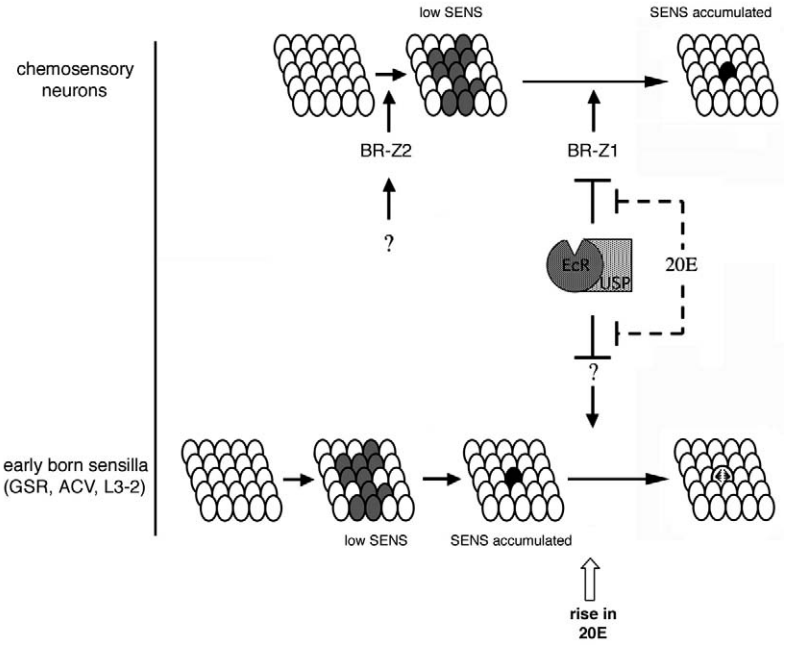

Fig. 7. Repression by the unliganded ecdysone receptor is lifted at different steps in neurogenesis for different sets of SOPs. For the chemosensory neurons of the margin we speculate that BR-Z2 activates SENS whereas BR-Z1 is required for the accumulation of SENS in the mature SOP. BR-Z1 is repressed by the unliganded EcR/USP complex. The control of *br-Z2* expression has not been established. The early born SOPs accumulate SENS prior to the rise of 20E and are independent of BR function. EcR/USP repression inhibits the division of the SOP. The rise in 20E relieves repression and allows the SOP to divide. Grey represents low levels of SENS protein [adapted from Jafar-Nejad et al. (Jafar-Nejad et al., 2003)]; black shows the mature SOPs that have accumulated SENS. Open arrow marks the rise in 20E.

as well, but since BR-Z3 is normally expressed at very low levels in the wing disc, we think it plays a minor role. BR-Z1 then is needed for the accumulation of SENS in the mature SOPs.

In summary the above genetic interactions suggest that unliganded EcR/USP represses BR expression that is required for *sens* activation and the formation of the mature SOPs in the margin.

Sensory neuron differentiation is controlled by a steroid-dependent gate

The SOPs are born in a specific temporal sequence in the wing disc (Huang et al., 1991). The first SOPs arise in the third instar, 20-30 hours before pupariation; they include GSR, ACV and L3-2 along the third vein (Fig. 4A). The SOPs of the margin arise later, at 10-12 hours before pupariation, so they are at a very different stage from that of the early born SOPs at the time metamorphosis begins. Since the unliganded receptor is acting as a repressor we postulate that the block must be occurring at different times during the progression of sensory organ differentiation for these two groups of sensilla (Fig. 7). Based on our genetic studies, the ecdysone-sensitive arrest for the chemosensory sensilla of the margin occurs in the up-regulation of SENS as the SOP is undergoing maturation. For the early born sensilla, however, SENS levels are already elevated before the rise in 20E and are not dependent on BR function. For these early born sensilla the ecdysone-sensitive arrest occurs after high SENS expression but prior to the division of the SOP (Fig. 7). We think that for different sets of sensilla the imposition of an ecdysone-sensitive arrest at different points in development is important to coordinate the differentiation of the sensilla. Such a mechanism would ensure that the outgrowing axons begin to elongate in a choreographed manner leading to the correct axon pathways and to their finding of the correct targets in the CNS according to their physiological function (Palka et al., 1986). This idea is supported by the observation that the axons of sensilla forced to differentiate precociously by the absence of a functional ecdysone receptor or by early expression of BR-Z1 or SENS often take abnormal routes (for example, Fig. 3D′).

A recent study by Niwa et al. (Niwa et al., 2004) demonstrated that ecdysone is also acting as a timer for the formation of the chordotonal and Johnston's organ as well as for the initiation of the morphogenetic furrow. These structures arise early in the third instar (80 hours after egg laying) and appear to be under the control of the small ecdysone peak at that time (Andres et al., 1993). In the case of the leg chordotonal organ, ecdysone appears to be controlling the proneural gene *atonal* (*ato*). We do not know yet if this control also occurs via de-repression as we see for the wing.

The subsequent progression of the morphogenetic furrow is also dependent on ecdysone (Brennan et al., 1998). This action of ecdysone has been proposed not to occur via EcR (Brennan et al., 2001). However, Zelhof et al. (Zelhof et al., 1997) showed that loss of USP leads to an advancement of the furrow and precocious differentiation of the photoreceptors. Bateman and McNeill (Bateman and McNeill, 2004) recently reported that the progression of the morphogenetic furrow, as well as the timing of differentiation of the chordotonal organs in the leg, are controlled by the insulin receptor (InR)/Tor pathway, with increased InR signaling leading to precocious differentiation. In the wing margin, by contrast, increasing or decreasing InR signaling did not affect the timing of differentiation of the chemosensory neurons (M.S., unpublished). Thus there must be multiple temporal control mechanisms for sensory structures. Our results have demonstrated repression of sensory organs by the unliganded ecdysone receptor at the end of the third instar, but do not rule out additional steps controlled by ecdysone or other factors. It remains to be elucidated which timer(s) is used when, and for which sensory structures.

In holometabolous insects functional larval tissues are replaced by the differentiating imaginal ones. The endocrine system is acting on larval tissues composed of differentiated

cells that are thus in an equivalent state to initiate programs such as cell death and neuronal remodeling. Here EcR/USP's role is activational (Yin and Thummel, 2005; Lee et al., 2000; Schubiger et al., 1998). For the differentiation of the imaginal tissues the endocrine system faces a varied cellular landscape where some cells may still be dividing while other have begun to differentiate. In these tissues the unliganded receptor acts as a repressor to interrupt the sequence of differentiation at different points in order to coordinate the response to the rising 20E titers. Release of repression by 20E may therefore function as a 'gate' at the onset of metamorphosis and thus would enable development of imaginal tissues to be coordinated and tightly controlled by the rising ecdysone titers. In metamorphosing amphibians we see a similar situation with functional larval tissues such as the tail and the gills dying and adult limbs and lungs developing in response to thyroid hormone. We would not be surprised to find that the thyroid hormone receptor is activational in the larval tissues but that the forming adult tissues are controlled through de-repression.

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