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Suppressed production of methyl farnesoid hormones yields developmental defects and lethality in *Drosophila* larvae

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ABSTRACT

A long-unresolved question in the developmental biology of *Drosophila melanogaster* has been whether methyl farnesoid hormones secreted by the ring gland are necessary for larval maturation and metamorphosis. In this study, we have used RNAi techniques to inhibit 3-Hydroxy-3-Methylglutaryl CoA Reductase (HMGCR) expression selectively in the corpora allatal cells that produce the circulating farnesoid hormones. The developing larvae manifest a number of developmental, metabolic and morphogenetic derangements. These defects included the exhibition of an "ultraspiracle" death phenotype at the 1st to 2nd instar larval molt, similar to that exhibited by animals that are null for the farnesoid receptor ultraspiracle. The few larvae surviving past a second lethal period at the 2nd to 3rd instar larval molt, again with "ultraspiracle" phenotype, often became developmentally arrested after either attaining a misformed puparium or after formation of the white pupa. Survival past the "ultraspiracle" lethal phenotype could be rescued by dietary provision of an endogenous dedicated precursor to the three naturally secreted methyl farnesoid hormones. In addition to these developmental and morphogenetic defects, most larvae that survived to the late second instar exhibited a posterior-originating melanization of the tracheal system. These results support the hypothesis that larval methyl farnesoid hormones are necessary for larval survival and morphogenetic transformation through the larval and pupal metamorphic processes.

1. Introduction

Hormonal signaling is a significant component of the regulation of cellular differentiation and tissue maturation in both vertebrates and invertebrates. In vertebrate systems, isoprene-based retinoid and steroid hormones each have been shown to affect embryonic development and reproductive maturation (Daftary and Taylor, 2006).

In the case of retinoids, several independent experimental paradigms to manipulate either the ligand, or its receptor, similarly support a role of endogenous retinoid in embryonic tissue differentiation. First, application of exogenous retinoic acid induces clear teratogenic effects on the embryonic formation of limbs and the central nervous system (Collins and Mao, 1999). Second, elegant surgical explantation techniques to detect secretion of biologically active retinoid (Sonneveld et al., 1998) have supported the hypothesis of an endogenous paracrine contribution of retinoic acid (Maden, 2001). Third, confirmatory morphogenetic outcomes were observed when molecular genetics was used to block endogenous retinoid biosynthesis by retinaldehyde dehydrogenase 2 (Ji et al., 2006). From the other direction of experimental paradigm, i.e., manipulation of the ligand's receptor, molecular genetics to remove the retinoic acid receptor again supported a model of paracrine activation of the retinoic acid receptor to regulate embryonic differentiation (Maden, 2006).

A similar line of investigation has been used in *Drosophila melanogaster* (hereafter: *Drosophila*) and other invertebrate model systems, to test the role of steroid and isoprene-derived farnesoid hormones in insect embryonic development and tissue differentiation. These farnesoid hormones are synthesized in a pathway that involves 3-Hydroxy-3-Methylglutaryl CoA Reductase (HMGCR, Bellés et al., 2005). Classical endocrine organ (ring gland) transplantation experiments evidenced that a hormone (that was later identified as 20-hydroxy ecdysone; 20E) was necessary for both larval molting and to drive the maturation of imaginal discs at

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the end of larval development (Bodenstein, 1944; Jones and Jones, 2007; Vogt, 1943). Molecular genetic experiments in *Drosophila* to block enzymes in the 20E biosynthetic pathway yielded early death of larvae (Henrich et al., 1993). Similarly, when biosynthesis of 20E was conditionally blocked during the metamorphic third larval instar, morphogenetic derangement of certain nerves and of imaginal structures was observed (Brennan et al., 1998; Li and Cooper, 2001). From the alternative direction of the ecdysone receptor, rather than its ligand, *Drosophila* null mutants of ecdysone receptor exhibit death during larval development, including an "ultraspiracle"-like failure to ecdyse the apolysed prior instar cuticle (Li and Bender, 2000).

To the considerable frustration of the field, it has been more difficult to experimentally address the role of farnesoid hormones in the larval development and morphogenetic transformation of *Drosophila* (Riddiford, 1993, 2008). On the basis of studies in other surgically tractable model insects, the first known farnesoid hormone in *Drosophila* (methyl epoxyfarnesoate, JH III) was hypothesized to function in metamorphosis by way of its decrease or absence at metamorphosis (Gilbert et al., 2000). Indeed, morphogenetic derangements can be obtained by exogenous JH treatment of *Drosophila* larvae at the onset of the larval to pupal transition (Postlethwait, 1974; Riddiford and Ashburner, 1991; Wozniak et al., 2004). However, for reasons of the small size of *Drosophila* larvae and the surgical difficulty in excising the juvenile hormone secreting cells from the ring gland, the corresponding experiment to surgically remove the endogenous hormone could not be done.

Experimental dissection later became more complicated by the discovery that the endocrine source secretes not one, but three farnesoids: methyl farnesoate, methyl epoxyfarnesoate (=JH III), and methyl bisepoxyfarnesoate (Richard et al., 1989a; hereafter referred to as 'farnesoid hormones' or 'methyl farnesoids'). Yet further exacerbating the situation has been that although nuclear hormone receptors for retinoic acid and 20E were firmly identified two decades ago, identification of nuclear hormone receptors for these three *Drosophila* farnesoids in larvae has been comparatively delayed. Only more recently has the *Drosophila* ortholog of vertebrate nuclear hormone receptor RXR been show to bind methyl farnesoate with nanomolar affinity (Jones et al., 2006), while the *Drosophila methoprene tolerant* (MET) protein has been reported to similarly bind JH III (Miura et al., 2005). However, while larvae carrying null mutation for the *Drosophila* RXR ortholog die with an "ultraspiracle" phenotype, most null mutant larvae for MET survive to adulthood (Wilson et al., 2006). Hence, the actual role of farnesoid hormones in the larval development and metamorphosis of *Drosophila* remains inadequately understood.

In this study, we have used RNAi molecular genetic approaches targeted to the ring gland to selectively block the endogenous biosynthesis of the three methyl farnesoids secreted by the *Drosophila* ring gland. We report here the resultant survival, behavioral, morphogenetic and other phenotypic outcomes. These outcomes include the presentation of an apparent "ultraspiracle" death phenotype, similar to that exhibited by larvae carrying a null mutation for the *Drosophila* RXR. The death with "ultraspiracle" phenotype can be rescued by exogenously supplying a dedicated endogenous farnesoid precursor to the three secreted farnesoid hormones.

2. Methods and materials

2.1. Fly strains

The fly strains used in these studies were maintained on standard food medium at 25 °C. The wild-type background used in the present study was the *yellow white* (*yw*) strain. In order to accomplish reduction in expression of the enzyme 3-Hydroxy-3-Methylglutaryl CoA Reductase (HMGCR) specifically in the corpora allatal cells, a binary UAS-based system was used. The fly line used here (Di11) carrying the transgene with a promoter for expression of the Gal4 protein specifically in the corpora allata cells was characterized previously (Belgacem and Martin, 2007). The specificity of the expression of this RNAi only in the corpora allatal cells ensures that the HMGCR-dependent pathways in all other larval body tissues (which lead to products other than methyl farnesoid hormones) are left unsuppressed by the RNAi. Hence, the phenotypic outcomes observed will be arising specifically from suppressing the HMGCR-dependent pathway of the corpora allatal cells that leads to the production by those cells of the three methyl farnesoid hormones. From a fly strain (10367-R3) previously reported as transformed with a UAS-driven transgene encoding expression of RNAi against HMGCR (p[UAS-RNAi-HMGCR] (a courtesy of R. Ueda, NIG, Japan, to J.R.M.) (Belgacem and Martin, 2007)) we recovered upon outcrossing to *yw* two independent transformant lines. Both lines performed similarly upon crossing into a background containing the Di11 transgene (the latter characterized by Belgacem and Martin (2007)), so the results with only one of these (HMGR(r)) are reported here. The Di11 transgenic chromosome is homozygous lethal, but can be maintained over a chromosome 3 *Ser* balancer marked with an actin-driven green fluorescent protein (GFP). For subsequent collection of hemolymph samples and hormone treatment experiments, these flies were crossed to the strain homozygous for the RNAi-HMGCR expression transgene. At the time of collection of newly hatched 1st instar larvae, those larvae visually expressing GFP under proper illumination were manually removed.

2.2. Experimental rearing conditions

Newly hatching 1st instar larvae (<4 h old) were collected and reared individually at 25 °C in 10 × 75 mm glass vials containing standard *Drosophila* diet. Larvae were observed and scored periodically for survival and expression of other phenotypes described here.

For studies on dietary provision of various farnesoid-related compounds to fly larvae expressing RNAi-HMGCR, three independent replicates were performed. Each replicate consisted of at least 11 larvae being reared individually in glass vials of food, onto which had been layered and dried 6.25 μl of an ethanolic solution containing 125 nmol of the given compound. Each larva was examined daily for the following parameters (a) the developmental stage of death, (b) the status of ecdysis before death, (c) the location of the body at death (in the food, on the food, on the glass wall of the vial or at the interface of the food and the glass wall) and (d) for the location of the ecdysed prior instar larval mouthparts (whether attached to the dead larva, visibly separated or not from the dead larva by more than one body length away, or not present on either the food or the wall of the glass vial, i.e., located down in the food). When the larva died on the glass wall of the vial, it could be easily determined whether the caste prior cuticle was still attached to the new body. This status could also be observationally determined when the new larva had died on the food. However, in a minority of cases, the roughened/wet food surface prevented clear observation of the point of attachment. On the basis of some preliminary assessments, we decided that for the purpose of the studies here, it was reasonable to presume that mouthparts less than one body length away from the body of the dead larva were still attached to the larva.

For the studies on hemolymph farnesoid concentrations, wild-type and experimental 3rd instar larvae were reared as respective batch cohorts on standard diet, and recovered from the diet for extraction at the time indicated in the text. Some parallel experimental larvae were reared on food containing blue dye, which con-