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Local requirement of the *Drosophila* insulin binding protein imp-L2 in coordinating developmental progression with nutritional conditions ☆

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ABSTRACT

In *Drosophila*, growth takes place during the larval stages until the formation of the pupa. Starvation delays pupariation to allow prolonged feeding, ensuring that the animal reaches an appropriate size to form a fertile adult. Pupariation is induced by a peak of the steroid hormone ecdysone produced by the prothoracic gland (PG) after larvae have reached a certain body mass. Local downregulation of the insulin/insulin-like growth factor signaling (IIS) activity in the PG interferes with ecdysone production, indicating that IIS activity in the PG couples the nutritional state to development. However, the underlying mechanism is not well understood. In this study we show that the secreted Imaginal morphogenesis protein-Late 2 (Imp-L2), a growth inhibitor in *Drosophila*, is involved in this process. Imp-L2 inhibits the activity of the *Drosophila* insulin-like peptides by direct binding and is expressed by specific cells in the brain, the ring gland, the gut and the fat body. We demonstrate that Imp-L2 is required to regulate and adapt developmental timing to nutritional conditions by regulating IIS activity in the PG. Increasing *Imp-L2* expression at its endogenous sites using an Imp-L2-Gal4 driver delays pupariation, while *Imp-L2* mutants exhibit a slight acceleration of development. These effects are strongly enhanced by starvation and are accompanied by massive alterations of ecdysone production resulting most likely from increased Imp-L2 production by neurons directly contacting the PG and not from elevated Imp-L2 levels in the hemolymph. Taken together our results suggest that *Imp-L2*-expressing neurons sense the nutritional state of *Drosophila* larvae and coordinate dietary information and ecdysone production to adjust developmental timing under starvation conditions.

Introduction

The evolutionarily well conserved insulin/insulin-like growth factor signaling (IIS) pathway plays important roles in diverse biological processes, such as growth, energy metabolism and development. Since animals are constantly exposed to environmental changes, the ability to adapt quickly to those changes is essential for their survival. Insulin-like growth factors are involved in the adaptation to nutritional restriction by coupling information about nutrient availability with growth and energy metabolism (Edgar, 2006; Wang and Hung, 2006).

In mammals IIS ligands include the insulin-like growth factors -I and –II (IGF-I and –II) controlling growth rates during development, and insulin controlling carbohydrate and lipid metabolism. In *Drosophila,* eight insulin-like peptides (Dilp1-8) that share homology with vertebrate IGF-I and insulin have been identified as ligands of a unique insulin receptor (InR) (Colombani et al., 2012; Geminard et al., 2006; Rulifson et al., 2002; Brogiolo et al., 2001). Four out of the eight Dilps (Dilp−1,–2, −3 and −5) are secreted into the hemolymph by two clusters of insulin producing cells (IPCs) located in each hemisphere of the brain (Ikeya et al., 2002; Brogiolo et al., 2001). Binding of Dilps to the insulin receptor in peripheral tissues activates intracellular signal transduction cascades including PI3-kinase and the Target of Rapamycin (TOR), leading to systemic growth activation.

Abbreviations: AED, after egg deposition; CC, corpora cardiac; Dilp, *Drosophila* insulin-like peptide; IGF, insulin-like growth factors; IGFBP, insulin-like growth factor binding proteins; IIS, insulin/insulin-like growth factor signaling; Imp-L2, Imaginal morphogenesis factor-Late2; InR, insulin receptor; IPC, insulin producing cells; LOF, loss of function; NPF, neuropeptide F; PG, prothoracic gland; PTTH, prothoracicotrophic hormone; TOR, Target of Rapamycin; SOG, subesophageal ganglion; 20E, 20-hydroxyecdysone

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Activity, stability and availability of IGFs are modulated by insulin-like growth factor binding proteins (IGFBPs) (Hwa et al., 1999). Furthermore, additional IGFBP-related proteins, such as the tumor suppressor IGFBP-7, have been identified in mammals (Wajapeyee et al., 2008; Hwa et al., 1999).

In *Drosophila,* only one Dilp-binding protein, the Imaginal morphogenesis factor-Late2 (Imp-L2), has been identified. Imp-L2, a member of the immunoglobulin superfamily, shares partial sequence homology with IGFBP-7 and binds IGF-I in vitro (Sloth Andersen et al., 2000; Yamanaka et al., 1997; Garbe et al., 1993). More recent studies showed that secreted Imp-L2 binds native Dilp-2 and -5 and thereby controls growth systemically as a negative regulator of IIS activity (Alic et al., 2011; Honegger et al., 2008). Decreasing IIS activity by either repression of InR or ablation of the IPCs leads to growth rate reduction and delayed development (Shingleton et al., 2005; Rulifson et al., 2002). Conversely, loss of Imp-L2 function leads to increased body size and starvation sensitivity (Honegger et al., 2008). However, Imp-L2 shows a complex expression pattern, suggesting that it serves additional local functions.

In *Drosophila* as in other holometabolous insects, the steroid hormone ecdysone regulates development by controlling the larval/pupal transition and molting (Marchal et al., 2010). Ecdysone production and release is mainly regulated by the prothoracicotrophic hormone (PTTH) (Mcbrayer et al., 2007; Nijhout, 2003). PTTH is produced by two pairs of neurons in the larval brain that target the PG. After larvae have reached critical weight, which is defined as the size that is sufficient to initiate metamorphosis in the absence of nutrition, PTTH is released and triggers ecdysone production. Ablation of those neurons leads to a massive decrease in ecdysone production and therefore to delayed pupariation (Mcbrayer et al., 2007). Notably, starvation does not affect PTTH levels, indicating that PTTH signaling is not able to couple development to nutrient availabilities. Conversely, tissue specific down-regulation of IIS activity in the PG, the source of ecdysone production, delays pupariation (Caldwell et al., 2005; Mirth et al., 2005), suggesting that IIS is involved in adapting developmental timing to nutritional changes. However, the mechanism behind this adaptation process is not fully understood.

In this study we show that the Dilp-2 binding protein Imp-L2 locally regulates IIS activity in the PG. We observed neurons, distinct from the PTTH expressing ones, that express Imp-L2 and project to the PG. Increased Imp-L2 production by these neurons severely delays pupariation by stalling ecdysone production, while Imp-L2 loss of function leads to elevated ecdysone production. Moreover, we show that the starvation-induced developmental delay is reduced in *Imp-L2* loss-of-function animals at the expense of survival, indicating that Imp-L2 controls ecdysone production in a nutrition-dependent manner to couple development to nutrient availability.

Results

Expression pattern of the Imp-L2 isoforms

Imp-L2 shows a diverse expression pattern. In situ hybridizations and immunohistochemistry revealed *Imp-L2* expression in the corpora cardiaca (CC) portion of the ring gland, in approximately 20 enteroendocrine cells in the anterior midgut and in distinct neurons in both brain hemispheres and the subesophageal ganglion (SOG) (Honegger et al., 2008). In addition, low levels of *Imp-L2* mRNA were detected in the prothoracic gland (PG) portion of the ring gland (Fig. S1A). This diverse expression pattern may underlie tissue-specific roles of Imp-L2, however, studies to elucidate local actions of Imp-L2 have not been carried out yet.

Since ubiquitous overexpression of Imp-L2 with strong drivers like actin-Gal4 leads to lethality (Honegger et al., 2008), we aimed to express Imp-L2 from its endogenous sites to analyze its function. Thus, we created two different Imp-L2-Gal4 lines, Imp-L2-RA-Gal4 and Imp-L2-RC-Gal4, representing transcript-specific driver lines of Imp-L2. To visualize their expression patterns, we crossed them to flies carrying a *CD8-GFP* gene under UAS control (UAS-CD8-GFP). CD8-GFP is membrane localized and thus visualizes the projection patterns of the Imp-L2 expressing cells. We stained for Imp-L2 protein to check the faithfulness of the driver lines. Imp-L2-RA-Gal4 is expressed in all cells positive for Imp-L2 staining (the anterior midgut, the CC, the glia cells and neurons in both brain hemispheres and the SOG) (Fig. 1A–D). We also observed projections of Imp-L2-expressing neurons on the PG (Fig. 1E). Additional GFP signal was detected in two tissues that did not reliably stain for Imp-L2 protein: the fat bodies of wandering but not feeding third instar larvae (Fig. S1B, C) and a few single PG cells (Fig. 1F). Imp-L2-RC-Gal4 appears to express exclusively in the PG. In all larval stages, Impl-L2-RC-Gal4 drives stronger GFP expression than Imp-L2-RA-Gal4 (Fig. 1H). However, we only sporadically detected low Imp-L2 protein levels in the PG even though *Imp-L2* mRNA was clearly present (Fig. S1A). Using a GFP reporter construct containing the small second intron of *Imp-L2,* we discovered that the cis regulatory sequences of *Imp-L2-RC* reside in this intron and drive GFP expression in the PG (Fig. 1G). Presumably, Imp-L2 protein expression in the PG and the fat body is below detection level, or the protein is secreted into the hemolymph immediately after production. Since the Imp-L2-RA-Gal4 line drives expression in all cells shown to be positive for Imp-L2 protein, it constitutes a powerful tool for further analysis of Imp-L2 function in overexpression experiments.

Imp-L2 is required to regulate and adapt developmental timing to nutritional conditions

In *Drosophila,* nutritional restriction delays pupariation due to an extended feeding phase required to attain critical weight. To test whether Imp-L2, as a negative regulator of IIS activity, is involved in this adaptation process we analyzed the developmental timing of *Imp-L2* mutant and Imp-L2-overexpressing larvae under normal and reduced food conditions. Overexpression of Imp-L2 using the Imp-L2-RA-Gal4 line (Imp-L2-RA > Imp-L2) elevates Imp-L2 levels specifically at the endogenous sites of *Imp-L2* production. Under normal food conditions neither Imp-L2 overexpression nor *Imp-L2* loss of function (LOF) affected developmental timing during the first larval instar (L1). However, the second instar (L2) period was prolonged by six hours in Imp-L2-RA-GAL4/UAS-Imp-L2 animals, whereas *Imp-L2* mutant larvae behaved like the control (Fig. 2A). The major effect occurred in the third larval stage (L3), where *Imp-L2* mutant larvae pupariated six hours earlier than control larvae, whereas Imp-L2-RA > Imp-L2 larvae were approximately 45 h delayed (Fig. 2B). Notably, these effects were even more striking under starvation conditions (10% yeast), in which *Imp-L2* mutant larvae pupariated 22 h earlier and Imp-L2-RA > Imp-L2 72 h later than control larvae (Fig. 2C). Hence, starvation-induced developmental delay is reduced by 64% in Imp-L2 mutants and increased by 75% in Imp-L2-RA > Imp-L2 larvae. On the other hand, survival rates of *Imp-L2* mutant larvae are decreased by 60% compared to the control (Fig. 2D). Thus, the lower Imp-L2 levels are, the earlier pupariation takes place under limited food conditions. However, early pupariation comes at the expense of larval survival rates, indicating that Imp-L2 is required for proper adjustment to nutritional changes and is essential for survival under adverse food conditions.