The IGFBP7 homolog Imp-L2 promotes insulin signaling in distinct neurons of the *Drosophila* brain

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Accepted 22 March 2013
Journal of Cell Science 126, 2571–2576

doi: 10.1242/jcs.120261

Summary

In *Drosophila*, Insulin-like peptide 2 (Dilp-2) is expressed by insulin-producing cells in the brain, and is secreted into the hemolymph to activate insulin signaling systemically. Within the brain, however, a more local activation of insulin signaling may be required to couple behavioral and physiological traits to nutritional inputs. We show that a small subset of neurons in the larval brain has high Dilp-2-mediated insulin signaling activity. This local insulin signaling activation is accompanied by selective Dilp-2 uptake and depends on the expression of the Imaginal morphogenesis protein-late 2 (Imp-L2) in the target neurons. We suggest that Imp-L2 acts as a licensing factor for neuronal IIS activation through Dilp-2 to further increase the precision of insulin activity in the brain.

Key words: *Drosophila*, Imp-L2, Insulin signaling, Brain

Introduction

Animals are constantly exposed to environmental changes that they need to adapt to in order to survive. In insects, hormones such as insulin-like growth ligands and adipokinetic hormones (AKH) (Grönke et al., 2007) act systemically to couple information about nutrient availability with growth and energy metabolism (Edgar, 2006; Wang et al., 2006). In *Drosophila*, eight insulin-like peptides (Dilp-1 to -8) that share homology with vertebrate IGF-I and insulin have been identified as ligands of a unique insulin receptor (InR) (Brogiolo et al., 2001; Colombani et al., 2012; Garelli et al., 2012; Geminard et al., 2006; Rulifson et al., 2002). 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 (Brogiolo et al., 2001; Ikeya et al., 2002). Binding of the Dilp proteins to InR in peripheral tissues stimulates systemic growth by activating an intracellular signal transduction cascade composed of PI3-kinase, protein kinase B (PKB) and Target of rapamycin (TOR). In the brain, a more local activation of insulin signaling (IIS) may be required since IIS activity in distinct neurons regulates behavioral and physiological traits. For instance, the receptor of neuropeptide F (NPFR) involved in food choice in relation to starvation is negatively regulated by IIS (Wu et al., 2003). Furthermore, neurons expressing the neuropeptide Hugin are involved in feeding regulation and send axons to the IPCs (Melcher and Pankratz, 2005). How secreted Dilp proteins trigger the local activation of IIS in distinct neurons remains unknown.

In this study we investigated a small number of neurons with elevated IIS activity in the larval brain that are directly contacted by the IPCs and take up Dilp-2. All neurons that show high IIS activity are positive for the Dilp-2-binding protein Imp-L2. In *Imp-L2*-deficient larvae high levels of IIS and uptake Dilp-2 are not detectable, but overexpression of Imp-L2 is sufficient to induce Dilp-2 uptake in specific neurons. We conclude that Imp-L2, a negative regulator of the humoral Dilp-2 response in peripheral tissues (Honegger et al., 2008), is required for Dilp-2-mediated activation of IIS in neurons, adding a layer of regulation to the control of cell-specific IIS activity in the larval brain.

Results and Discussion

Dilp-2 activates IIS in distinct neurons of the larval brain in an Imp-L2-dependent manner

We found that only a small subset of cells in the larval brain shows high IIS activity, as indicated by phosphorylated PKB (pPKB) antibody staining (Fig. 1A). Interestingly, these are cells expressing *Imp-L2*, a negative regulator of insulin signaling (Fig. 1A′), which are located at the IPC projection sites, e.g. the protocerebrum, the subesophageal ganglion and the corpora cardiaca (Ikeya et al., 2002) (see also Fig. 1B,C). In the subesophageal ganglion, Imp-L2-positive cells partially overlap with cells expressing the neuropeptide Hugin (Fig. 1C).

We posit that Imp-L2 production makes cells in the brain ring gland complex (BRC) responsive to activation by Dilp-2. The evidence for this is fourfold. (1) All cells positive for pPKB express Imp-L2 (Fig. 1A,A′), and Imp-L2-expressing cells are located at Dilp-2 secretion sites (Fig. 1C). (2) All Imp-L2-expressing cells take up Dilp-2, because they stain positive for the Dilp-2 antibody (Fig. 1D–D′′) even though *Dilp-2* mRNA is only detected in the IPCs (Ikeya et al., 2002). This effect is Dilp-2 specific, because Dilp-3 and Dilp-5 are exclusively detected in the IPCs where they are produced (Fig. 1E,F). (3) Imp-L2 expression is necessary for Dilp-2 uptake and IIS activity in these cells because high IIS activity as well as Dilp-2 uptake are absent in *Imp-L2*-deficient brains (Fig. 1G–I). (4) Imp-L2 overexpression in a subset of Hugin cells that are normally

Imp-L2 negative is sufficient to induce Dilp-2 uptake (Fig. 1J–L′).

Our results indicate that Dilp-2 and Imp-L2 function together to locally enhance IIS activity in distinct neurons. This local IIS-promoting function of Imp-L2 contrasts with its Dilp-2-antagonizing and IIS-inhibiting function in the systemic response (Honegger et al., 2008). In mammals, where seven different insulin/insulin-like growth factor binding proteins (IGFBP1–7)

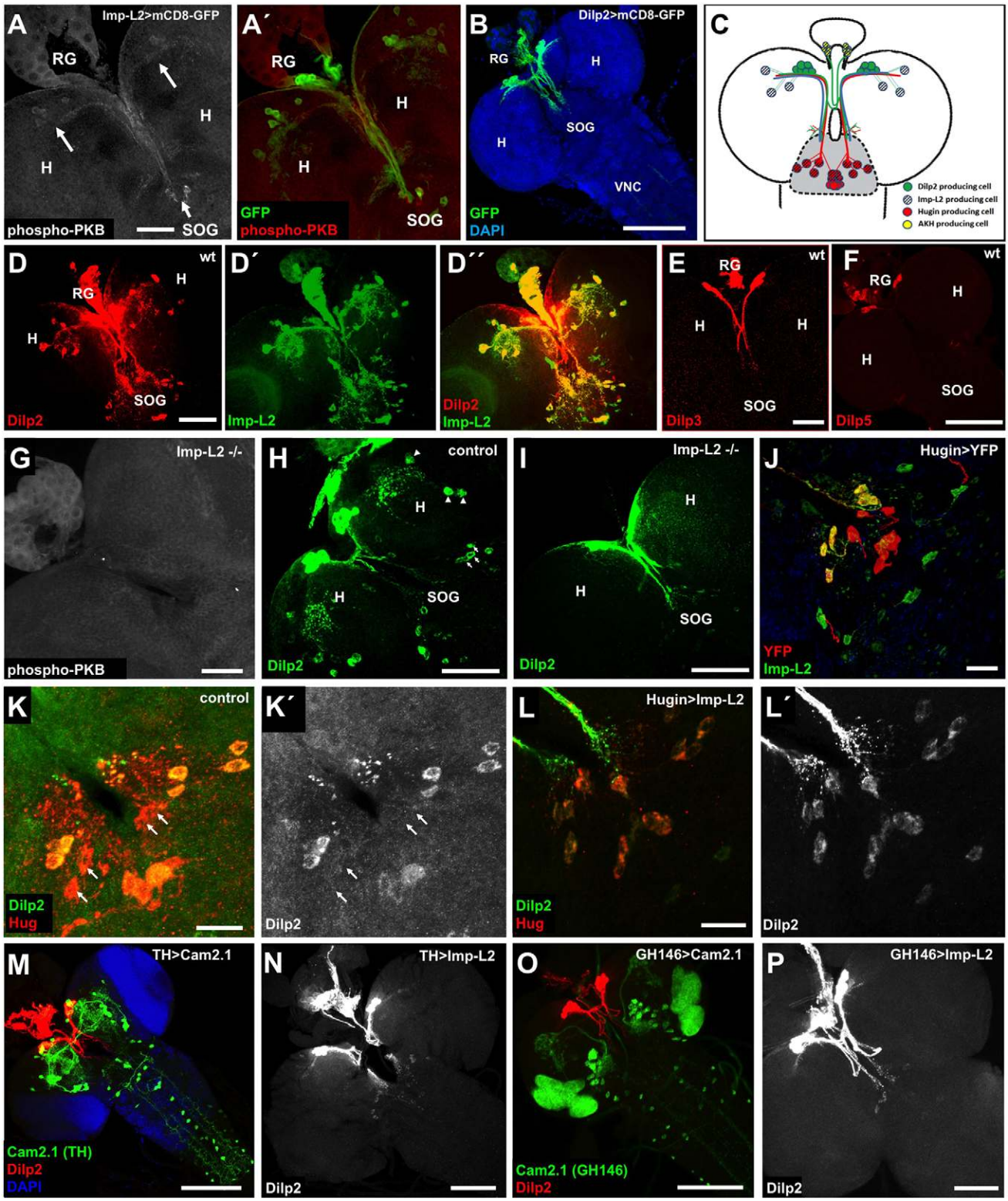

Fig. 1. See next page for legend.

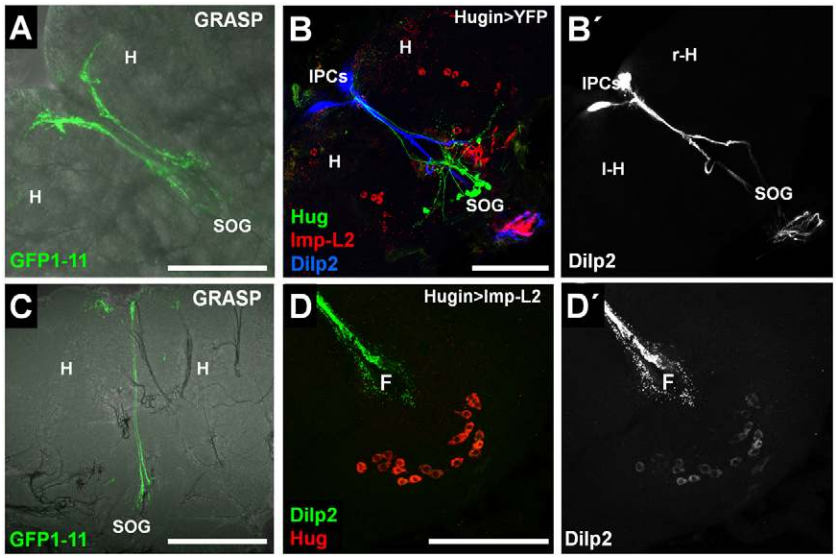

Fig. 2. Direct cellular connection with the IPCs is required for Dilp-2 uptake. (**A**) The GRASP technique detects direct cell contacts between IPCs and Hugin neurons (CD4::GFP11 under the control of a *Hugin* promoter and CD4::GFP1–10 under the control of the *Dilp-2* promoter). Larval brain shows GFP signal (green) where both GFPs physically interact. (**B**,**B′**) Adult brains expressing YFP (green) using Hugin-Gal4, stained for Imp-L2 (red) and Dilp-2 (blue in B, gray/white in B′). Hugin cells are Imp-L2 negative in the adult CNS, and Dilp-2 staining only marks the IPCs. (**C**) Adult brain examined using the GRASP technique. The GFP signal demonstrates that Hugin cells physically interact with IPCs. (**D**,**D′**) Dilp-2 staining (green in D, gray/white in D′) of an adult hug-Gal4/UAS-Imp-L2 animal shows that Imp-L2 overexpression is sufficient to promote Dilp-2 uptake into Hugin cells (red). Scale bars: 100 μm (A–C) and 50 μm (D,D′). F, foramen; H, hemisphere; SOG, subesophageal ganglion.

modulate the activity of IIS by binding IGF-I and IGF-II (Hwa et al., 1999; Jones and Clemmons, 1995), a similar dual function of IGFBPs was observed. Although IGFBP-4 and IGFBP-6 exclusively act as growth repressors, IGFBP-1, -2, -3 and -5 exhibit growth-inhibiting and growth-promoting functions (McCusker et al., 1990; Yin et al., 2004). This indicates the conservation of context-dependent opposite functions of insulin/insulin-like growth factor binding proteins.

Fig. 1. Imp-L2 expression makes the neurons responsive to targeting by Dilp-2. (**A**) Anti-phosphorylated-PKB (pPKB) antibody labels cells with high IIS activity (arrows) in the larval brain. Imp-L2-expressing cells (marked by GFP in Imp-L2-RA-Gal4>CD8-GFP animals; see Materials and Methods) are pPKB positive (**A′**). (**B**) Larval brain of Dilp-2>CD8-GFP animals, demonstrating that IPCs project to the hemisphere (H), subesophageal ganglion (SOG) and ring gland (RG). (**C**) Diagram of larval brain showing IPCs (green), Hugin cells (red), corpora cardiaca (yellow) and Imp-L2 expressing cells (hatched). 12 out of the 20 Hugin cells are Imp-L2 positive. IPCs project to sites of Imp-L2 expression. (**D–D′′**) Co-labeling of Dilp-2 (D, red) and Imp-L2 (D′, green) shows major overlap (D′′) indicating Dilp-2 uptake by Imp-L2 neurons. (**E**,**F**). Such uptake of Dilp-3 (E) and Dilp-5 (F) is not seen. (**G**) pPKB staining of *Imp-L2*$^{-/-}$ larval brains. Although in Imp-L2 mutants there is overall increased IIS activity (Honegger et al., 2008), no pPKB-positive cells are detected, indicating that Imp-L2 expression is required for high IIS activity in the brain. (**H**,**I**) Dilp-2 uptake is absent in *Imp-L2*-deficient brains. Arrows mark a subset of Hugin neurons, arrowheads some uncharacterized neurons in the protocerebrum. (**J**) Imp-L2 staining (green) in Hugin cells (red). Only a subset of Hugin neurons expresses Imp-L2. (**K**,**K′**) Subesophageal ganglion of control larva (hug-Gal4/+) stained against Dilp-2 (green in K, gray/white in K′) and Hugin (red). Only Imp-L2-positive Hugin cells take up Dilp-2. Arrows mark a subset of Hugin neurons. (**L**,**L′**) Uptake of Dilp-2 (green in L, gray/white in L′) is massively increased in the subesophageal ganglion of hug-Gal4/UAS-Imp-L2 larvae. Hugin cells that are normally Imp-L2 and Dilp-2 negative take up Dilp-2 in this setting. (**M**) Expression of Chameleon2.1 under the control of the tyrosine hydroxylase (TH) promoter (GFP, green; Dilp-2, red). (**N**) Overexpression of Imp-L2 using TH-Gal4 (Dilp-2, gray/white). (**O**) Expression of Chameleon2.1 under the control of GH146-Gal4 (GFP, green; Dilp-2, red). (**P**) Overexpression of Imp-L2 using GH146-Gal4 (Dilp-2, gray/white). Scale bars: 100 μm (B,F), 50 μm (A,D,E,G,H,I,M,O), 50 μm (N,P) and 20 μm (J,K,L). H, hemisphere; RG, ring gland; VNC, ventral nerve cord.

Local Imp-L2 expression in the brain facilitates activation of IIS by promoting Dilp-2 binding to its receptor

Overexpression of Imp-L2 in Hugin-positive cells induces Dilp-2 protein uptake in all Hugin neurons, indicating that Imp-L2 expression enables these cells to take up Dilp-2. However, the presence of Imp-L2 protein per se is not sufficient to allow Dilp-2 uptake, since overexpression of Imp-L2 with various other neuronal Gal4 lines did not lead to this effect (Fig. 1M–P). Unlike the neurons targeted by these lines, the cellular extensions of the Hugin neurons are in very close proximity to the IPC network. Using the GFP reconstitution across synaptic partners (GRASP) technique (Gordon and Scott, 2009) we detected direct cell contacts between IPCs and Hugin cells. We applied a dual expression system comprising a Dilp-2-Gal4 driving UAS-CD4::spGFP1–10 and a hug1.2-LexA driving LexAop-CD4::spGFP11, resulting in a GFP signal only where both GFP parts physically interact (Fig. 2A). Thus, in addition to Imp-L2 expression, a direct cellular contact to the IPCs may be required for Dilp-2 uptake. Indeed, in adult brains where the Imp-L2 neuronal network is not connected to the IPCs, Dilp-2 uptake cannot be detected (Fig. 2B,B′). Hugin neurons in the adult brain physically interact with the IPCs (Fig. 2C) but are, unlike in the larval brain, Imp-L2 negative (Fig. 2B). Overexpression of Imp-L2 in Hugin neurons of adults is sufficient to induce Dilp-2 uptake (Fig. 2D,D′). Thus, Imp-L2 expression and the physical proximity to the IPC network are required for Dilp-2 uptake.

Furthermore, Dilp-2 uptake depends on an intact InR, since overexpression of the full-length receptor in Hugin cells massively increases Dilp-2 uptake (Fig. 3A–B′). By contrast, a truncated InR lacking the ligand-binding domain does not induce Dilp-2 uptake (Fig. 3C,C′), although it is sufficient to activate IIS in these cells (Wittwer et al., 2005). InR-mediated uptake of Dilp-2 depends on Imp-L2 expression because InR overexpression in Hugin cells of *Imp-L2* mutants does not induce Dilp-2 uptake (Fig. 3D,D′). Overexpression of Imp-L2 or dp110 in Hugin cells led to equally increased phospho-PKB staining (Fig. 3E,F), validating that Imp-L2 expression increases IIS activity by mediating Dilp-2 uptake.

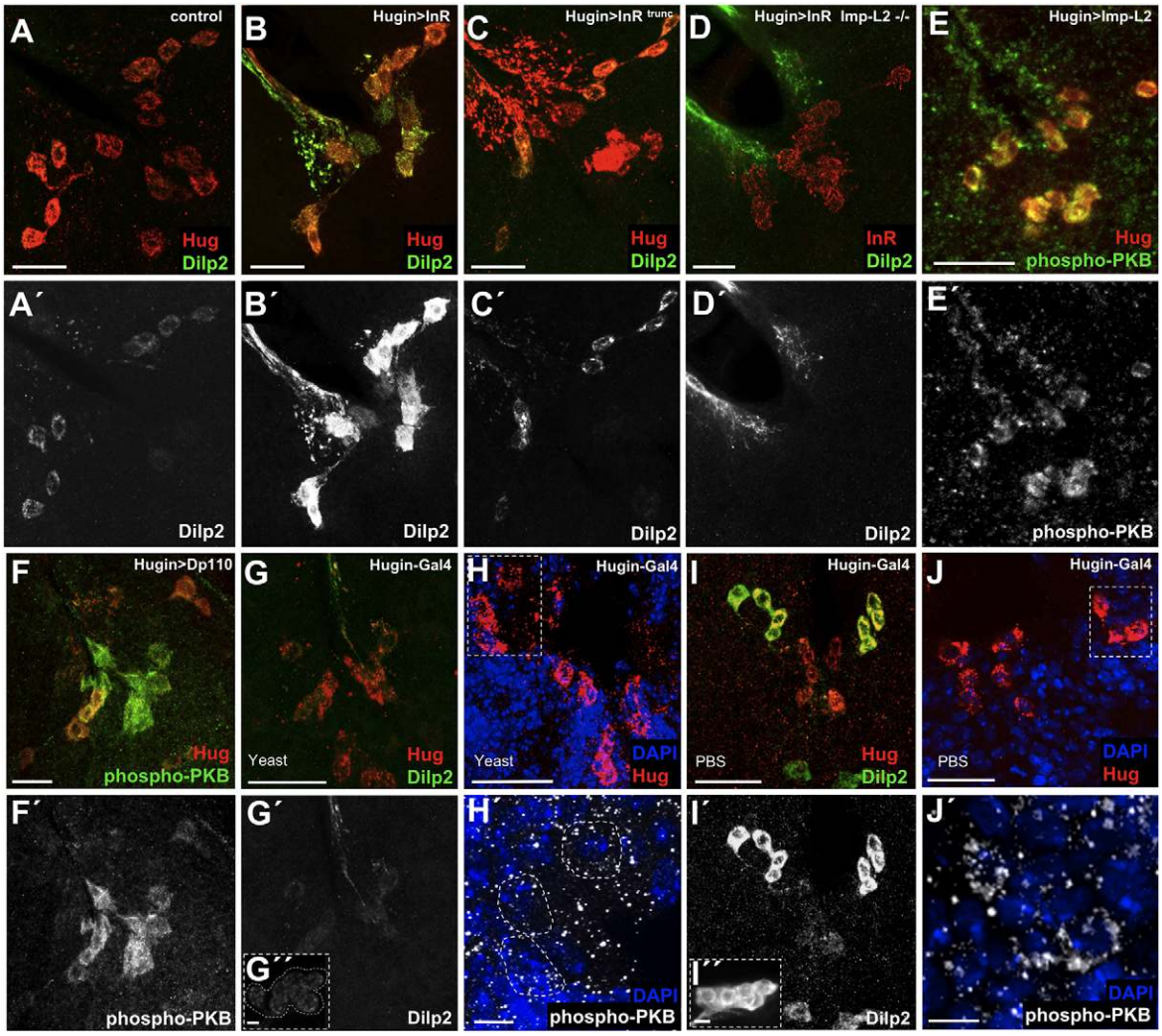

Fig. 3. Dilp-2 uptake is mediated by InR and increases IIS in Imp-L2-positive neurons. (**A–C′**) Hugin (red) and Dilp-2 (green in A,B,C, grey/white in A′,B′,C′) staining in the subesophageal ganglion. (A–C′) Overexpression of InR in Hugin cells increases Dilp-2 uptake (control: hug-Gal4/+), which is not seen in cells overexpressing a truncated form of InR lacking the ligand binding domain, suggesting that Dilp-2 binding to InR is essential for its uptake. (**D**,**D′**) Overexpression of InR in Hugin cells in an *Imp-L2* mutant background (*Imp-L2-MG2*, *hug-Gal4*/*Imp-L2-def42*) does not enhance Dilp-2 uptake, indicating that InR overexpression cannot overcome the lack of Imp-L2. Staining against InR (red) confirms efficient InR overexpression (Dilp-2, green). (**E–F**) Hugin (red) and phospho-PKB (green in E, F, gray/white in E′,F′) in the subesophageal ganglia of larvae overexpressing Imp-L2 (E,E′) or dp110 (F,F′) under the control of Hug-Gal4. (**G**,**J′**) Staining of larval brains for Hugin, Dilp-2 or phospho-PKB under starved (PBS) and yeast-fed conditions. (G,I) Hugin (red) and Dilp-2 (green in G,I, gray/white in G′,I′). IPCs are shown in the insets (G′′,I′′). Dilp-2 signals increase in the IPCs and in Hugin cells upon starvation. (H,J) A protein-rich diet (H) decreases, whereas starvation (J) enhances IIS in Hugin cells, as indicated by phospho-PKB staining. (H′) Magnification of boxed area in H (cells are circled by the dashed lines). (J′) magnification of boxed area in J (DAPI in blue). Scale bars: 20 μm (A–J), 5 μm (G′,H′,I′,J′).

Next, we tested whether neuronal Dilp-2 uptake is affected by different nutrient conditions. Indeed, a protein-rich diet decreased Dilp-2 uptake and therefore IIS (Fig. 3G,H), whereas starvation lead to increased Dilp-2 uptake and IIS activity in Imp-L2-expressing cells (Fig. 3I,J). Moreover, Dilp-2 signal in the IPCs was decreased in yeast-fed and increased in starved larvae (Fig. 3G′′,I′′), which is concordant with IPCs adapting Dilp-2 secretion to nutrition (Géminard et al., 2009). Thus, starvation decreases systemic IIS and increases IIS in Imp-L2-positive neurons, whereas a protein-rich diet has the opposite effect. Together our results may reflect a mechanism that activates certain neurons in the brain to locally enhance IIS under conditions where systemic IIS is decreased. Finally, we suggest that Dilp-2 uptake is mediated by endocytosis, since Dilp-2 and Imp-L2 co-localize with the late endosome marker Rab-7 (Fig. 4A–F).

The mechanism by which Imp-L2 facilitates Dilp-2 uptake is unknown. Considering that Imp-L2 is a secreted protein, it is surprising that only Imp-L2-expressing cells, but not the other cells adjacent to the IPCs can take up Dilp-2. In mammals, both secreted IGFPBs and membrane-bound IGFBPs were found *in vitro* (Hsu and Olefsky, 1992). If the same was true for the IGFBP7 homologue Imp-L2, one could argue that only membrane-associated Imp-L2, but not its secreted form can mediate Dilp-2 uptake. The affinity of IGFBP-5 to IGFs is reduced when bound to the extracellular matrix (ECM) or to glycosaminoglycans (Arai et al., 1996; Arai et al., 1994; Parker et al., 1998). Under this condition, IGF-R becomes a stronger

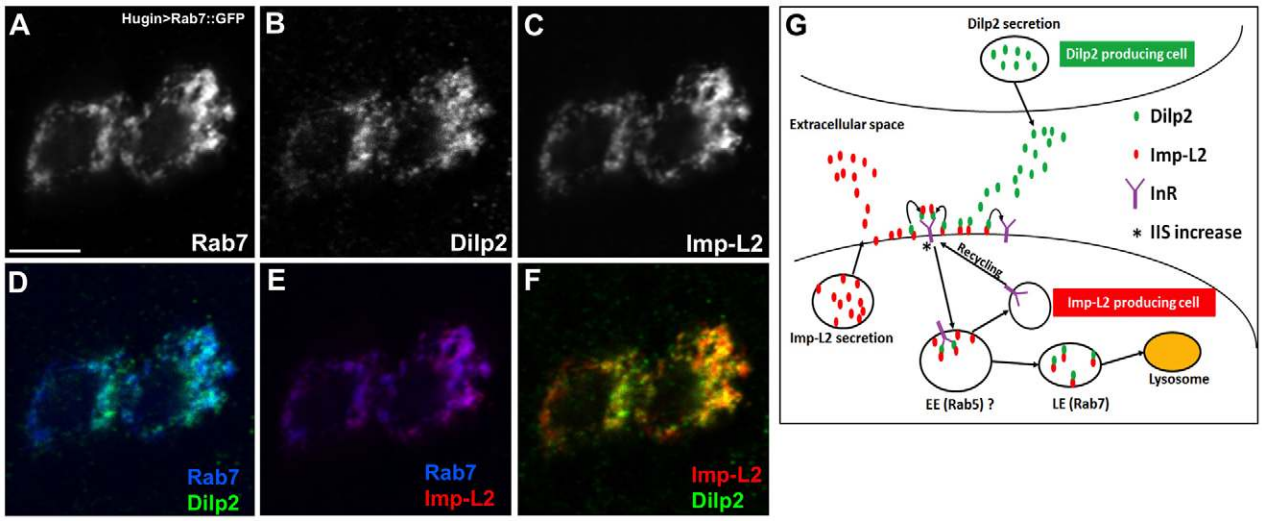

Fig. 4. Imp-L2 and Dilp-2 colocalize in late endosomes. (**A–F**) Expression of GFP–Rab7 in Hugin cells. Two lateral Hugin cells are shown. Stainings for Rab7 (A), Dilp-2 (B) and Imp-L2 (C) signals in gray/white. Merged channels of Rab7 (blue) and Dilp-2 (green; D), Rab7 (blue) and Imp-L2 (red; E) and Imp-L2 (red) and Dilp-2 (green; F). (**G**) Proposed model of Imp-L2 mediated Dilp-2 uptake. IPCs secrete Dilp-2 into the extracellular space where it binds to membrane-associated Imp-L2. Membrane bound Imp-L2 provides InR with its ligand, thereby activating IIS. Eventually, the entire complex becomes endocytosed by Imp-L2-expressing cells. InR is most likely recycled to the cell surface, while Imp-L2 and Dilp-2 enter the degradation pathway. Scale bar: 5 μm.

binding partner for IGFs than IGFBP-5. Thus, IGFBP-5 may exert its growth-promoting effect by increasing the bioavailability of IGF-I to the IGF-R after concentration of the IGFBP-5/IGF-I complex at the cell surface or the ECM.

Adapting this model to *Drosophila*, the following scenario may be envisioned (Fig. 4G). IPCs secrete Dilp-2 at sites of Imp-L2 production, where Dilp-2 becomes sequestered by membrane-associated Imp-L2. Hence, Dilp-2 is prevented from diffusing into all brain regions, thus locally increasing Dilp-2 concentrations. This scenario requires that membrane association of Imp-L2 lowers its affinity to Dilp-2, allowing Dilp-2 to bind more efficiently to InR. In this situation, Imp-L2 could act as a sink for Dilp-2, providing InR with higher ligand levels. Whether InR binds to and mediates the endocytosis of the Dilp-2–Imp-L2 complex, as suggested by the Dilp-2- and Imp-L2-positive late endosomes, or whether the complex dissociates upon ligand binding remains to be shown.

Whatever the precise mechanism is by which Imp-L2 mediates Dilp-2 uptake, our results demonstrate a novel function of insulin-binding proteins in contributing to the specificity by which neurons respond to Dilp-2. A physiological role may be to locally target neurons involved in coordinating metabolic status with feeding behavior, such as Hugin-, neuropeptide-F- and short-neuropeptide-F-expressing cells. For example, interference with InR in NPFR-expressing neurons makes larvae feed on normally rejected unattractive food. Conversely, upregulated IIS leads to food aversion in starved larvae (Wu et al., 2005). In this context, cells need to fulfill three separate criteria for a strong insulin response: direct contact to the IPCs, expression of InR on the surface and expression and secretion of the Dilp-2-binding protein, Imp-L2. The evolution of the Imp-L2 gene expression pattern thus provides an additional mechanism by which the insulin response in the brain is modulated.

Materials and Methods

Fly stocks and feeding experiments

Fly stocks used: *Imp-L2-def20*, *Imp-L2-def42*, *Imp-L2-MG2*, *UAS-sImp-L2* (Honegger et al., 2008), *HugS3-Gal4* (Melcher and Pankratz, 2005), *UAS-YFP*, *UAS-CD8-GFP*, *UAS-GFP-Rab7*, the *attP-86Fb* line, *UAS-Cam2.1*, *GH146-Gal4* and *TH-Gal4* (Bloomington, Indiana, USA, *UAS-InR* (Brogiolo et al., 2001), *UAS-InR^{trunc}* (Wittwer et al., 2005), *UAS-dp110* (Leevers et al., 1996). Three *Imp-L2* lines (*Imp-L2-def42*, *Imp-L2-def20* and *Imp-L2-MG2*) lack Dilp-2 uptake; however, only the *Imp-L2-def42* line is shown here. Imp-L2-RA-Gal4, a transcript-specific driver for Imp-L2-RA, drives expression in all cells positive for Imp-L2 protein (L.S-Z., K.K., E.H., H.S., Paola Cognigni, Stefan Christen, Uwe Sauer, and Irene Miguel-Aliaga, unpublished). The wild type used was the y w strain.

To measure Dilp-2 uptake into Hugin cells, animals were reared on apple agar plates with yeast for 72 hours before transfer to yeast on filter paper (protein-rich diet) or phosphate buffered saline (PBS) (starvation) for 14 hours.

Fluorescence microscopy

Antibody stainings were carried out as described previously (Melcher and Pankratz, 2005). Primary antibodies used were: anti-GFP (Abcam, ab6556 and ab13970), anti-InR (Cell Signaling, 3021S), anti-phospho-PKB (Cell Signaling, 4054S and 9271S, Fig. 1 and Fig. 3, respectively), anti-Dilp-2, anti-Dilp-3 and anti-Dilp-5 (E.H.), anti-Dilp-2, anti-Hugin (M.P.), anti-Imp-L2 (Linda Partridge, London, UK). Nuclei were stained with DAPI (Biostatus). Hugin and Dilp-2 antibodies were generated against the peptides QLQSNGEPAYRVRT and DMKALREYCSVVRN, respectively (Coring System Diagnostics).

Images were taken with a ZEISS LSM510 Meta, 710 and 780 or a Leica SPE confocal laser scanning microscope, processed in ZEN light edition (ZEISS) and readjusted for each color independently but always on the whole picture and set of experiment. The intensity of nonspecific background staining was lowered using the ‘dust and scratches’ filter in Adobe Photoshop.

Generation of fly lines

Hug1.2-LexA fly line was created by cloning a 1243 bp *Hugin* promoter fragment into the pBPnlsLexA::p65Uw vector (Addgene) (Pfeiffer et al., 2010) and injecting it into attP-86Fb embryos (Bischof et al., 2007).

Acknowledgements

We thank all members of the Hafen and Pankratz group for discussions and technical support, the Bloomington Stock Center for fly stocks and the Partridge lab for the Imp-L2 antibody.

Author contributions

R.B. and L.S-Z. contributed equally to this work. They designed the experiments and interpreted the data. E.H., M.J.P., K.K. and H.S. supervised the project. R.B., L.S-Z., M.P. and N.M. carried out confocal microscopy. L.S-Z. and M.P. generated transgenic flies, M.P. performed the GRASP analyses. N.M. performed the nutritional

assay. All authors wrote the manuscript, discussed the results and implications and commented on the manuscript at all stages.

Funding

This work was supported by grants from the Swiss National Science foundation [grant number SNF 31003AB_135699 to E.H.]; the Collaborative Research Center [grant number SFB 645 to M.J.P.]; and the Deutsche Forschungsgemeinschaft [grant number PA 787/6-1 to M.J.P.].

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