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RESEARCH TOPICS

THE COMING OF AGE OF INSULIN-SIGNALLING IN INSECTS

Topic Editors
Xanthe Vafopoulou and Colin G. Steel

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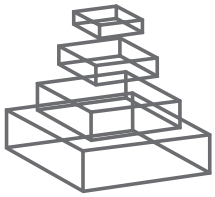

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THE COMING OF AGE OF INSULIN-SIGNALLING IN INSECTS

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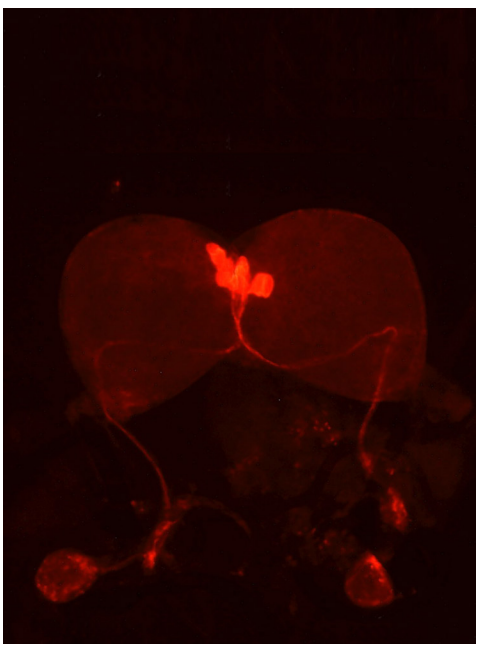

Bombyxin-producing neurosecretory cells. Whole-mount immunohistochemistry with anti-bombyxin-II mouse monoclonal antibody (A7B11) was performed on the brain-retrocerebral complex of the day-3 fifth instar larva of B. mori. BR, brain; CA, corpus allatum.

Image taken from: Mizoguchi A and Okamoto N (2013) Insulin-like and IGF-like peptides in the silkmoth Bombyx mori: discovery, structure, secretion, and function. *Front. Physiol.* 4:217. doi: 10.3389/fphys.2013.00217

The new millennium has seen a major paradigm shift in insect endocrinology. Great advancements are being made which establish that nutrition and growth play a central role in diverse cellular and physiological phenomena during insect development and reproduction. Nutrition affects rates of growth and is mainly regulated by the function of the pathway of insulin/insulin-like growth factor signalling. This pathway is highly conserved across species and ultimately regulates rates of cell growth and proliferation in growing organs. Insulin and insulin-like peptides (ILPs) are some of the best studied hormones in the animal kingdom and all share a common structural motif and initiate a wide range of closely similar physiological processes in higher organisms.

In insects, nutrition, via circulating sugar, promotes release of ILPs from brain neurosecretory cells into the haemolymph, which act on peripheral tissues and stimulate protein synthesis and cell growth. Therefore, insect ILPs are common mediators between nutrition and growth in insects and are functionally analogous to mammalian insulin. The 1980s and 1990s witnessed great progress in elucidation of the physiological and

molecular mechanism of action of numerous insect hormones involved in regulation of growth, development, reproduction and metabolism. But the signals for the initiation or termination of controlled events remained largely unknown. ILPs were first identified from the silkmoth Bombyx mori and were named bombyxins, but related peptides were soon found in numerous species and their functions elucidated. The insulin signalling pathway is now recognized as a central factor in the timing of cell proliferation, growth, longevity, reproduction, and reproductive diapause, as well as social behaviour. Recent work has revealed that the insulin signalling pathway is closely integrated with that of various other hormones, including ecdysteroids, the juvenile hormones and neuropeptide(s) such a prothoracicotropic hormone. In addition, the pathway is also linked with both circadian (daily) and photoperiodic (seasonal) clocks potentially providing a basis for its timing function.

This Research Topic aims to provide the only current collection of recent advances on insect ILPs. We encouraged submissions on all areas related to identification, characterization, regulation and physiological functions of insect ILPs. We welcomed both full and short reviews and original research articles.

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EDITORIAL
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The coming of age of insulin-signaling in insects

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Keywords: insulin-like proteins, interactions of signaling pathways, nutrition and metabolism, growth and development, timekeeping

Since the first isolation of insulin-like proteins (ILPs) from the silkmoth, *Bombyx mori* in 1987, discoveries of ILPs in other insects mushroomed and propelled intense studies of the functional roles of ILPs in numerous insect species. The primary sites of ILP production are brain neuroendocrine cells, but ILPs are also produced in other tissues. Synthesis of ILPs is regulated by many factors including the insulin/insulin-like growth factor signaling pathway (IIS) itself. ILPs can function in an autocrine, paracrine, and endocrine fashion and target a diversity of cells and tissues.

IIS is activated when ILPs bind to the insulin receptor (InR). IIS interacts with various major signaling pathways such as the Target of Rapamycin TOR (an important nutrient sensor) and FOXO (a regulator of stress tolerance, longevity, diapause, and growth). In insects, it also interacts with the signaling pathways of the developmentally significant hormones juvenile hormones (JHs) and ecdysteroids. The numerous interactions of IIS with other major signaling pathways results in a plethora of physiological actions in insects affecting a multitude of life events. The consensus is that the role of IIS in insects bears close similarities to its functional counterparts in the insulin/insulin-like growth factors of vertebrates.

The present Research topic is a compendium of 14 articles on the roles of IIS in a wide spectrum of functions. Of these, seven are reviews focused on different aspects of actions of IIS, five are original research and one is an editorial highlighting the contents of the present Topic. The IIS functions presented here include nutrition, growth and development, behavior, lifespan, semi-lethality, stress, dietary restriction, reproduction, axon guidance, and circadian and seasonal timekeeping. This Research Topic illustrates the complexity of the role of IIS in the detection of environmental cues, their translation into physiological adjustments of insects and the regulation of IIS by other physiological factors involving multiple interactions with other signaling pathways. It also raises stimulating questions for future directions of research in the role of IIS in insect physiology.

A historical account of the discovery of the first ILPs, the bombyxins, in the silkmoth is presented by Mizoguchi and Okamoto (2013). Bombyxins represent a large group of closely related peptides and the chemical characterization of these peptides and their genes as well as their sites of synthesis in the brain is detailed. They function as regulators of sugar homeostasis and promote cell division and tissue growth and interact with ecdysteroids and possibly JH during development. The recent discovery of a single chain bombyxin provided a link between insect ILPs and vertebrate insulin-like growth factors (IGFs).

The role of IIS in *Drosophila* has been extensively studied. Nässel et al. (2013) review the topography of neurons producing ILPs in the *Drosophila* brain (DILPs), their axonal projections and their regulation by factors including neurotransmitters, neuromodulators, hormones, and factors from the fat body. The functional role of DILPs in behavior, lifespan, resistance to stress, and starvation is also discussed. Individual DILPs perform different physiological roles during *Drosophila* development. A review by Kannan and Fridell (2013) pays particular attention to regulation of the differential expression of DILP2, 3, 5, and 6 during development and their regulatory functions in homeostasis, aging, and dietary restriction. DILP2 for example, which is expressed highly in the fly, regulates tissue growth and longevity. High blood levels of ILPs, can also be induced in *Drosophila* by over-expression of DILP2. In an original research article, Sato-Miyata et al. (2014) report that overexpression of DILP2 causes semi-lethality in flies and autophagy of fat body cells that can be rescued by a high protein diet. Li et al. (2014) show that the *Drosophila* insulin receptor (DInR) regulates axon guidance in photoreceptor cells in the developing nervous system by binding to specific adapter protein called Dreadlocks (Dock). However, mutations in DlnR of both putative Dock binding sites did not lead to defects in axon guidance. The authors suggest that Dock may be able to bind to multiple regions of DInR *in vivo* to ensure proper interaction with the receptor in order to achieve appropriate axon guidance.

IIS is also involved in regulation of female reproduction in conjunction with the JH and ecdysteroid signaling pathways. Badisco et al. (2013) review this extensively investigated area and summarize the roles of IIS in regulation of vitellogenesis, oogenesis, reproductive diapause, caste differentiation, and division of reproductive labor in various insects, primarily *Drosophila*. An insect also used as a model animal to study the integration of nutritional information and reproduction, is the mosquito. Hansen et al. (2014) review the complex interactions and cross-talk between complex signaling networks involving JH, ecdysteroids, nutrients, and IIS/TOR in the regulation of yolk protein precursor gene expression in mosquitoes.

IIS is also involved in the regulation of circadian and seasonal timekeeping at multiple levels of physiological organization. The relationship between metabolic state and the circadian clock in the brain is reviewed by Erion and Sehgal (2013). In *Drosophila*, nutrition and IIS influence the molecular oscillator in brain clock cells, thereby modulating daily activity rhythms and consequently affecting many key survival behaviors like feeding, sleep, reproduction, and maintenance of circadian rhythms. Vafopoulou and Steel (2014) report that *in vitro* treatment with bombyxin or

prothoracicotropic hormone (PTTH) induces expression of the clock protein PERIOD in *Rhodnius* circadian clock cells, demonstrating a direct effect of these hormones on the molecular oscillator and indicating interaction of the insulin and PTTH signaling pathways in the regulation of clock genes. The article also shows that the clock cells that control rhythmicity in hormones are themselves responsive to feedback from these same hormones by the IIS and PTTH signaling pathways. Sim and Denlinger (2013) review that the IIS/FOXO pathways influence many aspects of diapause, such as arrest of cell cycle and development, interference with life span, suppression of metabolism, fat body hypertrophy, enhanced stress tolerance, and probably enhanced innate immunity in various insects and in *C. elegans*. IIS involvement in diapause is executed in association with the JH signaling pathway.

Smith et al. (2014) investigate the possible involvement of IIS in the regulation of steroid synthesis by the prothoracic glands of *Manduca*. Unlike *Bombyx*, in which this process involves intersection of the IIS pathway with the PTTH signaling pathway, both bombyxin and *Manduca* ILP fail to stimulate steroid synthesis in *Manduca*. The authors conclude that steroid synthesis in *Manduca* is dependent on PTTH signaling and independent of IIS and does not require interaction of these two pathways.

The extensive variability of tissue responses to IIS raises the question of how and why insect tissues respond so differently to insulin. This is addressed in the review by Koyama et al. (2013). Based mainly on studies in *Drosophila*, but also in other insects, the authors summarize evidence that the IIS/TOR pathways regulate plasticity of organ growth and size during development. This plasticity depends on interactions between IIS/TOR pathway with those of ecdysteroid and JH, but that there are also differences in tissue sensitivities to the pathways.

In conclusion, IIS forms complex networks with several other signaling pathways. This complexity of pathway interplay and crossover introduces numerous variables that are difficult to determine or control during an experiment. Nijhout and Callier (2013) developed a scaled mathematical model of the insulin/TOR/MAPK signaling network that controls tissue growth that is a valuable tool to use when investigating the qualitative properties of this network.

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Insulin signaling and the regulation of insect diapause

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A rich chapter in the history of insect endocrinology has focused on hormonal control of diapause, especially the major roles played by juvenile hormones (JHs), ecdysteroids, and the neuropeptides that govern JH and ecdysteroid synthesis. More recently, experiments with adult diapause in *Drosophila melanogaster* and the mosquito *Culex pipiens*, and pupal diapause in the flesh fly *Sarcophaga crassipalpis* provide strong evidence that insulin signaling is also an important component of the regulatory pathway leading to the diapause phenotype. Insects produce many different insulin-like peptides (ILPs), and not all are involved in the diapause response; ILP-1 appears to be the one most closely linked to diapause in *C. pipiens*. Many steps in the pathway leading from perception of daylength (the primary environmental cue used to program diapause) to generation of the diapause phenotype remain unknown, but the role for insulin signaling in mosquito diapause appears to be upstream of JH, as evidenced by the fact that application of exogenous JH can rescue the effects of knocking down expression of ILP-1 or the Insulin Receptor. Fat accumulation, enhancement of stress tolerance, and other features of the diapause phenotype are likely linked to the insulin pathway through the action of a key transcription factor, FOXO. This review highlights many parallels for the role of insulin signaling as a regulator in insect diapause and dauer formation in the nematode *Caenorhabditis elegans*.

Keywords: diapause, dauer, insulin signaling, FOXO, *Culex pipiens*

INTRODUCTION

Diapause is a form of dormancy used widely by insects to survive adverse seasons. Unlike quiescence, defined as an immediate response to an unfavorable environmental stress, diapause is an anticipated, hormonally-regulated developmental arrest, frequently programmed by photoperiod. Within temperate zones, insects are temporally limited to just a few months of active development, while the remaining months are spent in diapause. Depending on the species, insect diapause can occur in embryos (e.g., the commercial silkmoth *Bombyx mori*), larvae (e.g., southwestern corn borer *Diatraea grandiosella*), pupae (e.g., flesh fly *Sarcophaga crassipalpis*) or adults (e.g., mosquito *Culex pipiens*). Among species in temperate zones, an overwintering diapause is most common, but a summer diapause can also occur (Masaki, 1980), and diapause is also well-documented among tropical species (Denlinger, 1986). In temperate zones the shortening day lengths and declining temperatures of late summer and early autumn provide the dominant environmental cues signaling the advent of winter (Tauber et al., 1986; Kostal and Denlinger, 2011), cues that set into motion a series of preparatory steps for successful overwintering.

The environmental cues used to program diapause are frequently received long before the actual inception of diapause. Depending on the species, the photoperiodic signals are received either through the eyes or directly by light-sensitive cells within the brain (Goto et al., 2010; Numata and Udaka, 2010). Most evidence suggests that the circadian clock is involved in distinguishing short from long days (Saunders, 2012; Goto, 2013; Meuti and Denlinger, 2013). The transduction pathway for photoperiodic stimuli engages neurons in the *pars intercerebralis*, *pars lateralis* and other domains within the brain (Shiga and Numata, 2000; Shimokawa et al., 2008) that release neuropeptides or growth factors into neighboring or remote cells to regulate development. Among the targets of these neuropeptides are endocrine glands such as the *corpora cardiaca*, the *corpora allata* and the prothoracic gland, organs that in turn synthesize and release hormones including juvenile hormones, ecdysteroids, adipokinetic hormone, as well as additional neuropeptides that impact insect diapause.

A functional module approach is a helpful way to view the diapause mechanism (Emerson et al., 2009; Bradshaw and Holzapfel, 2010). Three candidate modules are proposed: an input module that includes the functional timekeeping mechanism, an intermediate module linking photoperiodism to hormonal events, and an output module that includes the physiological responses. Modularity of this sort has been commonly invoked to interpret genetic mechanisms of embryonic development such as pattern formation and differentiation (Raff, 1996). Key components in a module are signaling cascades such as hedgehog, transforming growth factor (TGF-β) and insulin signaling (Cohen, 2003; Dupont and Holzenberger, 2003; Logan and Nusse, 2004; Bray, 2006; Kitisin et al., 2007). If we consider diapause as an alternative developmental program with separate functional modules, the application of this concept may be useful for dissecting molecular mechanisms of diapause programs.

Several recent reviews discuss regulatory features of diapause such as molecular regulation (Denlinger, 2002; Robich

and Denlinger, 2005; Macrae, 2010; Williams et al., 2010), hormonal control (Denlinger et al., 2012), the circadian clock and photoperiodism (Goto et al., 2010; Saunders, 2010; Kostal, 2011), and energy utilization (Hahn and Denlinger, 2007, 2011). One unifying theme for diapause in diverse species may be insulin signaling (Tatar and Yin, 2001; Williams et al., 2006; Sim and Denlinger, 2008, 2009a). This signaling pathway has been linked to diverse features of the diapause phenotype including arrested reproduction, extended lifespan, suppressed metabolism, fat hypertrophy and enhanced stress tolerance. Dauer formation in the nematode *Caenorhabditis elegans* (**Figure 1**) offers many parallels to insect diapause, including a role for insulin signaling, thus the comprehensive understanding of the molecular basis for dauer formation (Gottlieb and Ruvkun, 1994; Kimura et al., 1997; Apfeld and Kenyon, 1998) provides valuable insights for exploring general patterns of developmental arrest in invertebrate systems. The goal of this review is to summarize evidence linking insulin signaling to insect diapause and to thus create a foundation for developing a comprehensive view of the role of this pathway in shaping the complex diapause phenotype.

COMPONENTS OF THE INSULIN SIGNALING PATHWAY

Insulin signaling has been implicated as a regulator of diapause by observing the effects of this pathway on developmental and metabolic suppression (Apfeld and Kenyon, 1998; Tatar et al., 2001; Hahn and Denlinger, 2007; Sim and Denlinger, 2008; Ragland et al., 2010; Williams et al., 2010) and by observing naturally segregating variation of PI3K, a member of the insulin signaling pathway, in association with adult reproductive diapause in *Drosophila melanogaster* (Williams et al., 2006).

Seven genes encode insulin-like peptides in *Drosophila* (Brogiolo et al., 2001). All of these peptides have a domain structure that produces two chains, resulting in active dimer formation (Leevers, 2001). *Drosophila* insulin receptor (receptor tyrosine kinases) shares sequence similarity with human insulin receptor and can be activated by insulin (Fernandez et al., 1995; Chen et al., 1996). Activated receptor tyrosine kinases (RTKs) activate phosphatidylinositol 3-kinase (PI3K) through direct binding or through tyrosine phosphorylation of scaffolding adaptors, such as IRS1, which then bind and activate PI3K. PI3K phosphorylates phosphatidylinositol-4,5-bisphosphate (PIP_2) to generate phosphatidylinositol-3,4,5-trisphosphate (PIP_3) (Britton et al., 2002). In the end, PI3K targets two intracellular signaling proteins, Akt (also known as Protein Kinase B, PKB) and 3-phophoinositide-dependent protein kinase-1 (PDK-1); phosphorylation at serine and threonine residues activates Akt and PDK-1 (Taniguchi et al., 2006).

Akt plays a key role in multiple cellular processes including glucose metabolism, apoptosis, cell proliferation, transcription and cell migration (Hanada et al., 2004; Fayard et al., 2005). These various cellular processes are mediated by transcriptional factors and kinases which are activated by Akt phosphorylation. The downstream molecules of Akt include forkhead of transcriptional factors (FOXs), glycogen synthase kinase-3 (GSK-3), tuberous sclerosis complex (TSC1/2) and Rab-GTPase-activating protein (Rab-GAP) (Frame et al., 2001; Harris and Lawrence, 2003; Junger et al., 2003; Sano et al., 2003). In addition, a

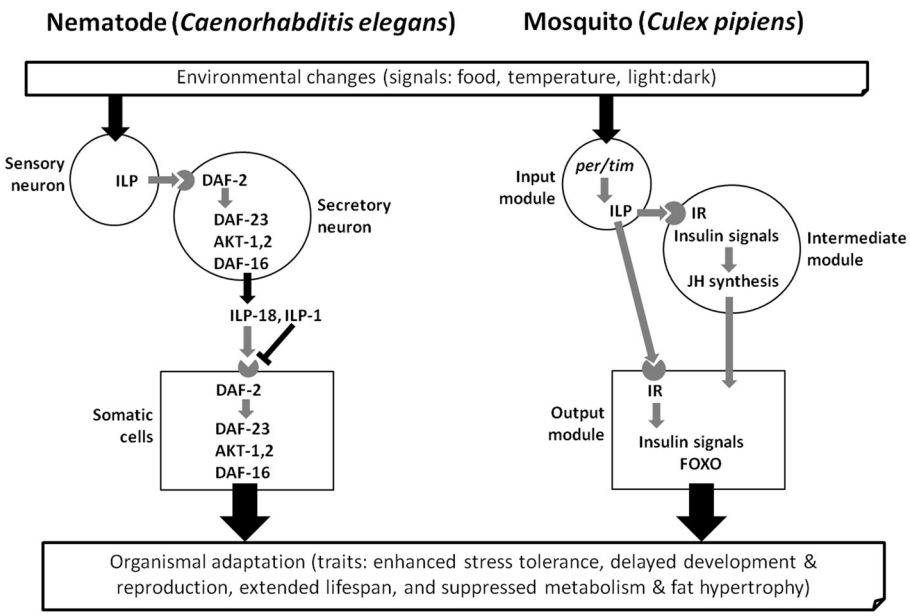

FIGURE 1 | Three conceptual modules (input, intermediate and output) that influence dauer larva formation in the nematode *Caenorhabditis elegans* and adult diapause of the mosquito *Culex pipiens*, based on references in the text. Gene activation (Black) and deactivation (Gray). Insulin-like peptide, ILP; DAF-2, insulin receptor (IR); DAF-23, phosphoinoitide 3-kinase; Akt, protein kinase B; DAF-16, forkhead transcription factor (FOXO); *per*/*tim*, core genes of molecular clock system; JH, juvenile hormone. The mosquito FOXO and insulin receptor (IR) are homologs of nematode DAF-16 and DAF-2, respectively.

number of upstream proteins regulate Akt; these include protein phosphatase 2A (PP2A), wideborst (Wdb), and a PH-domain leucine-rich repeat protein phosphatase (PHLPP) (Du et al., 2003; Vereshchagina et al., 2008).

Several proteins are involved in regulation of the insulin receptor and thus influence intracellular signaling components in the insulin signaling pathway. Protein Tyrosine Phosphatase 1B dephosphorylates active insulin receptor (Elchebly et al., 1999), while suppressors of cytokine signaling (SOCS)-1 and SOCS-3 can bind insulin receptor substrate, and attenuate insulin signals (Ueki et al., 2004). Other molecules including extracellular-signal-regulated kinase (ERK), Jun-N-terminal kinases, and kinase S6 attenuate the activity of insulin receptor (Miller et al., 1996; Bouzakri et al., 2003; Harrington et al., 2004). Phosphatase and tensin homolog (PTEN) can also inactivate PIP_3, a midpoint in the insulin signaling pathway (Goberdhan et al., 1999). Comprehensive reviews describe details of structure, signaling, function of insulin-like peptides, as well as other components of the insulin signaling pathway (Luckhart and Riehle, 2007; Antonova et al., 2012).

One outstanding question that remains is how input from the photoperiodic clock is linked to the insulin signaling pathway. Several candidate neuropeptides could serve as intermediaries. For example, Pigment Dispersing Factor (PDF) appears to be an output molecule of the circadian clock system in several insects and has been extensively studied in *D. melangaster* (Meelkop et al., 2011). Additional neuropeptides in flies, short neuropeptide F (sNPF), corazonin (CRZ) and drosulfakinins (DSKs), function in regulating insulin production, and thus could also serve an intermediary role. Some insulin-producing cells (IPCs) in the fly brain also coexpress receptors of both sNPF and CRZ neuropeptides, as well as the ligand for DSKs (Kapan et al., 2012; Soderberg et al., 2012). In these studies, knockdown of either sNPF or DSK decreases transcription of ILPs in the brain, suggesting a regulatory action of these two neuropeptides on IPCs. In *Rhodnius prolixus*, levels of prothoracicotrophic hormone (PTTH) oscillate in a circadian manner, suggesting a possible link between PTTH and clock functions as well (Vafopoulou et al., 2007, 2012). Yet, links between these neuropeptides, insulin signaling, and insect diapause remain to be determined.

CELL CYCLE AND DEVELOPMENTAL REGULATION

Arrest of the cell cycle and development are key characteristics of diapause. Pupal diapause in the flesh fly involves a G0/G1 cell cycle arrest that appears to be controlled by down regulation of *proliferating cell nuclear antigen* (Tammariello and Denlinger, 1998), a gene that controls the cell cycle by direct interaction with the cyclin/cdk complex (Watanabe et al., 1998). FOXO proteins have known roles in inducing cell cycle arrest. For example, in dauer larvae of *Caenorhabditis elegans* a FOXO homolog induces a G0/G1 cell cycle arrest through induction of Cip/Kip inhibitor, Cki-1 (Boxem and Van Den Heuvel, 2001). A similar role for FOXO is evident in *Drosophila* (Kramer et al., 2003), in which activated dFOXO promotes a G1 cell cycle arrest.

Drosophila females reared under low temperature and short daylength enter an adult reproductive diapause characterized by arrest of ovarian development in the previtellogenic stage, while non-diapausing females initiate vitellogenesis and complete ovarian development (Tatar et al., 2001). Development of *Drosophila* ovaries is regulated by an insulin signal in germ cells; dILPs specifically control the G2 phase of germ cell cycle via PI3K and dFOXO (LeFever and Drummond-Barbosa, 2005; Hsu et al., 2008). Regulation of ovarian development by insulin signaling is not limited to *Drosophila* but is also evident in the mosquito *Culex pipiens*. The insulin signal/FOXO pathway is central to initiation of the diapause program, including ovarian development arrest. A "diapause-like" ovarian arrest can be simulated in non-diapausing females by knocking down the insulin receptor (InR) using RNAi; this knock-down effect can be reversed with application of juvenile hormone, an endocrine stimulant well-known to terminate diapause in this species (Sim and Denlinger, 2008). Insulin-like peptide 1 (ILP-1) is the ILP most likely implicated in the diapause response of *Cx. pipiens* (Sim and Denlinger, 2009a).

In *C. elegans*, low food levels prompt synthesis of high levels of dauer pheromone, which in turn lead to dauer formation, rather than reproductive growth (Golden and Riddle, 1982, 1984). These environmental cues initially alter insulin signaling by regulating insulin-like peptide synthesis and secretion in specific subsets of sensory neurons. *C. elegans* has only a single insulin receptor (daf-2), but it has genes encoding 40 putative insulin-like peptides (Flatt et al., 2008a). Daf-2 is implicated in many genetically separable processes, including the dauer decision, lifespan control, and reproductive timing (Flatt et al., 2008b; Lee et al., 2008). Mutants with reduced daf-2 activity enter the dauer state, while increased insulin signaling promotes germ line proliferation, resulting in an increase of germ line stem cells. Many of the ILPs are expressed in sensory neurons and interneurons, where they encode distinct environmental information to regulate initiation and termination of dauer formation. For example, ILP-1(ins-1) induces dauer arrest under low food levels, and under favorable food conditions, daf-28 (insulin-like peptide) inhibits dauer arrest, whereas ILP-6 (ins-6) promotes the transition from the dauer state to normal reproductive growth (Cornils et al., 2011).

The finding that ILPs in *C. elegans* encode environmental cues used to regulate physiology also reflects what is found in insects, such as the fly *D. melanogaster* and the mosquito *C. pipiens*. *D. melanogaster* has genes encoding 7 ILPs, and as in *C. elegans,* the ILPs are expressed in different sensory neurons and interneurons. Interestingly, some of these neuronally-expressed ILPs (dilp-2, -3, -5) have been proposed to regulate growth and metabolism (Ikeya et al., 2002; Broughton et al., 2008; Zhang et al., 2009). We thus argue that insulin signals are likely used as mediators of a wide range of environmental cues, including those involved in regulating diverse forms of developmental arrest (**Figure 1**).

LIFESPAN EXTENSION

Genetic studies using the nematode *C. elegans* and the fruit fly *D. melanogaster* have identified several genes involved in extending lifespan. Work on the dauer stage of *C. elegans* is at the forefront of such research. Genome-wide RNAi screens identified key functional groups in the *C. elegans* insulin signaling pathway that contribute to lifespan extension (Hamilton et al., 2005). Most mutants with reduced insulin-like signaling have both dauer and

extended lifespan responses, but in some cases there are distinct differences when and where insulin/FOXO signals are activated. Most studies on daf-2 suggest it functions within the nervous system to regulate lifespan as well as dauer arrest (Kimura et al., 1997; Apfeld and Kenyon, 1998). Yet, several studies on the target of insulin signaling, daf-16 (aka forkhead of transcriptional factor FOXO), suggest that dauer arrest and lifespan are regulated by FOXO activity in a different way: FOXO within the nervous system has a stronger influence on dauer arrest than on lifespan, whereas intestinal FOXO plays a greater role in regulating lifespan than in regulating dauer arrest (Libina et al., 2003). Thus, FOXO activation in different tissues may have distinct phenotypic consequences. Furthermore, insulin signaling during larval development regulates dauer arrest without significantly impacting lifespan, whereas insulin-like signaling during adulthood regulates lifespan (Dillin et al., 2002). Daf-2 is thought to activate a conserved PI-3 kinase signaling pathway that affects lifespan, at least in part by regulating nuclear localization of daf-16 (FOXO). This transcription factor, FOXO, appears to extend lifespan by activating its downstream genes products such as superoxide dismutase, metallothionin, catalase, glutathione S-transferase, small heat shock proteins, and apolipoprotein (Vanfleteren and De Vreese, 1995; Honda and Honda, 1999; Barsyte et al., 2001; Sun et al., 2002; Walker and Lithgow, 2003).

Diapause incidence in *D. melanogaster* varies among populations (Schmidt et al., 2005; Williams et al., 2006). A transgenic study of Dp110 (phosphoinositide 3-kinase), a member of the insulin signaling pathway, supports the view that this gene, and hence the insulin signaling pathway, plays an important role in induction of reproductive diapause (Williams et al., 2006). Knock-down of genes encoding insulin-like peptides, insulin receptor and CHICO (IRS), and overexpression of the downstream transcription factor dFOXO, as well as inhibitor studies using the PIP_3 inhibitor PTEN, all reduce insulin signaling and subsequently extend lifespan (Clancy et al., 2001; Giannakou et al., 2004; Hwangbo et al., 2004; Giannakou and Partridge, 2007; Lee et al., 2008; Demontis and Perrimon, 2010; Gronke et al., 2010). Likewise, induction of the tuberous sclerosis complex (TSC1/2), kinase S6, the dFOXO regulated histone deacetylase Sir2, and the insulin signal suppressing pathway Jun-N-terminal kinase (JNK) extend lifespan (Kapahi et al., 2004; Partridge et al., 2005; Wang et al., 2005).

Furthermore, insulin signals appear to be a regulator of juvenile hormone synthesis (Flatt et al., 2005; Tu et al., 2005). In *Drosophila*, insulin receptors are present in the *corpora allata* (CA), the glands that synthesize JH (Belgacem and Martin, 2006), and suppression of the insulin signal correlates with low JH production (Tatar and Yin, 2001; Tu et al., 2005). Knock-down of the insulin receptor in the CA concurrently suppresses the gene encoding 3-hydroxy-3-methylglutargyl CoA Reductase (HMGCR), a key enzyme in JH synthesis (Belgacem and Martin, 2007). The subsequent shut-down of JH synthesis is a key signaling event triggering the onset of adult reproductive diapause in many insects (Denlinger et al., 2005), including *D. melanogaster*, the mosquito *Cx. pipiens* and the butterfly *Danaus plexippus* (Herman, 1981; Herman and Tatar, 2001; Sim and Denlinger, 2008). *D. melanogaster* selected to survive a high dose of JH analog overcame lifespan reduction when compared to flies not receiving JH (Flatt and Kawecki, 2007). These lines of evidence suggest that JH is involved in the trade-off between reproduction and extended lifespan through the insulin signaling pathway.

SUPPRESSED METABOLISM AND FAT HYPERTROPHY

At the initiation of diapause, metabolic processes are coordinately downregulated, thus enabling the overwintering insect to economically utilize its energy reserves, but in addition, this metabolic downregulation helps to minimize deficiencies in cellular processes that can cause cell death (Hand et al., 2011). Diapausing insects remain hypometabolic even after temperatures revert to conditions favorable for development. However, certain metabolic genes involved in the accumulation of energy reserves are highly upregulated, especially during the preparatory period of diapause. This energy storage is critical not only for surviving prolonged periods of developmental arrest but also for maximizing reproductive success once development resumes (Hahn and Denlinger, 2007). Carbohydrate sources such as nectar and rotten fruit are critical carbohydrate sources used for increasing energy reserves in adult diapausing females of the mosquito *Cx. pipiens*, the butterfly *Danaus plexippus,* and some other diapausing insects (Alonsomejia et al., 1997; Robich and Denlinger, 2005; Reynolds et al., 2012). We generated transcript profiles of thirty-two fat-related genes during diapause in the mosquito *Cx. pipiens* (Sim and Denlinger, 2009b), and among the genes upregulated in early diapause were *fatty acid synthase-1, -3*, and *fatty acid binding protein*, genes that contribute to accumulation of triacylglycerides in the fat body. This result is consistent with the observation that this mosquito switches from blood feeding to sugar feeding as a component of the diapause program and more than doubles its lipid reserves compared with females programmed for continuous development. When we knocked down *foxo* transcript by injection of dsRNA into these diapausing mosquitoes, we observed an immediate halt in the accumulation of lipid reserves (Sim and Denlinger, 2008). FOXO is normally activated by suppression of insulin signaling; thus FOXO may be involved in increasing transcript levels of genes involved in fatty acid synthesis, as observed in newly-emerged diapausing females. A transcriptome analysis of the Asian tiger mosquito, *Aedes albopictus,* also suggests the importance of FOXO during early diapause (Poelchau et al., 2011).

Insulin signaling and its target FOXO are implicated as a major regulator of diapause through effects on metabolic suppression, fat hypertrophy, and growth control (Puig et al., 2003; Williams et al., 2006; Hahn and Denlinger, 2007; Sim and Denlinger, 2009a,b; Ragland et al., 2010). A transcriptome analysis of diapause termination in the apple maggot fly, *Rhagoletis pomonella*, reveals the importance of the TOR signaling pathway, a pathway that interacts with insulin signaling (Ragland et al., 2011). TSC1 and TSC2, negative regulators of TOR signals and regulators of cell growth, were significantly upregulated in late diapause. FOXO and TOR pathways are both linked to insulin signaling and offer links for integrating metabolic and growth responses.

Among the *Drosophila* insulin-like peptides, ligands 1-5 are predicted to be closely related to mammalian insulin, while ligands 6 and 7 are more similar to IGF-1 and relaxin, respectively

(Brogiolo et al., 2001). Four of these insulin-like peptides, 1, 2, 3, and 5 are expressed in insulin-producing cells (IPCs) in the brain (Cao and Brown, 2001; Ikeya et al., 2002; Rulifson et al., 2002), and loss of insulin-like peptide-producing cells or mutations in the gene for *Drosophila* insulin receptor (dInR) or CHICO results in a significant increase in triacylglycerides (Bohni et al., 1999; Tatar et al., 2001; Broughton et al., 2005). By contrast, insulin is a positive regulator of fat cell mass, acting through changes in both cell number and lipid storage (Diangelo and Birnbaum, 2009). This evidence suggests that, unlike in mammals, different insect ILP may be involved in regulating distinct physiological processes such as energy metabolism, fat cell proliferation, lipid storage and other key traits for survival. Thus, it will be important to know when and where particular insulin-like peptides are suppressed or activated, and how the insulin-like peptides generate the increased fat cell mass and lipid storage in diapausing insects.

ENHANCED STRESS TOLERANCE

Diapausing insects are particularly well-adapted to survive low temperatures and other forms of environmental stress (Denlinger and Lee, 2010). Cold hardiness is frequently a component of the diapause program but is sometimes acquired after the onset of diapause, in direct response to low temperature (Denlinger, 1991). A variety of molecular mechanisms are used to either avoid or survive freezing (Michaud and Denlinger, 2006, 2007; Khani and Moharramipour, 2010; Vesala and Hoikkala, 2011). Suppression of the insulin signal appears to induce physiological responses promoting resistance to low temperature, oxidative stress, and pathogenic infections (Clancy et al., 2001; Broughton et al., 2005; Zhang et al., 2009; Felix et al., 2012). In *C. elegans*, dauer larvae differ from non-dauer larvae in aspects of metabolism related to cold tolerance. Genes involved in trehalose synthesis are upregulated in daf-2 (insulin receptor) mutants and dauers (Wang and Kim, 2003; McElwee et al., 2006; Shmookler Reis et al., 2011). Fatty acid desaturase genes are essential for cold tolerance in many animals, an effect promoted by the preservation of membrane fluidity at sub-zero temperatures (Gracey et al., 2004; Brock et al., 2007; Murray et al., 2007). Cold tolerance in dauers is enhanced by the overlapping effect of genes encoding fatty acid desaturase, targeted by insulin signal/FOXO, and genes involved in the cold-induced stress response (Savory et al., 2011).

Drosophila FOXO has a critical role in the systemic regulation of antioxidant enzymes, a response that acts through the insulin/FOXO signaling pathway in insulin-producing cells (IPCs) (Kops et al., 2002b; Hwangbo et al., 2004). FOXO activation subsequently increases stress tolerance through upregulation of superoxide dismutases (Kops et al., 2002a). This genetic regulation of antioxidant enzymes by FOXO is also noted in *C. elegans* (Vanfleteren and De Vreese, 1995) and the mosquito *Cx. pipiens* (Sim and Denlinger, 2011). Dauer worms and diapausing mosquitoes increase expression of the protective enzymes superoxide dismutase and catalase. In addition, several genome-wide studies indicate that detoxification/stress response genes are among the most common group of genes regulated by the insulin/FOXO signaling pathway (Murphy et al., 2003; Oh et al., 2006; Gershman et al., 2007).

The insulin signaling pathway also plays a critical role in regulation of innate immunity and lifespan in many insects (Luckhart and Riehle, 2007). However, there is a dichotomy in the functional role of insulin signals in activation of the immune response. For example, in the mosquito *Anopheles stephensi*, increased Akt/PKB signaling in the midgut significantly reduces malaria parasite development compared to control mosquitoes (Corby-Harris et al., 2010). The brain of the mosquito *Aedes aegypti* releases insulin-like peptides (ILPs) in response to a blood meal. In turn, the insulin signal induces hemocyte (immune cells) production, which serve as the first line of defense against pathogenic infections (Castillo et al., 2011). By contrast, in *C. elegans*, insulin signals are linked to both innate immunity and extended lifespan. The loss of function of insulin receptor (daf-2) results in decreased insulin signaling and enhanced resistance to pathogenic bacterial infection (Garsin et al., 2003). When forkhead transcription factor (daf-16), which is negatively regulated by the insulin signaling pathway in *C. elegans*, is suppressed the worms exhibit increased susceptibility to infection by pathogenic bacteria. Similar results were found in the fly *D. melanogaster*, in which there is a link between the Toll signaling pathway, the pathway that activates the innate immune response, and the insulin signaling pathway (Diangelo et al., 2009). These lines of evidence suggest that insulin/FOXO signaling in diapausing insects may be linked to induction of immune effectors that enhance resistance to pathogenic infection.

FUTURE DIRECTION

The evidence we present links the insulin/FOXO signaling pathway to insect diapause characteristics including cell cycle arrest, developmental arrest, extended lifespan, suppressed metabolism, fat hypertrophy, and enhanced stress tolerance. Although relatively few insect species have been examined, involvement of this pathway may emerge as one of the unifying themes of insect diapause. In life-history studies, the insulin/FOXO signaling pathway appears to also regulate growth, reproduction, and lifespan in numerous species including flies, worms and mosquitoes. Phenotypic plasticity observed in life-history traits provides insights for understanding relationships among diapause characteristics. Similar phenotypic plasticity of fitness factors is evident in diapause, i.e., trade-offs between/among diapause characteristics. For example, in adult reproductive diapause, extended lifespan is frequently coupled with arrested ovarian development and suppressed metabolic rate. Since insulin signaling plays a role in phenotypic plasticity among fitness factors, we propose that the insulin signal is a key regulator among diapause characteristics including suppressed growth and metabolism, enhanced stress tolerance, and extended lifespan. This idea raises several new questions. First, what is the nature and extent of the linkages among modules through insulin signals? Second, how are changes in insulin signals within one module coordinated with others, and what are the mechanisms that promote systemic changes? The answers to these questions are not simple, but recent advances in genomics and functional genetics provide new opportunities for testing hypotheses of this nature. Hopefully, such experiments will enable us to pinpoint the molecular mechanisms of phenotypic plasticity associated with insect diapause.

Several lines of evidence support our proposition. First, recent studies found that insulin signals can act locally as well as systemically in the fruit fly *D. melanogaster*. The glia provide local signals necessary for activation of neighboring neuroblasts, and interestingly the glia also produce insulin-like peptides (ILPs) that respond to signals from the fat body by binding to receptors on larval neuroblasts (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Considering the fact that the brain, endocrine organs (*corpora cardiac, corpora allata*, prothoracic gland), and fat body are all key organs essential to the diapause response, the presence of a systemic signaling system operating among these organs is likely to offer a conduit for cross-talk that may be critical for implementing and coordinating a successful diapause program (Xu et al., 2012). With the insulin signaling pathway being involved in so many aspects of the diapause phenotype, the local and systemic signals from different insulin-like peptides are promising candidates to explain molecular mechanisms used to generate this phenotype.

Secondly, modularity is a suitable model for viewing the complicated molecular mechanisms of diapause. Circadian clock oscillations (input module) are certainly functioning in the insect brain and likely contribute to photoperiodism (Ito et al., 2008; Ikeno et al., 2010). Interestingly, the clock genes have linkages to insulin signaling (Allen, 2007; Zheng and Sehgal, 2010). Diapause incidence in *Drosophila* is elevated when PI3-kinase, an insulin-regulated gene, is upregulated and is lowered when this gene is downregulated (Williams et al., 2006). This connection most likely acts through Susi, an inhibitor of insulin-regulated PI3-kinase. Susi shows a circadian pattern of expression that is high at night and low during the day (Claridge-Chang et al., 2001; McDonald et al., 2001; Wittwer et al., 2005). We suggest that insulin signaling is suppressed by long nightlengths (short daylengths), which in turn suppresses juvenile hormone synthesis within the *corpora allata* (intermediate module) (Hardie et al., 1985; Tatar et al., 2001; Tu et al., 2005). The fat body (output module) is crucial to important physiological functions including nutrient sensing, lipid storage, and endocrine signaling to the brain and reproductive organs. Additionally, the fat body is the nexus for lipid storage, arrested reproductive development, and induced stress tolerance during diapause. Insulin/FOXO appears to coordinate, or at least be involved in, the physiological responses during each module of adult reproductive diapause (Sim and Denlinger, 2008, 2009a, 2011, 2013). However, we still lack details and insight into how insulin signals affect each module of diapause at both local and systemic levels and to what extent insulin/Foxo signals are involved in diapauses of different developmental stages. Diapause appears to have evolved multiple times in insect lineages, thus we can very well-expect species variation in how this signaling pathway is exploited for regulating diapause in different species. The exciting prospect is that the pervasive influence of insulin signaling offers connections to insect diapause at many levels, from connections to photoperiodism through to the downstream generation of many of the phenotypic characteristics of the diapause state.

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Eat to reproduce: a key role for the insulin signaling pathway in adult insects

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Insects, like all heterotrophic organisms, acquire from their food the nutrients that are essential for anabolic processes that lead to growth (larval stages) or reproduction (adult stage). In adult females, this nutritional input is processed and results in a very specific output, i.e., the production of fully developed eggs ready for fertilization and deposition. An important role in this input-output transition is attributed to the insulin signaling pathway (ISP). The ISP is considered to act as a sensor of the organism's nutritional status and to stimulate the progression of anabolic events when the status is positive. In several insect species belonging to different orders, the ISP has been demonstrated to positively control vitellogenesis and oocyte growth. Whether or not ISP acts herein via a mediator action of lipophilic insect hormones (ecdysteroids and juvenile hormone) remains debatable and might be differently controlled in different insect orders. Most likely, insulin-related peptides, ecdysteroids and juvenile hormone are involved in a complex regulatory network, in which they mutually influence each other and in which the insect's nutritional status is a crucial determinant of the network's output. The current review will present an overview of the regulatory role of the ISP in female insect reproduction and its interaction with other pathways involving nutrients, lipophilic hormones and neuropeptides.

Keywords: insulin signaling pathway, neuropeptides, lipophilic hormones, nutritional status, female insect reproduction

INTRODUCTION

In order to maintain the existence of a given species and to pass on the genetic material that defines the species, all living organisms must be capable of producing viable offspring, in a process called "reproduction." As in many other animals, the embryos of most insect species develop within an egg, externally from the mother. It is therefore of crucial importance that the egg contains the necessary energy (nutrients), hormones and other components that are indispensable for embryonic development. These essential components are incorporated during oocyte development in the female insect's ovary. Synthesis and incorporation of these components require from the female a lot of energy, which she can only acquire by means of her nutritional input or by reallocation of previously stored energy-rich compounds. Although studied to a lesser extent, it is obvious that also in males the nutritional status is determinative for the development of viable sperm cells.

In an evolutionary conserved mechanism, increased insulin production and signaling—as a response to a positive nutritional status—tends to stimulate the start and progress of several anabolic processes, supporting growth (juveniles) and reproduction (adults). Multiple studies in different metazoan species have indeed demonstrated that not only the insulin-related peptides are evolutionary conserved, but also the components of their signaling pathway. As in other Metazoa, the insulin signaling pathway (ISP) is believed to exert a crucial role in a number of fundamental and interrelated physiological processes in insects (Claeys et al., 2002; Wu and Brown, 2006). In adult insects, the acquired nutritional input is processed and results in specific outputs, such as the production of mature gametes. With a specific focus on female reproductive physiology, the current review aims to illustrate the indispensable role of the ISP in this input-output transition.

Abbreviations: 20E, 20-hydroxyecdysone; AMPK, AMP-activated protein kinase; CA, corpora allata; CC, corpora cardiaca; dILP, Drosophila insulin-like peptide; DIR, Drosophila insulin receptor; Drk, downstream of receptor kinase; E, ecdysone; EGFR, epidermal growth factor receptor; ERK, extracellular signal-regulated kinase; FOXO, forkhead-related family of transcription factors; Grb2, growth factor receptor-bound protein 2; IGF, insulin-like growth factor; IGFBP, insulin-like growth factor-binding protein; ILP, insulin-like peptide; Imp-L2, imaginal morphogenesis protein—late 2; IR, insulin receptor; IRP, insulin-related peptide; IRS, insulin receptor substrate; ISP, insulin-signaling pathway; JH, juvenile hormone; MAPK, mitogen-activated protein kinase; MEK, mitogen-activated ERK-activating kinase; MIR, mosquito insulin receptor; OEH, ovary ecdysteroid hormone; PDK, phosphoinositide-dependent protein kinase; PI3K, phosphatidylinositol-3-kinase; PIP2, phosphatidylinositol-4,5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PKB, protein kinase B; PTEN, phosphatase and tensin homologue; RTK, receptor tyrosine kinase; S6K, ribosomal protein S6 kinase; SH2, Src-homology 2; Sos, Son of Sevenless; TOR, target of rapamycin; TSC, tuberous sclerosis

THE INSECT INSULIN SIGNALING PATHWAY (ISP)

The ISP is evolutionary conserved and has been functionally demonstrated in diverse protostomian and deuterostomian lineages (Tatar and Yin, 2001; Claeys et al., 2002; Burnell et al., 2005; Sherwood et al., 2006; Wu and Brown, 2006; Blumenthal, 2010; Fontana et al., 2010; Kawada et al., 2010; Fujisawa and Hayakawa, 2012). In **Figure 1** a schematic representation is given of the insulin signaling pathway as it has been described in mammals and for which orthologous components were identified

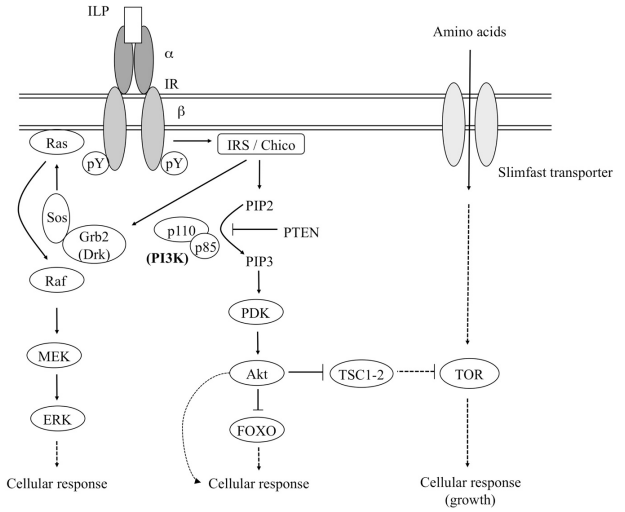

FIGURE 1 | Simplified schematic representation of the insulin and TOR signaling pathways as they have been described in mammals and for which orthologous components have been described in *D. melanogaster* and some other insects. For a detailed overview of the pathway components the reader is referred to the text. Dashed lines represent indirect interactions. Abbreviations: ILP, insulin-like peptide; IR, insulin receptor; IRS, insulin receptor substrate; Grb2, growth factor receptor bound protein-2 [the *Drosophila* ortholog of which is termed the "downstream of receptor kinase" (Drk)]; Sos, son of sevenless; MEK, mitogen-activated ERK-activating kinase; ERK, extracellular signal regulated kinase; PI3K, phosphatidylinositol-3-kinase; PIP2, phosphatidylinositol-4, 5-bisphosphate; PIP3, phosphatidylinositol-3,4,5-trisphosphate; PTEN, phosphatase and tensin homologue; PDK, phosphoinositide-dependent protein kinase; FOXO, forkhead box-related transcription factors, class O; TSC1-2, tuberous sclerosis 1-2 complex; TOR, target of rapamycin. [Figure adapted from Claeys et al. (2002).]

from *Drosophila melanogaster* and other insects. The ISP agonists in insects are generally termed "insulin-like peptides" (ILPs) or "insulin-related peptides" (IRPs). The insulin receptor (IR) is a transmembrane receptor tyrosine kinase (RTK) and consists of a dimer of two αβ-monomers. The α-subunits define the ligand binding specificity, whereas the β-subunits mediate the insulin(-like) signal to downstream cellular components. The IR makes use of the insulin receptor substrate (IRS) as an adaptor molecule to initiate the ISP (White, 1998). Upon binding of the hormone (insulin or a related peptide) to its receptor, the β-subunits undergo autophosphorylation at specific tyrosine residues. The activated RTK subsequently phosphorylates specific tyrosine residues of the IRS (Yenush et al., 1996). The *D. melanogaster* IR (DIR) gene encodes two DIR isoforms, one of which highly resembles the mammalian IR. The other isoform displays a C-terminal extension of about 300 amino acids that shows similarity to certain domains of the *D. melanogaster* IRS (which is termed Chico), and is also capable of activating downstream proteins in an IRS-independent manner (Fernandez et al., 1995). Whether the extended IR isoform also occurs in non-drosophilid insect species remains to be investigated.

The activated IRS recruits downstream factors toward the receptor-IRS complex. The phosphorylated tyrosine residues interact with specific "Src-homology 2" (SH2) domains in the Grb2 ("growth factor receptor bound protein-2") or phosphatidylinositol-3-kinase (PI3K) proteins (Blenis, 1993; Shepherd et al., 1998). [Src-homology domains are highly conserved non-catalytic structural domains that were initially described in the protein tyrosine kinase-encoding *src* oncogene. SH2 structures mediate high-affinity phosphotyrosine-dependent binding between proteins and are mostly involved in formation of signaling protein complexes at or near the plasma membrane (Shpakov and Pertseva, 2000).] Grb2 [the *Drosophila* ortholog of which is termed the "downstream of receptor kinase" (Drk) (Olivier et al., 1993)] and PI3K each initiate a separate signaling pathway, namely the Ras-MAPK ("mitogen activated protein kinase") and PI3K/PKB (PI3K/protein kinase B) pathway, respectively.

Following the activation of Grb2/Drk, an IRS-Grb2/Drk-Sos ("Son of Sevenless") complex is formed. Via Ras/Raf proteins, this complex activates the MEK/ERK ("mitogen-activated ERK-activating kinase/extracellular signal regulated kinase") signaling pathway, which controls many diverse cellular processes, such as proliferation, differentiation and development. Since the MEK/ERK signaling pathway is involved in many cellular processes, several different, cooperating mechanisms are necessary to determine the final outcome (Shaul and Seger, 2007).

Recruitment of PI3K (which is a dimer of a catalytic (p110) and a regulatory (p85) subunit) results in formation of the IRS-PI3K complex. Subsequently, PI3K catalyzes synthesis of PIP3 (phosphatidylinositol-3,4,5-trisphosphate) from PIP2 (phosphatidylinositol-4,5-bisphosphate). However, PTEN ("phosphatase and tensin homologue") can reverse this conversion and can again decrease the level of PIP3 in the cell. The "phosphoinositide-dependent protein kinase" (PDK) responds to the high PIP3 levels by recruiting the protein kinase B (PKB), which is also termed "Akt" (Alessi and Cohen, 1998; Shepherd et al., 1998). Akt (indirectly) affects—by phosphorylation—a number of downstream protein substrates, amongst which the TSC1-TSC2 ("tuberous sclerosis 1-2") complex (Avruch et al., 2005). Phosphorylation of the TSC1-TSC2 complex abolishes its (indirect) inhibitory action on the "target of rapamycin" (TOR) (Oldham et al., 2000; Huang and Manning, 2009). Akt and TOR are considered the "master regulator kinases" of the PI3K/PKB pathway.

By phosphorylation of downstream proteins, Akt indirectly stimulates and prevents anabolic and catabolic processes, respectively. Amongst the Akt targets are the "forkhead-related" FOXO family of transcription factors (Kramer et al., 2002). FOXO proteins are negatively regulated by the insulin signaling pathway, because Akt-mediated phosphorylation of FOXO molecules prevents them from being translocated to the nucleus (Lin et al., 1997; Ogg et al., 1997; Brunet et al., 1999; Kops et al., 1999). FOXO proteins regulate transcription of genes involved in stress resistance, DNA and protein repair and control of cell cycle (Daitoku and Fukamizu, 2007; Hedrick, 2009). In addition, they are indispensable in an organism's response to starvation, since they promote conservation of energy or even catabolism (Kramer et al., 2008). The effective activity of these factors is an important determinant of an organism's lifespan.

TOR activation occurs either as a downstream event in the ISP or, ISP-independently, by the availability of amino acids. In the latter case, the cellular uptake of amino acids by the Slimfast transporter indirectly results in TOR activation. Therefore, the TOR and ILP signaling pathways are considered as nutritional sensors at the cellular and systemic level, respectively. Depending on the available nutritional energy, the TOR signaling pathway drives the cellular decision whether to use energy and nutrients or whether to conserve them. TOR in its activated form stimulates protein synthesis, lipid synthesis and further uptake of nutrients, whereas it inhibits autophagy. Cell growth is thus the main output of active TOR signaling (Hietakangas and Cohen, 2009). It is in this context worth mentioning another evolutionary conserved energy sensing factor, namely the AMP-activated protein kinase (AMPK). Although mainly studied in vertebrate species, AMPK has in *Drosophila* accordingly been demonstrated to be activated upon low nutrient levels, which go accompanied by an increased AMP:ATP ratio (Pan and Hardie, 2002). Mammalian AMPK was shown to inhibit TOR signaling, by phosphorylation of both TSC2 (Inoki et al., 2003) and the TOR scaffold protein, Raptor (Gwinn et al., 2008). Studies in the nematode *Caenorhabditis elegans* indicate that AMPK-mediated inhibition of TOR also takes place in invertebrate species (Fukuyama et al., 2012).

THE INSECT'S NUTRITIONAL STATUS

THE FAT BODY

The fat body is an organ unique to insects that is indispensable for storage and release of energy reserves. It is distributed throughout the animal's body, mainly around the gut and the reproductive organs. In addition to being a storage organ, the fat body also plays a crucial role in the release of nutrients, in the synthesis of hemolymph proteins, in the endocrine system [for instance, expression of insulin-like peptides has been demonstrated in the fat body of some insect species (Badisco et al., 2008; Okamoto et al., 2009)], in the immune system (Ferrandon et al., 2007) and in the detoxification of nitrogen metabolism (Scaraffia et al., 2005). Because of its loose nature, the fat body is maximally exposed to the hemolymph, which is crucial for the functions it exerts. In addition, it is a heterogeneous organ being regionally differentiated into different types of cells that each exert specific tasks (Roma et al., 2010). The adipocytes (also termed "trophocytes") are the storage sites for carbohydrates, lipids and proteins and make out an important part of the fat body tissue. The size of the fat body is determined by the insect's life stage and is largely dependent on the amount of stored material. Information about the insect's nutritional status is thus not only available from the hemolymph, but also directly from the fat body.

Triglycerides have a higher caloric content per unit of weight than glycogen. Furthermore, a considerable source of water is released upon their oxidation. Therefore, the fat body has a higher capacity for lipogenesis from glucose than for glycogenesis, and accordingly lipids are the major components of the fat body (Inagaki and Yamashita, 1986; Zhou et al., 2004). A considerable part of an organism's amino acid reserves are stored as circulating storage proteins, such as hexamerins. These proteins are synthesized by the fat body and released into the hemolymph. When the amino acid reserves are needed, the storage proteins are taken up again by the fat body, in a receptor-mediated endocytotic process. Within the fat body cells, they are temporarily stored in protein granules. Subsequently, they are proteolytically degraded and serve as a source of amino acids (Haunerland, 1996). In all insect species, the period preceding vitellogenesis (i.e., yolk protein precursor production) is characterized by increased uptake of the storage proteins by the fat body, where this source of amino acids serves the synthesis of yolk proteins. Like growth, reproduction-related processes go accompanied with extensive protein synthesis, which is an energetically expensive process that depends upon the oxidation of specific compounds, such as carbohydrates and lipids. Therefore, strict (hormonal) control of storage and mobilization of energy reserves from the fat body is crucial for the correct progression of these reproductive anabolic processes. In addition, the fat body must be capable of integrating signals from other organs and those concerning the insect's nutritional status (**Figure 2**). [For a comprehensive review on the fat body, its biological functions and its regulation, we refer to Arrese and Soulages (2010).]

THE ISP ACTS AS A SENSOR OF NUTRITIONAL STATUS

Like all other animals, insects need to ingest food for the acquisition of energy and nutrients, in support of their metabolism. According to the physiological and developmental needs of the insect, the acquired nutrients can be differentially allocated to different organs, processes and/or metabolic pathways, enabling the insect to adjust its physiology according to its internal nutritional state. Nutrient sensing systems play an important role in this physiological adjustment process. An evolutionary conserved systemic nutrient sensor is the ISP. In general, this pathway is linked with the internal metabolic and nutritional state of metazoans and plays an important role in the induction of anabolic processes (Tatar and Yin, 2001; Burnell et al., 2005; Mukhopadhyay et al., 2006; Wu and Brown, 2006; Sim and Denlinger, 2008; Toivonen and Partridge, 2009; Fontana et al., 2010; Luedtke et al., 2010; Teleman, 2010).

Many reports confirm that also in insects, this general concept of ISP's role in controlling (certain aspects of) anabolism holds true. Nevertheless, clear functional differences have arisen between some of the ILPs of the different insect taxa, probably due to the long evolutionary history of the distinct insect orders, which has resulted in a variety of different life cycles, life history traits, feeding habits and feeding patterns. Moreover, gene duplication events that resulted in different numbers of paralogs in different taxa, might also have contributed to this [e.g., only 1 ILP (IRP) currently identified in locusts (Hetru et al., 1991; Badisco et al., 2008), while 8 ILPs have been demonstrated in *Drosophila* (Vanden Broeck, 2001; Colombani et al., 2012; Garelli et al., 2012; Nassel, 2012) and more than 30 seem to occur in *Bombyx mori* (Iwami, 2000; Aslam et al., 2011)]. In several insect species, the activity of the ILP/IR signaling system was found to be directly modulated in relation to the nutritional and feeding state, (e.g., changes in activity or in peptide/protein/transcript levels for ILP or some other ISP components: Masumura et al., 2000; Ikeya et al., 2002; Colombani et al., 2003; Puig and Tjian, 2006;

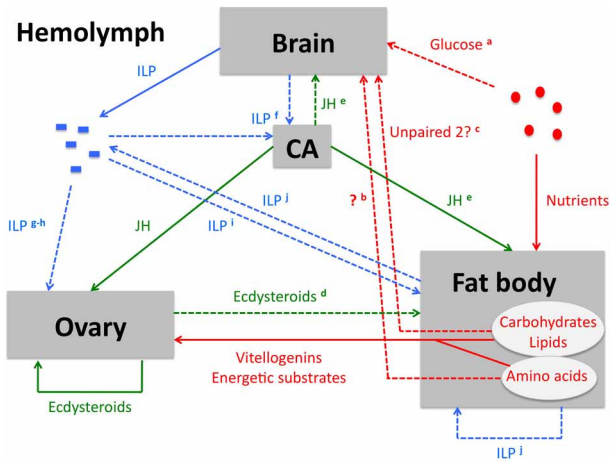

FIGURE 2 | Schematic overview of the key players in ISP-mediated control of female insect reproductive physiology. Solid lines represent a consensus, a process that has been demonstrated in several insect orders. [It needs to be emphasized that the role of ISP in honeybee reproduction strongly deviates from the consensus. Therefore, information from the honeybee is not taken into account in this overview.] Dashed lines represent processes that have only been demonstrated in a limited number of insect species or orders, or that may possibly act more indirectly than suggested on the figure. These lines go accompanied by a remark, indicated by a superscript character. **Nutrient-related (signaling) pathways (red arrows):** Upon digestion of food in the midgut, nutrients are absorbed by the midgut cells and subsequently released into the hemolymph. They are either directly used by the tissues as a source of metabolic energy or as a substrate in anabolic reactions, or they are stored in fat body cells. The fat body's nutritional stores may then be mobilized for the production of vitellogenins, energetic substrates and other metabolic products that serve the process of oogenesis within the ovary. The TOR signaling pathway constitutes a conserved cellular nutrient (amino acid)-sensing system (Hietakangas and Cohen, 2009) and is therefore indispensable in the control of vitellogenin synthesis in the fat body. [(a)] Glucose availability stimulates the *in vivo* release of bombyxin from the silkworm brain, although a direct effect on the brain has so far not been proven (Masumura et al., 2000). Although the isolated fruit fly brain does not seem to release ILPs in response to glucose *in vitro* (Geminard et al., 2009), it is worth noting that some electrical properties of ILP producing brain cells appear to be affected by the glucose (Fridell et al., 2009; Kreneisz et al., 2010). [(b)] A hitherto unknown humoral factor that stimulates ILP release from the fruit fly brain is released from the fat body via a TOR-mediated response to the presence of amino acids (Colombani et al., 2003; Geminard et al., 2009). [(c)] Similarly, the cytokine-like factor Unpaired 2 is produced in the fat body in response to dietary fats and sugar. Remarkably, Unpaired 2 also appears to induce the release of ILP from the fruit fly brain. Although Unpaired 2 has so far not been demonstrated in the fruit fly hemolymph circulation *in vivo*, the authors of the corresponding report suggested that this protein may act as a humoral factor (Rajan and Perrimon, 2012). **Lipophilic hormone signaling (green arrows):** Ecdysteroids are in adult insects mainly synthesized by the gonads. In females they fulfill auto- and paracrine roles in ovary and oocyte development. In addition, ecdysteroid conjugates are stored in the eggs as an embryonic source of these lipophilic hormones. Juvenile hormone is produced by the CA. It stimulates vitellogenin production by the fat body, as well as vitellogenin sequestration by the developing oocytes. [(d)] In dipteran species, an endocrine function for the ecdysteroids in the regulation of vitellogenin synthesis has been demonstrated (Huybrechts and De Loof, 1977). An endocrine role seems to be attributed to ecdysteroids in *B. mori* too, since in this insect they are capable of stimulating ILP synthesis in the fat body (Okamoto et al., 2009, 2011). Remarkably, the decline of

(Continued)

FIGURE 2 | Continued
ecdysteroids appears to be crucial for termination of vitellogenesis in both *A. aegypti* and *B. mori*, indicating that the outcome of ecdysteroid action in reproductive physiology is stage-dependent and species-specific (Dhadialla et al., 1998; Swevers and Iatrou, 2009; Bryant and Raikhel, 2011). [(e)] In the beetle *T. castaneum* a stimulatory effect of JH on expression of some ILP genes in brain and fat body was shown (Sheng et al., 2011). **ILP signaling (blue arrows):** A stimulatory role of insulin signaling on JH biosynthesis has been shown in several insect orders, [(f)] although it is not clear whether ILPs are directly delivered to the CA by projections of the CC or whether they are received from the circulating hemolymph (Tu et al., 2005; Belgacem and Martin, 2006). [(g)] A stimulatory effect of ILPs on ovarian ecdysteroidogenesis has so far only been demonstrated in dipteran species (Tu et al., 2002; Brown et al., 2008; Wen et al., 2010). [(h)] Direct ILP-mediated positive control of oogenesis has hitherto only been shown in *D. melanogaster* (Drummond-Barbosa and Spradling, 2001; LaFever and Drummond-Barbosa, 2005; Richard et al., 2005) and *T. castaneum* (Parthasarathy and Palli, 2011). [(i)] Similarly, direct ILP-mediated stimulation of vitellogenesis has so far only been observed in Diptera (Roy et al., 2007; Gulia-Nuss et al., 2011) and in *T. castaneum* (Parthasarathy and Palli, 2011; Sheng et al., 2011). [(j)] In some insect species, ILP synthesis also occurs in the fat body (Badisco et al., 2008; Okamoto et al., 2009). In the desert locust, *S. gregaria*, the expression levels in this tissue are temporally regulated during the reproductive cycle (Badisco et al., 2008). It is therefore possible that ILP produced by the fat body also acts, at least in this insect species, as a paracrine messenger that signals information about the nutritional status within this tissue and stimulates vitellogenin production (Badisco et al., 2011). Abbreviations: CA, *corpora allata*; CC, *corpora cardiaca*; ILP, insulin-like peptide; JH, juvenile hormone; TOR, target of rapamycin.

Arsic and Guerin, 2008). Other studies report that some insect ILPs are only released to signal nutrient availability under specific conditions, or following specific physiological or developmental behavioral events (e.g., only during molting or metamorphosis, although the feeding stage has already been finished) or for inducing growth under starvation conditions (Slaidina et al., 2009; Liu et al., 2010; Aslam et al., 2011).

In general, insect ILPs mainly originate from the central nervous system, but several ILPs (also) seem to be produced and released by the fat body, as well as by some other tissues (Mtioui et al., 1993; Iwami et al., 1996; Ikeya et al., 2002; Suenobu et al., 2004; Badisco et al., 2008; Okamoto et al., 2009; Nilsen et al., 2011; Okamoto et al., 2011). The function of ILPs in insect physiology has been extensively studied in the fruit fly *D. melanogaster*, which possesses (at least) eight ILPs (*Drosophila* ILPs: dILPs), some originating from the brain and other parts of the central nervous system, others from the midgut, fat body or imaginal discs (Colombani et al., 2012; Garelli et al., 2012; Nassel, 2012). It is worth noting that the central nervous system exerts the general control of the insects' body physiology, while the fat body acts as the main metabolic center (**Figure 2**). The fruit fly brain was found to release ILPs into the hemolymph in relation to nutritional state, although incubation of brains in the presence of nutrients did not result in ILP-release (Geminard et al., 2009). Instead, it was shown that, upon nutrient availability, the fat body releases a humoral factor that induces ILP-release from the brain cells. Since fat body-specific silencing of the Slimfast amino acid transporter or the TOR signaling resulted in decreased ILP release from the brain, as well as global growth defects and reduced PI3K signaling in peripheral tissues, it has been suggested that

this humoral factor is released by the fat body in response to the presence of amino acids (Colombani et al., 2003; Geminard et al., 2009). A recent study shows that the fruit fly fat body indeed produces a factor (the cytokine-like factor Unpaired 2) that is released in response to nutrient availability. Interestingly, although Unpaired 2 has so far not been demonstrated in the hemolymph circulation *in vivo*, fat body-derived Unpaired 2 was suggested to induce ILP secretion from the fruit fly brain by acting as a humoral factor. However, the fat body Unpaired 2 expression did not seem to be dependent on nutrient-derived proteins, but rather on dietary fats and sugar. These findings suggest that different ILP release-stimulating factors might be released from the fat body in response to the presence of different nutrients (Rajan and Perrimon, 2012) and that the fat body plays an important role in sensing and signaling the "systemic" nutritional state (**Figure 2**).

Previous reports could already link the insect ILPs with the presence of carbohydrates. The silkworm ILP, bombyxin, is *in vivo* released from the brain in response to glucose availability (Masumura et al., 2000). Although the fruit fly brain does not seem to readily release ILPs in the presence of glucose (Geminard et al., 2009), some electrical properties of ILP-producing cells change in the presence of glucose, further suggesting a link between ILPs and carbohydrate availability (Fridell et al., 2009; Kreneisz et al., 2010). Other studies demonstrate a link with fat availability and mobilization (Banerjee et al., 2012, 2013). Although much remains to be learned about the precise regulation of insect metabolism by the ISP, it seems that this pathway is not only linked with amino acid availability, but also with fat and sugar availability, which would correspond with its presumed role of nutrient sensor.

Most studies on insect ILPs focused on ILPs originating from the central nervous system, or used systemic experimental manipulations. Because of this, the precise role of ILPs originating from non-neuronal tissues remains underexposed. A study on the mealworm beetle, *Tenebrio molitor*, suggests that the midgut might release an ILP that influences glucose catabolism in the fat body (Mtioui et al., 1993). Several reports demonstrate that, at least in some insect species, the fat body itself can also be a source of ILPs (Badisco et al., 2008; Okamoto et al., 2009; Slaidina et al., 2009; Nilsen et al., 2011; Okamoto et al., 2011; Bai et al., 2012). The fruit fly dILP 6 is specifically released from the larval fat body to stimulate imaginal disc growth, in response to starvation or in specific periods of developmentally arrested feeding (Slaidina et al., 2009), and would affect fat body metabolism (Bai et al., 2012). ILP production directly by the fat body might thus constitute a(n) (alternative) nutrient sensing system that signals the nutrient storage information that is directly available from this tissue.

Since the ISP signals the "systemic" nutritional state of the insect, it may not be surprising that this pathway influences diverse physiological processes related to the acquisition, usage and metabolism of diet-derived nutrients. Besides affecting feeding behavior (Stafford et al., 2012; Zhao and Campos, 2012) and digestion (Gulia-Nuss et al., 2011) in some species, the ISP influences diverse anabolic processes, supporting reproduction, development and growth.

THE ISP AND FEMALE REPRODUCTIVE PHYSIOLOGY

KEY CONCEPTS OF FEMALE REPRODUCTIVE PHYSIOLOGY

Organs associated with reproductive physiology

Key organs in the female insect reproductive physiology are the ovaries and the fat body. Typical for insect ovaries are the two lateral oviducts that join into a common oviduct. Development of the oocytes takes place in tubular structures, termed ovarioles, which merge into the lateral oviducts. During development an oocyte moves in the anterior-posterior direction within the ovariole. Consequently, the most developed oocytes (terminal oocytes) are at the base of the ovariole, near the oviduct. The number of ovarioles is largely species-specific, but may also vary within one species (Büning, 1994). In many insect species, the terminal batch of oocytes in each ovariole develops synchronically and is released into the lateral oviduct, whereupon the next batch of oocytes can start expanding. Hence, egg production is usually a cyclic process. A crucial aspect of oocyte development is the accumulation of yolk proteins that serve as a source of nutrients for the developing embryo. Vitellogenins, the yolk protein precursors, are produced by the fat body and are released into the circulating hemolymph. The oocytes take them up by means of an endocytotic mechanism, mediated by a specific vitellogenin receptor that belongs to the class of low-density lipoprotein receptors (Raikhel and Dhadialla, 1992; Tufail and Takeda, 2009). In addition to providing yolk protein precursors, the fat body also delivers the energetic substrates and the building blocks necessary for anabolic processes that are to be executed within the ovary (**Figure 2**).

The lipophilic hormones

An indispensable role in communication between and within tissues associated with reproduction is attributed to the lipophilic hormones, ecdysteroids and juvenoids. Although well known for their critical role in larval development and metamorphosis, these hormones are synthesized again in the adult stage and contribute to the production of mature eggs. [Although less documented, they also contribute to reproductive events in males.] The term "ecdysteroids" is the covering name for a group of structurally similar, cholesterol-derived, insect hormones. Ecdysone (E) and 20-hydroxy-ecdysone (20E) are the physiologically most relevant ecdysteroids (Lafont and Kooman, 2009; Lafont et al., 2012). In the adult stage, ecdysteroids are mainly synthesized in the gonads (Brown et al., 2009), although other tissues have been suggested as additional sources of ecdysteroids in some insect species (e.g., Delbecque et al., 1990; Gillot and Ismail, 1995). Ecdysteroids have autocrine and paracrine regulatory roles in ovary and oocyte development, since they control ovary morphogenesis, differentiation of germ line stem cells and development of follicle cells (Parthasarathy et al., 2010; Gancz et al., 2011; Konig et al., 2011; Ting, 2013). In addition, conjugates are stored in the eggs as an embryonic source of ecdysteroids. An endocrine regulatory role has furthermore been observed in Diptera, where circulating ecdysteroids act upon the fat body to stimulate vitellogenin synthesis (Huybrechts and De Loof, 1977) (**Figure 2**). The cyclic progress of female mosquito (*Aedes aegypti*) reproduction is reflected in the appearance of the fat body, each cycle proceeding through the previtellogenic stage, the vitellogenic stage and a

temporary termination of vitellogenesis that is characterized by autophagy of the fat body cells. Interestingly, ecdysteroid signaling has in this organism also been shown to activate autophagy of fat body cells (Bryant and Raikhel, 2011). In addition, although the early stages of oogenesis, as well as initiation of vitellogenesis in *B. mori* require the presence of ecdysteroids, their absence seems to be necessary for completion of vitellogenesis and choriogenesis (Dhadialla et al., 1998; Swevers and Iatrou, 2009). These two examples indicate that the outcome of ecdysteroid action in reproductive physiology may be stage-dependent and species-specific.

Juvenile hormone (JH) belongs to the class of sesquiterpenoids and is produced by the *corpora allata* (CA). Like ecdysteroids, juvenoids occur in several isoforms, JH III being the most prevalent in insects (Darrouzet et al., 1997; Goodman and Granger, 2005; Kotaki et al., 2009). In many species, JH is known to stimulate vitellogenin production by the fat body, as well as vitellogenin sequestration by the growing oocytes (**Figure 2**) (Davey, 1981; Hartfelder, 2000; Fei et al., 2005). The latter is probably mediated by JH in two possible ways. First, JH promotes shrinkage of the follicle cells surrounding the developing oocyte and thus allows the yolk protein precursors to reach the oocyte (Tobe and Pratt, 1975). Second, some papers report on the stimulatory effect of JH analogs on the transcript levels of the vitellogenin receptor (Chen et al., 2004; Clifton and Noriega, 2012). In fact, JH has a regulatory function in many aspects of insect biology (in addition to reproduction), such as metabolism, immunity, stress tolerance and ageing, but also behavior, diapause, migration and (caste) polyphenisms, which makes that JH is also capable of indirectly influencing insect reproduction (reviewed by Simonet et al., 2004; Flatt et al., 2005; Verma, 2007). It is thus without doubt that JH is an important player in this physiological process.

ISP-MEDIATED CONTROL OF FEMALE REPRODUCTIVE PHYSIOLOGY

Interactions with ecdysteroid synthesis and signaling

As mentioned above, the lipophilic insect hormones, ecdysteroids and JH, regulate important aspects of insect reproductive physiology, and in this context multiple interactions with insect ISP have been reported. In addition to a regulatory activity of the ISP on the synthesis and release of the lipophilic hormones, ecdysteroids and JH were found to target some of the physiological processes that are also influenced by the ISP and, in some cases, even influence ILP levels and/or ISP activity (**Figure 2**).

The relationship between insect insulin signaling and ovarian ecdysteroid synthesis has mainly been investigated in dipteran species. Overall, insulin signaling seems to have a stimulatory effect on ecdysteroid production in the ovaries of these species. Fruit flies mutant in the IR indeed displayed impaired ovarian ecdysteroid synthesis (Tu et al., 2002). However, ovarian release and hemolymph levels of ecdysteroids as well as JH biosynthesis were merely not affected in homozygous Chico (the *Drosophila* IRS homolog) mutants, although oogenesis in these flies seemed to be perturbated (Richard et al., 2005). These findings might indicate a possible rescue mechanism for the Chico mutants in ecdysteroid and JH production, but not in oogenesis (see also Interactions with Juvenile Hormone Synthesis and Signaling).

The amino acids required for yolk protein production in mosquitoes are derived from a blood meal. Hence, the reproductive cycle in female mosquitoes is induced upon a positive change in the mosquito's systemic nutritional status. It had been observed that, in response to the blood meal, ecdysteroidogenic neurohormones are released (Lea and Brown, 1990). Later on, evidence was accumulating that insulin-like peptides were among them. First, a mosquito IR (MIR) was cloned from mosquito ovary mRNA and its transcript levels appeared to vary in function of the reproductive cycle (Graf et al., 1997). Second, upon a blood meal, MIR is mainly expressed in ovarian follicle cells, which are considered to be the production sites of ecdysteroids (Helbling and Graf, 1998). Third, ecdysteroid synthesis in ovaries of unfed mosquitoes could be stimulated by means of a porcine insulin treatment (Riehle and Brown, 2002). Fourth, by using specific insulin signaling inhibitors, bovine insulin-stimulated ecdysteroidogenesis was shown to act through the IR and the PI3K/PKB pathway (Riehle and Brown, 1999). And finally, an endogenous mosquito ILP (ILP3) has indeed been demonstrated to bind the MIR and to stimulate ovarian ecdysteroidogenesis (Brown et al., 2008; Wen et al., 2010). In addition to ILPs, another mosquito ecdysteroidogenic neurohormone is released upon ingestion of a blood meal. This "ovary ecdysteroidogenic hormone" (OEH) (Brown et al., 1998) displays sequence similarity to neuroparsins (Badisco et al., 2007).

Several functional interactions between the ecdysteroid pathway and the ISP have been reported in the context of growth and molting, demonstrating the complex relationships between both pathways (e.g., Orme and Leevers, 2005; Mirth and Riddiford, 2007; Francis et al., 2010; Walsh and Smith, 2011). Some reports describe molecular signal transduction components that take part in both pathways [e.g., the small GTPase Rab4b (Hou et al., 2012)], while others demonstrate functional interactions between components of both pathways, resulting in modulated activity of one of both pathways [e.g., interaction between FOXO and an ecdysone receptor coactivator (Francis et al., 2010); regulatory activity on insulin signaling by an ecdysone-repressed microRNA (Jin et al., 2012); ecdysone inhibiting insulin signaling (Colombani et al., 2005)]. Also in the regulation of insect reproductive physiology, multiple functional links between both pathways have been demonstrated. *In vitro* experiments using the yellow fever mosquito, *A. aegypti*, showed that only the combination of insulin and 20E stimulated expression of yolk protein precursors (Roy et al., 2007). Some reports describe that ecdysteroids can induce the expression of an IGF-like peptide in the silkworm *B. mori* (Okamoto et al., 2009, 2011). Interestingly, this peptide was suggested to be a growth factor for adult development, since it stimulated development of adult-specific structures [e.g., sperm duct, ejaculatory duct and several reproductive accessory glands (Okamoto et al., 2009)]. Other papers report on the nutritional status affecting ecdysone levels in adult females of the fruit fly *D. melanogaster*, resulting in effects on oogenesis and vitellogenesis (Bownes, 1989; Terashima and Bownes, 2005; Terashima et al., 2005). Whether this nutrient dependency of ecdysone levels in adult females of this species results from regulatory activity of insulin signaling in relation to nutrient availability remains to be investigated.

Interactions with juvenile hormone synthesis and signaling

Organisms displaying reduced insulin signaling are generally characterized by an extended life span, a phenomenon that in insects is often associated with reduced JH levels. Increased longevity in *Drosophila* IR mutants was indeed restored to wild type longevity upon JH (analog) treatment and the CA of the young adult mutants were proven to produce little JH (Tatar et al., 2001; Tatar, 2004). It has been suggested that JH deficiency upon reduced insulin signaling is not the result of impaired development of the CA tissue, but most probably relates to the disturbed neuroendocrine activation of the CA. The *Drosophila* IR is expressed in the CA, indicating a direct action of ILPs on this tissue (Belgacem and Martin, 2006). Either the ILPs are directly delivered to the CA by projections of the *corpora cardiaca* (CC) or they are received from the circulating hemolymph. It should however not be excluded that ILPs (also) act indirectly by affecting the production or activity of allatoregulatory peptides (Tu et al., 2005). As mentioned before, neither ecdysteroid nor JH levels appeared to be affected in homozygous *Drosophila* Chico mutants (Richard et al., 2005). Similarly, when studying the time-course of JH production during the first 10 days of *Drosophila* adulthood, IR mutations appeared to be more effective than Chico mutations in reduction of JH synthesis (Tu et al., 2005). It is in this context worth noting the more recent identification of the *Drosophila* adaptor protein Lnk, which appears to act in parallel to Chico in the ISP. It has been suggested that Lnk and Chico exert independent functions as well as partially overlapping functions (Werz et al., 2009). Therefore, it is not unlikely that Lnk rescues part of the defects resulting from mutations in Chico, although a role for the extended DIR isoform should also not be excluded.

Similarly as in *Drosophila*, the defects observed upon an RNAi-mediated knock-down of the IR in mosquitoes could be rescued by a JH treatment (Sim and Denlinger, 2008). It has furthermore been suggested that JH biosynthesis is dependent upon the insect's nutritional status. Hence, JH seems to act in concert with the insulin and TOR signaling pathways in regulating nutrient allocation in relation to reproductive physiology (Schal et al., 1997; Noriega, 2004; Hernandez-Martinez et al., 2007; Nouzova et al., 2011; Clifton and Noriega, 2012; Perez-Hedo et al., 2013). Further information that links the insulin/TOR signaling pathways, JH synthesis and nutrition comes from honeybee studies. A larva fed on nutrient-rich royal jelly will in normal circumstances develop into a queen bee, a process that is characterized by elevated JH titers. However, RNAi-mediated knock-down of either the IRS or TOR impeded royal jelly-fed larvae to develop into queens and resulted in the worker phenotype. Since application of JH was able to rescue the queen bee phenotype in either knock-down condition, this study offers an extra argument for a regulatory role of the ISP and TOR signaling in JH synthesis (Mutti et al., 2011). Interestingly, it was recently demonstrated in the German cockroach that insulin-mediated control of JH synthesis probably results from its inhibition of FOXO. RNAi-mediated knock-down of FOXO resulted in increased JH and vitellogenin production, even if the females had been starved. Under conditions of nutrient shortage, FOXO is suggested to translocate to the nucleus as a result of reduced ISP and, amongst others, to inhibit JH biosynthesis (Suren-Castillo et al., 2012).

In addition to ISP-mediated control of JH synthesis, some other functional interactions between the ISP and JH pathways have been described. In the beetle *Tribolium castaneum*, there is a stimulatory effect of JH on expression of some ILP genes in the fat body and brain of adult females. In addition, RNAi-based silencing of a JH biosynthesis enzyme and the JH receptor "methoprene-tolerant" caused decreased ILP gene expression (Sheng et al., 2011). Another example of a physiological process regulated by JH and ISP, is the report of Baumann et al. (2013), who show that the JH pathway and the ISP team up to stimulate lipid accumulation during tsetse fly lactation, although the actual nature of their interaction in exerting this effect is not clear yet (Attardo et al., 2012; Baumann et al., 2013).

Vitellogenesis

Studies in some insect species clearly demonstrate a direct stimulatory activity of ILPs, mostly in conjunction with lipophilic hormones, on vitellogenin synthesis by the fat body (**Figure 2**). Bovine insulin did indeed trigger yolk protein precursor production in *in vitro* mosquito fat body cultures only when it was applied together with 20E. Moreover, yolk protein precursor synthesis was impaired by supplying the medium with inhibitors of PI3K or TOR signaling (Roy et al., 2007). Similarly, *in vivo* knock-down of the MIR delayed expression of vitellogenin genes (Gulia-Nuss et al., 2011). These findings indicate that not only ovarian ecdysteroidogenesis, but also vitellogenesis, is controlled by the PI3K/PKB pathway. That control of vitellogenesis by ILPs is complex and is manifested at multiple levels has more recently also become clear by the observation that ILPs are likely to control blood meal digestion in mosquitoes. In addition to its stimulatory effect on ovarian ecdysteroidogenesis, *A. aegypti* ILP3 also stimulates trypsin-like expression in the midgut. Insulin signaling thus appears to synchronize the ecdysteroid-mediated start and progress of vitellogenesis with the availability of amino acids that are necessary for this process (Gulia-Nuss et al., 2011). In addition, the presence of amino acids is "sensed" by the TOR signaling pathway, which in turn stimulates transcription of vitellogenin genes (Hansen et al., 2004, 2005; Roy et al., 2007; Roy and Raikhel, 2011). Insulin and TOR signaling are thus both necessary in the control of vitellogenin synthesis in mosquitoes.

Similar observations have been made in the red flour beetle, *T. castaneum*, where knock-down of ISP and TOR signaling components mimicked the situation of starvation and led to severely reduced transcription of vitellogenin genes (Parthasarathy and Palli, 2011). The same study also demonstrated that vitellogenin transcript levels could not be restored in beetles mutant in the ecdysone receptor and in one of the JH biosynthesis enzymes that were re-fed after a period of starvation. These findings indicated that correctly functioning JH, ecdysteroid and insulin/TOR signaling pathways are crucial for vitellogenin gene expression. Most interestingly, silencing of JH biosynthesis and signaling components in *T. castaneum* did not only reduce ILP gene expression, but also resulted in FOXO-mediated inhibition of vitellogenin gene expression [the vitellogenin gene contains FOXO response elements] (Sheng et al., 2011). These findings show another example of direct ISP action in control of vitellogenesis by the fat body.

The stimulatory role of ILPs in vitellogenesis has also been shown in other insect species, although it remains to be proven whether they either directly or indirectly act upon the fat body. For instance, vitellogenin transcript levels were significantly reduced upon RNAi-mediated silencing of the IRP precursor transcript from the desert locust, *Schistocerca gregaria* (Badisco et al., 2011). It is worth noting that, in contrast to most other insect species, only one IRP has been identified in locust species. Moreover, the desert locust IRP is highly expressed in both brain and fat body, and expression in the latter is elevated during vitellogenesis (Badisco et al., 2008). Therefore, IRP produced by the fat body may act as a messenger that signals information about the nutrient reserves in this tissue and as an (alternative) system to sensing the locust's nutritional status contributing to the animal's metabolic control and energy homeostasis.

Oogenesis

In addition to controlling ecdysteroid synthesis in the ovary, which has been proven in some insect species, the ISP may also directly regulate correct progress of oogenesis within this tissue (**Figure 2**). A correctly functioning ISP appears to be necessary to regulate *Drosophila* egg production in response to dietary changes. Chico mutants displayed reduced proliferation of follicle stem cells and their egg chambers did not develop into the vitellogenic stage, even in the abundant presence of nutrients (Drummond-Barbosa and Spradling, 2001). It has later been demonstrated that germ line stem cell proliferation in response to nutrition is not solely controlled by insulin-dependent mechanisms, but requires other control mechanisms too. These mechanisms each act during specific phases of the cell cycle (Hsu et al., 2008). Interestingly, germ line cell division and cyst growth in *Drosophila* appear to be directly controlled by insulin derived from the central nervous system, indicating that information about the fly's nutritional status is possibly first processed by the central nervous system (LaFever and Drummond-Barbosa, 2005). Female sterility of Chico mutations appears to be autonomous to the ovary, since oocytes do not mature beyond the last previtellogenic stage although the flies display normal JH and ecdysteroid titers. Moreover, transplantation of wild type previtellogenic ovaries in the Chico mutant females resulted in normal oogenesis, indicating that the required systemic factors are present. In contrast, transplanted mutant ovaries into wild type females still displayed disturbed oogenesis. It is possible that a disturbed ISP in the ovaries leads to failure of yolk protein uptake (Richard et al., 2005). That yolk uptake by the oocytes is indeed likely to be mediated by ILPs was demonstrated in mosquitoes. *A. aegypti* ILP3, which is known to be produced in the brain, was able to rescue yolk protein uptake in decapitated females. At the moment, it is however not clear whether this effect is direct or either results from the ecdysteroidogenic activity of ILP3 (Brown et al., 2008). Similar observations have been done in *T. castaneum*, where knock-down of the IR, Chico or TOR resulted in total female sterility (no egg production) and severely reduced egg production was observed upon knock-down of Akt, PTEN, PI3K or S6K (one of the kinases phosphorylated by TOR). The observed defects that resulted from silencing these ISP components were due to impaired maturation of the primary oocytes and arrest of oocyte growth before migration of the follicles (Parthasarathy and Palli, 2011).

Female desert locusts that had been treated with IRP dsRNA were shown to have smaller terminal oocytes than the control animals, although it remains to be investigated whether this also involves a direct IRP action upon the ovary or either results from decreased vitellogenin transcription observed in the same animals (Badisco et al., 2011). Interestingly, a CC extract derived from a related locust species (*Locusta migratoria*) was capable of inducing ecdysteroid synthesis in previtellogenic ovaries of the blowfly, *Phormia regina*. There were arguments that the ecdysteroidogenic factor in this extract was likely to be an insulin-like substance (Maniere et al., 2009). Therefore, it is possible that locust IRP has ecdysteroidogenic activity on blowfly ovaries. However, it remains to be investigated whether this also holds true in locust species.

It was recently demonstrated in *Drosophila* that mutations in the IR and Chico did not exactly mimic the condition of starvation. Upon nutrient shortage, ovarian cells undergo programmed cell death and egg chambers degenerate. However, although their egg chambers developed abnormally, no programmed cell death was observed in the ovaries of IR and Chico mutants. Furthermore, these mutant flies even appeared resistant to starvation-induced programmed cell death. The ISP thus appears to be necessary for the correct response (programmed cell death) upon starvation, but it does certainly not act alone in control of this process. Mutations in TOR and S6K, on the other hand, did mimic starvation-induced programmed cell death. Most probably, the ISP and ISP-independent TOR signaling team up to mediate a correct programmed cell death response upon starvation (Pritchett and McCall, 2012).

Reproductive diapause

Several insect species are capable of transforming into some sort of reproductive resting stage—the so-called "reproductive diapause"—upon unfavorable environmental conditions. Reproductive diapause is in fact a type of phenotypic plasticity and is mainly elicited by alterations in temperature, photoperiod and/or food availability. It either enables an insect to survive adverse conditions or to postpone reproductive events until the environmental conditions are optimal for survival and development of the offspring. Depending on the insect species, reproductive diapause occurs in females and/or males (Pener, 1992). Since it has become clear that a crucial role in reproductive physiology is attributed to the ISP, it is not surprising that reduced insulin signaling is one of the key mechanisms controlling diapause. The low JH titer observed in diapausing insects is considered to be the causative factor and may result from reduced insulin signaling (Tatar and Yin, 2001). Diapause-like phenotypes as well as JH deficiency were indeed displayed by fruit flies mutant in the IR or Chico (Chen et al., 1996; Bohni et al., 1999; Suren-Castillo et al., 2012). A similar phenotype resulting from either ILP1 (Sim and Denlinger, 2009) or IR knock-down (Sim and Denlinger, 2008) in the mosquito *Culex pipiens* could be rescued by administration of JH. The same study pointed to a key role for FOXO, since a knockdown of this molecule prevented the mosquitoes from entering the reproductive diapause state (Sim and Denlinger, 2008). FOXO proteins are generally associated

with stress resistance, determination of life span and response to starvation. They promote conservation of energy or, when necessary, even catabolic processes. Reproductively diapausing insects are characterized by lipid accumulation in the fat body (triglycerides have a higher caloric content per unit of weight than glycogen). FOXO seems to be abundantly present in the fat body of diapausing *C. pipiens* and is considered to be critical for lipid sequestration. Interestingly, JH application suppressed FOXO in diapause-destined mosquitoes, indicating that probably both the ISP and JH are capable of suppressing FOXO. This study also indicates that either FOXO and JH interact in a complex way or that their interaction is species-specific (e.g., in the German cockroach, FOXO is suggested to be an inhibitor of JH synthesis). Shortage of JH may be a causative factor for FOXO activation in mosquitoes. In addition, low JH titers in mosquitoes are suggested to reduce the fat body response to ecdysteroids and therefore to lead to fat body hypertrophy rather than vitellogenin synthesis (Sim and Denlinger, 2013).

Caste differentiation and division of reproductive labor

Typical for eusocial insects, such as the honeybee (*Apis mellifera*), is the division of reproductive labor within the colony. Honeybees display a pronounced type of phenotypic plasticity, which is reflected in the occurrence of two specific phenotypes—queens and workers—each optimized for the specific tasks they exert. Under normal circumstances, the colony's queen is long-lived and is the only female that reproduces, whereas the workers have a much shorter lifespan and exert other tasks, such as nursing the developing juveniles or foraging. A female will develop into a queen only when she has been fed the nutrient-rich royal jelly during the larval stages. Queen development is characterized by elevated JH titers during the fourth and fifth instars. The ISP acts also in this specific case most probably as a sensor of nutritional status and is believed to constitute the link between nutrition and developmental fate of the honeybee.

Higher transcript levels of ILPs and IRs have been observed in queen larvae when compared to worker larvae. However, this situation changes further in the developmental progress when ILP levels appear to be higher in workers (Wheeler et al., 2006; de Azevedo and Hartfelder, 2008). Knock-down of the IRS caused development of the worker phenotype even when the larvae where fed on rich diet (Wolschin et al., 2011). A complementary study aimed at blocking the nutrient sensing system by a knock-down of IRS or TOR in royal jelly-fed larvae. Knock-down of IRS and TOR (both individually and simultaneously) clearly prevented the increase in JH titers necessary for queen development and resulted in the worker phenotype (Mutti et al., 2011). It should however be noted that the ISP might not be the only signaling pathway that is at play in caste differentiation. Royalactin, a protein present in royal jelly was demonstrated to trigger several of the queen bee-related characteristics and these effects appeared to be mediated by the epidermal growth factor receptor (EGFR) signaling (Kamakura, 2011). Interestingly, in mammals, IRS has been shown to mediate EGFR signaling too (Fujioka and Ui, 2001; Fujioka et al., 2001) and similarities or interplay between the ISP and EGFR signaling would therefore not be unlikely in honeybee caste differentiation in response to a rich diet (Mutti et al., 2011).

In addition to the conserved nutrient-sensing mechanism during larval development, other species-specific ISP-related mechanisms are probably at stake in the control of egg production in reproducing queens. Indeed, the role of the ISP in control of reproduction in the adult honeybee queen appears to strongly deviate from the conserved role in most other insects. ILP transcript levels seem to be lower in adult queens than in adult workers, which could explain their extended life span (reduced insulin signaling is generally associated with increased longevity, a phenomenon that appears to be evolutionary conserved). In contrast to many other insect species, lowered ILP transcript levels (and thus likely a reduced ISP) do not result in reproductive defects in the honeybee queen (Corona et al., 2007). In addition, some other contradictions are observed in honeybees. First, although queen bees do have a higher nutritional status throughout their lifetime than workers, their adult ILP transcript levels are lower. These findings suggest that the theory of increased insulin signaling activity upon a better nutritional status does not apply to honeybees. Second, whereas JH is well-known for its stimulatory effect on vitellogenin synthesis in several insect species, it suppressed vitellogenin transcription in honeybees. Interestingly, honeybee vitellogenin, which is abundantly present in the reproducing queen, was shown to act as an anti-oxidant. This may be an alternative explanation for the increased longevity of honeybee queens (Seehuus et al., 2006). In addition, honeybee vitellogenin also appeared to affect the levels of ILPs. These findings indicated that, at least in honeybees, vitellogenin is more than just a yolk protein precursor but is likely to function as a signaling molecule too. It was therefore suggested that the ISP, JH and vitellogenin act in a complex regulatory network that regulates honeybee longevity and reproduction (Corona et al., 2007). Studies of the IRs in two ant species suggested that active insulin signaling is required for correct progress of the reproductive physiology in these social insects (Okada et al., 2010; Lu and Pietrantonio, 2011). Therefore, the somewhat contradictory role of the ISP in honeybees is probably restricted to a minority of (social) insect species.

Other roles of insulin in reproduction-associated processes

The production of cuticular hydrocarbons (many of which are pheromones) in *Drosophila* also seems to depend on insulin signaling. In a process that probably (also) involves the nutrient-dependent TOR signaling, the ISP stimulates transcription of the cuticular hydrocarbon synthesis enzymes. These findings illustrate that fruit flies that have a positive nutritional status and thus also a healthy and vital appearance, do probably display a higher sexual attractiveness (Kuo et al., 2012).

Only recently, light has been shed on the role of the ISP in controlling reproduction in viviparous insects. In tsetse flies, there is only one, intra-uterinely developing, larva per reproductive cycle. The developing larva is nourished by a milk-like substance secreted from a specialized gland (the so-called "milk gland"). The milk-like substance has a high lipid content, which has been derived from the mother's lipid reserves. Interestingly, lipid allocation during lactating (larvigenesis) and non-lactating (oogenesis and embryogenesis) periods is controlled by insulin

and JH signaling. More specifically, JH and the ISP suppress lipolysis during the non-lactating periods (Baumann et al., 2013).

THE ISP IN INTERACTION WITH OTHER HORMONES/PATHWAYS

As previously mentioned, the ISP influences multiple aspects of insect physiology. This pathway exerts its biological function not by acting alone, but by interacting with other (neuro)endocrine pathways. As has become clear from the sections "Interactions with ecdysteroid / juvenile hormone synthesis and signaling," the ISP acts in concert with the lipophilic hormones to control multiple aspects of insect reproductive physiology. In most insect species, the ISP appears to have a stimulatory effect on lipophilic hormone synthesis. However, the situation appears to be more complex, since *vice versa* ecdysteroids [e.g., *B. mori* (Okamoto et al., 2009, 2011)] and JH [e.g., *T. castaneum* (Sheng et al., 2011)] have in some species been demonstrated to affect ILP expression. In addition, it is also likely that the ISP and the lipophilic hormone signaling pathways act in parallel in control of certain processes. Hence, control of insect reproductive physiology by the ISP and the lipophilic hormone pathways appears to be complex. Moreover, a species-specific and stage-dependent outcome of insulin, ecdysteroid and JH signaling should not be excluded.

Since the insect ISP is involved in the regulation of diverse physiological processes, it may also not be surprising that this pathway displays interactions with several other neuropeptide pathways. Most functional connections between the ISP and other neuropeptidergic pathways have been demonstrated in the context of nutrient homeostasis, metabolism and feeding. Indeed, several neuropeptides modulate ILP release and/or expression in the fruit fly brain [tachykinin-related peptide (Birse et al., 2011); sulfakinin (Soderberg et al., 2012); short neuropeptide F (Lee et al., 2008, 2009); and possibly also corazonin (Kapan et al., 2012)]. In other cases, the ISP itself seems to influence the expression or activity of other neuropeptide pathways. This is the case for the signaling pathways of sulfakinin (insect homolog of the vertebrate cholecystokinin), which is expressed in many of the insulin-expressing cells of the fruit fly brain (Soderberg et al., 2012), and neuropeptide F (Wu et al., 2005; Lingo et al., 2007). Because of the functional links between metabolism and reproductive physiology, these pathway interactions might (indirectly) take part in the regulation of reproductive physiology. Some reports suggest interactions between ISP and other neurohormonal pathways that have been (more directly) associated with reproductive physiology. Neuroparsins constitute a family of arthropod-specific neurohormones (Badisco et al., 2007). Based on their sequence similarity with insulin-like growth factor binding proteins (IGFBPs), neuroparsins were suggested to be potential modulators of ILP functioning, an idea that was supported by the observation of *in vitro* binding of locust neuroparsin and IRP (Badisco et al., 2008). Since some neuroparsins display anti-gonadotropic activity in locusts (Girardie et al., 1987; Badisco et al., 2011), it is possible that these act as (negative) regulators of ILP-signaling (Badisco et al., 2011). However, as it is also the case for vertebrate IGFBPs, other modes of action may occur. It is worth noting that the mosquito neuroparsin-like factor, OEH, is a gonadotropic factor and appears to act in parallel with ILP(s) (Brown et al., 1998). Some insect species do not seem to possess neuroparsin-like molecules [e.g., several *Drosophila* species (Veenstra, 2010)], although the actual occurrence of these factors remains uncertain. In *D. melanogaster* and the moth *Spodoptera frugiperda*, yet another factor with apparent sequence homology to vertebrate IGFBP has been implicated in the regulation of insulin signaling (Sloth Andersen et al., 2000; Honegger et al., 2008). This factor, named "Imaginal morphogenesis protein-Late 2" (Imp-L2), binds endogenous ILPs and in this way, modulates insulin signaling (Alic et al., 2011; Bader et al., 2013).

In addition to the above-mentioned interactions with neuropeptide and lipophilic hormone pathways, some studies suggest that the ISP in the fruit fly brain also displays functional links with specific neurotransmitter pathways [e.g., involving serotonin (Ruaud and Thummel, 2008; Luo et al., 2012), octopamine (Crocker et al., 2010; Erion et al., 2012), and γ-aminobutyric acid (Enell et al., 2010)]. However, these interactions were not studied in relation to reproductive processes, and it is therefore not clear whether they are also of importance for the control of reproductive physiology.

THE ISP AND MALE REPRODUCTIVE PHYSIOLOGY

As discussed above, many studies have already analyzed the role of the ISP as a nutrient-dependent regulator of female insect reproductive physiology. Although less documented than its role in females, the insect ISP also coordinates several aspects of male reproductive physiology according to the insect's nutritional state. Insulin signaling was found to regulate spermatogenesis in male fruit fly testes (Ueishi et al., 2009; McLeod et al., 2010; Wang et al., 2011). Besides directly inducing maintenance and proliferation of germ line stem cells in relation to nutrient availability, the ISP also affected spermatocyte growth in testes of male *D. melanogaster* (Ueishi et al., 2009; McLeod et al., 2010; Wang et al., 2011). A study in other *Drosophila* species suggested that the ISP also influences the growth of male external genitalia (Masly et al., 2011). Studies on different species of horned beetles suggested that the ISP also mediates the growth of the horns that are used by males of these species for competing with rival males, when protecting female mating partners (Emlen et al., 2012; Lavine et al., 2013). For several species of horned beetles, an interesting male reproductive dimorphism, associated with different reproductive strategies (reproductive trade-off) has been described. Larger males develop large horns for competing with other males, while small males instead choose to additionally invest in their testes and copulatory organs, in order to increase their copulation success when performing "sneak copulations" (Emlen, 1997; Tomkins and Simmons, 2000; Simmons and Emlen, 2006). This reproductive dimorphism seems to be mediated by insulin signaling, in relation to the male's nutritional status. Depending on nutrient availability and resulting body size, insulin signaling (through FOXO) regulates the growth of horns for competition or instead of testes and copulatory organs (Snell-Rood and Moczek, 2012; Lavine et al., 2013). When comparing with the literature on female insect reproductive physiology, it seems that ISP function in relation to male reproductive physiology has received little attention so far. The diversity of physiological processes affected

by ISP in female insects suggests that several target processes of the ISP in adult males may remain to be identified.

CONCLUSIONS

The current review illustrates that insulin signaling, in response to the insect's nutritional status, contributes at different levels to the control of reproductive physiology. In addition to lipophilic hormone synthesis, vitellogenesis and oogenesis, the ISP may also regulate reproductive diapause, caste development, pheromone production and even lactation physiology in viviparous insects. Energy derived from nutrition must not only be invested in development of reproductive structures, but also needs to be incorporated in the eggs as a source of nutrition for the developing embryo. Therefore, one of the most important tasks of the ISP is the allocation of energy to specific ongoing processes related to reproductive physiology. However, the ISP does not act alone in control of the aforementioned processes. It is part of a complex interaction network that also involves the lipophilic hormones and other (neuro)peptides, and in which the different components act in parallel and/or mutually interact with each other and with the available nutrients.

So far, studies relating the insect ISP to reproductive physiology have mainly been conducted in dipteran species and to a much lesser extent in other insect species. That the findings from dipteran insect studies cannot always be extrapolated to other species is nicely illustrated by the fact that some of the established relationships involving nutrition, insulin signaling and vitellogenin synthesis seem to be inverted in honeybees. Thus, a lot remains to be learned from insects belonging to non-dipteran orders.

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Mechanisms regulating nutrition-dependent developmental plasticity through organ-specific effects in insects

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Nutrition, via the insulin/insulin-like growth factor (IIS)/Target of Rapamycin (TOR) signaling pathway, can provide a strong molding force for determining animal size and shape. For instance, nutrition induces a disproportionate increase in the size of male horns in dung and rhinoceros beetles, or mandibles in staghorn or horned flour beetles, relative to body size. In these species, well-fed male larvae produce adults with greatly enlarged horns or mandibles, whereas males that are starved or poorly fed as larvae bear much more modest appendages. Changes in IIS/TOR signaling plays a key role in appendage development by regulating growth in the horn and mandible primordia. In contrast, changes in the IIS/TOR pathway produce minimal effects on the size of other adult structures, such as the male genitalia in fruit flies and dung beetles. The horn, mandible and genitalia illustrate that although all tissues are exposed to the same hormonal environment within the larval body, the extent to which insulin can induce growth is organ specific. In addition, the IIS/TOR pathway affects body size and shape by controlling production of metamorphic hormones important for regulating developmental timing, like the steroid molting hormone ecdysone and sesquiterpenoid hormone juvenile hormone. In this review, we discuss recent results from *Drosophila* and other insects that highlight mechanisms allowing tissues to differ in their sensitivity to IIS/TOR and the potential consequences of these differences on body size and shape.

Keywords: IIS/TOR signaling, nutritional plasticity, body/organ size, relative organ growth, organ-specific sensitivity, ecdysone, juvenile hormone

Much of the diversity seen across insect species is generated by changes in organ size and shape relative to the whole body (Emlen and Nijhout, 2000; Shingleton et al., 2008). Even within a species, we find dramatic examples where the size of an organ changes disproportionately with increasing body size. In dung beetles, the relative size of male horns, used in courtship battles to gain access to females, varies disproportionately with body size (Arrow, 1951; Emlen, 1994, 1997a). Small-bodied males have very small horns similar to those of females. In contrast, above a threshold body size, males develop much larger horns (Emlen, 1997b). Although differential growth between the organs and the whole body appears more dramatic in polyphenic insects like dung beetles, similar phenomena occur on a subtler scale in all insects to shape the final adult form (Shingleton et al., 2007, 2008).

Although we are beginning to elucidate the mechanisms regulating whole body size, understanding differences in the relative growth of organs poses a new challenge. The regulation of the relative size of organs must, in part, be determined by the mechanisms that govern body size. However, because organs differ in their scaling relationships with body size, there must also be additional organ-specific mechanisms for growth. Furthermore, because organs do not grow at the same rate throughout development, we expect the organ's response to a given growth factor to change over developmental time.

Here, we review the findings over the past fifteen years that describe how body size changes in response to an environmental cue, nutrition, through the action of the insulin/insulin-like growth factor signaling (IIS)/target of rapamycin (TOR) signaling pathway. Further, we will discuss how the IIS/TOR pathway affects endocrine tissues to regulate the production of two metamorphic hormones: the steroid molting hormone ecdysone and the sesquiterpenoid hormone juvenile hormone (JH). Finally, we present our hypothesis explaining how the interplay between the IIS/TOR pathway and the regulation of tissue growth by ecdysone and JH might act to mould organism shape. This hypothesis serves as a framework for evolutionary/developmental studies of nutrition-based phenotypic plasticity.

NUTRITION-DEPENDENT SIGNALING VIA THE IIS/TOR PATHWAY

Body size is a function of larval nutrition in insects. Once larvae initiate metamorphosis, adult body size becomes fixed, as insects do not feed during the pupal stages and the sclerotized outer skeleton of the adult body does not permit further growth. In insects that undergo complete metamorphosis (holometabolous insects), many adult organs develop inside the larval body as imaginal primordia or discs. As the larva eats, these imaginal tissues grow and respond to the same cues that control whole body growth.

In organisms ranging from insects to humans, the IIS/TOR pathway regulates growth in response to nutrition (**Figure 1**). In the fruit fly, *Drosophila melanogaster*, rich nutritional environments cause a set of neurosecretory cells in the brain, the insulin producing cells (IPCs), to produce and secrete three insulin-like peptides (ILPs), ILP2, ILP3, and ILP5 (Brogiolo et al., 2001; Ikeya et al., 2002). Starvation represses both the synthesis and secretion of these ILPs (Brogiolo et al., 2001; Ikeya et al., 2002; Géminard et al., 2009). Further, ablating the IPCs genocopies the effects of starvation (Rulifson et al., 2002; Broughton et al., 2005), suggesting that the ILPs produced in the IPCs are major mediators of nutrition-dependent growth.

Although the functions of the IIS/TOR pathway are conserved across insects, the number of ILPs varies greatly between species. There are eight ILPs in *Drosophila* and the mosquito, *Aedes aegypti* (Brogiolo et al., 2001; Riehle et al., 2006; Colombani et al., 2012; Garelli et al., 2012), two in the honeybee, *Apis mellifera* (Corona et al., 2007), and thirty two ILPs in the silkworm, *Bombyx mori* (Iwami, 2000).

Nutrition-dependent ILP production in the IPCs is thought to regulate most growth. Nevertheless, there are several additional sources of ILPs important for stage- or tissue-specific growth. For instance, the mid gut, imaginal discs, ventral nerve cord, and salivary glands also express ILPs in *Drosophila* (Brogiolo et al., 2001), and these ILPs are thought to have systemic effects

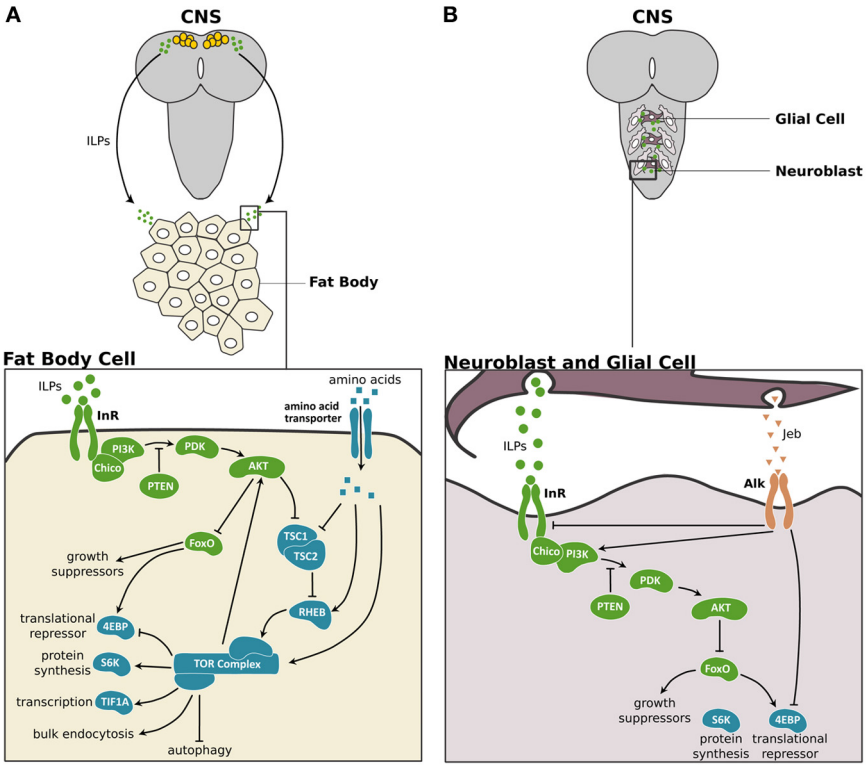

FIGURE 1 | The IIS/TOR signaling pathway in fat body and the central nervous system of *Drosophila*. (A) In *Drosophila*, three of the eight ILPs, ILP2, ILP3, and ILP5 are expressed in a set of neurosecretory cells in the central nervous system (CNS) (Brogiolo et al., 2001; Ikeya et al., 2002). These ILPs activate insulin/insulin-like growth factor signaling (IIS) in peripheral tissues like the fat body cells. In the fat body, the ILPs bind to and activate the Insulin Receptor (InR), which in turn activates Chico, the insulin receptor substrate. This activation initiates a phosphokinase signal transduction cascade that involves phosphatidylinositide 3-kinase (PI3K), the phosphoinositide-dependent protein kinase 1 (PDK) and the protein kinase Akt (Sarbassov et al., 2005). The phosphatase and tensin homolog (PTEN) catalysis the reverse reaction promoted by PI3K, thereby inhibiting the IIS pathway (Goberdhan et al., 1999; Gao et al., 2000). When activated, Akt promotes cell growth by inhibiting the transcription factor Forkhead Box class O (FoxO) (Junger et al., 2003), which activates the 4E-Binding Protein (4EBP), a translational repressor (Miron and Sonenberg, 2001). Furthermore, Akt suppresses the negative regulators of the Target of Rapamycin (TOR) pathway, Tuberous Sclerosis Complex 1 and 2 (TSC1/2) (Gao and Pan, 2001; Gao et al., 2002). TOR activity itself is enhanced by high concentrations of intracellular amino acids (Gao et al., 2002) via the Ras Homolog Enhanced in Brain (RHEB; Garami et al., 2003). TOR activity promotes cell growth by enhancing translation and ribosome biogenesis through inhibition of the 4EBP and activation of the ribosomal protein S6 kinase (S6K). TOR also stimulates rRNA synthesis by activating the Transcriptional Intermediary Factor 1A (TIF-1A) (Hietakangas and Cohen, 2007). Lastly, TOR promotes bulk endocytosis and inhibits autophagy. TOR feeds back on the IIS pathway by regulating Akt in a cell autonomous manner (Hietakangas and Cohen, 2007). **(B)** In the CNS two ILPs, ILP3 and ILP6, are secreted by surface glia and activate the IIS pathway in the neuroblasts to regulate the growth of this tissue (Chell and Brand, 2010; Sousa-Nunes et al., 2011). The anaplastic lymphoma kinase (Alk) and its ligand, Jelly belly (Jeb) promote growth of neuroblasts in starved larvae (Cheng et al., 2011). Alk promotes CNS growth in starved conditions by suppressing InR and directly stimulating PI3K activity. IIS components are shown in green, TOR pathway components are shown in blue and Jeb/Alk components are shown in orange.

on growth. In the *Drosophila* central nervous system (CNS), growth of the neuroblasts results from local ILP production in the glia, and not from the IPCs (Chell and Brand, 2010; Sousa-Nunes et al., 2011) (**Figure 1B**). Furthermore, when larvae stop feeding at the onset of metamorphosis, tissue growth is sustained through the secretion of ILP6 primarily by the fat body (Okamoto et al., 2009b; Slaidina et al., 2009). Thus, the pool of ILPs that mediates growth is diverse, both in its spatial and temporal expression.

Irrespective of the source, all ILPs are thought to bind to the Insulin Receptor (InR). Dipterans and lepidopterans have one InR (Graf et al., 1997; Tatar et al., 2001; Koyama et al., 2008), whereas hymenopterans have two (Corona et al., 2007; Lu and Pietrantonio, 2011). By binding to InR, ILPs activate a series of kinases such as Akt (Sarbassov et al., 2005) to promote growth (**Figure 1**; for more details see Nijhout et al., 2013).

The insulin pathway interacts with two additional nutrition sensitive pathways, the TOR and AMP-activated protein kinase (AMPK) pathways, to regulate growth. The TOR pathway responds directly to intracellular amino acid concentrations to regulate Akt in a cell autonomous manner (Hietakangas and Cohen, 2007) (**Figure 1A**). In addition, insulin signaling itself acts through Akt to suppress the negative regulators of TOR signaling, Tuberous Sclerosis Complex 1 and 2 (TSC1/2) (Gao and Pan, 2001; Gao et al., 2002). Because these two pathways converge in function, they are often referred to as the IIS/TOR pathway.

The AMPK pathway senses energy levels in the cell by responding to intracellular adenosine nucleotide levels to regulate growth and metabolism in *Drosophila* larvae (Braco et al., 2012; Mihaylova and Shaw, 2012). In *Drosophila* larvae, blocking AMPK signaling appears to regulate growth by affecting contraction of the visceral muscle, thereby interfering with gut function (Bland et al., 2010). In mammals, AMPK signaling interacts with IIS/TOR by regulating TSC1/2 (Mihaylova and Shaw, 2012). Thus, AMPK is also considered part of this signaling network, although a direct molecular link has yet to be established in *Drosophila*.

In response to IIS/TOR signaling, Akt acts on a series of downstream targets, thereby inducing organ growth. For example, Akt indirectly activates S6 kinase (S6K), which enhances ribosome synthesis (Miron et al., 2003) and cell growth (Montagne et al., 1999; Garami et al., 2003). Akt also phosphorylates the transcription factor Forkhead Box class O (FoxO), a negative regulator of growth (Junger et al., 2003). In well-fed larvae, phosphorylated FoxO is excluded from the nucleus thereby allowing growth to proceed (Junger et al., 2003). In starved larvae, unphosphorylated FoxO remains in the nucleus and acts on its targets to suppress growth (Junger et al., 2003). FoxO transcriptionally regulates the translational repressor, 4E-Binding Protein (4EBP) (Miron and Sonenberg, 2001).

The IIS/TOR pathway also regulates the production of ILPs in *Drosophila*. Amino acid sensing in the fat body via the TOR pathway controls ILP synthesis and secretion in the IPCs (Britton and Edgar, 1998; Colombani et al., 2003; Géminard et al., 2009). Although the nature of the signal produced in response to amino acids is unknown, in response to dietary sugars and lipids, the fat body secretes a type I cytokine, Unpaired 2 (Upd2) (Rajan and Perrimon, 2012). In adults, Upd2 regulates ILP secretion (Rajan and Perrimon, 2012). Thus, the fat body regulates ILP secretion in response to a number of dietary macronutrients, fine-tuning body size regulation to the nutritional environment.

TISSUE-SPECIFIC SENSITIVITIES TO IIS/TOR SIGNALING

The IIS/TOR pathway has unequal effects on the growth of different organs (**Figure 2**). In *Drosophila*, nutrition affects the size of the wings, palps and legs in proportion to body size through the activities of IIS/TOR signaling (Shingleton et al., 2005, 2009). Other organs are less sensitive to changes in nutrition. For instance, the size of the CNS and the male genitalia varies little with nutritional changes in body size (Shingleton et al., 2005; Cheng et al., 2011; Tang et al., 2011). The mechanisms that allow organs to become less sensitive to nutrition are presumably an adapted response to spare the effects of poor nutrition in organs where changes in size interferes with their function (Shingleton, 2010).

Organs become less sensitive to nutrition through at least two mechanisms. The CNS in *Drosophila* is protected from reductions in its size due to starvation through the activity of a "backdoor mechanism." In starved larvae, glial cells secrete Jelly belly (Jeb), which binds to its receptor, anaplastic lymphoma kinase (Alk), and activates the IIS/TOR pathway downstream of InR (Cheng et al., 2011) (**Figure 1B**). In this manner, the CNS maintains its growth under starvation conditions. The genital disc uses an alternative mechanism to reduce its plasticity in response to nutrition. In *Drosophila,* the genital disc expresses low levels of FoxO mRNA and in starved larvae the genital disc shows low levels of FoxO activity (Tang et al., 2011). As a consequence, starvation has little effect on genital size (Shingleton et al., 2005, 2009;

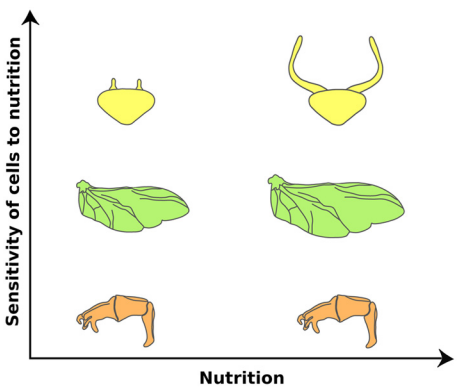

FIGURE 2 | Organs differ in their sensitivities to nutrition in the dung beetle, *Onthophagus taurus*. Nutrition affects the size of the wings (green) in proportion to the body. Other organs, such as the genital disc (orange), are less sensitive to changes in nutrition. This reduction of nutrition sensitivity is achieved by retaining high IIS/TOR signaling activity in low nutritional environments. In contrast, horn discs (yellow) show enhanced response to nutrition. Large males have disproportionately larger horns than small males. This hyperplastic response appears to be due to changes in organ sensitivity to nutrition via enhancing the IIS/TOR signaling activity. (Modified from Emlen et al., 2012).

Tang et al., 2011). Overexpressing FoxO in this tissue increases its sensitivity to nutrition and results in smaller genitalia (Tang et al., 2011). Despite the differences in mechanisms between the CNS and genital disc, ultimately these organs are protected from the effects of starvation by retaining high levels of the IIS/TOR activity independent of nutritional conditions.

Other organs, such as horns in dung and rhinoceros beetles (Emlen, 1997b; Emlen et al., 2012) and mandibles in stag and broad horned flour beetles (Okada and Miyatake, 2010; Gotoh et al., 2011), show exaggerated responses to nutrition. In these organisms, large males have disproportionately larger horns or mandibles for their body size than small males. In the case of the dung and rhinoceros beetles, this hyperplastic response is caused by elevated sensitivity to changes in IIS/TOR signaling. Knocking down InR in rhinoceros beetles shows little effect on genital size, moderate effects on wing size, and more dramatic effects on horn size (Emlen et al., 2012) (**Figure 2**). Changing the level of activity of IIS/TOR appears to be a common mechanism for regulating the degree of plasticity in organ size. In *Drosophila*, changes in the expression of either FoxO or InR generate disproportionate growth of the wing in relation to body size (Shingleton and Tang, 2012), indicating that modulating IIS/TOR signaling at several levels of its action can produce exaggerated organ growth (Shingleton and Frankino, 2012; Shingleton and Tang, 2012).

Even in organs that scale more-or-less proportionally with body size, IIS/TOR signaling affects their growth at different points during development. In many insects, the imaginal tissues do not undergo substantial growth until after the onset of metamorphosis (Kurushima and Ohtaki, 1975; Truman et al., 2006). These organs appear less sensitive to IIS/TOR signaling in the larval feeding period (Nijhout and Grunert, 2010). Organs that grow in the larval stages can still differ in their sensitivity to nutrition at different periods of development. In *Drosophila*, the wing imaginal discs grow throughout the third (final) larval instar. Starving early third instar larvae significantly compromises wing disc growth (Shingleton et al., 2007; Mirth and Shingleton, 2012). Later in development, starvation has a much more modest effect on the growth of the wing discs (Shingleton et al., 2008). These differences in IIS/TOR sensitivity underlie differences in organ growth. Further, changes in IIS/TOR sensitivity are likely to result from cues from the metamorphic hormones.

IIS/TOR SIGNALING AND ECDYSONE

The IIS/TOR pathway controls the growth of all organs, including the endocrine organs that produce hormones necessary for coordinating nutrition-dependent developmental plasticity (Hartfelder and Engels, 1998). In insects, these organs include those that synthesize the metamorphic hormones, ecdysone and JH (Hartfelder, 2000; Flatt et al., 2005; Mirth and Riddiford, 2007; Mirth and Shingleton, 2012).

Ecdysone is produced by the prothoracic glands (PGs) via a series of cytochrome P450 enzymes, the so-called Halloween genes, from a cholesterol precursor (Gilbert et al., 2002). Once synthesized, ecdysone is released from the PGs and converted to its biologically more active form, 20-hydroxyecdysone, in peripheral tissues such as fat body (Bollenbacher et al., 1977; Petryk et al., 2003). Hereafter, we refer to both forms as ecdysone.

The primary action of ecdysone is to control the larval/nymphal and metamorphic molts. Before each molt, the neuropeptide prothoracicotropic hormone (PTTH) is released to stimulate a rise in ecdysone synthesis (Truman, 1972; McBrayer et al., 2007). In the final instar of lepidopterans, coleopterans and dipterans, additional pulses of ecdysone stimulate other types of developmental transitions important for determining body size and developmental timing (Bollenbacher et al., 1981; Emlen and Nijhout, 1999; Warren et al., 2006). Many, if not all, of these pulses are regulated by environmental stimuli, such as nutrition (Emlen and Nijhout, 1999; Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Layalle et al., 2008; Gu et al., 2009; Walkiewicz and Stern, 2009; Walsh and Smith, 2011).

One such pulse occurs early in the third instar of *Drosophila*. Nutrition-dependent secretion of ILPs activates the IIS/TOR pathway in the PGs and upregulates the expression of the Halloween genes *phantom* (*phm*) and *disembodied* (*dib*) (Colombani et al., 2005; Layalle et al., 2008; Walkiewicz and Stern, 2009). Repressing the IIS/TOR pathway in the PGs reduces both *phm* and *dib* transcription (Colombani et al., 2005; Layalle et al., 2008; Walkiewicz and Stern, 2009) and ecdysone concentration in early third instar larvae (Colombani et al., 2005; Mirth et al., 2005) (**Figure 3A**). Similarly, the IIS/TOR pathway regulates ecdysone synthesis in the final instar of other insect species, such as in the tobacco hornworm, *Manduca sexta* (Walsh and Smith, 2011; Kemirembe et al., 2012) and *Bombyx* (Gu et al., 2009). Collectively, the data outlined above indicate that the IIS/TOR pathway regulates ecdysone synthesis in response to nutrition at specific stages of development.

This stage-specific action of IIS/TOR signaling on ecdysone synthesis allows nutrition to regulate the progression and outcome of particular developmental transitions. For example, in *Drosophila*, the duration of the growth period is determined by a developmental event, known as critical weight, early in the third larval instar (Beadle et al., 1938; Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Shingleton et al., 2005). Larvae starved before reaching critical weight delay both patterning in the wing discs and the onset of metamorphosis (Beadle et al., 1938; Mirth et al., 2005; Shingleton et al., 2005; Mirth et al., 2009). Larvae starved after critical weight show normal timing in the patterning of their wing discs relative to fed larvae and initiate metamorphosis early (Beadle et al., 1938; Mirth et al., 2005; Shingleton et al., 2005; Mirth et al., 2009). Critical weight coincides with a small nutrition-sensitive ecdysone pulse known as the critical weight pulse (Warren et al., 2006; Mirth and Riddiford, 2007). By regulating ecdysone synthesis at this stage, the IIS/TOR pathway affects the progression of tissue patterning, the length of the growth period and final body size (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Layalle et al., 2008; Mirth et al., 2009).

Ecdysone, in turn, regulates the IIS/TOR pathway throughout the body. In feeding *Drosophila* larvae, ecdysone represses TOR signaling in the fat body, which in turn produces an unknown signal that regulates ILP production in the IPCs, and hence controls systemic growth (Britton and Edgar, 1998; Colombani et al., 2003, 2005; Rusten et al., 2004; Delanoue et al., 2010). At the onset of wandering, ecdysone stimulates the production of ILP6 in the

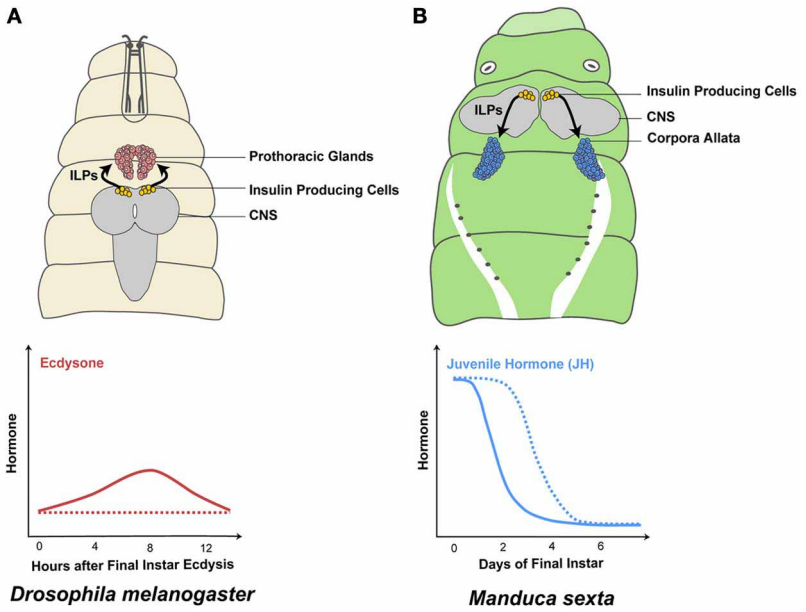

FIGURE 3 | The IIS/TOR pathway regulates metamorphic hormone synthesis. Insulin-like peptide (ILP) secretion by the insulin producing cells in the central nervous system (CNS) depends on nutrition (Masumura et al., 2000; Brogiolo et al., 2001; Ikeya et al., 2002; Colombani et al., 2003). In well-fed final instar *Drosophila melanogaster* larvae, the prothoracic glands increase ecdysone synthesis in response to high concentrations of ILPs in the hemolymph (solid line in **A**) (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Warren et al., 2006). In contrast, when larvae are starved early in the final instar, ecdysone synthesis is reduced/delayed (dashed line in **A**) (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Juvenile hormone (JH) is produced in the corpora allata and the rate of JH synthesis and degradation changes in response to nutrition. Well-fed *Manduca sexta* larvae show a decrease in JH concentrations on day 1 (solid line in **B**) (Fain and Riddiford, 1975). In contrast, when *Manduca* larvae are starved at the onset of the final larval instar, JH concentrations remain high until they feed (dashed line in **B**) (Cymborowski et al., 1982).

fat body to control organ growth in non-feeding stages (Okamoto et al., 2009b; Slaidina et al., 2009). Similarly, ecdysone stimulates the production of an ILP during adult development in *Bombyx* (Okamoto et al., 2009a). Thus, ecdysone can both stimulate and suppress IIS/TOR signaling in the same tissue depending on the stage.

The signaling pathway activated by ecdysone also shows several levels of interaction with the IIS/TOR pathway. Ecdysone binds to a heterodimeric nuclear hormone receptor, Ecdysone Receptor (EcR) and Ultraspiracle (Usp) (Yao et al., 1992, 1993; Talbot et al., 1993). The action of this receptor is mediated through a number of co-activators/repressors. The expression of one of the co-activators of EcR, DOR, is regulated by the IIS/TOR pathway (Francis et al., 2010). Furthermore, HR3, the product of an ecdysone response gene, regulates cell-autonomous growth through S6K activity (Montagne et al., 2010). This tightly woven net of interactions between IIS/TOR and ecdysone signaling pathways allows for fine-scale regulation of nutrition-dependent responses both between organs and across developmental stages.

IIS/TOR SIGNALING AND JH

Considerably less is known about the pathways involved in JH signaling than for ecdysone signaling. Nevertheless, recent data have uncovered some of the mechanisms of JH action. JH determines the nature of molts by modulating the function of ecdysone. JH is known as a "*status quo*" hormone, because it prevents progression to the next life stage between molts (Riddiford, 1996). In holometabolous insects, JH concentration is high during early larval instars to prevent pupation. Only when JH concentration drops at the final larval instar do larvae undergo metamorphosis. JH is produced in the corpora allata (CA) and the removal of the CA from young larvae induces precocious metamorphosis (Williams, 1961).

Besides the *status quo* function, JH also works as a growth regulator. JH and its mimics inhibit cell proliferation in the absence of ecdysone in a lepidopteran imaginal disc-derived cell line (Oberlander et al., 2000) and in the wing discs of *Bombyx* (Koyama et al., 2004a). In the ventral diaphragm myoblasts of *Manduca*, high concentration of ecdysone suppresses cell proliferation but the same amount of ecdysone stimulates cell proliferation in the presence of JH after the wandering stage (Champlin et al., 1999), suggesting that the effects of JH on cell proliferation might be due to modulating the effects of ecdysone.

In *Drosophila*, JH binds to one of two basic helix-loop-helix Per-Arnt-Sim (bHLH-PAS) receptors with partially redundant functions: Methoprene-tolerant (Met) (Miura et al., 2005; Charles et al., 2011) and Germ cell-expressed (Gce) (Baumann et al., 2010). In other insects, JH signaling appears to occur through a single receptor, commonly referred to as Met, which appears to be the ancestral gene that duplicated to give rise to Met and Gce (Baumann et al., 2010). *Drosophila* Met null mutants are smaller than normal (Belgacem and Martin, 2007) as are animals in which the CA is ablated (Riddiford et al., 2010). Furthermore, knocking down Met produces small precocious adults in the red

flour beetle, *Tribolium castaneum* (Konopova and Jindra, 2007) and the true bug, *Pyrrhocoris apterus* (Konopova et al., 2011). Thus, JH regulates body size in many insects.

The rates of JH synthesis and degradation change in response to nutrition. In *Manduca* larvae, feeding at the beginning of the final larval instar causes JH concentration to decline rapidly (Fain and Riddiford, 1975). In contrast, JH concentration remains high in starved larvae due to increased JH synthesis and to suppression of the JH degradation cascade (Cymborowski et al., 1982; Lee and Horodyski, 2006) (**Figure 3B**). Similarly, in adult females of *Aedes* increased IIS/TOR signaling after a blood meal causes JH levels to decline rapidly by down-regulating JH synthesis genes (Noriega, 2004; Perez-Hedo et al., 2013). Mutations in InR result in low JH concentrations in adult *Drosophila* (Tatar et al., 2001; Tu et al., 2005). Finally, knocking down FoxO suppresses starvation-dependent inhibition of JH synthesis in the German cockroach, *Blattella germanica* (Suren-Castillo et al., 2012). Therefore, IIS/TOR signaling regulates JH signaling by controlling JH synthesis and degradation in a nutrition-dependent manner.

JH, in turn, regulates the IIS/TOR pathway. Knocking down an enzyme important for JH synthesis, JH acid methyltransferase, or knocking down Met decreases ILP expression in *Tribolium* (Sheng et al., 2011). Much like ecdysone, JH both regulates and is regulated by the IIS/TOR pathway.

NUTRITION-DEPENDENT TISSUE PLASTICITY IN RESPONSE TO ECDYSONE AND JH

Clearly, organs differ in their sensitivity to nutrition and IIS/TOR signaling, and this regulates their organ-specific growth rates. A growing body of evidence supports the hypothesis that this difference in sensitivity across organs and between stages results, in many cases, from interactions between IIS/TOR signaling and ecdysone/JH signaling pathways.

Ecdysone regulates the growth of various tissues—for example, the imaginal discs, the fat body, and the developing ovaries—in different manners (Hodin and Riddiford, 1998; Colombani et al., 2005; Mirth et al., 2009; Gancz et al., 2011). Ecdysone stimulates tissue growth and cell proliferation in the imaginal discs of *Precis coenia* (the buckeye butterfly), *Manduca*, and *Bombyx* (Champlin and Truman, 1998a,b; Nijhout and Grunert, 2002, 2010; Koyama et al., 2004a; Nijhout et al., 2007; Mirth et al., 2009). Similarly, ecdysone signaling in the *Drosophila* ovaries positively regulates ovariole number, the main determinant of ovary size (Gancz et al., 2011). In the fat body of *Drosophila*, ecdysone signaling suppresses growth by activating FoxO activity (Colombani et al., 2005; Delanoue et al., 2010). Thus, ecdysone alters growth in an organ-specific manner.

Even within the same tissue, ecdysone induces different effects depending on its concentration. Although at low concentrations ecdysone induces cell division and growth in imaginal discs, high concentrations of ecdysone tend to inhibit imaginal cell proliferation and induce differentiation (Champlin and Truman, 1998a,b; Nijhout and Grunert, 2002, 2010; Koyama et al., 2004a; Nijhout et al., 2007).

Furthermore, within the same tissue growth can rely either on IIS/TOR or ecdysone signaling depending on the stage of development. In *Drosophila*, cell divisions in the neural epithelia of the optic lobe depend on the IIS/TOR pathway early in larval development (Lanet et al., 2013). Later on in development, neural proliferation relies on ecdysone signaling, but not on IIS/TOR (Lanet et al., 2013). In the optic lobe, this ensures that proliferation necessary for the differentiation of neuronal types is protected from nutritional variation.

Finally, the effects of ecdysone on growth can depend on the nutritional context. In *Drosophila*, increasing ecdysone concentrations suppresses larval growth rate in suboptimal nutritional conditions (Colombani et al., 2005; Mirth et al., 2005). These effects on growth rate are eliminated under optimal nutritional conditions, when larvae are supplemented with live yeast. Similarly, in lepidopterans, wing imaginal discs show the greatest amount of growth in the presence of both insulin and ecdysone (Nijhout and Grunert, 2002; Nijhout et al., 2007). These data show that IIS/TOR and ecdysone signaling interact to regulate growth.

In *Manduca,* JH also regulates the growth of imaginal discs in response to nutrition. Starvation represses the growth of the wing discs, and eye and leg primordia (Macwhinnie et al., 2005; Truman et al., 2006). Eliminating JH by removing the CA partially restores disc growth even in starved larvae (Truman et al., 2006). The effects of JH on tissue growth are overridden by insulin; wing discs cultured in the presence of JH alone show reduced growth whereas when they are cultured with JH and insulin, growth rates are restored (Koyama et al., 2008).

Sensitivity to JH varies between organs. In lepidopterans, wing discs become committed to form pupal tissue shortly after the molt to the final larval instar (Ohtaki et al., 1986; Kremen and Nijhout, 1989, 1998; Obara et al., 2002; Koyama et al., 2004b). Once the discs are committed, JH no longer reverses their developmental fates. The epidermis loses sensitivity to JH much later, shortly before the onset of metamorphosis (Riddiford, 1976, 1978; Ohtaki et al., 1986; Kremen and Nijhout, 1989, 1998). This difference in timing of commitment causes organs to differ in their response to nutritional stimuli. For instance, starvation at the middle of the final instar often produces animals with larval epidermis and pupal appendages.

A HYPOTHESIS FOR REGULATING ORGAN PLASTICITY

From the studies outlined above, it is clear that in insects the IIS/TOR pathway and ecdysone and JH pathways interact to control the growth of organs in a manner appropriate for the tissue and the developmental stage. For organs that show hyperplastic responses to nutrition, like beetle horns and mandibles, these mechanisms of interaction control switches for the growth of exaggerated traits.

We propose that nutrition, via IIS/TOR signaling, regulates ecdysone and JH synthesis, which in turn regulates IIS/TOR sensitivity in developing organs (**Figure 5**). For example, below a threshold body size, horn size in male dung beetles varies little with nutrition (Emlen, 1994), suggesting they are less sensitive to IIS/TOR signaling. Above the threshold body size, horns become hypersensitive to nutrition (Emlen, 1994). This developmental switch in sensitivity to nutrition between small and large males correlates with stage-dependent changes in metamorphic hormone concentration. A pulse of ecdysone

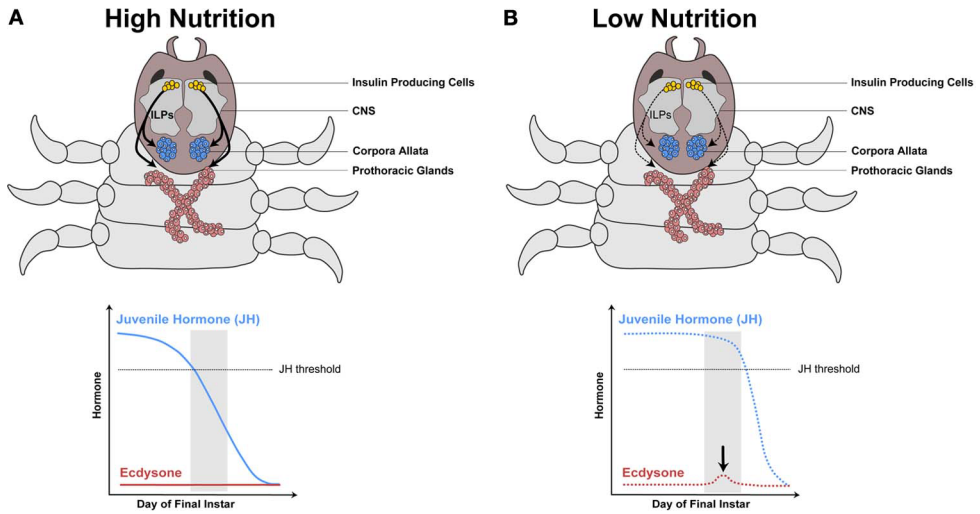

FIGURE 4 | Model hypothesizing how the IIS/TOR pathway regulates hormone synthesis in the dung beetle, *Onthophagus taurus*. In rich nutritional environments **(A)** higher concentration of insulin-like peptides (ILPs, curved black arrows) are secreted from the insulin producing cells in the central nervous system (CNS). The prothoracic glands and corpora allata grow in response to these high concentrations of ILPs, which alters the synthesis of ecdysone and juvenile hormone (JH), respectively. **(A)** In well-fed larvae, JH concentrations decline rapidly below a threshold (dashed gray line), as a consequence ecdysone does not rise during a critical stage near the end of the final larval instar (gray box). **(B)** In contrast, starved larvae have slower rates of JH decline, which triggers a small pulse of ecdysone at this stage. [Modified from Emlen and Allen (2003), Emlen et al. (2007)].

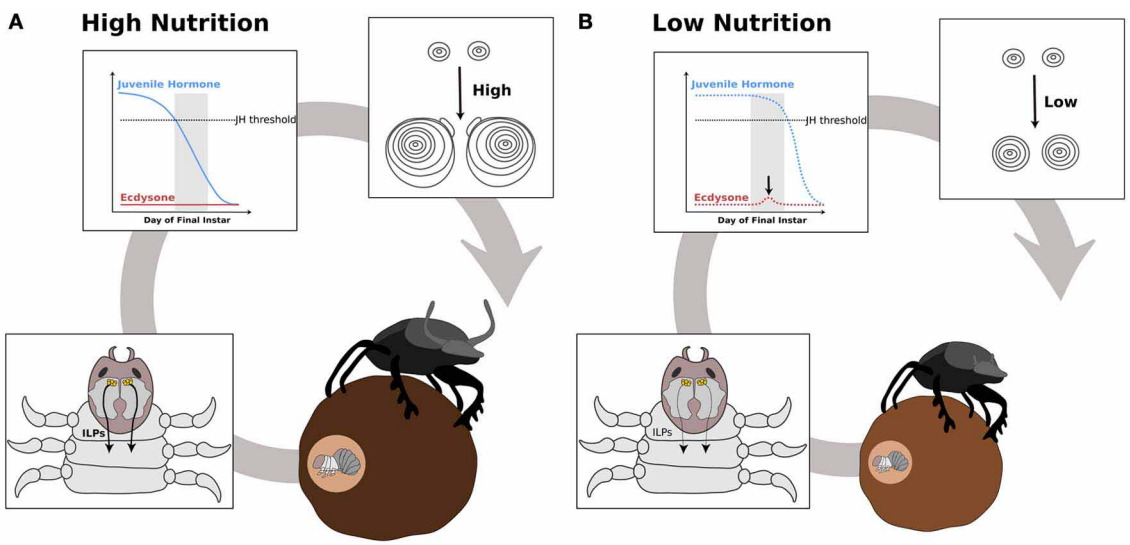

FIGURE 5 | Hypothesis outlining the role of ecdysone and juvenile hormone in switching nutrition sensitivity in the developing horns of the dung beetle, *Onthophagus taurus*. Beetle horn growth depends on larval nutrition. In well-fed larvae **(A)**, the secretion of insulin-like peptides (ILPs, curved black arrows) from the central nervous system (CNS) increases as the larvae feed (A′). At a critical stage near the end of the final instar (gray box), juvenile hormone (JH) concentration falls below threshold and ecdysone synthesis is not induced (A″). This results in high sensitivity to IIS/TOR signaling in the horn discs (A‴). As a consequence, the horn discs grow more relative to their body size (A⁗). In poorly fed larvae **(B)**, ILP secretion (black curved arrows) is low (B′). Thus, JH concentration remains high during the critical stage (B″). This high level of JH appears to trigger a small pulse of ecdysone (black arrow in B″), which leads to reduced IIS/TOR sensitivity in the horn disc (B‴). The horn discs grow slowly giving rise to a small pair of horns (B⁗).

occurs in the middle of the final instar in small male and female dung beetles of the species *Onthophagus taurus* (Emlen and Nijhout, 2001). Large males do not show this pulse (Emlen and Nijhout, 2001) (**Figure 4**). Topical application of JH at this time represses horn growth (Emlen and Nijhout, 2001), presumably by inducing the ecdysone pulse. This ecdysone pulse appears to regulate JH concentrations and/or sensitivity in later stages of development. Conversely, at the onset of metamorphosis, application of JH induces horn development in small males (Emlen and Nijhout, 1999). The exaggerated

mandibles of male stag beetles, *Cyclommatus metallifer*, and horned flour beetles, *Gnatocerus cornutus*, are likewise sensitive to JH (Gotoh et al., 2011; Okada et al., 2012). Application of JH mimics induces large mandibles relative to body size for both species (Gotoh et al., 2011; Okada et al., 2012). Furthermore, in the horned flour beetle, while JH increases relative mandible size, it decreases the relative size of other structures such as the elytra and hind wings (Okada et al., 2012). This suggests that IIS/TOR signaling modifies JH and ecdysone concentrations in response to nutrition (**Figure 5**). These changes in metamorphic hormone concentrations alter body size and shape by reprogramming tissue-specific sensitivities to IIS/TOR to change the relative growth of tissues (**Figure 5**).

Social insects provide another dramatic example of how IIS/TOR signaling regulates hormone production to control body size and relative organ growth. In *Apis*, caste differentiation is determined primarily by a special nutritional component called royal jelly (Haydak, 1970; Wheeler et al., 2006; de Azevedo and Hartfelder, 2008; Wolschin et al., 2011). Larvae that are fed royal jelly differentiate into queens. If not, they develop into workers. Queens and workers differ in both size and shape: queens are larger and, because they are specialized for reproduction, their abdomens and ovaries are disproportionately larger in size compared to workers.

Royalactin, a component of royal jelly, induces queen differentiation (Kamakura, 2011) by increasing the overall concentration of JH (Rembold, 1987; Rachinsky et al., 1990). Furthermore, it induces a queen-specific JH peak in the early fifth (final) instar (Rembold, 1987; Rachinsky et al., 1990). These differences are presumably induced by accelerated growth in the CA in larvae fed royal jelly (Dogra et al., 1977), which stimulates higher rates of JH synthesis (Rachinsky and Hartfelder, 1990). Topical application of JH in larvae that are not fed royal jelly induces queen-like phenotypes (Wirtz, 1973; Nijhout and Wheeler, 1982).

Royal jelly regulates JH synthesis through the IIS/TOR pathway (Wheeler et al., 2006; de Azevedo and Hartfelder, 2008; Wolschin et al., 2011). By knocking down components of the IIS/TOR pathway to reduce its activity, the queen-specific JH pulse disappears and accordingly, the whole body of these animals becomes reprogrammed to produce worker-like phenotypes (Patel et al., 2007; Mutti et al., 2011). Topical application of JH rescues these phenotypes. Interestingly, it appears that IIS/TOR signaling regulates differential DNA methylation between queens and workers. In addition, some of these differentially methylated genes are JH-related genes (Foret et al., 2012). Finally, knocking down DNA methyltransferase induces queen-like morphology (Kucharski et al., 2008). Thus, in honeybees, a specific nutritional component induces epigenetic changes that control JH concentrations. These changes in JH reshape both whole body and relative organ growth to generate the different morphs.

CONCLUSIONS

Over the past fifteen years, the IIS/TOR pathway has emerged as the principal pathway regulating plasticity in body size. However, the final shape of an adult insect requires both regulating overall body size and the relative growth of organs. Organs achieve specific rates of growth by differing in their sensitivities to IIS/TOR signaling both between organs and over developmental time. Further, the IIS/TOR signaling pathway regulates relative organ growth both through direct action on organs and indirectly by regulating metamorphic hormone synthesis, which have their own organ- and stage-specific effects on growth. Thus, we propose that nutrition-sensitive production of ecdysone and/or JH might act to switch organ-specific growth rates by altering their sensitivity to IIS/TOR signaling itself. This interplay between IIS/TOR and the metamorphic hormones provides a potent mechanism to dramatically alter body shape in polyphenic insects, like beetles and social insects.

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A new mathematical approach for qualitative modeling of the insulin-TOR-MAPK network

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In this paper we develop a novel mathematical model of the insulin-TOR-MAPK signaling network that controls growth. Most data on the properties of the insulin and MAPK signaling networks are static and the responses to experimental interventions, such as knockouts, overexpression, and hormonal input are typically reported as scaled quantities. The modeling paradigm we develop here uses scaled variables and is ideally suited to simulate systems in which much of the available data are scaled. Our mathematical representation of signaling networks provides a way to reconcile theory and experiments, thus leading to a better understanding of the properties and function of these signaling networks. We test the performance of the model against a broad diversity of experimental data. The model correctly reproduces experimental insulin dose-response relationships. We study the interaction between insulin and MAPK signaling in the control of protein synthesis, and the interactions between amino acids, insulin and TOR signaling. We study the effects of variation in FOXO expression on protein synthesis and glucose transport capacity, and show that a FOXO knockout can partially rescue protein synthesis capacity of an insulin receptor (INR) knockout. We conclude that the modeling paradigm we develop provides a simple tool to investigate the qualitative properties of signaling networks.

Keywords: mathematical model, sigmoid, insulin, TOR, MAPK, FOXO, growth

INTRODUCTION

Signaling through the insulin pathway is a major regulator of growth in a broad diversity of vertebrates and invertebrates ranging from humans and mice, to worms and flies (Oldham and Hafen, 2003; Taniguchi et al., 2006; Sutter et al., 2007; So et al., 2011). It regulates diverse processes such as blood glucose homeostasis, differentiation, growth, and senescence (Tatar et al., 2003). The pathway is also frequently misregulated in diseases such as diabetes and different types of cancers. In some cells, insulin regulates the uptake of glucose (Furtado et al., 2003), in others it is a general regulator of protein synthesis and cell growth (Colombani et al., 2003). Among the great discoveries is the interaction between insulin signaling and the Target of Rapamycin (TOR), which mediates between the insulin signal and the utilization of amino acids in protein synthesis and growth (Brogiolo et al., 2001; Oldham and Hafen, 2003; Avruch et al., 2006; Grewal, 2009; Kim and Guan, 2011). The insulin network also interacts with members of the FOXO family of transcriptional regulators in the control of cell growth and cell proliferation (Jünger et al., 2003; Puig et al., 2003; Southgate et al., 2007; Tang et al., 2011; Snell-Rood and Moczek, 2012). The insulin signal is able to stimulate upstream members of Ras/Raf-mediated MAPK cascades (Seger and Krebs, 1995), which also lead to cellular growth.

Experimental work to understand the properties and function of the insulin signaling network has tended to focus on the roles of one or two components of the network at a time, for example by knocking out or overexpressing one component and examining the phenotypic effect. The overall network is a conceptual framework built up of a large set of individual experiences and understandings. The network is becoming increasingly complex and it is difficult to know how the various components interact with each other. Experimentally, it is impractical, and in most cases impossible, to control for all potential variables, nor is it possible to determine to what degree an experimental result is a consequence of the particular background in which it was performed. Indeed, the functional consequence of a mutation is largely dependent on the background in which it occurs (Dworkin et al., 2009; Chandler et al., 2013). Alternatively, if the experiment was done under highly controlled conditions, *in vitro*, on a subset of the system, it is not possible to say how that subset would actually perform when embedded within the more complex network with all its many inputs, interactions, and feedback loops. With very simple systems it is possible to perform thought experiments that examine the logic of a network diagram by simply stepping through it. But when the system is large, with positive and negative feedback loops and a mixture of activating and inhibitory interactions, thought experiments generally fail. In addition, although our understanding of the components and overall structure of signal transduction networks is growing rapidly, the dynamical properties of these networks remain largely unknown. This is important because the explanation of some cellular phenomenon lies not in the components but in the dynamics of the system that led to the phenomenon (Wolkenhauer et al., 2004).

A grasp of mechanics and combinatorial possibilities of interactions between signaling molecules is insufficient for

understanding cellular responses or changes in physiological states. This is because the set of interactions that actually make a functional difference to the cell are a small subset of all the molecular interactions that occur; furthermore, detailed knowledge of local interactions may not be sufficient to understand the global dynamics of the network. Uncovering which components and which dynamics are functionally important is one of the most challenging and important questions for understanding the function and evolution of these signaling networks.

One solution to understanding the properties of a complex network is through mathematical modeling. A mathematical model is nothing more than a quantitative, simplified abstraction of the structure and kinetics of the system. It has two advantages: one is that a mathematical model is completely explicit in what is included and what is not, something an experimental system seldom if ever can be, and second that it allows one to examine whether the network elucidated by experimentation indeed has the properties we assume it does.

There are many approaches to mathematical modeling. Boolean models can describe the logic of a regulatory network, differential equation models can describe the kinetics of a network, and statistical models can describe the patterns of correlation and covariance within a network. Several investigators have developed differential equation-based mathematical models for different portions of the insulin signaling network (Sedaghat et al., 2002). Some of these models reproduce selected experimental data well (Sedaghat et al., 2002), while others do not attempt to do so and are mostly concerned with the general or the formal properties of the model mechanism (Alon, 2006; Vinod and Venkatesh, 2009).

The most common problem encountered in building a mathematical model is the dearth of data on the kinetics of the reactions. Most data are static and record the response to an insulin stimulus as "fold-activation" or "percent of control value" or "percent of maximum response." Thus response data are typically reported as scaled quantities. The modeling paradigm we develop here uses scaled variables and is ideally suited to simulate systems in which much of the available data are scaled. We develop a simple network modeling paradigm, inspired by Boolean, genetic, and neural network models (Reinitz and Sharp, 1995; Vohradsky, 2001; Jaeger et al., 2004; Faure et al., 2006; Martin et al., 2007), that can be used to study the qualitative behavior of complex networks, and apply it to a study of the insulin signaling network. Abstracting the network in a way that is consistent with the available data opens possibilities to reconcile experiments with theory, and thus improves our understanding of the structure and function of signaling networks. A well-validated mathematical model is also a useful adjunct tool for the experimental biologist because it allows for quick and inexpensive testing of alternative hypotheses and may provide suggestions for experimental design.

METHODS

STRUCTURE OF THE NETWORK

The insulin signaling network we model is illustrated in **Figure 1**. Insulin binds the insulin receptor (INR), causing it to become phosphorylated and thus activated (Oldham and Hafen, 2003). INR has multiple phosphorylation sites which become autophosphorylated upon binding of insulin. The activated INR recruits Insulin Receptor Substrate adaptor proteins (IRS) to the cell membrane. The IRS proteins bind to phosophoinositide 3-kinase (PI3K) and to phosphatidylinositol(3,4,5)-triphosphate (PIP3), and recruit both to the membrane. Biochemically, PI3K becomes activated when it interacts with specific phosphotyrosine motifs in the IRS (Taniguchi et al., 2006). Activated PI3K phosphorylates phosphatidylinositol(4,5)-biphosphate (PIP2, also at the cell membrane) into PIP3. The tumor suppressor gene phosphatase tensin homolog deleted on chromosome 10 (PTEN) catalyzes the opposite reaction, dephosphorylating PIP3 to PIP2. PTEN reduces the amount of PIP3, a substrate necessary for the activation of protein kinase B (PKB, also called Akt) (Brazil and Hemmings, 2001). Through this mechanism, PTEN is an antagonist to insulin signaling. PTEN is one of the most commonly lost tumor suppressor genes in human cancer. It has relatively high constitutive phosphatase activity (Leslie and Downes, 2004). PIP3 facilitates the phosphorylation of protein kinase C (PKC) by PDK1, thus activating PKC (Standaert et al., 2001; Taniguchi et al., 2006). PIP3 also causes an increase in autophosphorylation of PKC, independent of PDK1, possibly by inducing conformational changes in PKC (Standaert et al., 2001). PKC phosphorylates p70 ribosomal kinase S6K (Valovka et al., 2003). S6K is involved in the regulation of cell cycle and growth (Montagne et al., 1999; Valovka et al., 2003). S6K phosphorylates the ribosomal protein S6, and controls the translation of a class of mRNAs that encode ribosomal proteins and elongation factors (Jefferies et al., 1997).

PIP3 phosphorylates and activates PKB/Akt. PKB has several targets including FOXO, a constitutively active transcription factor (Southgate et al., 2007) that is involved in the cellular response to nutritional conditions (Gershman et al., 2007). When FOXO is phosphorylated by PKB, it is translocated from the nucleus to the cytoplasm, where it can no longer activate transcription (Essers

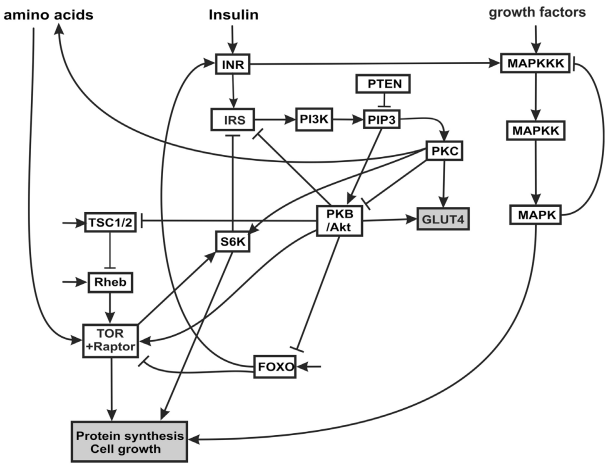

FIGURE 1 | Insulin and MAPK signaling network. The acronyms for the components of the network and the rationale for the connectivity are explained in the text.

et al., 2005; Aoyama et al., 2006). Under extreme starvation conditions, FOXO upregulates the expression (but not the activation) of the INR, increasing cell sensitivity to insulin, and allowing a fast response to insulin after feeding (Jünger et al., 2003; Puig and Tijan, 2005).

PKB also activates TOR, a serine/threonine kinase that regulates growth in response to nutritional conditions. TOR is required for response to amino acids (Hara et al., 1998). TOR is activated by PKB and positively regulates cell growth via two principal targets, S6K and 4E-BP (Hay and Sonenberg, 2004; Sarbassov et al., 2005). S6K also phosphorylates the INR, decreasing the interaction of INR with its substrate (IRS) and inhibiting insulin signaling (Harrington et al., 2005). The proximal regulator of TOR is a small GTPase, Ras homology enriched in brain (Rheb), which binds to the TOR catalytic domain and activates TOR (Avruch et al., 2006). Amino acid withdrawal interferes with the interaction of Rheb and TOR-raptor, indicating that the Rheb-TOR interaction is responsible for the sensitivity of the TOR pathway to the presence or absence of amino acids. Rheb is negatively regulated by the Tuberous sclerosis complex proteins, composed of TSC1 (hamartin) and TSC2 (tuberin) (Manning and Cantley, 2003). The disease known as tuberous sclerosis is an autosomal dominant disorder associated with benign tumors that is the result of inherited mutations in the TSC1 or TSC2 genes. PKB phosphorylates TSC2 (Choo et al., 2006) inhibiting the function of the TSC1-TSC2 complex (Choo et al., 2006). Thus PKB signaling releases the inhibition of Rheb and activates TOR signaling. The TSC complex is necessary for the downregulation of TOR in response to hypoxia (Ellisen, 2005).

Many growth factors signal through the MAPK cascade. The MAPK cascade is a highly conserved signaling pathway and is a major regulator of growth and differentiation. MAPK cascades typically have three levels (Huang and Ferrell, 1996). The terminal member of the cascade is a MAPK (e.g., ERK, JNK, p34, p42), which is activated by a MAPK kinase (MAPKK: e.g., MEK, EKK), which in turn is activated by a MAPKK kinase (MAPKKK; e.f Raf, Mos). The MAPKKK can be activated in several ways: most commonly by external signals such as epidermal growth factor (EGFR) via a G-protein-coupled receptor complex, and also by insulin signaling (Oldham and Hafen, 2003). The terminal MAPK translocates to the nucleus where it phosphorylates transcriptional regulators for protein synthesis, growth and differentiation.

STRUCTURE OF THE MODEL

In modeling this system we omit consideration of multiple phosphorylation steps, equilibrium reactions between kinases and phosphatases, and translocations between cytosolic and nuclear compartments. We consider only the activity level of the various kinases and other components in the network. Unlike a Boolean network, in which each element is either on or off, each element in our scheme can have a continuum of activity between zero (inactive) and one (maximum activity). The activity level of a node in the network is a function of activating and inhibitory inputs. We assume that activation follows a sigmoid trajectory, with little activity at low input levels and saturating at high input levels.

The time-dependent activation equation for each node in the network is the logistic

$$\frac{dy}{dt} = ay(1 - y/b) \tag{1}$$

where a is the rate of increase and b is the ceiling. Over time y levels off at the value of b. Graphs of this time-dependent response, and the effects of parameters a and b, are shown in **Figure 2**. The value of b is a sigmoidal function of input, so that at low input b (and consequently the maximum value of y) is small and rises sigmoidally to a value of 1 as input increases. The equation for b is a solution to the logistic, as follows

$$b = \frac{1}{1 + e^{-\frac{\text{input}-0.5}{c}}} \tag{2}$$

where the 0.5 ensures the inflection point is at an input of 0.5, and c sets the steepness of the transition, with smaller values producing a more switch-like transition. The curves are symmetrical around the inflection point. Graphs of this function for different values of c are shown in **Figure 3**.

This pair of functions thus scales the value of y between 0 and 1, for a range of inputs that are scaled from 0 to1. The value of y then becomes part of the input to the next step in the network. Multiple (activating and inhibiting) inputs are summed as follows: activators are averaged, and inhibitors are averaged and subtracted from the activator mean.

Thus the input function looks like:

$$\text{input} = \frac{1}{n}\sum_{i=1}^{n} W_i \,^{*}\text{activator}_i - \frac{1}{m}\sum_{i=1}^{m} W_i \,^{*}\text{inhibitor}_i, \tag{3}$$

where W_i represent the weight of each activator or inhibitor. The weights were chosen in such a way that the model reproduced experimental data (Sedaghat et al., 2002; Danielsson et al., 2005).

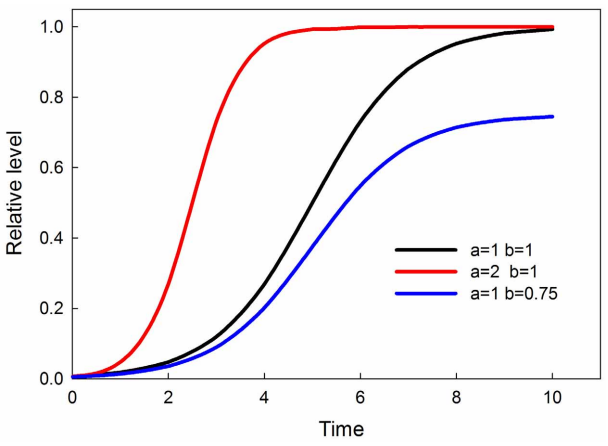

FIGURE 2 | Time-dependent sigmoids (effect of parameters *a* and *b*). Parameter *a* controls when the response attains saturation. Parameter *b* controls the saturation point of the response.

Values for the weights used in the present model, and the values of all other parameters, are given in **Table 1**. There is no information available on how multiple inputs are integrated, so we assume a linear weighing scheme. The linear weighing scheme is therefore a hypothesis about how things could work, and as more data become available it might have to be modified. We show that the selection of weights and parameter values in **Table 1** is also sufficient to enable the model to reproduce a broad diversity of experimental results.

The model consists of a set of coupled equations of the form of Equation (1), one for each node in the network, with the values of b represented by Equation (2) and the inputs by Equation (3). Most nodes are inactive unless activated, with the exception of TOR, Rheb and TSC, which are constitutively active unless inhibited. There are three external inputs: insulin, amino acids, and growth factors that activate the MAPK cascade.

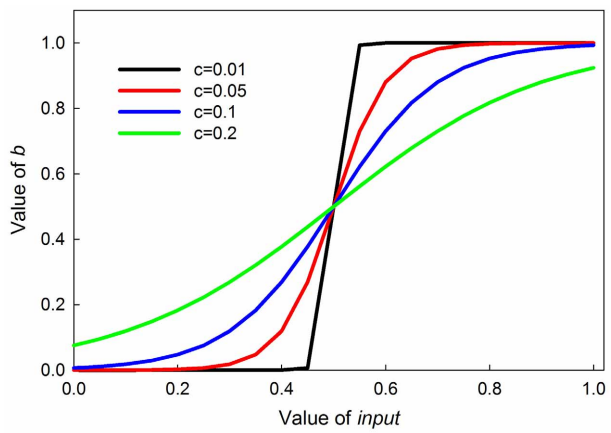

FIGURE 3 | Graphs of the value of *b* in Equation (2) as a function parameter *c*. Parameter c controls the steepness of the sigmoid; the smaller c is, the steeper the sigmoid.

RESULTS AND DISCUSSION

THE MAPK CASCADE AND SWITCH-LIKE BEHAVIOR

The MAPK phosphorylation cascade is one of the most prevalent signal transduction pathways, typically mediating between a G-protein coupled surface receptor for a growth signal and a transcriptional regulator that affects growth and cell proliferation. The MAPK cascade can also be activated by insulin signaling via the stimulation of the upstream kinase (e.g., Raf) via INR (Oldham and Hafen, 2003). MAPK cascades have either three or four levels with multiple phosphorylation steps at each level (Huang and Ferrell, 1996). This structure sharpens the response to a graded signal and makes the response increasingly more switch-like at successively lower levels of the cascade (Huang and Ferrell, 1996). In our model we do not explicitly model phosphorylation and dephosphorylation steps but instead model the transition between an active and inactive kinase, using our sigmoid formalism. **Figure 4** illustrates the behavior of the 3-step MAPK cascade we model to a linear graded input and shows the expected switch-like behavior. The increasing steepness of the response emerges from the fact that each lower step in the cascade is responding to a sigmoidal input in a sigmoidal fashion. MAPK cascades may have a negative feedback regulation by the last to the first member in the cascade (Brondello et al., 1997; Keyse, 2000; Kholodenko, 2000; Asthagiri and Lauffenburger, 2001; Nijhout et al., 2003). **Figure 5** shows the dose-response behavior of our model when such a feedback is included, using the same kinetic parameters as in **Figure 4**. Including the feedback makes the response of the terminal kinase less switch-like.

INSULIN DOSE-RESPONSE

In our model insulin concentration can vary from zero (no insulin) to 1 (maximum or saturating insulin). Dose-response curves are typically hyperbolic on linear axes and sigmoidal when plotted on a logarithmic x-axis. In our scheme all axes are linear and the linear x-axis must therefore represent a logarithmic

Table 1 | Parameter values and input functions used in the model.

Variable	Activators	Inhibitors	Input function	*c*	*a*
INR	Insulin, FOXO		(insulin.*1+ FOXO.*0.144)./2	0.15	1
IRS	INR	PKB, S6K	INR.*2−(0.1.*PKB + 0.2.*S6K)./2	0.15	1
PI3K	IRS		IRS.*1	0.2	1
PIP3	PI3K		PI3K.*1	0.2	1
PKC	PIP3		PIP3.*1	0.25	1
PKB	PIP3	PKC	PIP3.*1−0.5.*PKC	0.15	1
GLUT4	PKC, PKB		(PKC.*3 + PKB.*4)./2	0.05	1
TSC	Constitutive	PKB	1–0.7.*PKB	0.1	0.5
Rheb	Constitutive	TSC	1–0.5.*TSC	0.1	1
TOR	Rheb, amino acids, PKB	FOXO	(rheb.*3 + 1.*aminoAcids + 2.*PKB)./3–2*FOXO)	0.1	1
S6K	TOR, PKC		(TOR.*1.5 + PKC.*0.5)./2	0.1	1
Protein synthesis	TOR, S6K, MAPKKK		(S6K.*1 + TOR.*1 + MAPK.*1.2)./3	0.1	1
FOXO	Constitutive	PKB	1–0.9.*PKB	0.1	0.5
MAPK	MAPKK		MAPKK	0.1	1
MAPKK	MAPKKK		MAPKKK	0.15	1
MAPKKK	INR, EGF growth factors	MAPK	(egfsignal + PI3K)/2–0.3.*MAPK	0.2	1

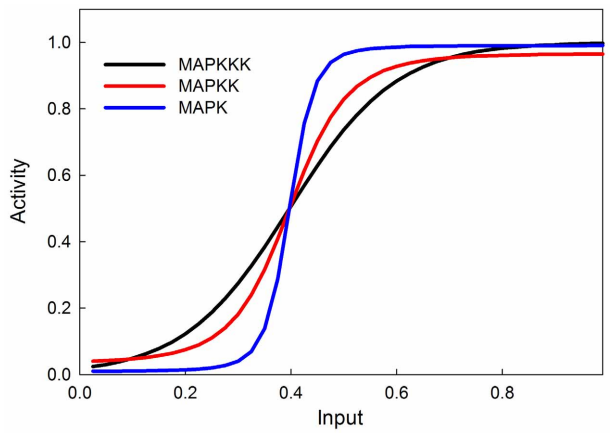

FIGURE 4 | MAPK cascade simulation shows that the signal becomes more switchlike as it travels down the MAPK cascade, as shown in Huang and Ferrell (1996).

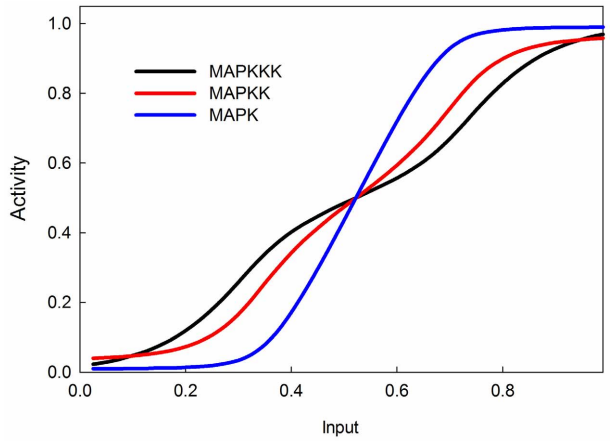

FIGURE 5 | MAPK cascade with negative feedback from MAPK to MAPKKK. The negative feedback makes the response less switchlike.

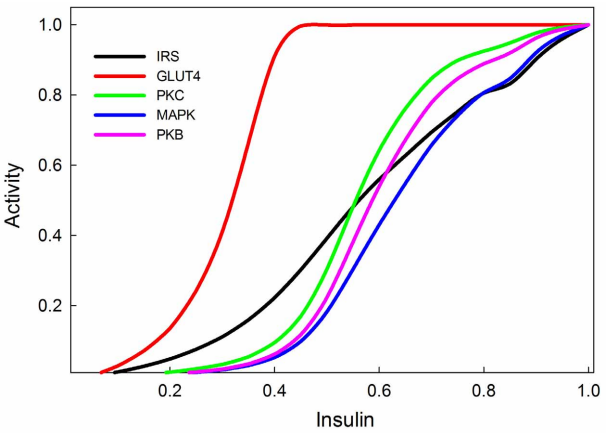

FIGURE 6 | Insulin dose response curves of various components of the insulin and MAPK signaling pathways replicating (Stagsted et al., 1993; Sedaghat et al., 2002; Danielsson et al., 2005). The GLUT4 transporters are the most sensitive to insulin signaling, and achieve their maximum response at relatively low levels of insulin stimulation (0.4).

insulin concentration scale. The dose-response of INR phosphorylation to insulin in experimental settings spans about 4-orders of magnitude of insulin concentration (from 10^{-11} to 10^{-7} M) (Stagsted et al., 1993; Kurtzhals et al., 2000; Sedaghat et al., 2002; Danielsson et al., 2005). Thus on our linear scale 0.25 units correspond approximately to one decade on a logarithmic scale.

Dose-response curves for active IRS, active PI3K, PKB, GLUT4, and MAPK, as functions of insulin concentration are shown in **Figure 6**. The activities are scaled to the maximal response and these relative responses closely resemble the empirical data of Stagsted et al. (1993); Danielsson et al. (2005), except for the response of PKB, which in experimental data appears to saturate at somewhat lower concentrations of insulin than it does in our model.

AMINO ACIDS, PROTEIN SYNTHESIS, AND TOR KNOCKOUT

Normal growth requires, in addition to insulin signaling, amino acid signaling through TOR (Hay and Sonenberg, 2004; Martin and Hall, 2005; Kim and Guan, 2011). In our model, amino acids activate TOR/Raptor directly. In addition, insulin signaling is known to stimulate the import into the cell of neutral amino acids that are handled by the system-A transporter (Kilberg, 1982; McDowell et al., 1998; Biolo et al., 1999; Jones et al., 2006). The exact mechanism is not fully understood, and here we model it as an effect of PKC. Thus growth is regulated by three interacting pathways, MAPK, insulin, and TOR (**Figure 7**). MAPK and insulin signaling can stimulate growth autonomously, but TOR requires activation by insulin signaling in order to be sensitive to stimulation by amino acids.

PTEN, A TUMOR SUPPRESSOR GENE

PTEN is a well-known tumor suppressor gene, and many cancers are associated with a reduction in PTEN activity (Leslie and Downes, 2004; Nassif et al., 2004; Song et al., 2012). We show with our model that knocking out PTEN increases protein synthesis at lower insulin levels (**Figure 8A**), and increases insulin sensitivity of GLUT4 activation (**Figure 8B**). This is consistent with data showing that PTEN haploinsufficiency increases the probability of developing tumors, and also increases insulin sensitivity (protects against diabetes) (Pal et al., 2013). Overexpression of PTEN, by contrast, greatly reduces both proteins synthesis and GLUT4 activation, consistent with its role as a tumor suppressor (Zhao et al., 2005).

FOXO: A REGULATOR OF PHENOTYPIC PLASTICITY?

Insulin signaling regulates growth and size in response to nutrition (Oldham and Hafen, 2003; Grewal, 2009), but few morphological traits scale isometrically: morphological traits commonly scale hyper- or hypo-allometrically with size. These scaling

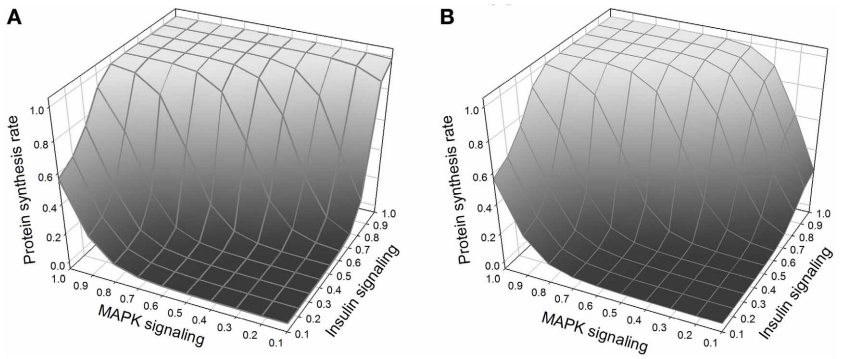

FIGURE 7 | (A) Protein synthesis as a function of MAPK and insulin. MAPK and insulin act synergistically, and the maximum protein synthesis occurs when both pathways are activated. Nevertheless, at low levels of MAPK signaling, insulin is still able to stimulate protein synthesis. **(B)** Same figure but with TOR knockout. The effect of the TOR knockout on protein synthesis is only noticeable when there is weak MAPK signaling (right corner of the figure); when TOR is knocked out and MAPK signaling is low, insulin alone cannot stimulate protein synthesis.

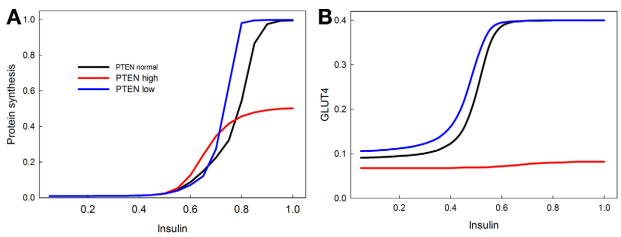

FIGURE 8 | Effect of PTEN over expression and knock-down on the sensitivity of insulin stimulated protein synthesis (A) and GLUT4 activation (B). PTEN overexpression reduces the maximum level of protein synthesis, and completely abrogates the response of GLUT4 to insulin stimulation. PTEN underexpression does not have as dramatic an effect, but does increase the sensitivity of protein synthesis in response to insulin especially at high insulin levels. In contrast, the stimulatory effect of PTEN underexpression on GLUT 4 sensitivity is mainly observed at low insulin levels.

relationships must arise from differential sensitivity of tissues to systemic growth factors and nutritional availability.

Recent evidence indicates that differential trait growth is a consequence of differential sensitivity to insulin, regulated by FOXO (Tang et al., 2011; Snell-Rood and Moczek, 2012; Shingleton and Frankino, 2013). Genitalia of flies (Tang et al., 2011) and of beetles (Snell-Rood and Moczek, 2012) are relatively insensitive to nutrition (or lack thereof). In contrast, wings and horns are more sensitive to nutrition (Tang et al., 2011; Emlen et al., 2012). In beetles, knockdown of FOXO using RNAi causes slight but significant overgrowth of the genitalia (Snell-Rood and Moczek, 2012), indicating that in normal growth conditions, FOXO represses insulin-stimulated growth in that tissue. Loss of FOXO causes genitalia and wings to both scale isometrically with body size (Tang et al., 2011), indicating that FOXO is necessary to maintain trait-specific sensitivity to insulin.

In normal growth conditions, increased expression of FOXO decreases body and organ size (Jünger et al., 2003; Puig et al., 2003). In poor nutritional conditions or in Insulin Receptor (InR) mutants, growth is inhibited, and this reduction in growth is partially rescued by knocking out FOXO (Tang et al., 2011). A similar phenomenon has been observed in *C. elegans*: mutants in Daf-2 (INR homolog) arrest growth at the dauer stage, but null mutations in Daf-16 (FOXO homolog) suppress the effects of mutations in Daf-2, rescuing growth (Ogg et al., 1997). This suggests that in both flies and worms, FOXO knockout rescues InR mutants.

We tested the model against these predictions. Specifically we tested the following predictions: (1) *FOXO overexpression* should decrease body size/protein synthesis under any nutritional condition. Indeed, **Figure 9** shows the result of overexpressing FOXO: protein synthesis is reduced as a function of all insulin inputs, but the effect is particularly strong at high insulin levels (in good nutritional conditions). (2) *FOXO knockout*. Under high insulin signaling conditions/high nutritional conditions, FOXO knockout should slightly stimulate protein synthesis. Under low insulin signaling conditions, FOXO knockout flies should increase protein synthesis relative to wild type flies. Indeed, **Figure 9** shows the result of knocking out FOXO. Protein synthesis is increased at low insulin levels, indicating that FOXO knockout compensates for poor nutritional conditions. In high nutritional conditions, protein synthesis is close to wild type, if not slightly enhanced, consistent with the slight increase in size of the beetle genitalia in FOXO knock-down animals (Snell-Rood and Moczek, 2012). (3) *FOXO and InR double knockout*. InR knockout should reduce protein synthesis, and this reduction should be partially rescued by the FOXO knockout. Indeed, **Figure 10** shows that protein synthesis is strongly reduced in the InR knockout and that protein synthesis is rescued in the double FOXO-InR knockout. Thus, the model is consistent with previous observations and appears to summarize the developmental mechanism by which FOXO regulates sensitivity to insulin input.

HYPOXIA

Hypoxia decreases body size in a variety of species, although hyperoxia often does not have a symmetrical effect (Harrison et al., 2010; Harrison and Haddad, 2011). The mechanisms by which hypoxia affect growth and size are incompletely understood. Oxygen affects multiple physiological processes and is sensed by a variety of signaling pathways, including the Hypoxia

Inducible Factor (HIF) (Gorr et al., 2006) and nitric oxide (NO)/cyclic GMP pathway (Davies, 2000).

HIF and HIF targets interact with components of the insulin signaling pathway; specifically, oxygen affects the inhibition of TSC by Akt (Brugarolas et al., 2004; Deyoung et al., 2008). In normoxic conditions, insulin causes Akt to phosphorylate TSC2. This phosphorylation promotes the binding of TSC2 to its inhibitor 14-3-3 (Deyoung et al., 2008), thereby inhibiting the dimerization and activation of the TSC1/2 complex. In response to hypoxia, REDD1 is induced and sequesters the inhibitor 14-3-3. This releases TSC2 to dimerize with TSC1 and thus promote TSC1/2 function and inhibition of TOR. Thus, in low oxygen conditions, protein synthesis is reduced. Because TSC1/2 is already constitutively active at low insulin signaling, the effects of hypoxia on TSC1/2 will be most apparent at high levels of insulin signaling.

Our model shows that low oxygen levels decrease protein synthesis, and the effect is strongest at high levels of insulin (**Figure 11**). The reason for this effect is that at low levels of insulin, Akt is not stimulated, so Akt is not strongly inhibiting TSC, and the removal of this inhibition has no effect. Hypoxia has the strongest effect on growth when insulin signaling (and Akt) activity is high. In our model, hyperoxia only marginally stimulates protein synthesis beyond its normoxic range. This is consistent with the observation that hyperoxia does not have strong stimulating effects on growth and size in a variety of species (including *Drosophila* and *Manduca*). The fact that the effect of hypoxia is a disinhibition could possibly explain the asymmetrical response of growth to hypoxia and hyperoxia. The double inhibition (Akt inhibits TSC, TSC inhibits TOR) is relieved in hypoxia, but it is unclear whether hyperoxia will significantly enhance the inhibition of TSC by Akt. If this inhibition is already strong in normoxia, then hyperoxia provides no additional benefit. This could explain the asymmetrical growth response to hypoxia/hyperoxia observed in many insect species.

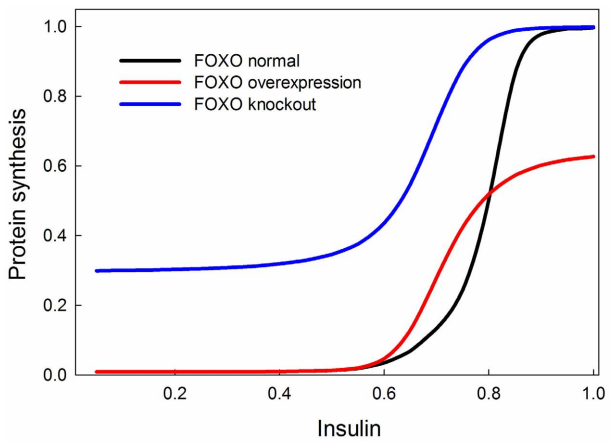

FIGURE 9 | Effect of overexpression and knockout of FOXO on insulin-stimulated protein synthesis. FOXO knockout increases protein synthesis especially at low levels of insulin stimulation. On the other hand, FOXO overexpression primarily affects protein synthesis when insulin signaling is high. These two observations can be explained by the fact that FOXO inhibits TOR and this inhibition is inhibited by PKB when insulin is high. At low insulin levels, FOXO should inhibit TOR and protein synthesis, and hence the knockout relieves this inhibition. At high insulin levels, FOXO should be inhibited by PKB and therefore not have an inhibitory effect on protein synthesis, but FOXO overexpression prevents PKB from entirely relieving the constitutive inhibition of protein synthesis by FOXO.

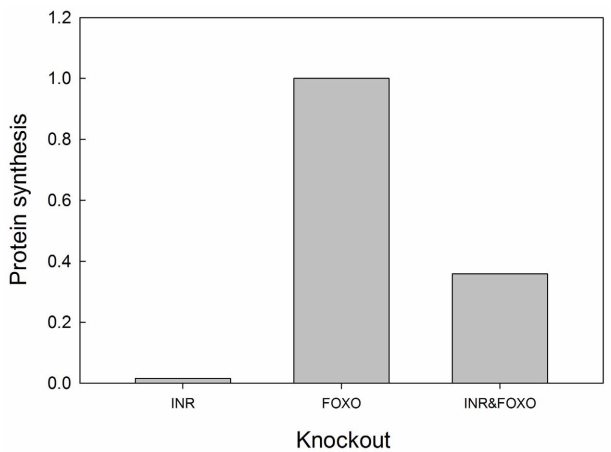

FIGURE 10 | FOXO knockout rescues the growth defects of INR mutants. In an INR deficient mutant, protein synthesis is severely depressed and regulated primarily by MAPK (activation of MAPK is set at 0.1 in this experiment). FOXO knockout alone has no effect on protein synthesis, but can partially rescue protein synthesis in an INR knockout. Protein synthesis values are scaled to those of a "wild type." Input into the MAPK pathway is 0.1.

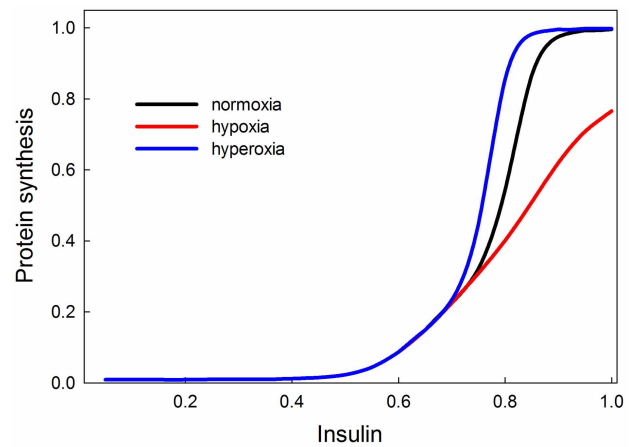

FIGURE 11 | Effects of oxygen on protein synthesis (growth), mediated by the insulin and TOR signaling pathways are most strongly observed at high insulin signaling levels (>0.7). This suggests that in poor nutritional conditions, hypoxia or hyperoxia are unlikely to have strong effects on growth and size; only in good nutritional conditions will oxygen show an effect. Hypoxia decreases the rate of insulin-stimulated protein synthesis. Hyperoxia causes protein synthesis to reach its saturating rate at a slightly lower level of insulin signaling, so it has a slight stimulatory effect for insulin between 0.7 and 0.8. At levels of insulin signaling >0.8, hyperoxia provides no additional stimulation of growth because growth rate has attained its maximum.

CONCLUSIONS AND SIGNIFICANCE

We have developed a simple and easy-to-implement mathematical model for investigating the logical sufficiency and qualitative behavior of signaling pathways. This model is particularly useful to simulate experimental data that are scaled or normalized, which are the norm in studies of signaling pathways. We use this model to study the behavior of an integrated insulin-TOR-MAPK pathway and compare the results to a broad diversity of experimental data.

Our model provides a simple and easy-to-implement tool for investigating the dynamics of a system that integrates multiple graded inputs and produces a specific output from a set of possible responses. The kinetics of signal transduction pathways are inherently non-linear and our model embraces this nonlinearity by assuming that the strength of a response (e.g., the activation of a kinase), is a sigmoid function of the combined activating and inhibitory inputs, so that at low input there is no response and at high input the response saturates. The model correctly simulates the ultrasensitivity and switch-like behavior of the MAPK cascade. We show that the model also correctly simulates published dose-response curves of INR, PKC, and GLUT4 to insulin input.

We used the model to simulate several experimental observations. TOR has been widely described as a "sensor" for amino acid input and inactivation of TOR by mutation or by rapamycin reduces growth and body size. When we inactivated TOR in our model we found a reduction in insulin-induced protein synthesis (which we use as a proxy for growth), but, as expected, no effect on MAPK-induced protein synthesis. Thus in cases where growth is controlled jointly by insulin and MAPK signaling, the effect of TOR will depend on the relative role of insulin.

Growth depends not only on hormone signaling but also on an adequate supply of amino acids. Amino acids can directly stimulate the TOR branch of the insulin signaling pathway, but insulin signaling also enhances the uptake of certain amino acids. In our model we found a hyperbolic relationship between amino acid availability and S6K activity in the presence of constant insulin signaling, but no unique effect of amino acids on protein synthesis. Protein synthesis required both insulin and amino acids, but increasing amino acid levels did not increase protein synthesis under constant insulin. In accord with experimental findings we found that overexpression of FOXO decreased insulin-stimulated protein synthesis, whereas a FOXO knockout made protein synthesis more sensitive to insulin levels. Likewise, knockout of FOXO can rescue protein synthesis deficiency caused by an INR knockout. Finally, knockout of PTEN and TSC, both well-known tumor suppressors, greatly increased insulin-driven protein synthesis.

The network we simulated here leaves out most of the biochemical details of the insulin and MAPK signaling pathways, and thus can only simulate the qualitative behavior of the network. Even so, the fit to experimental data is excellent (which are typically also qualitative), which suggest that the structure of the network, and the way in which we modeled it, are sufficient to explain the phenomena we studied here.

A natural question that arises when developing a mathematical model is whether all the necessary players and all their interactions have been included. No network is ever complete, and investigators continually find new players and new interactions. But it is not always clear whether each newly discovered item is critical for the normal operation of the network, or whether it is important only under a particular set of conditions, or whether it has only a minor effect. Moreover, investigators often publish small abbreviated models of hypothetical mechanism suggested by their experimental data but seldom investigate whether the proposed mechanism is sufficient to produce the observed behavior. The current modeling paradigm provides a simple tool to do such a test. One of the uses of a mathematical model is to investigate whether such a hypothetical network actually has the expected behavior. A mathematical model can say whether the system that is being modeled is sufficient to produce the observed biological behaviors found under experimental conditions. And when this is not the case, experimentation with the model can often lead the way to discovery of what is missing.

ACKNOWLEDGMENTS

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Synergistic induction of the clock protein PERIOD by insulin-like peptide and prothoracicotropic hormone in *Rhodnius prolixus* (Hemiptera): implications for convergence of hormone signaling pathways

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We showed previously that release of the cerebral neurohormones, bombyxin (an insulin-like peptide, ILP) and prothoracicotropic hormone (PTTH) from the brain have strong circadian rhythms, driven by master clock cells in the brain. These neurohormone rhythms synchronize the photosensitive brain clock with the photosensitive peripheral clock in the cells of the prothoracic glands (PGs), in which both regulate steroidogenesis. Here, using immunohistochemistry and confocal laser scanning microscopy, we show these neurohormones likely act on clock cells in the brain and PGs by regulating expression of PERIOD (PER) protein. PER is severely reduced in the nuclei of all clock cells in continuous light, but on transfer of tissues to darkness *in vitro*, it is rapidly induced. A 4h pulse of either PTTH or ILPs to brain and PGs *in vitro* both rapidly and highly significantly induce PER in the nuclei of clock cells. Administration of both neurohormones together induces more PER than does either alone and even more than does transfer to darkness, at least in PG cells. These are clearly non-steroidogenic actions of these peptides. In the peripheral oscillators salivary gland (SG) and fat body cells, neither bombyxin nor PTTH nor darkness induced PER, but a combination of both bombyxin and PTTH induced PER. Thus, PTTH and ILPs exert synergistic actions on induction of PER in both clock cells and peripheral oscillators, implying their signaling pathways converge, but in different ways in different cell types. We infer clock cells are able to integrate light cycle information with internal signals from hormones.

Keywords: insulin signaling, PTTH signaling, daily cycling, rhythm, pigment dispersing factor, PDF, lateral neurons

INTRODUCTION

In the blood sucking bug *Rhodnius prolixus*, insulin-like peptides (ILPs) are synthesized in identified neuroendocrine cells in the brain and are released into the haemolymph with a circadian rhythm in both the larval and adult stages (Vafopoulou and Steel, 2002, 2012b). A blood meal elicits rapid and dramatic release of ILPs indicating a role of ILPs in nutrient sensing (Vafopoulou and Steel, 2012b). In *Rhodnius*, bombyxin, the first ILP isolated from insects (Nagasawa et al., 1988), possesses mild ecdysteroidogenic activity on prothoracic glands (PGs) (Vafopoulou and Steel, 1997). The daily rhythm of synthesis and release of ILPs is tightly coupled to the daily rhythm of production and release of another cerebral neurohormone, the prothoracicotropic hormone, PTTH (Vafopoulou and Steel, 1996a,b). The only known function of PTTH is regulation of the synthesis of ecdysteroids by the paired PGs. PGs are photosensitive circadian clocks (Vafopoulou and Steel, 1992, 1998) which generate the circadian rhythm of ecdysteroid synthesis and release (Vafopoulou and Steel, 1991). Thus, all three hormones ILPs, PTTH and ecdysteroids are released with a synchronous daily rhythm. The circadian timing system in the brain has been described in detail (Vafopoulou et al., 2010; Vafopoulou and Steel, 2012a) and is the primary regulator of all three rhythms. Within the brain, axonal projections from the clock cells make intimate associations with both the ILP neurons (Vafopoulou and Steel, 2012b) and the PTTH neurons (Vafopoulou et al., 2007), revealing nervous pathways by which the brain timing system can drive rhythmic release of these neuropeptides. The PTTH rhythm acts on the PGs, in which it entrains the rhythm of steroidogenesis generated by the PG clock (Pelc and Steel, 1997; Vafopoulou and Steel, 1999), suggesting it may act on the clock in the PG cells. Since ILPs also possess steroidogenic activity on PGs, these too may influence the PG clock. Thus, existing evidence suggests that brain neuropeptides act as "internal messengers of time" (internal Zeitgebers). The present work provides direct evidence that both bombyxin and PTTH do indeed act on the canonical clock protein PERIOD (PER) in the molecular clock in the PGs and also in the clock cells in the brain. Their actions on PER induction are shown to be synergistic, which indicates convergence in the signaling pathways for the two peptides at some point(s).

The broader significance of clock control of rhythmicity in these hormones resides in the functional importance of the resulting circadian rhythm in circulating ecdysteroids. Ecdysteroids are of central importance in the coordination both of development (in the larva) and of reproduction (in the adult) [e.g., reviews by Marchal et al. (2010), Schwedes and Carney (2012)].

Almost all insect cells possess the ecdysteroid receptor (EcR), and we have shown that circadian cycling of EcR occurs in numerous cell types (Vafopoulou and Steel, 2006). This finding shows that many cell types respond to the rhythm of circulating ecdysteroids. We have suggested (Steel and Vafopoulou, 2006) that rhythmic ecdysteroid responses serve first, to orchestrate cellular events in target cells around a circadian cycle and second, to synchronize rhythmicities in distant target cells with each other, thereby creating internal order throughout the animal.

The present work also shows that this temporal order is not generated exclusively by ecdysteroids. We show that certain tissues possess peripheral oscillators (these do not free run in aperiodic conditions and so are driven oscillators, not clocks), specifically salivary glands (SGs) and fat body (FB), which are driven directly by ILPs and PTTH from the brain. The response to the neuropeptides also involves their action on PER in these cell types, but in a different manner from their action on the PG and brain clock cells, that is also indicative of convergence in the signaling pathways for the neurohormones.

MATERIALS AND METHODS

ANIMALS, TISSUES AND *in vitro* INCUBATIONS

Rhodnius larvae were raised at 28 ± 0.5°C in 12 h light: 12 h dark (12L:12D). Only male 5th (last) instar larvae were used in experiments. Unfed larvae exist in a state of developmental arrest and larval-adult development is initiated by a large blood meal. Animals raised in 12L:12D were used as controls to determine daily cycling of PER in various cell types (see below).

Experimental animals were transferred to continuous light (LL) 3 weeks prior to feeding. This long exposure to LL abolishes all known circadian rhythms of synthesis and release of several cerebral neuropeptides and synthesis and haemolymph titer of ecdysteroids (Vafopoulou and Steel, 1992, 2001). These larvae are called LL larvae. At day 12 after feeding of LL larvae, tissues were dissected and subjected to various treatments *in vitro*. Eight animals were sacrificed per experiment. For experiments with brains, the number of brains used is stated in Results. Tissues included the brain, PGs, SGs and pieces of abdominal FB. For *in vitro* incubations of LL tissues, all tissues with a certain cell type were pooled and each tissue type was incubated individually in incubation chambers for 4 h *in vitro* in 100 µl *Rhodnius* saline (Lane et al., 1975), containing glucose and antibiotics under constant gentle agitation. Incubations were: (A) Incubation in saline in light (controls). (B) Incubation in saline in dark. (C) Incubation in the presence of crude protein extract from brains of unfed fifth instar larvae (one brain equivalent per incubation chamber); these extracts were shown previously to contain biologically active PTTH and ILPs (see Vafopoulou and Steel, 1996a, 2002, 2012a). Extraction of proteins from brains has been described before (Vafopoulou and Steel, 1996a). (D) Incubation in the presence of bombyxin. (E) Incubation in the presence of PTTH. (F) Incubation in the presence of both bombyxin and PTTH. (G) Synthetic Pigment Dispersing Factor of *Uca pugilator* (PDF) (see below) was employed as control peptide in the above experiments. All tissues were subjected simultaneously to different treatments. After termination of incubation, all tissues were processed simultaneously for immunohistochemistry.

ANTIBODIES, PEPTIDES, AND CHEMICALS

A rabbit polyclonal antibody to PER was used which was prepared against a 14-amino acid sequence corresponding to the protein-protein dimerization motif of the PAS region of PER (residues 605–618; KSSTETPPSYNQLN; known as peptide PER-S, bleed S80-3) of *Drosophila* and was a generous gift of Dr. Kathleen Siwicki (Swarthmore College, Swarthmore, PA). The specificity of binding of this antibody to *Drosophila* PER was demonstrated by Siwicki et al. (1988). The 14-amino acid sequence of PER-S is highly conserved among PER proteins. This antibody recognizes the native PER of *Rhodnius* (Vafopoulou et al., 2010) and was used at a dilution of 1:1000.

A guinea pig polyclonal antibody was prepared against a custom-made, synthetic peptide for the complete amino acid sequence of *Uca pugilator* pigment dispersing hormone (PDH) (NSELINSILGLPKVMDA) (GenScript, Piscataway, NJ). The insect homologs of PDH are known as pigment dispersing factors (PDFs). PDF antibodies have been used extensively to trace axonal projections of clock cells in the brains of various insects including *Rhodnius* (Vafopoulou et al., 2010; Vafopoulou and Steel, 2012a). The specificity of this antibody to recognize *Rhodnius* PDF was determined by double immunochemistry with an anti-PDF used extensively before by us (Vafopoulou et al., 2007, 2010; Vafopoulou and Steel, 2012a,b) as well by many other laboratories. This antibody was used to trace the axonal projections of the *Rhodnius* brain clock cells and produced a staining pattern that completely co-localized with the staining pattern produced by the previously used anti-PDF. This antibody was used at a dilution of 1:500.

Recombinant *Bombyx* PTTH (PTTH) (expressed in *E. coli*) (Ishibashi et al., 1994) and synthetic bombyxin-II (Nagasawa et al., 1988) were generous gifts from A. Mizoguchi (Nagoya University, Nagoya, Japan). These peptides exhibit steroidogenic activity in the PGs of *Rhodnius* (Vafopoulou and Steel, 1997) and antibodies against these peptides were used to immunologically identify the *Rhodnius* PTTH and ILPs (Vafopoulou and Steel, 2002) and to localize the neurons producing them in the brain (Vafopoulou et al., 2007; Vafopoulou and Steel, 2012b). PTTH was used at 8 ng/ml dilution and bombyxin was used at 300 ng/ml dilution in *Rhodnius* saline. These concentrations are the lowest that induce maximal stimulation of ecdysteroid synthesis by PG cells *in vitro* in published dose-response curves (Vafopoulou and Steel, 1997). For combination treatments with both bombyxin and PTTH, the peptides were used at the above concentrations. As control, synthetic PDF was used at its haemolymph concentration of 1 nM (Persson et al., 2001) and at 10 nM concentrations. No effect of PDF on PER was seen in any experiment, hence these data are not included in Results.

Goat anti-rabbit and goat anti-guinea pig IgGs conjugated to the green fluorophore fluorescein isothiocyanate (FITC) used in immunohistochemistry were purchased from Sigma–Aldrich (St. Louis, MO). Vectashield mounting medium was purchased from Vector Laboratories, Burlington ON, Canada.

IMMUNOHISTOCHEMISTRY, IMAGE COLLECTION, AND STATISTICAL ANALYSES

All tissues were prepared according to an established protocol (e.g., Vafopoulou and Steel, 2012a). Briefly, issues were fixed in freshly prepared 4% paraformaldehyde in phosphate-buffered saline (PBS, pH 7.2) for 2 h at room temperature, washed thoroughly in PBS (pH 7.2) and preincubated in 5% control serum containing 1% Triton X-100 as a permeabilizing agent. Tissues were then incubated for 24 h in solutions containing the primary antibodies at 4°C. Tissues were thoroughly washed in PBS and incubated overnight in the secondary antibody generated in goats. All secondary antibodies were used at 1:200 dilution. After thorough washing in PBS, tissues were mounted in Vectashield. In technical controls, the primary antibody was replaced with non-immune serum or the secondary antibody was replaced with PBS. Fluorescence levels in these controls were indistinguishable from the background and no autofluorescence was detected. Digital optical sections at 1 μm distances were viewed with an Olympus FV300 confocal laser scanning microscope. Microscope parameters were kept constant. Images were processed using ImageJ (1.47 q) (NIH, Bethesda, MD) and Adobe Photoshop CS5 (San Jose, CA). The digitized images were modified only to adjust contrast and merge files.

Pixel intensity of fluorescence in nuclei and cytoplasm of randomly selected incubated cells was quantified using the line tool in Image J on original images from the microscope. The length of the line was kept constant in all measurements. Mean pixel intensity was calculated for all cell types and all different treatments as follows: four cells were selected randomly from a particular tissue from a single animal and pixel intensities were measured separately for nuclei and cytoplasm. Cells were chosen using randomization procedures that vary somewhat between tissue types because structure of the tissues do not permit the same procedure for all cell types. For example, PG cells form a line along the edges of the prothoracic lobe; we picked every 4th cell in a row. Fat body cells are spread on a one-cell thick sheet; we used a grid with designated spots in which the cells were counted in each preparation. The same grid was used in all experiments. SG cells are spread as single cell sheets over the outer surface of the SG. We picked every 4th cell on a straight line longitudinally. Then, the average values from these four measurements of fluorescence intensities were separately calculated for nuclei and cytoplasm. These averages represented values corresponding to a single animal. Since eight animals were used per treatment, the mean pixel intensity (±s.e.m.) of nucleus and cytoplasm was calculated from eight averages corresponding to eight animals. This was done for each cell type and for each treatment. For brains, because 12 or 15 animals were used as brain donors, 12 or 15 such averages (see Results) were used to calculate mean pixel intensities of cytoplasmic fluorescence in LNs per treatment. Only LNs in the right optic lobe were used for measurements. Comparison between treatments of a particular cell type were made using two non-parametric tests, the Mann–Whitney *U*-test (Mann and Whitney, 1947) and the Kruskal-Wallis 3- or 4-point test (Kruskal and Wallis, 1952). Background fluorescence levels were minimal and were obtained from adjacent regions in the same preparations that lacked evident fluorescence. These values were subtracted from all measurements before statistical analysis.

RESULTS

CYCLING OF PER IN CELLS OF ENTRAINED ANIMALS

Three cell types, PG, SG, and FB cells were examined for PER cycling using immunohistochemistry on day 12 after a blood meal of LD animals. All three cell types exhibited a clear daily rhythm in both abundance and cellular location of PER fluorescence (**Figures 1A–D** for PG cells; **E–H** for FB cells, and **I–L** for SG cells). Nuclear PER fluorescence in PG cells was intense during early scotophase at 1 h after lights-off (AZT 1) and declined in middle scotophase at 7 h after lights-off (AZT 7), indicating depletion of the fluorescent material during the scotophase (**Table 1**). This decrease in intensity from AZT 1 to AZT 7 was highly statistically significant ($P < 0.01$). Fluorescence intensity continued to decline during the photophase showing that PER in PG nuclei undergoes cycling during the course of a day with a peak in early scotophase. The same pattern of changes during the course of a day in nuclear fluorescence intensity was also observed in FB (**Table 2**; $P < 0.01$ when AZT1 was compared with AZT 7) and SG cells (**Table 3**; $P < 0.05$ when AZT1 was compared with AZT7). Therefore, all three cell types exhibit daily cycling of nuclear PER with peaks in early scotophase, that is synchronous in all cell types. Nuclear PER fluorescence peaked in all three cell types around the time of dusk.

Cytoplasmic PER fluorescence also showed daily cycling in all three cell types, but the phase of the daily rhythm differed between cell types. In the cytoplasm of PGs (**Table 1**), PER fluorescence intensity also cycled. It increased to a peak at AZT 7, which was significantly higher than the immediately preceding point AZT 1 and the following point AZT 19 ($P < 0.01$ for both comparisons). These analyses reveal that when cytoplasmic PER increased to a peak, nuclear PER declined to a trough and when nuclear PER increased to a peak, cytoplasmic PER decreased to a trough. All this suggests daily cycling of PER between the two cellular compartments in PG cells. Cytoplasmic PER fluorescence also cycled in FB cells (**Table 2**) and SG cells (**Table 3**) with troughs at AZT 7, which were highly significant when compared to the preceding point AZT 1 ($P < 0.01$ for both cell types) and marginally significant when compared to the following point AZT 19 ($P = 0.05$ for either cell type). Therefore, cytoplasmic PER exhibited a daily rhythm in fluorescence intensity in FB and SG cells that was in synchrony with their rhythms in nuclear PER fluorescence intensity. Thus, regulation of movement of PER in FB and SG cells seem to differ from that in PG cells (see Discussion).

DEPLETION OF PER AND ABOLITION OF ITS CYCLING IN CHRONIC LL *in vivo*

The experimental treatments described in the following sections involving transfer to darkness and the effects of neuropeptides all employ animals that had been maintained for 3 or more weeks in LL (see Methods). Therefore, it was first necessary to document the behavior of PER in the cells of these LL animals. Chronic exposure to light depleted PER by approximately 30–50%

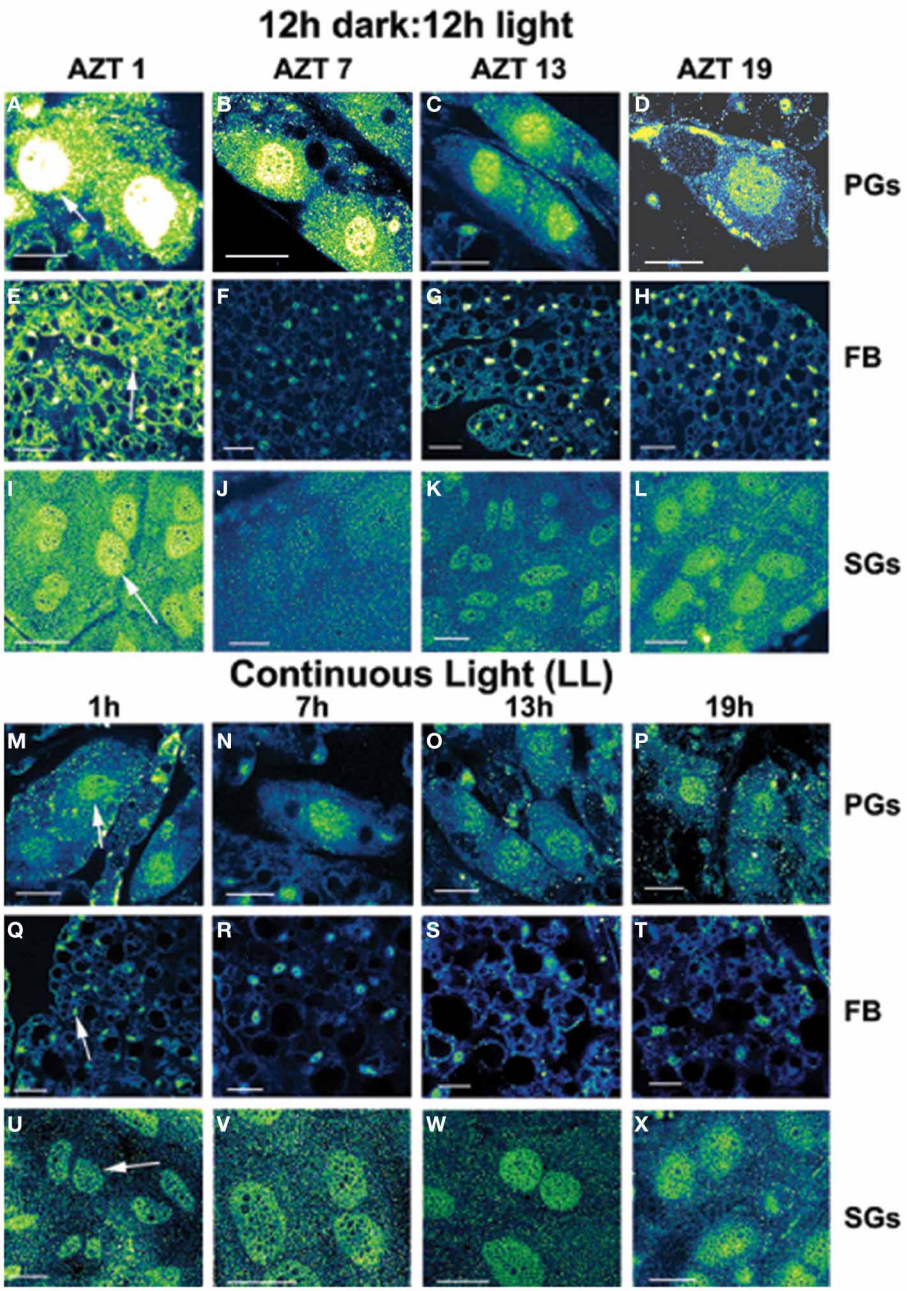

FIGURE 1 | Daily cycling *in vivo* in abundance and nuclear localization of PER immunofluorescence (A–L). Green/yellow/white shows fluorescence. Tissue samples were dissected at four times during day 12 after a blood meal: 1 h after lights-off (AZT 1), 7 h after lights-off (AZT 7), 1 h after lights-on (AZT 13) and 7 h after lights-on (AZT 19). **(A–D)** show PG cells, **(E–H)** show FB cells and **(I–L)** show SG cells. Note the cycling of nuclear PER with peaks at AZT 1 (early scotophase). Abolition of daily cycling of PER after exposure to chronic LL *in vivo* **(M–X)**. Tissue samples were dissected at 6 h intervals in a 24 h period at 1, 7 13, and 19 h. **(M–P)** show PG cells, **(Q–T)** show FB cells and **(U–X)** show SG cells. Arrows in **(A,E,I,M,Q,U)** show fluorescent nuclei. Scale bars = 10 μm

(depending on the cell type) throughout a day and abolished the daily peak of PER fluorescence in both nuclei and cytoplasm of all three cell types (**Figures 1M–P** for PGs; **Q–T** for FB; **U–X** for SGs). However, LL treatment did not completely eliminate PER from any cell type (see Discussion). Kruskal-Wallace analysis of the LL data (**Table 1** for PGs; **Table 2** for FB; **Table 3** for SGs) revealed no significant variation in fluorescence intensities over the course of a day in either the nucleus or the cytoplasm in any of the three cell types. Therefore, chronic exposure to LL abolished PER rhythmicity in all three tissues. Controls for the experiments below consisted of incubation of the above cell types in light for 4 h *in vitro* (**Figures 2A** for PGs, **G**, for fat body, **M**, for SGs).

Table 1 | PG cells (mean relative PER fluorescence ± SEM every 6 h throughout a day).

PG CELLS IN 12D:12L		
AZT	**Nucleus**	**Cytoplasm**
1	3508 ± 205	2263 ± 255
7	2531 ± 140	2953 ± 207
13	2621 ± 167	1914 ± 177
19	2198 ± 212	1946 ± 152
PG CELLS IN LL		
Hours in a 24 h cycle	**Nucleus**	**Cytoplasm**
1	1832 ± 151	1866 ± 175
7	1825 ± 118	1854 ± 92
13	1811 ± 190	1798 ± 120
19	1819 ± 96	1877 ± 111

N = 8 animals.

Table 2 | FB cells (mean relative PER fluorescence ± SEM every 6 h throughout a day).

FB CELLS IN 12D:12L		
AZT	**Nucleus**	**Cytoplasm**
1	2960 ± 42	2889 ± 160
7	2197 ± 112	1729 ± 98
13	2613 ± 218	2320 ± 112
19	2736 ± 220	2238 ± 130
FB CELLS IN LL		
Hours in a 24 h cycle	**Nucleus**	**Cytoplasm**
1	2307 ± 198	1500 ± 101
7	2176 ± 103	1526 ± 122
13	2238 ± 162	1490 ± 115
17	2336 ± 164	1503 ± 141

N = 8 animals.

Table 3 | SG cells (mean relative PER fluorescence ± SEM every 6 h throughout a day).

SG CELLS IN 12D:12L		
AZT	**Nucleus**	**Cytoplasm**
1	2708 ± 169	2137 ± 137
7	1301 ± 128	1099 ± 83
13	1927 ± 136	1524 ± 116
19	2373 ± 144	1567 ± 189
SG CELLS IN LL		
Hours in a 24 h cycle	**Nucleus**	**Cytoplasm**
1	1918 ± 127	1629 ± 86
7	1944 ± 188	1719 ± 93
13	1990 ± 184	1612 ± 101
19	1873 ± 125	1697 ± 94

N = 8 animals.

PER INDUCTION IN PG CELLS *in vitro*

Transfer to darkness

It is known that steroid synthesis by the PG cells becomes arrhythmic in prolonged LL and that rhythmicity is re-initiated within 4 h of transfer to darkness *in vitro* (Vafopoulou and Steel, 1998). However, it has not been shown that this effect is mediated by the action of the light cue on the clock in the PG cells. Here, we found the level of PER fluorescence in PGs transferred from LL to darkness increased rapidly in both cytoplasm and nucleus ($P < 0.01$ for both compartments) (**Figure 2B**) to levels comparable to the daily peak values of entrained animals (**Table 4**). Since the levels of PER in LL are low (**Figure 2A**; **Table 1**), we infer that transfer to darkness re-initiates production of PER and its rapid entry into to the nucleus.

Action of neuropeptides

All four neuropeptide treatments (brain extract, bombyxin, PTTH, and bombyxin plus PTTH) induced significant increases in the level of both nuclear and cytoplasmic PER fluorescence (**Figures 2C–F**; **Table 4**) ($P < 0.01$ for both nucleus and cytoplasm when compared to control LL nucleus and cytoplasm). This increase was about 2–2.5-fold dependent on the treatment. That both bombyxin and PTTH independently induced PER indicates that the signaling pathways of these two neuropeptides are involved in PER expression (see Discussion). It appears that darkness was marginally more effective than either neuropeptide ($P = 0.05$ when nuclear and cytoplasmic PER fluorescence intensities after transfer to dark were compared to the corresponding nuclear and cytoplasmic fluorescence intensities after treatment with these neuropeptides). Importantly, exposure of PG cells to both neuropeptides simultaneously (**Figure 2F**, **Table 4**), or to brain extract (**Figure 2C**; **Table 4**), induced an increase in PER fluorescence to a significantly higher level in both nuclear and cytoplasmic fluorescence intensities when these values were compared to the corresponding values of intensities in nucleus and cytoplasm of PG cells treated by either neuropeptide alone ($P < 0.01$ for all comparisons) and even exceeded the level induced by transfer to darkness ($P < 0.05$ for both comparisons). The finding that nuclear and cytoplasmic levels of PER fluorescence both increase very rapidly following all of these treatments implies swift translocation to the nucleus of newly induced PER.

PER INDUCTION IN FB AND SG CELLS *in vitro*

Transfer to darkness

Neither FB nor SG cells responded to transfer to darkness with any change in the level of PER fluorescence in either nuclei or cytoplasm (**Figures 2H,N** respectively; **Table 4**) ($P > 0.2$ in all comparisons). Thus, PER levels in both cell types are insensitive to lights-off.

Action of neuropeptides

Treatment with bombyxin or PTTH had no effect on the level of either nuclear or cytoplasmic PER in either FB (**Figures 2J,K** respectively) or SG (**Figures 2P,Q** respectively) cells ($P > 0.2$ for all comparisons) (**Table 4**). However, simultaneous treatment

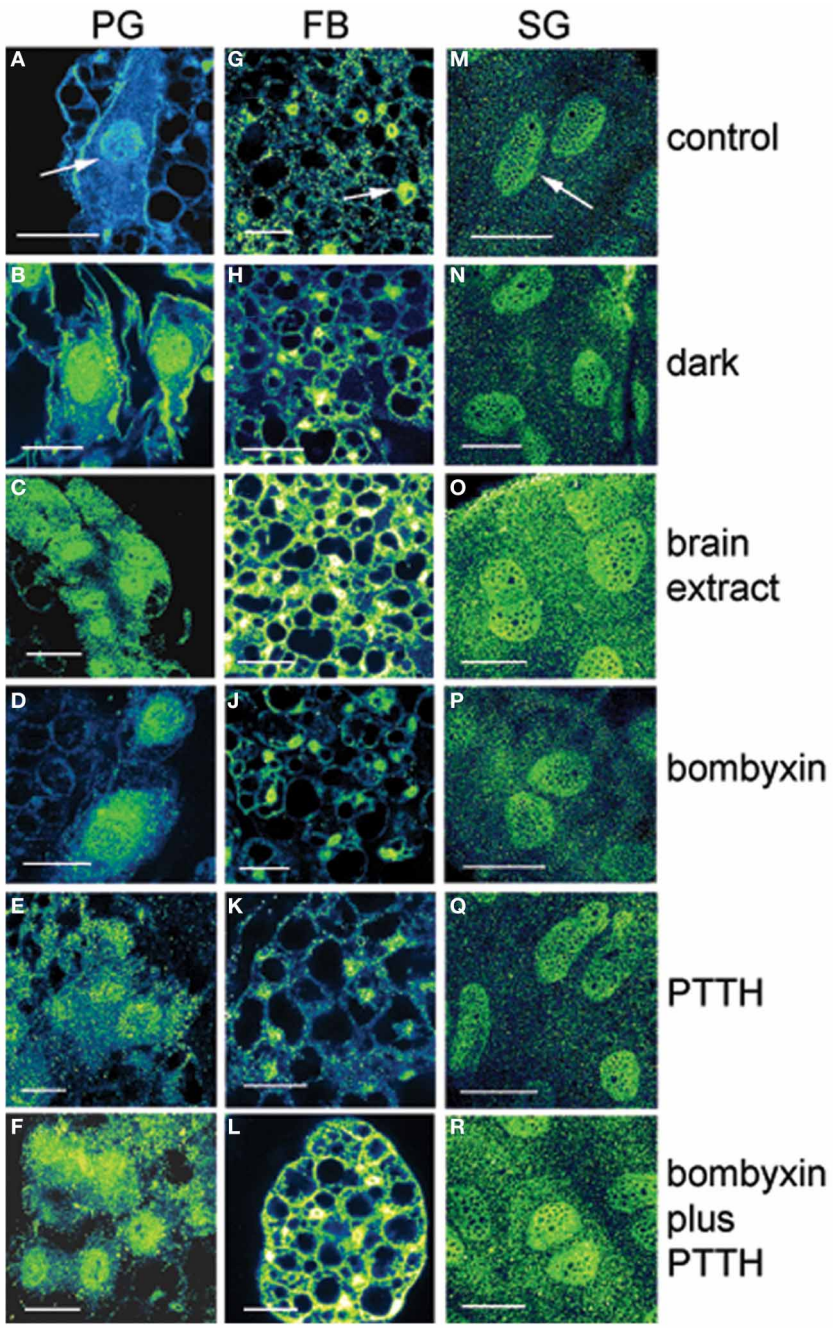

FIGURE 2 | Effects of various treatments *in vitro* on PER immunofluorescence in PG cells (A–F), FB cells (G–L), and SG cells (M–R) from arrhythmic LL animals at day 12 after feeding. Fluorescence in shown as green/yellow. Arrows in **(A,G,M)** show nuclei. Controls were incubated in light. In PG cells note the substantial increase in nuclear immunofluorescence intensity following all treatments compared to controls. Note that transfer to dark and treatments with bombyxin or PTTH had no effect in FB and SG cells. Note that treatment with brain extract, which contains both ILP and PTTH, and combination treatment with both bombyxin (an ILP) and PTTH increased substantially both nuclear and cytoplasmic fluorescence intensity in all three cell types when compared with other treatments. Scale bars = 10 μm.

with both bombyxin and PTTH induced significant increases in PER fluorescence in both nuclei ($P < 0.01$ for either cell type) and cytoplasm ($P < 0.05$ for either cell type) when values were compared to corresponding LL values for either cell type (**Figure 2L** for FB cells and **Figure 2R** for SG cells) Brain extract was also as effective as this combination of neuropeptides. These findings indicate that simultaneous activation of both the insulin and PTTH signaling pathways is required to cause upregulation of PER expression in FB and SG cells (see Discussion).

Table 4 | Mean relative PER fluorescence ± SEM in PG, FB and SG cells following various *in vitro* treatments.

Treatment	Nucleus	Cytoplasm
PG CELLS		
Control	1424 ± 48	1283 ± 79
Darkness	3247 ± 197	2146 ± 144
Brain extract	3012 + 168	2388 ± 158
Bombyxin	2718 ± 109	2234 ± 68
PTTH	2265 ± 79	2481 ± 105
Bombyxin plus PTTH	3494 ± 58	2866 ± 134
FB CELLS		
Control	2238 ± 264	1442 ± 149
Darkness	2150 ± 223	1529 ± 124
Brain extract	3084 ± 187	1952 ± 100
Bombyxin	2453 ± 272	1626 ± 134
PTTH	2469 ± 283	1481 ± 264
Bombyxin plus PTTH	3192 ± 94	1854 ± 59
SG CELLS		
Control	1806 ± 78	1557 ± 60
Darkness	1780 ± 130	1535 ± 79
Brain extract	2185 ± 54	1934 ± 83
Bombyxin	1893 ± 53	1465 ± 60
PTTH	1883 ± 153	1558 ± 90
Bombyxin plus PTTH	2378 ± 73	1905 ± 55

N = 8 animals.

PER AND PDF INDUCTION IN BRAIN CLOCK CELLS

Vafopoulou et al. (2010) showed that PER fluorescence in *Rhodnius* brain clock cells showed circadian cycling in 12L:12D; the main group of clock cells (lateral clock neurons; LNs) were also filled with PDF, which enabled tracing of their axons. **Figure 3A** shows the location of LNs stained with anti-PER (**Figure 3A**) and anti-PDF (**Figure 3B**) on the border of the optic lobe and protocerebrum of a control scotophase animal entrained in 12L:12D at day 12 after a blood meal. We have shown previously (and discussed in depth) that PER fluorescence in LNs is exclusively cytoplasmic (Vafopoulou et al., 2010; Vafopoulou and Steel, 2012a,b). Here, we first examined the effect of transfer of 12 such animals to LL for 3 weeks on levels of PER and PDF in LNs *in vivo*. All brains were completely devoid of PER fluorescence and 10 of the 12 were also devoid of PDF fluorescence. Two of the 12 brains showed trace levels of PDF in their somata and none in their axons. **Figure 3C** shows an LL brain stained with anti-PDF in which PDF fluorescence in LNs is reduced to background levels. PER fluorescence in LNs is similarly reduced to background levels (not shown). Thus, chronic LL completely abolishes PER in brain clock cells and PDF is abolished or drastically reduced.

Brains were excised from these LL animals, incubated *in vitro* and challenged with transfer to darkness or with neuropeptides.

Transfer to darkness

A group of 15 LL brains was transferred to darkness and PER fluorescence was examined after 4 h incubation *in vitro*.

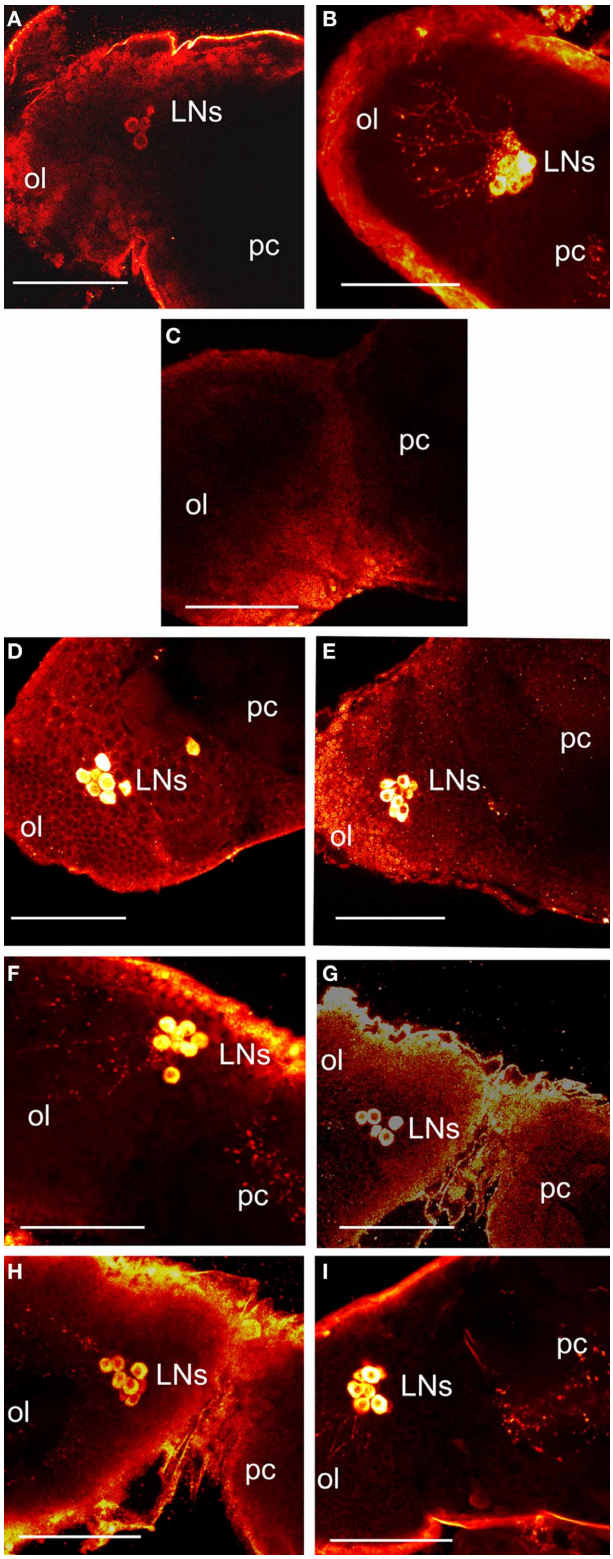

FIGURE 3 | Images of control and treated brains 12 days after a blood meal. All images are a dorsal view of the right brain hemisphere. **(A–C)** show *in vivo* brains. Control scotophase brains from entrained animals in 12L:12D stained with anti-PER **(A)** or anti-PDF **(B)**. Fluorescence is shown

(Continued)

FIGURE 3 | Continued
as yellow/white. Images show the lateral clock neurons (LNs) at the junction of protocerebrum (pc) and optic lobe (ol) and their axonal projections. **(C)** a brain from animals transferred to LL for 3 weeks and stained with anti-PDF; note absence of PDF fluorescence in LNs. LL brains similarly stained with anti-PER also showed no staining in the LNs (not shown). **(D–I)** show LL brains incubated *in vitro*. **(D–E)** show brains stained with anti-PER. **(D)** LL brain transferred to darkness. **(E)** brain challenged with bombyxin. Note induction of PER (compare with **C**). **(F–I)** show brains stained with anti-PDF. **(F)** brain transferred to darkness. **(G)** brain challenged with bombyxin. **(H)** brain challenged with anti-PTTH. **(I)** brain challenged with bombyxin plus PTTH. Scale bars = 10 μm.

In all 15 brains, PER fluorescence re-appeared in the somata of LNs (**Figure 3D**). In addition, PDF fluorescence was also restored and had migrated down the axons in all brains (**Figure 3F**). Therefore, transfer to darkness induced both PER and PDF fluorescence within 4 h.

Action of neuropeptides

Groups of 15 LL brains were incubated *in vitro* with either bombyxin or PTTH or both neuropeptides together and then stained for PER (**Figure 3E**) or PDF (**Figures 3G–I**). All neuropeptides induced strong PER fluorescence in LNs (**Figure 3E** shows incubation with bombyxin; images from incubations with PTTH or bombyxin plus PTTH are not shown). All neuropeptides also induced strong PDF fluorescence (**Figure 3G**, incubation with bombyxin; **Figure 3H**, incubation with PTTH; **Figure 3I**, incubation with bombyxin plus PTTH). The pattern and intensity of induced fluorescence were closely similar to that normally seen *in vivo* in scotophase brains from animals entrained in 12L:12D, and similar to that induced by transfer to darkness (**Table 5**). Of particular interest is the finding that incubation with both bombyxin and PTTH together induced significantly greater fluorescence in LNs than did incubation with either neuropeptide alone ($P < 0.01$ for both comparisons). Therefore, both signaling pathways are involved in the expression of both PER and PDF (see Discussion).

Table 5 | Mean relative PER fluorescence ± SEM in LNs following various *in vitro* treatments.

Treatment	Cytoplasm
LNs	
Control	646 ± 12
Darkness	1802 ± 51
Brain extract	1933 ± 28
Bombyxin	1907 ± 38
PTTH	1690 ± 59
Bombyxin plus PTTH	2011 ± 44

N = 15 animals.

DISCUSSION

INDUCTION OF PER BY NEUROHORMONES AND PHOTIC CUES IN VARIOUS CELL TYPES

The present paper demonstrates that the neurohormones bombyxin (an ILP), and PTTH play an important role in the regulation of the clock protein PER in various tissue types of *Rhodnius*. Insulin and members of the family of insulin/insulin-like growth factors target a wide variety of tissues and exhibit a plethora of effects such as mediation of nutrition on cell growth, development, longevity, senescence and metabolic homeostasis of insects (reviews by Wu and Brown, 2006; Hunt et al., 2007; Nässel and Winther, 2010; Shingleton, 2010; Teleman, 2010, and articles in this Research Topic). On the other hand, the only documented target of PTTH is the PGs and knowledge of its signaling pathway derives exclusively from studies of its ecdysteroidogenic action on PGs. These two seemingly unrelated signal transduction systems are both involved in the regulation of PER. Moreover, this regulation appears to be achieved by convergence of the signaling pathways for these hormones as discussed below. The details of this convergence vary with the cell type under study. Consequently, we introduce the information of PER regulation first in the context of the differing characteristics of PER expression in the different cell types, before presenting a more generalized interpretation.

All tissues studied here (brain, PG, FB, SG) are known to play roles in circadian timekeeping and to express PER cyclically. But these roles differ greatly between tissues, and thus so does the significance of the action of neurohormones on PER expression in them.

The molecular mechanism of circadian timekeeping was first elucidated in specific neurons of the brain of *Drosophila*. Briefly, "clock genes" (such as *per*) are cyclically transcribed as a result of indirect stimulation and inhibition by feedback from their own protein products (e.g., PER) within a complex molecular oscillator (MO) (review by Hardin, 2011). PER and *per* play critical, central roles and are regarded as canonical components of the MO. Thus, circadian cycling of PER is viewed as a necessary feature of the MO. All four tissue types examined here exhibited robust daily cycling of PER in both abundance and cellular localization, with peak nuclear localization in the scotophase of all types.

The brain clock system of *Rhodnius* (Vafopoulou et al., 2010; Vafopoulou and Steel, 2012a) is very similar in organization to that of *Drosophila* (reviews by Helfrich-Förster, 2003, 2005; Nitabach and Taghert, 2008). One group of clock cells in *Drosophila* (LNs) is photosensitive (Klarsfeld et al., 2011) and is traditionally regarded as the pacemaker (Vossall and Young, 1995). *Rhodnius* LNs exhibit circadian cycling of PER (Vafopoulou et al., 2010) and also express the neuropeptide PDF, which fills their axonal projections (Vafopoulou et al., 2007, 2010; Vafopoulou and Steel, 2012a). PDF has been considered an output protein of the brain clock (review by Taghert and Shafer, 2006; Helfrich-Förster, 2009). PDF-filled axons project to and make intimate associations with axonal projections from both the ILP (Vafopoulou and Steel, 2012b) and PTTH (Vafopoulou et al., 2007) neurons, providing a neural pathway for driving the known rhythmic release of these neuropeptides into the circulation (Vafopoulou and Steel, 1996a, 2002). Here, we found that chronic LL completely abolished fluorescence due to both PER and PDF in the LNs. Transfer of brains to darkness *in vitro* restored both PER and PDF within 4 h. We infer that *Rhodnius* LNs are photosensitive and that a light cue induces both PER

and PDF. This conclusion implies that *pdf* transcription resumes when the MO is restarted by a cue from either light or a neurohormone, i.e., *pdf* is a clock-controlled gene. The effects of the neurohormones bombyxin and PTTH on the brain clock *in vitro* were particularly remarkable. Both hormones promptly induced both PER and PDF fluorescence to levels typical of normal scotophase animals *in vivo*, i.e., both hormones acted on the brain similarly to darkness. Since the brain clock itself controls the rhythmic release of both these hormones, these findings indicate a "temporal feedback" whereby the released hormones act back on the clock in the LNs to affect PER expression. We are unaware of any prior evidence that brain neurohormones act back on the brain clock in insects. Clearly, the brain clock is responsive not only to external light signals but also to internal hormonal signals as well (see Discussion below).

The PG cells of *Rhodnius* are well documented peripheral clocks (Vafopoulou and Steel, 1992, 1998) that rhythmically synthesize ecdysteroids. Rhythmic synthesis persists *in vitro* (Vafopoulou and Steel, 1992) and can be reinitiated by transfer of arrhythmic LL PGs *in vitro* to darkness (Vafopoulou and Steel, 1998). PG cells were severely depleted of PER and its daily rhythmicity was abolished by chronic exposure to LL. Transfer of these PGs to darkness *in vitro* resulted in prompt and massive increases in both nuclear and cytoplasmic PER in PG cells. Continued examination of these tissues for several days *in vitro* showed that induced PER exhibited a free-running rhythm in abundance and nuclear location (unpublished). Thus, the present data show that re-initiation of the ecdysteroid synthesis rhythm is mediated by the action of the light cue on a PER-based MO in the PG cells. Bombyxin and PTTH are known to act as stimulators of ecdysteroid synthesis in PGs of *Rhodnius*, with bombyxin being less effective than PTTH (Vafopoulou and Steel, 1997). The action of PTTH is believed to be control of the phase of the PG clock (Pelc and Steel, 1997). Here, we found that both neurohormones (administered separately *in vitro*) induced substantial increases in both nuclear and cytoplasmic PER fluorescence in PG cells, but not to the levels induced by darkness or to those seen *in vivo*. However, when both peptides were administered together, the resulting levels of PER fluorescence exceeded both those induced by darkness and those seen *in vivo* (see Discussion below).

The status of SG and FB cells as circadian clocks is much less clear than the above. Here, we found that both cell types exhibit daily cycling of PER *in vivo*, but cycling does not persist *in vitro* (unpublished), implying that these cell types are oscillators that are driven by cues received *in vivo* and are not self-sustained clocks. This view is supported by the finding that cytoplasmic and nuclear PER fluorescence in FB and SG cells cycle in synchrony with each other, in contrast to the asynchronous behavior seen in brain LN and PG cells. Such synchrony is not consistent with daily migration of PER between cytoplasm and nucleus. Nevertheless, PER levels in these cell types were drastically depleted in chronic LL, as in the other tissue types. But, unlike the other tissue types, transfer to darkness *in vitro* did not restore PER fluorescence in either cytoplasm or nucleus. We conclude that light cues have no effect on PER in these tissues. Further, neither treatment with bombyxin nor PTTH *in vitro* induced PER fluorescence. However, treatment with both peptides simultaneously did increase PER fluorescence in both nuclei and cytoplasm in both SG and FB cells. Thus, induction of PER expression in these cells requires simultaneous exposure to both neuropeptides to induce PER expression (see Discussion below).

POTENTIAL MECHANISM OF INDUCTION OF PER BY NEUROHORMONES

Both bombyxin and PTTH rapidly induce PER when applied *in vitro* to either brains or PGs. A direct effect of bombyxin and PTTH on the clock cells is probable, most obviously in the case of their action on PGs, in which all cells are clock cells (Vafopoulou and Steel, 1998). In the brain, the possibility of hormone action on interneurons cannot be excluded. Nevertheless, in both tissues the actions must be mediated via cellular signaling pathways, whether within the clock cells themselves or within neurons that control them.

Signal transduction of insulin is activated by binding of insulin to its cognate receptor which leads to a cascade of phosphorylations of a series of intracellular signal transducer enzymes such as the phosphoionositide kinase 3 (PI3 K), which in turn activates a cytosolic serine-threonine kinase (Akt), also called protein kinase B (PKB). This pathway interacts with the TOR pathway (target of rapamycin) through activation by phosphorylation of TOR which regulates functions such as cell growth, differentiation and survival (e.g., Nojima et al., 2003; Tokunaga et al., 2004). Subsequently, Akt inhibits by phosphorylation the nuclear translocation of the transcription factor Forkhead fox O (FOXO); FOXO regulates metabolism, growth, energy homeostasis, stress responses, life span and senescence (for references see Puig and Mattila, 2011). It is reasonable to assume that the action of bombyxin on *Rhodnius* PGs is mediated by a typical insulin signaling pathway as has been shown in other insect systems (e.g., Nijhout et al., 2007; Nagata et al., 2008).

There is currently no evidence that the signaling pathways for insulin or PTTH influence PER expression. However, there is evidence that the insulin signaling pathway affects another canonical clock protein, TIMELESS (TIM) (Zheng and Sehgal, 2010). In the *Drosophila* brain, increased TOR signaling, a pathway that interacts with the insulin pathway, resulted in TIM accumulation in the nuclei of small lateral ventral clock neurons (LN_vs), a subgroup of LNs (Zheng and Sehgal, 2010). In addition, genetic manipulation of the expression of Akt and TOR in the LN_vs, which control the activity rhythm of *Drosophila,* revealed that both enzymes are involved in the regulation of period length of the activity rhythm (Zheng and Sehgal, 2010). This relationship could be reciprocal resulting in a functional cross-talk between components of the clock and insulin signaling as found in a human cell line by Zhang et al. (2009). In this study, use of a large scale screening of individual small interfering RNAs, a large number of knockdown genes and protein interaction network analysis identified a strong relationship between the insulin signaling pathway and the circadian clock. In this system, downregulation of multiple components of the insulin signaling pathway changed the period length of the clock. Conversely, transcription

of multiple components of the insulin signaling pathway was regulated by the clock. Therefore, the insulin transduction system and the molecular clock interact in a variety of animal systems.

With respect to PTTH, its only documented action is on PGs. The classical PTTH signaling pathway is unrelated to the insulin signaling pathway. It involves the binding of PTTH to its cognate receptor, Torso, a receptor tyrosine kinase (Rewitz et al., 2009b) which initiates an extensive signaling transduction network (Rewitz et al., 2009b; Marchal et al., 2010) that is mediated by an increase in intracellular in Ca^{++} and an increase Ca^{++}/calmodulin-dependent cAMP (Smith et al., 1984, 1985; Gu et al., 1998, 2010), which results in a cascade of protein phosphorylations and leads to translation of several enzymes involved in steroidogenesis (review by Marchal et al., 2010). In addition, a recent quantitative phosphoproteomics approach in *Manduca sexta* revealed involvement of PTTH in the phosphoryation of many proteins unrelated to those involved in the classical PTTH signaling pathway (Rewitz et al., 2009a). These proteins regulate several different signaling pathways and include, among others, rate limiting enzymes involved in transcription and translation. These effects are seen not only in PGs but in a variety of other tissues suggesting that PTTH participates in several signaling pathways and in multiple cell types (Rybczynski et al., 2009). The present finding that PTTH induces PER is the first evidence of involvement of PTTH in regulation of a specific cellular activity that does not occur solely in PGs and is not directly related to ecdysteroidogenesis.

SYNERGISM BETWEEN ILP AND PTTH IN INDUCTION OF PER

The present findings broadly illustrate that bombyxin and PTTH are both able to induce PER in a variety of different cell types. But in some cell types they act independently (brain, PGs), in some they act additively (PGs) and in others are both required simultaneously (SGs and FB). It is therefore possible that the signaling pathways for these hormones may possess varying degrees of convergence in different cell types. There is strong evidence of convergence of the two pathways in PGs, which could explain the synergistic action of the two hormones on PER. Recent studies on PTTH-mediated ecdysteroidogenesis in *Bombyx* PGs revealed the existence of a second PTTH signaling pathway that involves the PI3K/Akt/TOR sequence (Gu et al., 2010, 2011, 2012). Bovine insulin also stimulated PGs by the PI3K/Akt/TOR signaling sequence (Gu et al., 2009, 2012). Therefore, both insulin and PTTH signaling pathways converge at the level of PI3K/Akt/TOR sequence. In addition, the ribosomal S6 kinase (S6K), a downstream component of the TOR pathway, regulates glycogen synthase kinase 3β (GSK3β) along with Akt. GSK3β is a key enzyme in the regulation of clock proteins in both mammals (e.g., Iitaka et al., 2005; Yin et al., 2006; Sahar et al., 2009; Spengler et al., 2009) and *Drosophila* (Martinek et al., 2001). Interestingly, S6K and its ribosomal protein substrate S6 are both downstream targets of the classical PTTH signaling pathway that controls ecdysteroidogenesis in *Manduca* (Song and Gilbert, 1997). Rapamycin, a specific inhibitor of TOR, also inhibited the PTTH-mediated phosphorylation of S6K and ecdysteroidogenesis (Song and Gilbert, 1994, 1995; Gu et al., 2012). Therefore, the insulin and PTTH signaling pathways could also converge at the level of S6K. Therefore, both pathways converge at multiple points. We infer that there is extensive cross talk between insulin and PTTH signaling involved in the regulation of PER in PGs.

By contrast, when SG or FB cells were exposed to both bombyxin and PTTH there was a significant increase in PER in both nuclei and cytoplasm, even though these cells did not respond at all to treatments with either peptide alone. This finding shows that PER expression in cells that are peripheral oscillators is only elicited by simultaneous stimulation by ILP and PTTH and suggests that both signaling pathways, when activated, can act cooperatively to stimulate PER production in peripheral oscillators. This response would enable these peripheral oscillators to orchestrate their internal activities with respect to circadian time and also to coordinate these activities with other cells that are also responsive to both hormones.

IMPLICATIONS FOR TEMPORAL ORDER

The present findings show that rhythmicity in brain neurohormones can no longer be explained solely in terms of their release being driven by a brain clock system that is entrained by light cues from the external world. We have shown that the release of neurohormones acts back on the brain to influence the behavior of PER in the MO of the clock cells that drive these rhythms. The precise significance of this "temporal feedback" remains unknown, but could serve as a mechanism that helps to stabilize the daily timing of release of various rhythmic neurohormones, which would ensure synchrony in the rhythmic responses of target tissues. Even the further downstream hormone rhythms such as the ecdysteroid circadian rhythm have been found to participate in this temporal feedback, since daily shuttling of the EcR was previously reported (Vafopoulou and Steel, 2006) in both the brain neuroendocrine cells and in the clock cells themselves in the brain. Consequently, the brain clock cells are receiving rhythmic signals from at least three circulating hormones (ILP, PTTH, and ecdysteroids) and therefore appear to be integrating external light cycle information with internal information from hormone rhythms. The complexity and intricacy of these interactions between hormone rhythms and the clock system attests to the importance of precisely regulated endocrine rhythms in animal physiology. We have discussed previously (Steel and Vafopoulou, 2006) that tightly organized rhythmicity in the endocrine system is essential to the appropriate orchestration of daily events within cells and also to the synchrony of the various tissues and organs within the animal both with each other and with the external world.

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Factors that regulate insulin producing cells and their output in *Drosophila*

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Insulin-like peptides (ILPs) and growth factors (IGFs) not only regulate development, growth, reproduction, metabolism, stress resistance, and lifespan, but also certain behaviors and cognitive functions. ILPs, IGFs, their tyrosine kinase receptors and downstream signaling components have been largely conserved over animal evolution. Eight ILPs have been identified in *Drosophila* (DILP1-8) and they display cell and stage-specific expression patterns. Only one insulin receptor, dInR, is known in *Drosophila* and most other invertebrates. Nevertheless, the different DILPs are independently regulated transcriptionally and appear to have distinct functions, although some functional redundancy has been revealed. This review summarizes what is known about regulation of production and release of DILPs in *Drosophila* with focus on insulin signaling in the daily life of the fly. Under what conditions are DILP-producing cells (IPCs) activated and which factors have been identified in control of IPC activity in larvae and adult flies? The brain IPCs that produce DILP2, 3 and 5 are indirectly targeted by DILP6 and a leptin-like factor from the fat body, as well as directly by a few neurotransmitters and neuropeptides. Serotonin, octopamine, GABA, short neuropeptide F (sNPF), corazonin and tachykinin-related peptide have been identified in *Drosophila* as regulators of IPCs. The GABAergic cells that inhibit IPCs and DILP release are in turn targeted by a leptin-like peptide (unpaired 2) from the fat body, and the IPC-stimulating corazonin/sNPF neurons may be targeted by gut-derived peptides. We also discuss physiological conditions under which IPC activity may be regulated, including nutritional states, stress and diapause induction.

Keywords: insulin receptor, neuropeptide, metabolism, neuromodulation, insulin signaling, insulin release

INTRODUCTION

Insulin and IGF signaling (IIS) play pivotal roles during development and growth, but also in daily life of the mature organism where they regulate metabolism, reproduction, stress responses and other processes influencing aging and lifespan (Brogiolo et al., 2001; Giannakou and Partridge, 2007; Grönke et al., 2010; Teleman, 2010; Antonova et al., 2012; Itskov and Ribeiro, 2013). The molecular components of the IIS pathway are well-conserved over evolution, although the complexity is somewhat larger in mammals than in invertebrates, mainly due to increased numbers of genes encoding receptor types and downstream signaling elements (Brogiolo et al., 2001; Garofalo, 2002; Teleman, 2010). This review focuses on *Drosophila* and the reader is referred to some relevant reviews for other insects or invertebrates (Claeys et al., 2002; Kaletsky and Murphy, 2010; Antonova et al., 2012; Lapierre and Hansen, 2012; Badisco et al., 2013; Mizoguchi and Okamoto, 2013).

In *Drosophila* there are eight insulin-like peptides (ILPs), designated DILP1—8, but only one known receptor, dInR (Fernandez et al., 1995; Brogiolo et al., 2001; Grönke et al., 2010; Colombani et al., 2012; Garelli et al., 2012). The different DILPs are produced in various cell types and tissues in specific spatio-temporal patterns during development and in the adult fly and thus seem to play different functional roles. In the present account we will mainly deal with DILP function in the adult fly and only briefly discuss larval and developmental roles of these peptide hormones. Thus, we focus primarily on DILP2, 3, 5, 6, and 7 that have established roles in adult physiology, and highlight what is known about the regulation of production and release of these peptides. Of these, especially DILP2, 3 and 5 from the insulin producing cells (IPCs) of the brain have been the subject of many investigations. It should be made clear that whereas the functional roles of DILPs and IIS in *Drosophila* have been under intense study, the control of DILP production and release by extrinsic factors has only recently received some attention. There are on the other hand a large number of studies showing that genetic manipulations of IPCs affect transcription of the three brain-derived DILPs. We summarize here what is known about secreted factors, such as neurotransmitters, neuromodulators and hormones, that regulate activity in IPCs and thereby production and/or release of DILPs and co-expressed hormones. Also other factors and physiological conditions that affect IPC activity and DILP gene transcription will be discussed. As will be seen in this review there is a major gap in our knowledge on the integrated role of neuronal systems that regulate IPCs and physiological conditions requiring activation or inactivation of insulin signaling. In other words there is a need to identify conditions and signals that activate the different neuronal pathways that control IPCs.

ANATOMY AND ORGANIZATION OF INSULIN PRODUCING CELLS

In larvae and adults of *Drosophila* DILP2, 3 and 5 are produced in a set of 14 median neurosecretory cells, IPCs, in the brain (Brogiolo et al., 2001; Cao and Brown, 2001; Rulifson et al., 2002; Geminard et al., 2009), *Dilp1* transcript was detected in larval IPCs (Rulifson et al., 2002), DILP7 in about 20 neurons of the abdominal neuromeres of the fused thoracic-abdominal ganglia (Brogiolo et al., 2001; Miguel-Aliaga et al., 2008; Yang et al., 2008) and DILP6 mainly in adipose cells of the fat body, but also in larval salivary glands and heart (Okamoto et al., 2009; Slaidina et al., 2009) (**Table 1**). These production sites are shown in **Figure 1**. Furthermore, DILP3 is produced in muscle cells of the adult midgut and DILP5 in follicle cells of the ovary as well as principal cells of the renal tubules (Brogiolo et al., 2001; Veenstra et al., 2008; Söderberg et al., 2011). DILP8 has been detected in the imaginal discs (bags of progenitor cells of adult tissues) of larvae (Colombani et al., 2012; Garelli et al., 2012). An early study employed *in situ* hybridization to reveal the following additional expression sites in the third instar larva: *Dilp2* in imaginal discs and salivary glands, and *Dilp4* in the embryonic anterior midgut and mesoderm (Brogiolo et al., 2001). Finally, DILP6, and maybe DILP2, are expressed in glial cells in the CNS of early larval stages where they play roles in neuroblast reactivation (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Consulting the FlyAtlas gene expression database [flyatlas.gla.ac.uk; (Chintapalli et al., 2007)] there are no records for *Dilp1* and *4* expression in any larval or adult tissue, whereas the distribution of the other *Dilps* is largely confirmed. According to FlyAtlas *Dilp8* transcript is enriched in adult ovaries. The DILP1–8 expression patterns are summarized in **Table 1**.

The 14 brain IPCs (**Figures 1**, **2A**) are embedded in a cluster of median neurosecretory cells (MNCs) in the pars intercerebralis (PI). The morphology of the brain IPCs has been described from immunolabeling with several DILP antisera and from use of *Dilp2*-Gal4-driven GFP and it seems that each of the 14 brain IPCs coexpresses DILP2, 3 and 5 (Brogiolo et al., 2001; Cao and Brown, 2001; Ikeya et al., 2002; Rulifson et al., 2002; Broughton et al., 2005; Geminard et al., 2009). In larvae the IPCs additionally produce DILP1 (Rulifson et al., 2002). Recently it was shown that many of the brain IPCs also coexpress drosulfakinin (DSK), a cholecystokinin-like peptide (Söderberg et al., 2012). Available data suggest that these IPCs all share the same morphology. They have cell bodies located in the PI, two sets of branches in the PI,

Table 1 | Location of insulin-producing cells in *Drosophila*.

DILPs	Location		Axon terminations	References
	Larvae	Adult		
DILP 1	IPCs	–	–	Rulifson et al., 2002
DILP 2	IPCs Imaginal discs Salivary glands Glial cells of CNS	IPCs	Brain neuropil Corpora cardiaca Anterior aorta Proventriculus Crop	Rulifson et al., 2002; Brogiolo et al., 2001; Cao and Brown, 2001
DILP 3	IPCs	IPCs Muscle cells of midgut	Corpora cardiaca Anterior aorta Proventriculus Crop	Rulifson et al., 2002; Brogiolo et al., 2001; Veenstra et al., 2008
DILP 4	Anterior midgut	–	–	Brogiolo et al., 2001
DILP 5	IPCs Principal cells in renal Tubules	IPCs Follicle cells of ovary Principal cells in renal Tubules	Corpora cardiaca Anterior aorta Proventriculus Crop	Rulifson et al., 2002; Brogiolo et al., 2001; Söderberg et al., 2011
DILP 6	Adipose cells Salivary glands Heart Glial cells of CNS	Adipose cells	–	Okamoto et al., 2009; Slaidina et al., 2009
DILP 7	Abdominal neuromeres	Abdominal neuromeres	Brain neuropil Hindgut Reproductive tract	Miguel-Aliaga et al., 2008; Yang et al., 2008
DILP 8	Imaginal discs	Ovary	–	Garelli et al., 2012; Colombani et al., 2012; FlyAtlas gene expression data base shows ovary expression

IPCs, insulin producing cells.

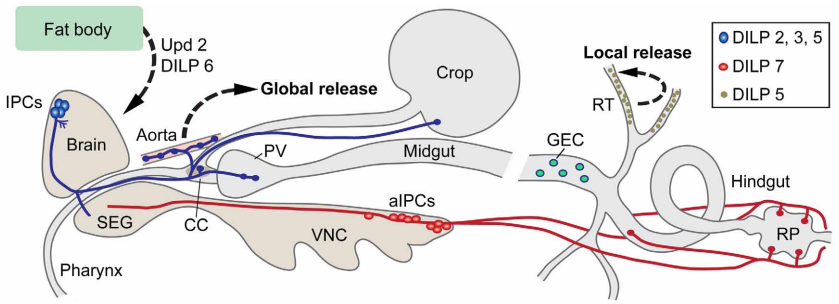

FIGURE 1 | Insulin-producing cells (IPCs) in the *Drosophila* nervous system and gut. A set of 14 IPCs (blue) in pars intercerebralis of the brain send axons to the tritocerebrum (adjacent to the subesophageal ganglion, SEG), to the corpora cardiaca (CC) with associated aorta, proventriculus (PV), and the crop. Likely release sites for circulating *Drosophila* insulin-like peptides (DILPs) are in CC, aorta, PV, and crop. These IPCs produce DILP2, 3, and 5. The branches in protocerebrum (near cell bodies) and tritocerebrum of the brain could be dendritic and/or represent further release sites. A second set of 20 cells (aIPCs; red) is found in the abdominal neuromeres of the ventral nerve cord (VNC). These produce DILP7 and supply axon terminations to the hindgut including the rectal papillae (RP), and in females to reproductive organs (not shown here). At least two of the aIPCs send axons to the SEG. It is not clear whether their branches in the SEG are dendrites or release sites (or both). Additionally the principal cells (green) of the renal tubules (RT) produce DILP5. This DILP may act locally in the tubules. DILP6 is produced in fat body cells in the head and abdomen. The fat body also releases a leptin-like peptide, unpaired 2 (Upd2). Both DILP6 and Upd2 regulate IPC activity. In the midgut there are peptidergic endocrine cells (GEC) that may release peptides into the circulation to target cells in renal tubules and brain. This figure is updated and modified from Nässel (2012) which was partly based on Cognigni et al. (2011).

another set in tritocerebrum and axons extending to varicose terminations in the corpora cardiaca, anterior aorta, proventriculus and crop (**Figures 1**, **2**). However, no attempts have been made so far to analyze individual IPCs, which leaves the possibility that some of the 14 neurons have more restricted morphologies. The dendrites, or at least major input sites, of the IPCs have not been identified conclusively in any insect. Thus, it is not known whether the branches in the PI and tritocerebrum (**Figure 2**) are dendritic or maybe a mix of input sites and peptide release sites. In published accounts antisera to DILPs immunolabel both the IPC branches in the PI and in the tritocerebrum, which suggests that DILPs are stored and maybe released within the brain. In other words it is possible that DILPs are released both into the circulation from neurohemal releases sites and in a paracrine fashion within the brain. A recent study indeed suggests that at least DILP2 is released within the brain neuropil and acts on other neurons of the larval brain (Bader et al., 2013).

There are also a few reports on *Dilp2*-Gal4 expressing neurons in the larval and adult abdominal and thoracic ganglia (Kaplan et al., 2008; Agrawal et al., 2010). However, the expression of DILP2 peptide or *Dilp2* transcript in these cells has not been confirmed, so it is possible that this extra *Dilp2*-Gal4 expression lacks fidelity.

DILP7 is produced in at least two types of neurons in the ventral nerve cord (**Figure 1**). There are several sets of DILP7 neurons (dMP2) in abdominal neuromeres A6–9, some of which are efferent with axons that terminate on the hindgut. One pair of DILP7 expressing interneurons (DP) in A1 arborize in the abdominal neuropil and send axons to the brain (Miguel-Aliaga et al., 2008). In the third instar larva the axons of the DP neurons terminate close to the protocerebral branches of the IPCs (Miguel-Aliaga et al., 2008; Nässel et al., 2008) and in the adult brain these axons impinge on the ventral portion of the tritocerebral processes of the IPCs (Cognigni et al., 2011). The DPs, but none of the other DILP7-expressing neurons, coexpress short neuropeptide F (sNPF), and weak *Cha*-Gal4 expression, that indicates presence of choline acetyltransferase and thus suggests a cholinergic phenotype (Nässel et al., 2008). It is therefore possible that the larval DPs modulate the IPCs by means of DILP7 and sNPF (and possibly acetylcholine). The DILP7 producing neurons are likely to release peptide in a paracrine fashion within the CNS and at the hindgut structures (Miguel-Aliaga et al., 2008; Cognigni et al., 2011). A role of DILP7 has also been detected in reproductive behavior in egg laying, correlated with DILP7 containing axons innervating the female reproductive tract (Yang et al., 2008).

It can be noted that the IPCs are a subpopulation of the MNCs and that other MNCs with similar morphology produce distinct neurohormones such as the peptides myosuppressin, and diuretic hormones 31 and 44 (Park et al., 2008). In addition there are bilateral clusters of peptide-producing lateral neurosecretory cells (LNCs; **Figure 3**) that also send axons to the corpora cardiaca/allata, aorta and anterior gut structures (often referred to as the retrocerebral complex). The LNCs in some cases have collateral processes that superimpose the proto- and tritocerebral processes of the median neurosecretory cells (**Figures 2B,C**, **3**) or converge with them in the retrocerebral complex (see Homberg et al., 1991; Shiga et al., 2000; Siegmund and Korge, 2001; Hamanaka et al., 2007; Kapan et al., 2012). Several of the LNCs produce peptides that are released as circulating hormones, others may produce peptides that act more locally as regulators of release from MNCs (detailed in the next section). Among the peptide hormones produced by LNCs in *Drosophila* are prothoracicotropic hormone (PTTH; in larvae only), corazonin, and ion transport peptide (ITP) (McBrayer et al., 2007; Dircksen et al., 2008; Lee et al., 2008a; Kapan et al., 2012). Thus, the MNCs and LNCs constitute groups of neurons that may play roles reminiscent of hypothalamic neurons of vertebrates (Scharrer, 1972, 1987; Hartenstein, 2006).

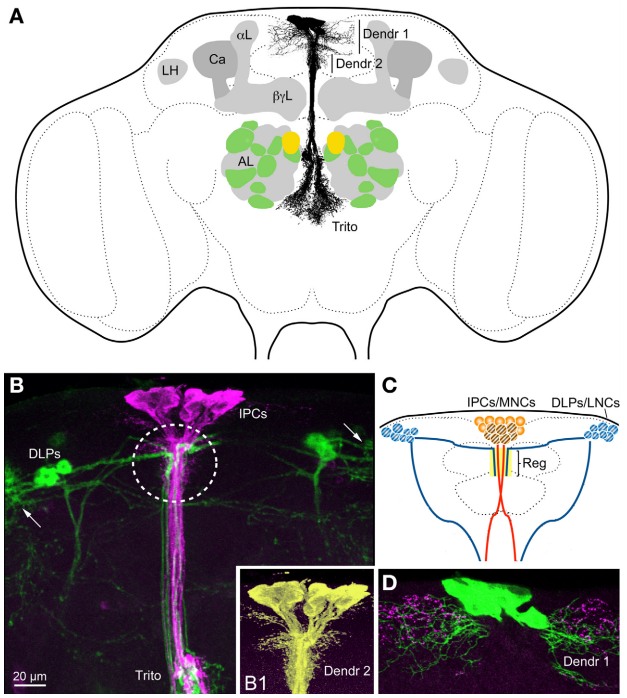

FIGURE 2 | Insulin-producing cells (IPCs) and other neurons in the *Drosophila* brain. (A) The IPCs are seen with their cell bodies dorsally, two sets of presumed dendrites (Dendr 1 and 2) in the pars intercerebralis and processes branching in the (tritocerebrum Trito). It is not known whether these branches are dendrites or axon terminations, or both. The axons that exit to the corpora cardiaca and aorta are not displayed (they exit above the tritocerebrum, in a direction toward the reader). The antennal lobes (AL) are depicted with the anterior 10 (green and yellow) of the about 14 glomeruli that contain olfactory sensory neurons (OSNs) expressing short neuropeptide F (sNPF). The yellow glomeruli are DM1 that receive OSNs expressing odorant receptor Or42b and sNPF, known to be essential for food search. These sNPF-expressing OSNs also express the insulin receptor (dInR) and the sNPF receptor. DILPs are known to modulate odor sensitivity of these OSNs (Root et al., 2011). The mushroom bodies with calyx (Ca), α-, β- and γ-lobes (αL, βγL) and the lateral horn (LH) are also depicted. The mushroom bodies also seem to be targeted by DILPs, at least in larvae (Zhao and Campos, 2012). **(B)** The IPCs (magenta, anti-DILP2) and corazonin-expressing DLP neurons (GFP, green) converge medially in the pars intercerebralis (encircled) and in the tritocerebrum (Trito). The DLPs are known to regulate IPC activity (Kapan et al., 2012). Arrows indicate the likely dendrites of the DLPs. **(B1)** Detail of IPCs (enhanced color) visualizing the short dendrites (Dendr 2) that seem to receive inputs from DLPs. **(C)** Schematic depiction of IPCs, DLPs and their point of convergence in the pars intercerebralis (Reg). The IPCs are located in the MNC cluster and the DLPs among the LNCs. For further details see **Figure 3**. **(D)** The IPCs (green) may receive inputs from serotonin-producing neuron branches (magenta) both at the long dendrites (Dendr 1) and the short (not shown here). Panel **(B)** is altered from Kapan et al. (2012) and 2D from Luo et al. (2012).

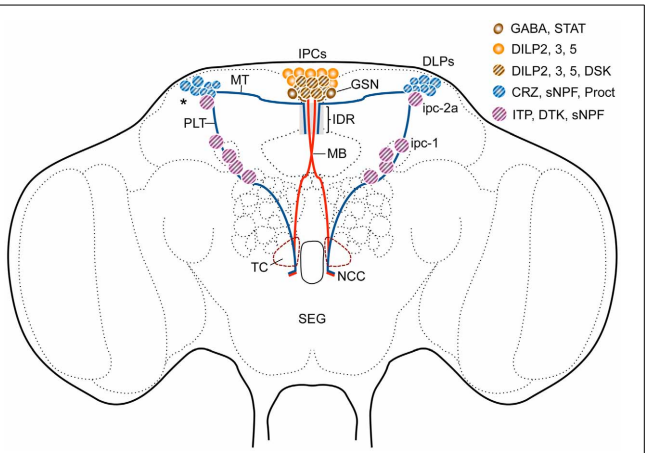

FIGURE 3 | Sets of peptidergic neurons in the *Drosophila* brain that are associated with the IPCs. Some of the IPCs co-express drosulfakinins (DSK1 and 2), peptides that induce satiety (Söderberg et al., 2012). The IPCs are regulated by DLP neurons that produce short neuropeptide F (sNPF), corazonin (CRZ) and proctolin (Proct) (Isaac et al., 2004; Kapan et al., 2012) as well as GABAergic neurons (GSN) that in turn receive leptin-like (Upd2) signals from the fat body (Rajan and Perrimon, 2012). The GSNs express a Upd2-activated Jak/Stat receptor (Dome). The interactions between DLPs and GSNs and IPCs probably occur dorso-medially (IDR) in the pars intercerebralis and maybe in the tritocerebrum (TC). Another set of lateral neurosecretory cells (LNCs), designated ipc-1 and ipc-2a, express the peptides sNPF, *Drosophila* tachykinin (DTK) and ion transport peptide (ITP) (Kahsai et al., 2010). These neurons, like the DLPs, are parts of the LNC clusters and have axon terminations in sites overlapping those of the IPCs and the DLPs in the corpora cardiaca and anterior aorta. Further abbreviations: STAT, Jak/stat receptor Dome; MT, medially projecting axon tract; PLT, posterior lateral axon tract; MB, median bundle; NCC, nerves to corpora cardiaca. Asterisk indicates region where dendrites of DLPs arborize.

BRIEF SUMMARY OF FUNCTIONAL ROLES OF IPCs AND DILPs

There is an extensive literature on the functional roles of DILPs and more specifically DILPs released from the brain IPCs (reviewed by e. g., Géminard et al., 2006; Giannakou and Partridge, 2007; Grönke et al., 2010; Teleman, 2010; Antonova et al., 2012; Itskov and Ribeiro, 2013). Hence, only a brief summary is provided here to put the subsequent discussion into context. Developmental aspects of DILP signaling (including growth) are not considered here.

Genetic ablation of IPCs, or other manipulations that diminish DILP signaling from these cells affect carbohydrate and lipid metabolism. Thus, fasting glucose levels in the hemolymph increase after IPC ablation, as seen also in diabetic mammals (Rulifson et al., 2002; Broughton et al., 2005). Assays of stored carbohydrates in whole body extracts revealed an increase in both trehalose and glycogen (Broughton et al., 2005). Also triacylglycerol stores increase in flies with decreased IPC activity (Broughton et al., 2005; Slack et al., 2010). Interestingly depletion of DILP2, but not DILP3 and 5 in IPCs, affects stored trehalose, but not the other circulating or stored compounds, suggesting compensation by the other two DILPs (Broughton et al., 2008). Indeed, DILP2 knockdown leads to increased levels of DILP3 and 5.

One of the early findings on insulin signaling in *Drosophila* was that diminished insulin-receptor activity increases lifespan (Clancy et al., 2001; Tatar et al., 2001). It is sufficient to ablate the IPCs to extend both median and maximal lifespan of flies (Broughton et al., 2005). Mated females extended their median lifespans by 33.5% and males by 10.5%. The mortality started later in life of aging IPC-deleted flies, but thereafter at the same rate as in control flies (Broughton et al., 2005). There seems to be a

link between dietary restriction, extended lifespan and functional IPCs (Broughton et al., 2010). In control flies a diluted protein (yeast) content in the food extends lifespan by 12–20%. However, IPC ablation renders flies less responsive to dietary restriction in terms of longevity (Broughton et al., 2010).

Responsiveness to stress is likely to, at least partly, be related to lifespan. Oxidative stress certainly appears to be a factor that affects longevity (Broughton et al., 2005; Kenyon, 2005). Diminished signaling from IPCs increases resistance to oxidative stress (Broughton et al., 2005) and it is known that Jun-N-terminal Kinase (JNK) signaling in IPCs is required for adaptive responses to stress (Karpac et al., 2009). A panel of single and combinatory *Dilp* mutants was tested for stress resistance. It was found that only *Dilp2,3,5* and *Dilp1-4* mutant flies display resistance to paraquat-induced oxidative stress, suggesting a requirement of DILP2 and 3 for the stress response (Grönke et al., 2010). Resistance to starvation (and dry starvation) is also dependent on DILP signaling and the IPCs, and flies display increased resistance after inactivated signaling (Broughton et al., 2005); starved *Dilp1-4* mutants display increased survival by 18% (Grönke et al., 2010). On the other hand, the resistance to temperature stress did not increase after diminished insulin signaling (or IPC activity): recovery from cold and heat knockdown was tested (Broughton et al., 2005).

Fecundity is dependent on intact DILP signaling from the IPCs and probably the fat body (Broughton et al., 2005; Grönke et al., 2010). These authors found that ablation of IPCs or genetic diminishment of DILPs 2, 3, 5, and 6 reduce life-time fecundity. Generally, diminished systemic insulin signaling increases life span on the cost of fecundity.

Foraging and feeding are in several ways dependent on DILP signaling. At the sensory level DILPs play a role in regulating strength of odor signals via action on dInR expressed in olfactory sensory neurons (OSNs) of the antennae. It was shown that the receptor of the peptide sNPF (sNPFR) is down-regulated in OSNs in fed flies where circulating insulin levels are increased (Root et al., 2011). The diminished sNPFR expression presynaptically in OSNs decreases synaptic transmission to second order olfactory neurons and hence sensitivity to food odors. This renders the flies less attracted to food sources and food search diminishes. Antennal lobe structures (glomeruli with sNPF/sNPFR expressing OSNs) are shown in **Figure 2A**. The next level of DILP action is in brain circuits where *Drosophila* neuropeptide F (NPF) and its receptor (NPFR) play important roles in feeding (Wu et al., 2003, 2005a,b). These authors showed that NPF signaling is critical for choice of food in relation to hunger. Also the NPFR is negatively regulated by DILP signaling and interference with the dInR in NPFR-expressing neurons produced behavioral effects on feeding (Wu et al., 2005a,b). Down-regulation of DILP signaling to NPFR neurons leads to a phenotype where fed larvae feed on non-palatable food that is normally rejected, and upregulated DILP signaling induced food aversion in starved larvae. Therefore, it seems that DILPs released at feeding act as a satiety signal via the NPF system (Wu et al., 2005a,b). It has also been shown that silencing or ablating IPCs, and thus decreasing DILP signaling, leads to increased feeding, especially under poor nutritional conditions (Cognigni et al., 2011; Söderberg et al., 2012). Furthermore, sNPF is known to regulate feeding; in part this may be by activating the IPCs and insulin signaling (Lee et al., 2004, 2008b; Hong et al., 2012). This will be further discussed in the next section.

A recent study implicated a role of insulin signaling in feeding by means of action in mushroom body circuits in the brain of *Drosophila* larvae (Zhao and Campos, 2012). Mushroom body structures are shown in **Figure 2A**. These authors found that knockdown of insulin signaling to intrinsic mushroom body neurons (Kenyon cells) by expression of dominant negative forms of dInR and PI3K (phosphoinositide 3 kinase) reduced food intake in larvae and diminished growth. This diminished signaling did not delay larval development, but affected cell proliferation. However, the mushroom body development, as monitored by size and shape of their lobes, was not affected by the manipulations of PI3k and the downstream FOXO (Zhao and Campos, 2012). If the development of the mushroom body neurons was not affected, then it might be that synaptic activity in this brain center was altered. Since the intrinsic Kenyon cells employ sNPF as a functional neuromodulator (Johard et al., 2008; Knapek et al., 2013) it might be that the sNPF signaling is diminished by insulin action, similar to the antennal OSNs (Root et al., 2011).

There are several indications that insulin signaling plays a role in induction and maintenance of diapause in insects, including species of *Drosophila* (Tatar and Yin, 2001; Hahn and Denlinger, 2011; Antonova et al., 2012; Sim and Denlinger, 2013). When kept at about 11°C and short day conditions *Drosophila melanogaster* females display adult reproductive diapause. This diapause is an overwintering strategy for many insects, characterized by arrested development and reallocation of metabolism and physiology from reproduction to somatic maintenance (Tatar and Yin, 2001; MacRae, 2010; Hahn and Denlinger, 2011). Disruption of various components of the insulin-signaling pathway in *D. melanogaster* shuts down reproduction and increases energy stores, inducing a physiologic state similar to the natural adult diapause in other *Drosophila* species (Tatar and Yin, 2001; Salminen et al., 2012). It has also been shown that naturally occurring polymorphisms in genes encoding several insulin signaling components affect diapause induction (Williams et al., 2006; Fabian et al., 2012). The mechanisms of DILP and juvenile hormone signaling in *D. melanogaster* reproductive diapause are yet to be elucidated.

Finally, the IPCs may play a role in sleep regulation (Crocker et al., 2010). These authors found that activation of the IPCs by expressing a constitutively active depolarizing sodium channel reduced night-time sleep and conversely a hyperpolarizing potassium channel decreased sleep. They also showed that an octopamine receptor (OAMB) expressed by the IPCs mediates this effect on sleep, such that octopamine has a wake-promoting effect (Crocker et al., 2010). A null mutation in the OAMB receptor results in increased sleep, and a specific rescue with wild type OAMB only in the IPCs restored normal sleep levels. This study, however, did not test whether DILPs play a role in the regulation of sleep, but a later paper actually shows that DILPs do not mediate the octopamine-effects on sleep/wake (Hong et al., 2012). It can be noted that the IPCs, and maybe even insulin signaling, seem to be of importance for general locomotor activity in flies (Belgacem and Martin, 2002, 2006; Jones et al., 2009).

The above examples of various roles of IPCs and actions of DILPs beg the question: how are the IPCs and DILP release regulated? Can we identify circulating signals that act on the IPCs, and are there neuronal pathways directly devoted to regulation of these cells?

WHAT CONTROLS RELEASE OF IPCs FROM CNS NEURONS?

A key trigger of DILP release from IPCs in feeding stages of *Drosophila* appears to be intake of nutrition. The post-feeding increase in circulating glucose and amino acid levels is sensed by the fat body and as a consequence signals are released into the circulation and reach the brain and the IPCs. This was first studied in larvae where nutrient sensing occurs in the fat body and a fat body-secreted factor activates DILP release (Geminard et al., 2009). The fat body nutritional sensor is the amino acid transporter slimfast, which activates the TOR (target of rapamycin) pathway (Colombani et al., 2003). In the adult fly a similar mechanism has been proposed, and the fat body-derived factor identified as a leptin-like molecule, unpaired 2 (Upd2), that indirectly activates the IPCs by lifting a tonic inhibition from specific GABAergic neurons (Rajan and Perrimon, 2012). This will be dealt with in more detail below. The IPCs do not seem to display clear cell-autonomous nutrient sensitivity, in contrast to the larval secretory cells producing the glucagon-like adipokinetic hormone (AKH) (Kim and Rulifson, 2004). Some evidence, however, exists that the adult IPCs have autonomous glucose sensing by means of glucose uptake, glycolysis and subsequent ATP inactivation of ATP-sensitive potassium (K^{ATP}) channels similar to mammalian pancreatic beta cells (Kreneisz et al., 2010). However, no coupling to regulation of DILP release was made in that study. Another membrane-associated channel has been implicated in adult IPCs in regulation of DILP signaling. This is the calcium-activated potassium channel Slowpoke (SLO) that is known to be regulated by a binding partner, the SLO-binding protein (SLOB) (Sheldon et al., 2011). Both SLO and SLOB are expressed by the IPCs, and when their expression is diminished in these cells, *Dilp3* transcript level decreases, energy is stored, and DILP signaling is changed. It was proposed that SLO and SLOB modulate the action potential duration in the IPCs and thus have a possible role in release of DILPs (Sheldon et al., 2011).

A novel nutrient sensor was revealed recently: the fructose sensitive gustatory receptor Gr43a (Miyamoto et al., 2012). In addition to its expression in sensory cells of the proboscis, Gr43a was found in four pairs of neurons in the region of LNCs of the brain. The Gr43a expressing brain neurons can sense circulating fructose and were shown to regulate food intake in a satiety-dependent manner (Miyamoto et al., 2012). Fructose levels increase drastically and transiently in the circulation after a carbohydrate meal, although glucose and trehalose are the predominant hemolymph carbohydrates (Miyamoto et al., 2012). These authors propose that dietary sucrose can be converted to fructose and glucose in the fly and also that fructose is a normal component in *Drosophila's* fruit diet. Possibly the Gr43a-expressing neurons interact with neurons in the LNC cluster that in turn affect feeding circuits or maybe insulin signaling via IPCs.

Recently a receptor resembling the mammalian adiponectin receptor was identified in *Drosophila* IPCs (Kwak et al., 2013). It was proposed that this receptor regulates IPC activity, DILP2 release and that it may respond to another adipokine signal from the fat body. These authors could, however, not identify a adiponectin-like ligand in *Drosophila*.

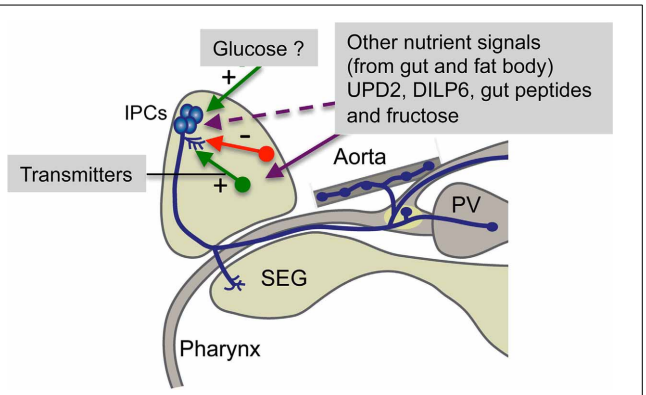

FIGURE 4 | Summary of factors regulating brain IPCs. The brain IPCs are regulated by neurotransmitters (including neuropeptides) from neurons in the brain (red and green arrows) in a stimulatory (+) or inhibitory (–) fashion. These neurons are in turn likely to be activated by nutritional signals from the circulation, fat body or intestine. Some of these signals may act directly on the IPCs. UPD2 inactivates GABAergic neurons that then relieves tonic inhibition of IPCs (Rajan and Perrimon, 2012) and circulating fructose acts on brain neurons expressing the fructose receptor Gr43a (Miyamoto et al., 2012). Gut peptides include allatostatin A, DH31 and tachykinins (DTKs), known to have receptors on neurons regulating IPCs or directly on IPCs (Johnson et al., 2005; Veenstra, 2009; Birse et al., 2011). Circulating glucose has been proposed to be sensed by IPCs via uptake, entering glycolysis and the resulting ATP blocking ATP-sensitive K^+ channels on IPCs leading to membrane depolarization and subsequent opening of voltage sensitive Ca^{2+} channels (Kreneisz et al., 2010). PV, proventriculus; SEG, subesophageal ganglion. Not shown here is the expression of an adiponectin-like receptor in IPCs (Kwak et al., 2013). This receptor may be targeted by a hitherto unidentified adipokine signal from the fat body.

In mammals release of insulin is modulated by several hormones and neurotransmitters, as well as feed-back from circulating insulin (Drucker et al., 1987; Aspinwall et al., 1999; Adeghate et al., 2001; Dong et al., 2006; Sonoda et al., 2008). Similarly, the *Drosophila* IPCs appear to be regulated by neurons producing neuropeptides, monoamines and GABA and receive DILP feedback. Roles of sNPF, corazonin *Drosophila* tachykinin, GABA, serotonin and octopamine in IPC regulation are discussed in the following. A summarizing diagram of pathways that regulate IPCs is shown in **Figure 4**.

A role of sNPF has been proposed in regulation of feeding, growth and insulin production (Lee et al., 2004, 2008b; Hong et al., 2012). Those studies did not clarify which sNPF expressing neurons are responsible for the regulation of IPCs. In the larva there are two candidate systems of sNPF neurons: (1) the two DP neurons of the first abdominal neuromere that express DILP7 and sNPF (Miguel-Aliaga et al., 2008; Nässel et al., 2008), and (2) a set of lateral brain neurons (DLPs; among the LNCs; see **Figures 2B,C**, **3**) that send axonal processes to the presumed dendrite region of the IPCs and to the corpora cardiaca region of the ring gland where the IPC axons terminate (Nässel et al., 2008). In the adult fly the sNPF expressing DLPs coexpress the neuropeptide corazonin and their axons project to the IPC branches in pars intercerebralis, tritocerebrum and

corpora cardiaca (Kapan et al., 2012). It was shown by targeted RNA interference that both sNPF and corazonin from the DLPs stimulate the IPCs and thereby affect carbohydrate and lipid levels (Kapan et al., 2012). After sNPF knockdown in DLPs the transcript levels of *Dilp2* and *5* decrease (*Dilp3* not affected), whereas corazonin-RNAi in the same cells does not affect *Dilp* transcription. Thus, the two peptides co-released from DLPs act in different ways on the IPCs, and corazonin additionally appears to be released into the hemolymph to act on the fat body (Kapan et al., 2012). The DLPs have been shown to express receptors for allatostatin A and diuretic hormones 31 and 44 (Johnson et al., 2005; Veenstra, 2009). The former two peptides are produced by endocrine cells of the midgut (Veenstra et al., 2008) and are possibly released into the circulation during feeding to target the DLPs. If this is the case, then the midgut endocrines may act as nutrient sensors or monitor gut distension during feeding (see Miyamoto et al., 2013). It can also be noted here that the fructose sensing Gr43a-expressing neurons in the area of LNCs might be in a position to act directly on the DLPs (Miyamoto et al., 2012).

The next neurotransmitter to be proposed in the regulation of IPCs was serotonin (5-hydroxy tryptamine; 5-HT). Serotonergic neurons were found to express *Drosophila* NS3, a nucleostemin family GTPase (Kaplan et al., 2008). NS3 manipulations affect growth in *Drosophila* via insulin signaling (Kaplan et al., 2008). When an *ns3* mutant was rescued by expressing *ns3* in serotonergic neurons the growth defects were reversed. These authors suggested that NS3 acts in serotonergic neurons in regulation of insulin signaling and thereby control of organismal growth (Kaplan et al., 2008). The direct connection between serotonergic neurons and IPCs was, however, not revealed in the study. We therefore screened for serotonin receptors on IPCs and found that out of the four known receptors in *Drosophila* only 5-HT_{1A} is expressed in these cells (Luo et al., 2012).

Targeted knock-down of the 5-HT_{1A} receptor in IPCs produced phenotypes that suggest an effect on insulin signaling (Luo et al., 2012). Diminished receptor levels decreased survival of flies at starvation and altered lipid levels, but the responses to temperature stress were the opposite of what was expected for a presumed action of an inhibitory 5-HT_{1A} receptor. The 5-HT_{1A} knockdown flies displayed decreased survival at 39°C and required longer to recover from cold coma induced by exposure to 0°C (Luo et al., 2012). This suggested that the receptor normally has a stimulatory effect on insulin signaling, since an earlier study had shown that decreased insulin release resulting from IPC ablation led to decreased tolerance to heat and cold treatment (Broughton et al., 2005). We speculate that the 5-HT_{1A} receptor is indeed inhibitory and inhibits adenylate cyclase (AC) and protein kinase A (PKA) and subsequently inactivates cAMP response element binding protein (CREB) (see Nichols and Nichols, 2008). Activated CREB is known to inhibit insulin signaling (Walkiewicz and Stern, 2009), and therefore inhibition of AC, PKA, and CREB would stimulate insulin signaling. The adverse effect of 5-HT_{1A} knockdown on survival at starvation and on lipid levels could be caused by an increase in locomotor activity as a response to starvation (Lee and Park, 2004), where more energy is consumed and thus overrides the insulin-mediated effect on starvation resistance.

The inhibitory neurotransmitter GABA acts via ionotropic or metabotropic receptors, but only the metabotropic $GABA_B$ receptor (GBR) was detected on the *Drosophila* IPCs (Enell et al., 2010). Targeted knockdown of the GBR in IPCs resulted in phenotypes indicating that its role is to inhibit production and/or release of DILPs. Flies with diminished GBR in IPCs displayed shorter lifespan than controls, decreased starvation resistance and altered carbohydrate and lipid metabolism (Enell et al., 2010). The ionotropic $GABA_A$ receptor subunit RDL was not found on the IPCs, although it is otherwise very widespread. This study indicated that GABA inhibits activity in the IPCs via its metabotropic receptor, but it was not shown what triggers GABA signaling to the IPCs. The regulation of GABAergic neurons acting on the IPCs was, however, clarified in a later study. It was found that the leptin-like Upd2 (Unpaired 2; related to type 1 cytokines) is released from the fat body after feeding and acts on its receptor Dome that is expressed on a few GABAergic neurons adjacent to the IPCs (Rajan and Perrimon, 2012) (**Figure 3**). Upd2 activates JAK/STAT signaling in the Dome-expressing GABAergic neurons and thereby lifts the tonic inhibition of the IPCs and allows DILP release. Thus, a nutrient-triggered signal from the fat body acts indirectly via GABAergic neurons to induce systemic DILP signaling. This model requires that Upd2 can pass through the blood-brain barrier to be able to act on GABAergic neurons in the brain.

Another modulatory control of IPCs is by means of peptide products of the *Drosophila* tachykinin (DTK) precursor gene (*Dtk*). Six DTK peptides have been identified, five of which are expressed in the CNS (Siviter et al., 2000; Winther et al., 2003). We found that one of the two known DTK receptors, DTKR, is expressed in IPCs and that knockdown of this receptor affects the IPCs (Birse et al., 2011). Diminishment of DTKR expression on IPCs results in decreased starvation resistance, and a more rapid decrease of whole body trehalose, but has no effect on lipid levels. These results suggest that also DTKR inhibits insulin signaling from the IPCs. An important finding in this context was that DTKR knockdown in IPCs affects *Dilp* transcript levels in brains of fed and starved flies. It could be shown that only *Dilp2* and *Dilp3*, but not *Dilp5* transcripts were affected by receptor knockdown (Birse et al., 2011). After DTKR-RNAi the *Dilp2* and *3* transcripts both increased in fed flies, whereas after 24 h starvation the *Dilp3* transcript decreased and *Dilp2* increased. This suggests that the DTKR activation induces transcriptional effects that are differential for the three *Dilp* genes. Similar individual regulation of *Dilp* transcription in the brain IPCs has been shown in multiple other experiments: for instance after nutritional restriction, manipulations of JNK signaling or SLO/SLOB expression in IPCs, NS3 in serotonergic neurons and sNPF in DLP neurons (Broughton et al., 2008, 2010; Kaplan et al., 2008; Karpac et al., 2009; Sheldon et al., 2011; Kapan et al., 2012). We will return to the possible role of differential transcriptional control of *Dilps* in the concluding section.

A further neurotransmitter/neuromodulator that has been suggested to modulate IPC activity is octopamine (Crocker et al., 2010). It was shown that the IPCs express one of the octopamine receptors, OAMB, and knockdown of this receptor altered sleep-wake patterns in the flies. Stimulation of the IPCs

with octopamine increased cAMP in these neurons and the wake-promoting effect of octopamine appears to be dependent on PKA activation in the IPCs (Crocker et al., 2010). These authors, however, did not provide any evidence that octopaminergic activation of the IPCs affects systemic insulin signaling.

There is some evidence that various DILPs may provide feedback onto the brain IPCs. Experiments show that overexpression of *Dilp6* in the fat body of adult flies leads to extended lifespan, but also to a repression of brain levels of *Dilp2* and *5* transcript and a decrease in circulating DILP2 levels (Bai et al., 2012). These authors suggest that DILP6 release from the fat body may act locally on dInR in the fat body (in autocrine fashion), but also repress IIS in other tissues by inhibiting DILP2 release from the IPCs. This would suggest an inhibitory action of DILP6 on IPCs directly or indirectly via other neurons. The latter is possible since increased expression of DILP6 in fat body suppresses sNPF expression in the brain (Bai et al., 2012), and sNPF is known to regulate the IPCs and *Dilp* transcription (Lee et al., 2008b; Kapan et al., 2012). Another set of experiments suggests that DILPs released from the IPCs may feed back to the IPCs. Knockdown of *Dilp2* leads to an increase in *Dilp3* and *5* mRNA, and via other manipulations it was shown that at least *Dilp3* transcription is regulated via autocrine DILP action (Broughton et al., 2008). This autocrine or paracrine regulation of *Dilp3* (via the dInR and FOXO) may involve DILP2, 3 and 5 (Broughton et al., 2008; Grönke et al., 2010). As mentioned earlier there might also be a communication between IPCs and two neurons in the abdominal ganglion expressing DILP7 (Miguel-Aliaga et al., 2008; Cognigni et al., 2011), but the functional connections have not yet been demonstrated.

Finally, it has been suggested that there may be direct functional connections between IPCs and cells that produce AKH in the corpora cardiaca (Rulifson et al., 2002; Buch et al., 2008). Ablation of AKH-producing cells leads to increased *Dilp3* mRNA, but unchanged levels of *Dilp2* and *5,* and ablation of IPCs resulted in increased Akh transcript (Buch et al., 2008).

At present the only modulatory neuronal pathway that has been placed in a functional context with respect to systemic insulin signaling is the GABAergic one (Rajan and Perrimon, 2011). Here it was shown that food intake leads to the release of a fat body-derived leptin-like factor that triggers inhibition of specific GABAergic neurons that in turn lift a tonic inhibition of the IPCs. This disinhibition presumably facilitates DILP release. It is important for the near future to investigate how the other peptidergic and aminergic neuronal systems are targeted by systemic signals that reflect levels of nutrients, stress and other factors that trigger or inhibit insulin signaling. There are some additional proteins that recently have been implicated in regulation of DILP release from IPCs in *Drosophila* that also need to be linked to upstream systemic signals: an adiponectin receptor and the protein interacting with C kinase 1 (PICK 1), both of which are expressed in IPCs (Holst et al., 2013; Kwak et al., 2013).

CONTROL OF RELEASE OF DILPs FROM GLIAL CELLS AND OTHER SOURCES

Above we have discussed DILP producing neurons in the brain and how they may be regulated. What about DILP production/release in other cell types? It has been reported that during larval development DILP2 and 6 are expressed in glial cells of the ventral nerve cord (Chell and Brand, 2010). Some of these glial cells are located at the surface of the ventral nerve cord, but underneath the basement membrane (i.e., inside the blood brain barrier). The DILP producing glial cells are in contact with neuroblasts (neuronal stem cells) and it was shown that DILPs via the dInR and PI3K reactivate these dormant neuroblasts in a nutrient dependent manner (Chell and Brand, 2010; Sousa-Nunes et al., 2011). *Dilp6* transcription increases in the glia of larvae 24 h post-hatching and is dependent on amino acids in the food. Thus, for increase in DILP expression, and its paracrine release, a nutrient-dependent signal from the fat body is required to act on the glial cells (Chell and Brand, 2010; Sousa-Nunes et al., 2011). This signal obviously has to pass through the blood-brain barrier, similar to the leptin-like Upd2 described earlier (Rajan and Perrimon, 2012) and mechanisms for such a passage are not known in *Drosophila*.

Another case of nutrient-dependent activation of stem cells by paracrine DILP release was demonstrated in the *Drosophila* intestine (O'Brien et al., 2011). In the adult fly midgut stem cells proliferate and thus ensure growth of the intestine under good nutritional conditions. It was shown that DILP3 is expressed by muscle fibers in a region of the midgut rich in stem cells (Veenstra et al., 2008; O'Brien et al., 2011). The expression of *Dilp3* transcript increases in fed flies during adult gut growth, and in a temporal fashion matching the dynamics of stem cell proliferation. Experiments showed that DILP3 in the stem cell niche is necessary for cell proliferation (O'Brien et al., 2011). These authors propose that DILP3 production and release may depend on local nutrient sensing in the intestine, but that circulating DILPs may provide additional activation of gut stem cells.

Finally, it was shown that principal cells of the *Drosophila* Malpighian tubules express DILP5 both in larvae and adults (Söderberg et al., 2011). Malpighian tubules regulate water and ion homeostasis, but may also play roles in immune responses and oxidative stress (Dow, 2009). The DILP5 levels in the principal cells are dependent on desiccation stress, but also on signaling by tachykinins (DTKs) via their receptor DTKR (Söderberg et al., 2011). Thus, DTKR is expressed in principal cells and so is the dInR. Targeted knockdown of DTKR, DILP5 or the dInR in principal cells or mutation of *Dilp5* resulted in increased survival at desiccation, starvation and oxidative stress, whereas over-expression of these components produced the opposite phenotype (Söderberg et al., 2011). Therefore, various stressors seem to induce hormonal release of DTKs from the intestine that act on the renal tubules to regulate local DILP5 signaling and thus functions of Malpighian tubules related to overcoming oxidative stress.

CONCLUSIONS AND OUTLOOK

The IPCs of the brain have a central role at the interface between nutritional status of the fly and control of homeostasis, physiology, and behavior. Systemic insulin signaling therefore regulates a wide array of functions in the daily life of an organism, including responses to various types of stressors. Importantly IPCs, and other sources of DILPs, also regulate mechanisms in

growth and aging, and thereby life span. DILP signaling can be both systemic and paracrine. Systemic DILP signaling is primarily by means of the IPCs that release DILP2, 3 and 5, or the fat body releasing DILP6 (Brogiolo et al., 2001; Rulifson et al., 2002; Okamoto et al., 2009; Slaidina et al., 2009; Bai et al., 2012). Paracrine signaling has been suggested within the brain for DILP2 from IPCs (Bader et al., 2013), for DILP2 and 6 from glial cells to neuroblasts (Chell and Brand, 2010; Sousa-Nunes et al., 2011), for DILP3 from midgut muscle fibers to gut stem cells (O'Brien et al., 2011) and for DILP5 acting locally in Malpighian tubules (Söderberg et al., 2011).

The IPCs also express the cholecystokinin-like peptides DSK 1 and 2 that seem to induce satiety in flies (Söderberg et al., 2012). Most likely the DILPs and DSKs act in a synchronized fashion on different targets after onset of feeding. However, the IPCs have been implicated in some activities that might not necessarily involve release of DILPs into the circulation. For instance the sleep-wake regulation by octopaminergic inputs to the IPCs (Crocker et al., 2010) could be by means of either paracrine release of DILPs into the brain or by central release of some other colocalized substance (DSK or other neurotransmitters). Another likely paracrine action of DILPs is in regulation of NPF expressing neurons in feeding circuits (Wu et al., 2005a,b). Further indication of paracrine CNS action is the recent finding that DILP2 activates a small set of identified neurons in the brain, as monitored by PKB phosphorylation (Bader et al., 2013). These brain neurons responding to DILP2 include the hugin-expressing neurons known to regulate feeding. The hugin neurons also take up DILP2, probably via dInR internalization, and express an insulin binding protein (Imp-L2). These examples of possible paracrine DILP signaling in the brain suggest roles in modulation of behavior that involve various central neuronal circuits and clearly deserve further study. Can we expect that the dInR is expressed in neurons of dedicated brain circuits, as seems to be the case in mammals (Laron, 2009; Fernandez and Torres-Aleman, 2012)?

A somewhat puzzling feature of IPCs is that they co-express three different *Dilp* genes that are subject to individual transcriptional regulation. Different stimuli affect the transcription of these three genes in a multitude of combinations. However, most likely a depolarization of the IPCs induces release of all the peptides in whatever stoichiometric ratio they are stored. Since the DILPs 2, 3 and 5 have partly redundant functions, and only one form of dInR has been clearly identified (Brogiolo et al., 2001; Broughton et al., 2008; Grönke et al., 2010) one may wonder why the ratio between DILPs is variable. Do the individual DILPs have distinct functions? To test this it is important to investigate whether there are additional dInR subtypes, as in mammals, or if the different DILPs at least have different affinities for, or activities, on the single known receptor.

Finally, it is important to obtain an integrated view of the neuronal systems that regulate the IPCs and the physiological conditions that induce or inhibit release of DILPs. At present we know of several sets of neurons that utilize GABA, monoamines and neuropeptides to regulate activity in the IPCs, but only for the GABAergic neurons we have some clues what triggers the system. Therefore, it is important to further analyze the anatomy of the neuronal circuits that target the IPCs and to search for circulating (and other) signals that use these circuits to mediate aspects of the metabolic and physiological status of the organism.

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Insulin-like and IGF-like peptides in the silkmoth *Bombyx mori*: discovery, structure, secretion, and function

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A quarter of a century has passed since bombyxin, the first insulin-like peptide identified in insects, was discovered in the silkmoth *Bombyx mori*. During these years, bombyxin has been studied for its structure, genes, distribution, hemolymph titers, secretion control, as well as physiological functions, thereby stimulating a wide range of studies on insulin-like peptides in other insects. Moreover, recent studies have identified a new class of insulin family peptides, IGF-like peptides, in *B. mori* and *Drosophila melanogaster*, broadening the base of the research area of the insulin-related peptides in insects. In this review, we describe the achievements of the studies on insulin-like and IGF-like peptides mainly in *B. mori* with short histories of their discovery. Our emphasis is that bombyxins, secreted by the brain neurosecretory cells, regulate nutrient-dependent growth and metabolism, whereas the IGF-like peptides, secreted by the fat body and other peripheral tissues, regulate stage-dependent growth of tissues.

Keywords: insulin-like peptide, IGF-like peptide, *Bombyx mori*, bombyxin, BIGFLP, dilp6, insect, growth

INTRODUCTION

THE AIM OF THIS REVIEW

Since 1984, when bombyxin was identified as the first insulin-like peptide in invertebrates (Nagasawa et al., 1984b), this brain neurosecretory hormone of the silkmoth *Bombyx mori* has been extensively characterized for its chemical nature, gene structure, distribution, secretion control, as well as physiological functions. In addition, another class of insulin-related peptides has recently been discovered again in *B. mori* and characterized (Okamoto et al., 2009a). The former is similar to vertebrate insulin in its two-chain structure and physiological functions, while the latter to vertebrate insulin-like growth factors (IGFs) in its single-chain structure, sites of production and physiological roles. In this article, we will provide an overview of the accumulated knowledge on these two classes of *Bombyx* insulin-related peptides and discuss the physiological significance of the presence of these hormones in insects. Recently, physiological functions and action mechanisms of insect insulin/IGF-like peptides have been actively studied in other insects, especially in the fruit fly *Drosophila melanogaster*, by using genetic approaches. In this review, however, we will concentrate on the achievements with *B. mori*, because comprehensive reviews exist for *Drosophila* and other insects (Garofalo, 2002; Oldham and Hafen, 2003; Tatar et al., 2003; Géminard et al., 2006; Wu and Brown, 2006; Broughton and Partridge, 2009; Grewal, 2009; Teleman, 2010; Grönke and Partridge, 2010; Antonova et al., 2012; Nässel, 2012; Hyun, 2013).

HISTORICAL BACKGROUND

Insulin is best known for its hypoglycemic action (Newsholme et al., 1992; Saltiel and Kahn, 2001). Even before the discovery of bombyxin, the existence of hypoglycemic hormones had been demonstrated in the honeybee *Apis mellifera* (Kramer et al., 1977, 1982; Bounias et al., 1986), the blowfly *Calliphora vomitoria* (Duve et al., 1979), the tobacco hornworm *Manduca sexta* (Tager et al., 1976; Kramer et al., 1982), the cockroach *Periplaneta americana* (Barrett and Loughton, 1987) and others (for a review, see Kramer, 1985). These hypoglycemic hormones were presumed to be insulin-related peptides, because insulin-immunoreactive substances had also been detected in many insects including above-mentioned species as well as the locust *Locusta migratoria* and the silkmoth *B. mori* by radioimmunoassay (RIA) (Ishay et al., 1976; Tager et al., 1976; Kramer et al., 1977; Duve et al., 1979; Orchard and Loughton, 1980; Kramer, 1985) and/or immunocytochemistry (Duve and Thorpe, 1979; Yui et al., 1980). These insulin-like peptides in most insects were localized in the neurosecretory cells of the brain and its neurohemal organs, corpora cardiaca (CC) and/or corpora allata (CA), and were therefore recognized as brain neurosecretory hormones. The insulin-immunoreactive material was purified and chemically characterized in *M. sexta* (Kramer et al., 1982) and *C. vomitoria* (Thorpe and Duve, 1984). Although the amino acid sequence was not determined, their molecular size and amino acid composition were similar to those of vertebrate insulins.

BOMBYX INSULIN-LIKE PEPTIDE (BOMBYXIN)

DISCOVERY OF BOMBYXIN

Bombyxin was initially called 4K-prothoracicotropic hormone (4K-PTTH), because it was purified as the brain neurosecretory hormone with MW of 4400 that stimulates the prothoracic glands (PGs) to release ecdysone (Nagasawa et al., 1984b, 1986). In this purification study, the adult heads of *B. mori* were used as the starting material for purification and the debrained dormant pupae of the heterologous moth *Samia cynthia ricini* were used for the bioassay of the hormone, because PTTH was believed

to be species-nonspecific between these two species and because *Samia* debrained pupae provided a more stable assay system than *Bombyx* debrained pupae (see Ishizaki, 2004 for more detailed history of bombyxin and PTTH purifications). In fact, crude extracts of *B. mori* brain were able to provoke adult development when injected into debrained dormant pupae of both *B. mori* and *S. cynthia ricini*. At the final stage of purification, however, the purified 4K-PTTH was found to be ineffective on the PGs of *B. mori* from which it was derived (Ishizaki et al., 1983), indicating that this peptide is not the true PTTH of *B. mori*. Nevertheless, the name 4K-PTTH was used for years thereafter, because it was actually highly active on the *Samia* PGs both *in vivo* and *in vitro* (Nagasawa et al., 1984a). This peptide was purified to homogeneity and its partial sequence determined in 1984 (Nagasawa et al., 1984a,b). Surprisingly, the N-terminal sequence of 4K-PTTH was structurally homologous to vertebrate insulin and IGF (see section Primary Structure). This was the first insulin-related peptide identified in invertebrates. Thus, this peptide was finally renamed bombyxin for *Bombyx* insulin (Mizoguchi et al., 1987).

PRIMARY STRUCTURE

4K-PTTH was purified to homogeneity through 15 steps of purification from 678,000 *Bombyx* adult heads, yielding three peaks on HPLC. Each peak contained 36–50 μg of peptide, and was sequenced separately. The N-terminal 19 amino acid sequences of these peptides, named 4K-PTTH-I, II, III, were similar to each other and even to the corresponding portions of human insulin and IGF-I (Nagasawa et al., 1984b). The complete amino acid sequence was determined later for 4K-PTTH-II (Nagasawa et al., 1986; for minor revision, see Nagasawa et al., 1988). It consisted of two non-identical peptide chains (A and B chains), like vertebrate insulin. The A and B chains consisted of 20 and 28 amino acid residues, respectively. 4K-PTTH-II showed high sequence identity (∼40%) with human insulin, and the positions of seven cysteine residues were completely identical with those in other insulin family peptides.

HIGHER STRUCTURE

The location of disulfide bridges in the molecule was determined through thermolysin digestion of 4K-PTTH-II (hereafter bombyxin-II) and subsequent chemical analyses (Nagasawa et al., 1988). Three disulfide bonds were linked in the same positions as in insulin.

A three-dimensional model of bombyxin-II was constructed using interactive computer graphics and energy minimization techniques (Jhoti et al., 1987), confirming the insulin-like structure of bombyxin-II (called PTTH-II in the paper). This model predicts that bombyxin-II is unlikely to form either dimers or hexamers, characteristic of human insulin and that a hydrophobic surface region of the peptide may be important in binding with other proteins.

In addition to bombyxin-I, -II, -III, two more bombyxin species have been isolated from *Bombyx* heads. The primary structure of bombyxin-IV was determined for both chains (Maruyama et al., 1988), and that of bombyxin-V for the B-chain (Jhoti et al., 1987). Bombyxin-IV had 62.5% sequence identity with bombyxin-II.

Bombyxin-II and -IV were chemically synthesized by solid phase peptide synthesis of the A- and B-chains followed by air oxidation (Nagasawa et al., 1988) or semicontrolled pairing of cysteine residues (Maruyama et al., 1990) in a yield of 4 and 8%, respectively. The synthetic bombyxin-II and -IV had nearly the same specific activity as the natural ones.

By using the synthetic bombyxin-II, its three-dimensional solution structure was determined by NMR measurements (Nagata et al., 1995a). Although the overall main-chain structure of bombyxin-II was similar to that of insulin, significant conformational and functional differences in their B-chain C-terminal portions were found. This part of bombyxin-II adopts an extension of the B-chain central helix like that of relaxin and is not required for bombyxin activity, contrasting with the corresponding part of insulin, which adopts a sharp turn and a β-strand and is essential for insulin activity. A further study employing chimeric molecules of bombyxin-II and human insulin suggested that the surface patch formed by the central part of the bombyxin-II B-chain is of critical importance for recognition of the bombyxin receptor (Nagata et al., 1995b).

BOMBYXIN GENES

Although the above purification study revealed an abundance of molecular forms of bombyxin, the subsequent molecular cloning studies have identified more genes encoding bombyxins. Thirty-two bombyxin-encoding genes have been cloned (Iwami et al., 1989, 1990; Kawakami et al., 1989; Kondo et al., 1996; Tsuzuki et al., 1997; Yoshida et al., 1997, 1998). In addition, some more bombyxin genes have been identified by the BLAST searches on the *Bombyx* genome (Aslam et al., 2011). The cloned genes are classified into 7 families, A–G, according to their sequence similarity. The families A, B, and C consist of 10, 12, and 6 genes, respectively, and each of the families D–G contains a single gene (Iwami, 2000). Among these genes, A6 and/or A7 encode bombyxin-II, and E1 encode bombyxin-IV. However, the genes coding for bombyxin-I, III, and V have not been identified. Most bombyxin genes encode polypeptides similar to preproinsulin, which consists of the signal peptide, B-chain, C-peptide, and A-chain, in this order, from the N-terminus, although several other genes are presumably pseudogenes (Kondo et al., 1996). Novel bombyxin genes recently identified in the *Bombyx* genome are classified into five different families, V–Z (Aslam et al., 2011). Each family contains a single gene except for family V, which includes two closely related genes. These new bombyxin genes also code for preproinsulin-like structure, similar to the family A–G genes. The similarities in the amino acid sequences of preprobombyxins between families are about 50% (Iwami, 2000). The sequence similarities are high in the A- and B-chains (domains) but low in the C-peptide (domain).

All six cysteines and some hydrophobic residues in the A- and B-domains responsible for the formation of a hydrophobic core are completely conserved in all probombyxins and insulin. The C-domains of all probombyxins are flanked by dibasic (monobasic in some cases) sites, suggesting that the C-domain is removed to generate mature bombyxins in the same way as insulin maturation. However, this may be the case only when the bombyxin genes are expressed in specific tissues such as the brain, from

which bombyxin-II and -IV were purified. These two bombyxins have been confirmed to consist of A- and B-chains like insulin, as described above. In 2009, however, a novel insulin family peptide containing the C-domain was purified from *Bombyx* pupal hemolymph [(Okamoto et al., 2009a); see section *Bombyx* IGF-Like Peptide (BIGFLP) for details]. This peptide, named *Bombyx* IGF-like peptide (BIGFLP), is the product of the bombyxin Y1 gene (Aslam et al., 2011), and its C-domain is also flanked by dibasic sites. However, this peptide is secreted with the C-domain intact. Its main production site is the fat body. It is probable that fat body cells lack the enzyme(s) that cleaves peptides at dibasic sites. The discovery of BIGFLP suggests that the mature form of bombyxin depends on the type of cells where the bombyxin gene is expressed.

A remarkable feature of most bombyxin genes is a lack of any introns (Iwami, 2000), since vertebrate insulins consistently have two introns at conserved sites, one in the 5′-UTR and the other in the C-domain (Steiner et al., 1985). However, three of the recently identified bombyxin genes were found to have introns. *Bombyxin-V1* and *-V2* each had one intervening intron of different length in the 5′-UTR, and *bombyxin-Z1* contained two introns in the 5′ UTR and one intron within the C-domain (Aslam et al., 2011).

In the *Bombyx* genome, most bombyxin genes are clustered in two segments on chromosome 11 (25 genes) and an unidentified chromosome (6 genes), while forming gene pairs, gene triplets, or single genes. Other genes are located singly on chromosome 1 (3 genes), chromosome 9 (2 genes), and chromosome 11 (1 gene) (Iwami, 2000; Aslam et al., 2011).

BOMBYXIN BIOSYNTHESIS AND RELEASE

BOMBYXIN-PRODUCING CELLS

Bombyxin-producing cells were first identified by immunocytochemistry using a mouse monoclonal antibody (AN-I) generated against a synthetic decapeptide corresponding to the N-terminal portion of the A-chain of bombyxin-I (Mizoguchi et al., 1987). This antibody immunostained four pairs of large mid-dorsal neurosecretory cells of the brain and thick and dense nerve fibers around the periphery of the CA, suggesting that bombyxin is produced by these neurosecretory cells, axonally transported to and released from the CA. The axonal pathway of the bombyxin-containing neurons was not clear in this study, because immunohistochemistry was performed on brain sections. However, the subsequent study employing intracellular injection of Lucifer yellow (Ichikawa, 1991) or whole-mount immunohistochemistry (**Figure 1**) confirmed the connection between the medial neurosecretory cells (MNCs) in the brain and the axon terminals in the CA. These studies revealed that the axons originating from the four MNCs are fasciculated, traverse the midline of the brain to enter the contralateral lobe, and proceed along the ventral surface of the brain toward the retrocerebral nerve. The axons descend down the nerve, pass through the CC, and terminate in the CA with branches. Lucifer yellow injection in bombyxin-producing cells also revealed thick ipsilateral and thin contralateral dendrites as well as an abundance of varicosities along the branched axons over the periphery of the CA (Ichikawa, 1991).

Developmentally, the number of bombyxin-producing neurosecretory cells in the brain is consistently four throughout

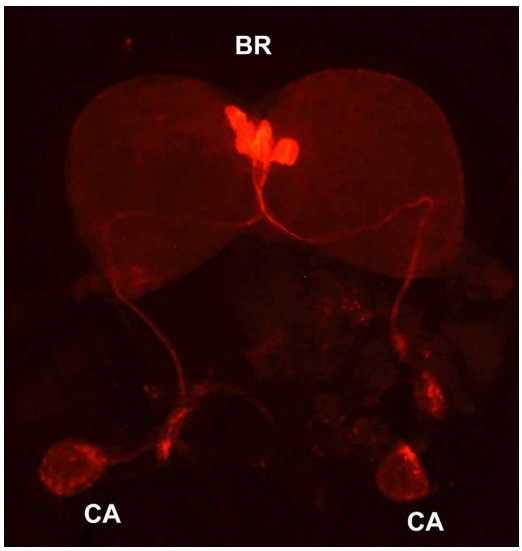

FIGURE 1 | Bombyxin-producing neurosecretory cells. Whole-mount immunohistochemistry with anti-bombyxin-II mouse monoclonal antibody (A7B11) was performed on the brain-retrocerebral complex of the day-3 fifth instar larva of *B. mori*. BR, brain; CA, corpus allatum.

postembryonic development from hatching through adult eclosion (Mizoguchi et al., 1990). However, the immunoreactivity of the cells has been shown to markedly change during *Bombyx* development, especially in the fifth instar. For the first three days of the instar, the four pairs of cells are heavily stained but thereafter the stainability is impaired. Interestingly, two of these cells in each brain hemisphere are relatively intensely stained whereas the other two cells stained poorly. This unequal stainability of the cells might represent a pulsatile secretion of bombyxin. The immunoreactivity becomes weakest on the day of wandering and is then gradually regained (Mizoguchi et al., 1990).

The bombyxin-producing cells in the brain have also been identified by *in situ* hybridization. The bombyxin genes of families A–F, V, W, and Y are all expressed in four pairs of MNCs of the fifth larval brain (Iwami, 2000; Aslam et al., 2011), indicating that these cells are the exclusive source of bombyxin in the brain.

Early investigations by Northern hybridization could not detect bombyxin expression in tissues other than brain (Adachi et al., 1989; Iwami et al., 1990). However, RT-PCR analyses have disclosed that bombyxin genes are ubiquitously expressed in larval tissues, although the level of expression is very low if compared to that in the brain (Iwami et al., 1996). Moreover, it has recently been shown that some "bombyxin" genes are expressed predominantly in the fat body and/or gonads in a stage specific manner. Such genes include bombxin-X1, Y1 and ZI genes (Aslam et al., 2011). However, calling them "bombyxin" genes is controversial, because one of the products of these genes has been shown to be more like IGF rather than insulin in its structural features and physiological roles [see section *Bombyx* IGF-Like Peptide (BIGFLP)]. If bombyxin is defined as a structurally and functionally insulin-like peptide of *B. mori*, the IGF-like peptide should be called by other name. In this review, we discriminate the insulin-related peptides produced mainly by peripheral tissues

from bombyxins, which are produced predominantly by the brain neurosecretory cells.

MEASUREMENT OF BOMBYXIN

Bombyxin titers in the hemolymph have been determined by RIA (Saegusa et al., 1992; Masumura et al., 2000) or time-resolved fluoroimmunoassay (TR-FIA) (Satake et al., 1997). RIA was developed using a mouse monoclonal antibody (M7H2) generated against pure native bombyxin-II and ^{125}I-labeled native bombyxin-II. The bombyxin concentration determined by this RIA was expressed by *Samia* units/ml, because a partially purified bombyxin was used as the standard, due to a paucity of the pure hormone. The lower limit of detection was equivalent to 0.2 *Samia* units (as determined by the *Samia* pupal assay) of bombyxin contained in the partially purified bombyxin. One *Samia* unit corresponds to 0.1 ng of bombyxin-I and -III and 0.4 ng of bombyxin-II (Nagasawa et al., 1984b). Therefore, the sensitivity of this RIA can be roughly estimated to be several tens of picograms, assuming that the anti-bombyxin-II antibody binds to any bombyxin species with the same affinity. In fact, however, the cross-reactivity of this antibody to other bombyxin species is not known. Nevertheless, this RIA provided a valuable opportunity to assess the physiological function of bombyxin and to analyze the regulation of its secretion. The titers of bombyxin immunoreactivity in the hemolymph were relatively constant during larval development with no correlation with ecdysteroid peaks, confirming that this hormone is not involved in the regulation of PG activity (Saegusa et al., 1992). Although the hemolymph titers of bombyxin immunoreactivity determined by RIA were remarkably high during pupa-adult development, this immunoreactivity in the pupal hemolymph was later proved not to represent bombyxin but to denote other bombyxin-related peptide. However, without this information about very high bombyxin titers in the pupal hemolymph, the recent discovery of an IGF-like peptide may not have been achieved [see section *Bombyx* IGF-Like Peptide (BIGFLP)]. The same RIA was applied to investigate the effects of starvation and feeding of larvae on bombyxin secretion (Masumura et al., 2000), revealing an important aspect of bombyxin secretion (see section Regulation of Bombyxin Release).

More recently, a TR-FIA based on a sandwich protocol has been developed using two kinds of monoclonal antibodies against synthetic bombyxin-II (A3A1 and A7B11), one of which was biotin-labeled and used as a tracer antibody, and the other used as a capture antibody (Satake et al., 1997). The lower limit of detection by this assay was 3 pg (bombyxin-II equivalents), much lower than that of the RIA. This TR-FIA was applied to measure bombyxin titers in the hemolymph of *Bombyx* adults, revealing that bombyxin is released immediately after eclosion in a sex-specific manner (Satake et al., 1999). The peak bombyxin titer was three times higher in males. However, the physiological relevance of the bombyxin release at this stage and its sex difference remains to be elucidated.

REGULATION OF BOMBYXIN RELEASE

The effects of starvation and refeeding on bombyxin secretion were investigated in *Bombyx* larvae. The release of bombyxin was monitored by measuring hemolymph titers and brain contents of bombyxin by RIA (Masumura et al., 2000). Following starvation for 3–6 h, the hemolymph titer decreased, while the brain content increased significantly. By contrast, refeeding after 6 h-starvation resulted in a quick recovery of the hemolymph bombyxin titer as well as a quick decrease in the brain bombyxin content. These results clearly demonstrated that bombyxin release is closely associated with feeding. In humans, insulin secretion is stimulated by hyperglycemia after a meal. The glucose levels in *Bombyx* hemolymph also change dramatically after starvation and refeeding. Therefore, the effect of glucose on bombyxin release was examined by injecting D-glucose into starved larvae. The brain bombyxin content decreased within 1 h after glucose injection in a dose-dependent manner. This result indicates that glucose serves as a common nutritional signal for inducing the release of both mammalian and insect insulins as a messenger for the "fed" state of the body.

It is unclear whether the bombyxin-producing cells directly respond to circulating glucose or not. In *D. melanogaster*, the secretion of insulin-like peptides (DILPs) from the brain also depends on the nutritional conditions, but the availability of nutrients is remotely sensed by the fat body, which in turn regulates DILP secretion through humoral factors (Géminard et al., 2009; Bai et al., 2012; Rajan and Perrimon, 2012). In this case, the major nutritional signal in the diet is not glucose but amino acids (Géminard et al., 2009).

BOMBYXIN ACTIONS

REGULATION OF TREHALOSE METABOLISM

When bombyxin was purified from *B. mori*, no physiological action of this peptide was known in *B. mori* from which it was derived (see section Discovery of Bombyxin). Prior to the identification of bombyxin as an insulin-related peptide, however, the presence in several insects of insulin-like hypoglycemic hormones had been repeatedly suggested (see section Historical Background). Thus, soon after a synthetic bombyxin-II was obtained (Nagata et al., 1992), the effects of bombyxin on sugar metabolism were investigated. Bombyxin-II lowered the concentration of the major hemolymph sugar, trehalose, in a dose-dependent manner when injected into neck-ligated *Bombyx* larvae (Satake et al., 1997).

Bombyxin-II injection also led to the elevation of trehalase activity in the midgut and muscle (Satake et al., 1997). Trehalase located on the cell surface is assumed to hydrolyze trehalose in the hemolymph to glucose for transport into the cells (Shimada and Yamashita, 1979; Azuma and Yamashita, 1985). Therefore, these observations suggest that bombyxin induces hypotrehalosemia by promoting the hydrolysis of hemolymph trehalose to glucose and thereby facilitating its incorporation into tissues. However, such effects of bombyxin on trehalose metabolism may be larval stage-specific, because bombyxin-II injection into adults did not result in hypotrehalosemia (Satake et al., 1999).

Glucose also constitutes the hemolymph sugar in *B. mori,* although its concentration is very low as compared with that of trehalose. Interestingly, bombyxin-II injection did not affect the glucose concentration in the hemolymph (Satake et al., 1997).

REGULATION OF GLYCOGEN METABOLISM

In mammals, insulin not only promotes glucose uptake by adipose tissue and muscle but also facilitates glycogen production in

some tissues including liver and muscle (Newsholme et al., 1992; Saltiel and Kahn, 2001). Therefore, the effect of bombyxin on tissue glycogen content was also examined. Unexpectedly, glycogen content in the fat body, a major glycogen storage tissue, was significantly decreased, but not increased, after bombyxin-II injection (Satake et al., 1997).

The effect of bombyxin on the activity of glycogen phosphorylase, a key enzyme of glycogenolysis, was also examined. When 10 ng of bombyxin-II was injected, the percentage of active glycogen phosphorylase was significantly increased, providing a biochemical basis for the glycogen-reducing effect of bombyxin (Satake et al., 1997).

It is worth noting that hemolymph trehalose and fat body glycogen are both major storage forms of carbohydrates in insect. Thus, the results of these investigations suggest that bombyxin likely functions to facilitate the use of energy reserves.

REGULATION OF TISSUE GROWTH

The growth-promoting action of bombyxin on wing imaginal discs has been demonstrated in the butterfly *Precis coenia* (Nijhout and Grunert, 2002). Although the wing disc of the final instar larva does not grow *in vitro* in the standard tissue culture medium without supplements, it does grow in the medium supplemented with an optimal concentration of 20-hydroxyecdysone (20E, 0.1 ng/ml) and with hemolymph taken from growing larvae. Assuming that the factor in the hemolymph responsible for the promoted growth of the disc is bombyxin, the effect of bombyxin-II on the disc growth *in vitro* was examined, using mitotic rate as an indicator of its growth. Bombyxin-II elevated the mitotic rate in a dose-dependent manner when added together with 0.1 mg/ml 20E to the culture. Moreover, the growth-promoting effect of the hemolymph was abolished when the hemolymph was pretreated with bombyxin antibodies (AN-I and guinea pig polyclonal antibody against synthetic bombyxin-II)-bound column to remove bombyxin, leading the authors to conclude that bombyxin is a growth factor for wing imaginal discs (Nijhout and Grunert, 2002). A similar effect of bombyxin-II on wing disc growth has also been reported in *M. sexta* (Nijhout et al., 2007).

Bombyxin-II also promotes cell proliferation in the hematopoietic organ (HPO) of *B. mori*. Cell proliferation in the larval HPOs cultured *in vitro* is markedly promoted by *B. mori* larval hemolymph. Thus, the active factor in the hemolymph has again been presumed to be bombyxin. When the HPOs from day-1 fifth instar larvae were cultured for 48 h with bombyxin-II, the number of discharged hemocytes increased in a dose-dependent manner (Nakahara et al., 2006). Because the hemocyte discharge is linked to cell proliferation (Nakahara et al., 2003), this result shows mitogenic activity for bombyxin-II. In contrast to the above study with the *Precis* wing discs, bombyxin-II was still effective in the absence of 20E. 20E was also mitogenic to the HPO irrespective of the presence of bombyxin-II. Despite the clear action of bombyxin-II on the HPO, the authors concluded that bombyxin is not the primary effector in the larval hemolymph, because anti-bombyxin-II antibodies (a mixture of M7H2, A3A1, A7B11, and guinea pig polyclonal antibody) were unable to neutralize the mitogenic activity of the hemolymph. However, this conclusion might be premature, because bombyxin is highly heterogeneous and the anti-bombyxin-II antibodies are only able to recognize a part of many bombyxin molecular species (Saegusa et al., 1992).

The effect of bombyxin on the systemic growth of lepidopteran insects including *B. mori* has not been investigated due to the difficulties in applying genetic approaches to analyze bombyxin actions in this order of insects (Terenius et al., 2011). However, such an effect of bombyxin homologues has been extensively studied in *D. melanogaster*, which has eight insulin-like peptides (DILP1-8) (Brogiolo et al., 2001; Colombani et al., 2012; Garelli et al., 2012). Among them, *dilp2, −3, and −5* are mainly expressed in the MNCs of the brain as with bombyxin (Brogiolo et al., 2001; Ikeya et al., 2002; Rulifson et al., 2002; Broughton et al., 2005; Nässel, 2012) and their products are thus thought to be functional homologues of bombyxins. When individual *dilps* (*dilp1-7*) were overexpressed, a proportionate increase in body size was observed in adult flies (Brogiolo et al., 2001; Ikeya et al., 2002), with *dilp2* showing the highest potency for growth promotion (Ikeya et al., 2002). The *dilp2*-overexpressed flies exhibited larger wing size due to increases in both cell size and cell number. In contrast, when the DILP-producing MNCs in the brain were genetically ablated using a *dilp2* promoter to express the apoptotic gene, *reaper*, larval growth was severely retarded, puparium formation delayed, and adult size reduced. All these phenotypes were rescued by ubiquitously expressing *dilp2* using heat-shock promoter (Rulifson et al., 2002). These results clearly demonstrate that the DILPs secreted from the MNCs are potent regulators of the systemic growth of *Drosophila*.

OTHER KNOWN ACTIONS

A highly purified preparation of bombyxin induced meiosis in the ovaries that were taken from young *Bombyx* larvae and cultured *in vitro* (Orikasa et al., 1993). However, the authors proposed that bombyxin merely stimulates the ovaries to produce ecdysteroids, which in turn induce meiosis of the ovarian cells, because 20E also induces meiosis at a very low concentration (Orikasa et al., 1993) and because the ovaries from the fourth instar *Bombyx* larvae secrete ecdysteroids *in vitro* (Orikasa, unpublished).

It has also been reported that synthetic bombyxin-II induces a series of morphological changes in BM-N4 cells (Tanaka et al., 1995), a cell line established from ovarian tissues of *B. mori* (Volkman and Goldsmith, 1982). After bombyxin administration, most of the cells became bigger and round in shape and aggregated to form clumps. Some other cells were tightly attached to the bottom of the culture flask forming a spindle-like or fibroblastic shape (Tanaka et al., 1995). These morphological changes were induced by bombyxin at a concentration of 1 nM or higher. Five other *Bombyx* cell lines, all of which were derived from embryos, did not respond to bombyxin at concentrations up to 1 μM.

A bombyxin-like peptide of *M. sexta* has been suggested to be involved in the pupal commitment of wing imaginal discs (Koyama et al., 2008). At the beginning of the final larval instar, wing discs are committed to initiate larval-pupal development. Juvenile hormone (JH) prevents this commitment in earlier instars and in starved final instar larvae, but nutrient intake overcomes this effect of JH in the latter (Truman et al., 2006).

Thus, an insulin/IGF signal was hypothesized to mediate the effect of nutrients. When wing discs from freshly molted final instar larvae were cultured *in vitro* with methoprene, a JH mimic, the expression level of *broad*, a molecular marker for pupal commitment, was suppressed. The suppression effect of JH, however, was overcome by the administration of partially purified *Manduca* bombyxin or bovine insulin, supporting the above-mentioned hypothesis.

As described in section Discovery of Bombyxin, bombyxin did not stimulate *Bombyx* PGs in the debrained pupal assay or in the conventional *in vitro* PG assay. Recently, however, it was demonstrated that bovine insulin stimulates ecdysteroidogenesis in *Bombyx* PGs during a long-term incubation period (Gu et al., 2009). A significant increase in ecdysteroid secretion by the PGs was observed after 8 h of incubation with insulin at a concentration of 1.7 μM or higher. In addition, insulin also stimulated DNA synthesis and cell viability of PGs, as assayed after 48 h of incubation. The PG-stimulating action of insulin was also verified by *in vivo* experiments: injection of insulin into day 6 last instar larvae significantly increased both the hemolymph ecdysteroid titers and *in vitro* ecdysteroidogenic activity of the PGs 24 h after the injection. Although bovine insulin was used in this study, the results strongly suggest that bombyxin, intrinsic insulin of *B. mori*, has the same long-term actions on the PGs. The effects of insulin-like peptides on ecdysteroidogenesis in PGs are also demonstrated in *D. melanogaster* (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005; Walkiewicz and Stern, 2009).

BOMBYX IGF-LIKE PEPTIDE (BIGFLP)

DISCOVERY OF BIGFLP

As described in section Measurement of Bombyxin, the hemolymph bombyxin titer, as measured by RIA, is remarkably high during metamorphosis (Saegusa et al., 1992). The titers were so high the authors suspected that the immunoreactivity in the pupal hemolymph did not represent bombyxin, because it was unlikely that only eight neurosecretory cells in the brain could secrete such large amounts of bombyxin. Therefore, pupal hemolymph was analyzed by Western blotting using M7H2 antibody, the same antibody as used for RIA, to estimate the molecular mass of the immunoreactive material. It was 8 kDa, slightly larger than that of bombyxin-II and similar to that of a bombyxin precursor polypeptide. Thus, it was decided to purify this peptide to determine its structure. The peptide was effectively enriched from pupal hemolymph using an M7H2 antibody-bound affinity column and further purified to homogeneity through only two steps of HPLC (Okamoto et al., 2009a). The amino acid sequence of the peptide was determined by a combination of N-terminal sequencing, *Bombyx* genome search and MALDI TOF-MS analysis. The peptide had high homology with bombyxins and most interestingly it contained the C-domain within the molecule. Although the C-domain was flanked by dibasic amino acid motifs, it was obvious that this peptide is secreted into hemolymph as a single chain peptide like IGFs, because it was purified from the hemolymph. For this and some other reasons described below, this peptide was named BIGFLP (Okamoto et al., 2009a).

BIGFLP-PRODUCING CELLS

Although all known bombyxins are mainly produced by MNCs in the brain (Mizoguchi et al., 1987; Iwami, 2000), expression analysis by real-time quantitative RT-PCR (qRT-PCR) revealed that BIGFLP is predominantly produced by the fat body (Okamoto et al., 2009a). The fat body is the functional equivalent of the vertebrate liver, which is the major source of circulating IGFs. BIGFLP gene expression in the fat body was very low during larval stages but dramatically increased during metamorphosis. Production of BIGFLP by the fat body was also confirmed by immunohistochemistry with a BIGFLP-specific monoclonal antibody (D7H3) generated against its C-domain (Okamoto et al., 2009a, 2011). BIGFLP-producing tissues and stages were systematically surveyed by means of immunohistochemistry, *in situ* hybridization, as well as qRT-PCR (Okamoto et al., 2011). These analyses revealed that BIGFLP is produced not only by the fat body but also by the brain and gonads in a stage-specific manner (Okamoto et al., 2011). The BIGFLP-producing cells in the brain were identical to the cells that produce bombyxins, but the temporal expression pattern of BIGFLP was clearly different from that of bombyxins. BIGFLP is produced in the brain only after the penultimate (fourth) instar stage. In the ovary, BIGFLP expression was observed in the ovariole sheath, which wraps around an array of follicles, during the wandering and early pupal stages. The sheath of testis also produces BIGFLP during pupa-adult development.

REGULATION OF BIGFLP PRODUCTION AND RELEASE

The temporal expression patterns of the BIGFLP gene in the fat body and gonads suggested that BIGFLP production in these organs is regulated by ecdysteroids, which are released concurrently. This hypothesis was verified though the experiments where these tissues were cultured *in vitro* with 20E (Okamoto et al., 2009a, 2011). In contrast, *BIGFLP* expression in the brain was not induced by 20E (Okamoto et al., 2011). These results indicate that the regulatory mechanisms of BIGFLP gene expression differ across tissues.

BIGFLP titers in the hemolymph were determined by TR-FIA using two antibodies, one of which (capture antibody) was a BIGFLP-specific mouse monoclonal antibody (D11E12) against its C-domain and the other (tracer antibody) was anti-bombyxin-II guinea pig polyclonal antibody that cross-reacts with BIGFLP. The BIGFLP titers were remarkably high, with the titer rising early in the pupal stage to 800 nM in females and 200 nM in males. These values are much more similar to those of IGFs in human adults (20–80 nM) (Humbel, 1990) than to those of bombyxin and insulin, which are of the order of 100 pM (Andersen et al., 1993; Satake et al., 1999).

BIGFLP ACTIONS

Lepidopteran pupae initiate adult development shortly after pupal ecdysis, when larval tissues degenerate, while adult tissues including reproductive system, imaginal discs and flight muscles undergo growth and differentiation. The temporal secretion pattern of BIGFLP suggested that BIGFLP regulates growth and development of adult tissues during metamorphosis. The potential growth-promoting effect of BIGFLP was investigated using an *in vitro* tissue culture system. When genital imaginal discs

were cultured with BIGFLP, the tissue size, protein content and cell number of the discs were significantly increased after 5 days. BIGFLP also promoted BrdU incorporation not only in genital discs but also in sperm ducts, flight muscle anlagen and wing discs. Interestingly, no or little promotion of BrdU incorporation by BIGFLP was observed in the fat body, midgut and epidermis, all of which are degenerated or reconstructed during adult development, suggesting that BIGFLP functions as a growth hormone to regulate adult tissue development in *B. mori* (Okamoto et al., 2009a).

Although the dominant source of BIGFLP in the hemolymph is the fat body, several other tissues including brain and gonads also produce BIGFLP (see section BIGFLP-Producing Cells). For example, the ovariole sheath produces BIGFLP during the period when the ovaries undergo rapid growth and development. BIGFLP expression is especially high in the area where follicles are immature. Interestingly, the *Bombyx* insulin-like receptor gene is also predominantly expressed in the early vitellogenic ovaries (Swevers and Iatrou, 2003). Thus, ovariole sheath-derived BIGFLP may regulate the early follicular growth in a paracrine manner. Thus, BIGFLP may exert its effects through both endocrine and paracrine pathways, as do the vertebrate IGFs.

IGF-LIKE PEPTIDE IN *DROSOPHILA*

CANDIDATES FOR IGF-LIKE PEPTIDES IN OTHER INSECTS

Are IGF-like peptides present in insects other than *B. mori*? Based on sequence homology, no obvious orthologs of BIGFLP have been identified in other insects, because the amino acid sequences of insect insulin-related peptides are highly diverged between insect orders, except for some critical residues necessary for tertiary structure formation. One of the characteristic features of IGFs as compared to proinsulin is a shortened C-domain. In this regard, some insulin-related peptides in insects are more similar to IGFs than to insulin (**Figure 2**). Such peptides are found in *D. melanogaster* (DILP6) (Brogiolo et al., 2001), *A. mellifera* (AmILP1) (Wheeler et al., 2006), the red flour beetle *Tribolium castaneum* (TcILP3) (Li et al., 2008), and the mosquito *Aedes aegypti* (AaegILP6) (Riehle et al., 2006).

Another characteristic feature of the IGF-like peptide in *B. mori* is a very high level of gene expression in the fat body during metamorphosis. Therefore, insulin-related genes in other insects showing such an expression pattern are good candidates for being IGF-like peptides. Although bombyxin genes and a majority of known insect insulin-related genes are mainly expressed in MNCs of the brain (Mizoguchi et al., 1987; Iwami, 2000; Brogiolo et al.,

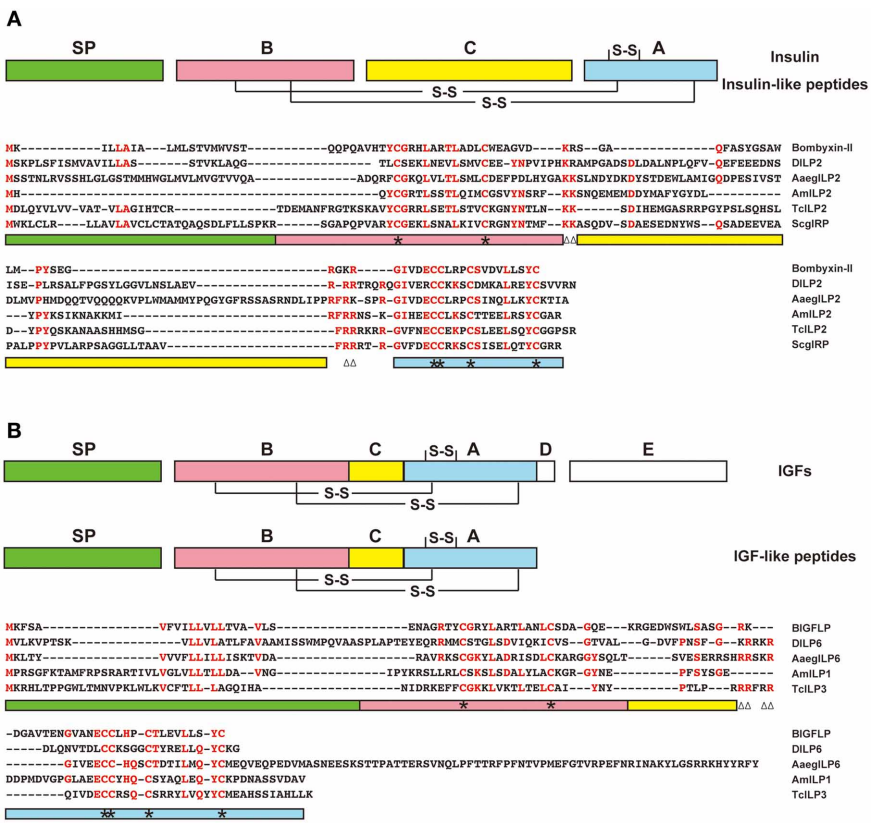

FIGURE 2 | Predicted insulin-like and IGF-like peptides in insects. Amino acid sequences of the representatives of putative insulin-like **(A)** and IGF-like **(B)** peptides from *B. mori* (bombyxin-II and BIGFLP), *D. melanogaster* (DILP2 and 6), *A. aegypti* (AaegILP2 and 6), *A. mellifera* (AmILP2 and 1), *T. castaneum* (TcILP2 and 3), and *Schistocerca gregaria* (ScgIRP) are aligned. Highly conserved amino acid residues are shown in red. Color bars below the alignment indicate the predicted domains in the precursor peptides: green, signal peptide; red, B-domain; yellow, C-domain; blue, A-domain. Asterisks denote cysteine residues, and paired triangles denote potential cleavage sites (dibasic amino acids).

2001; Ikeya et al., 2002; Rulifson et al., 2002; Broughton et al., 2005; Nässel, 2012), some insulin-related genes are expressed outside the brain (Brogiolo et al., 2001; Riehle et al., 2006).

DILP6 AS A *DROSOPHILA* IGF-LIKE PEPTIDE

In *D. melanogaster*, developmental changes in the gene expression of seven DILPs were investigated from embryo to adult (Okamoto et al., 2009b; Slaidina et al., 2009). These analyses revealed that only *dilp6* was expressed predominantly during the late third instar and during pupa-adult development, at remarkably high levels. Furthermore, *dilp6* exhibited the highest expression in the fat body. These characteristics of *dilp6* expression, together with the structural feature of DILP6, led to its identification as a *Drosophila* IGF-like peptide. Interestingly, *dilp6* expression in the fat body is induced *in vitro* by 20E (Okamoto et al., 2009b; Slaidina et al., 2009), as is *BIGFLP* (Okamoto et al., 2009a). The ecdysteroid-induced expression of *dilp6* in the fat body is not affected by cycloheximide, a protein synthesis inhibitor, suggesting that *dilp6* expression is directly induced by 20E (Okamoto et al., 2009b). The expression of *dilp6* in the fat body is also up-regulated by starvation of larvae through direct induction by Forkhead box O (FOXO) transcription factor, independent of the ecdysteroids (Slaidina et al., 2009), suggesting that DILP6 also plays some roles during starved conditions. A recent study has shown that *dilp6* expression in the adult fat body is also regulated by FOXO (Bai et al., 2012). This FOXO-inducible *dilp6* expression is suggested to be involved in the compensatory regulation between fat body-derived and brain-derived DILPs (Grönke et al., 2010; Bai et al., 2012).

DILP6 FUNCTIONS

Several research groups independently generated *dilp6* mutants and revealed that these mutants result in ∼10% reduced final adult body size and weight (Okamoto et al., 2009b; Slaidina et al., 2009; Zhang et al., 2009; Grönke et al., 2010). The hair density analysis demonstrated that *dilp6* mutants have normal cell size but reduced cell number, which likely accounts for the reduction in body size (Okamoto et al., 2009b). Based on the temporal *dilp6* expression pattern, DILP6 was expected to regulate growth during metamorphosis. The developmental changes in body weight were investigated in the *dilp6* mutants from the beginning of the 3rd instar to adult emergence (Okamoto et al., 2009b). During the feeding period in the 3rd instar, the growth rate was the same as controls. However, the difference in body weight between controls and *dilp6* mutants was manifested during the post-feeding period. The *dilp6* mutants lost weight at a higher rate and emerged as smaller adult flies. During the wandering and adult developmental stages, when animals never feed, the nutrient supply is limited to the stored nutrients that have been accumulated during the feeding period. Therefore, this *dilp6* mutant phenotype suggests a role for DILP6 in regulating the utilization efficiency of nutrient stores during the post-feeding stages. This phenotype is rescued by fat body-specific expression of *dilp6* during the post-feeding period (Okamoto et al., 2009b). Furthermore, when *dilp6* was overexpressed during the post-feeding period, the body weight of adult flies was increased compared with control animals, showing that the effect of DILP6 on adult size is closely related to its expression level (Okamoto et al., 2009b). Similar results were also obtained by using *Gal4/Gal80^{ts}* temporal conditional expression system to induce *dilp6* RNAi or overexpression during the post-feeding period (Slaidina et al., 2009). These studies demonstrate that DILP6 is involved in the regulation of postfeeding growth (Okamoto et al., 2009b; Slaidina et al., 2009).

Recent several studies have demonstrated that DILP6 act locally within the central nervous system (Chell and Brand, 2010; Sousa-Nunes et al., 2011; Avet-Rochex et al., 2012). A subset of glia expresses *dilp6* during the feeding stages of larvae, and its expression is inhibited by starvation. This nutrient-dependent expression and/or secretion of DILP6 activate the insulin/IGF signaling in the adjacent neuroblasts, thereby leading to their exit from quiescence (Chell and Brand, 2010; Sousa-Nunes et al., 2011). Moreover, DILP6 has also been suggested to regulate the proliferation of perineural and cortex glia in the larval brain (Avet-Rochex et al., 2012). Thus, the fat body-derived DILP6 promotes systemic growth in an endocrine fashion, while the glia-derived DILP6 regulates neuroblast reactivation and gliogenesis in a paracrine/autocrine manner.

CONCLUDING REMARKS

Two classes of insulin-related peptides were discovered and characterized in *B. mori*; one is bombyxin, which is a functional counterpart of vertebrate insulin, and the other is BIGFLP, which is more similar to IGFs rather than to insulin in the structural and biological traits. However, these two peptides are not

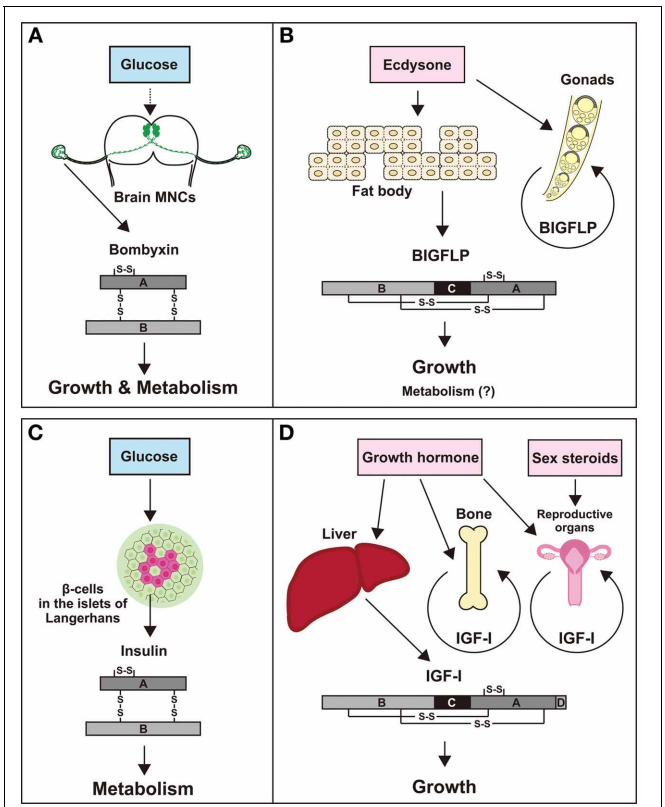

FIGURE 3 | Comparison of bombyxin and BIGFLP of *B. mori* with insulin and IGF-I of humans. High similarities are noted between bombyxin **(A)** and insulin **(C)** and between BIGFLP **(B)** and IGF-I **(D)** in the structural feature, sites of production, secretion control, and actions.

complete homologues of insulin and IGF, because they are presumed to share the same receptor (Okamoto et al., 2009b) and therefore have essentially the same actions, unlike insulin and IGF, each of which has its own receptor. Nevertheless, it is still conceivable that bombyxin and BIGFLP are functional counterparts of insulin and IGF, respectively, for the following reasons. (1) The essential role of insulin and bombyxin, both of which are secreted after food intake, is to convey information about the nutritional conditions of the body to the peripheral tissues. (2) IGF and BIGFLP are secreted to promote tissue growth under the control of specific hormones that are released at specific stages of development. (3) IGF and BIGFLP are mainly produced by homologous tissues, the liver and the fat body, respectively, and are also produced by other peripheral tissues. Thus, although the actions on target tissues may be the same, the physiological functions of the two hormones are distinct. It is likely that bombyxin serves as a link between food intake and larval growth, while BIGFLP plays a role in facilitating metamorphic growth under the control of the developmental signal, ecdysteroid. Both hormones may also regulate energy metabolism to support the growth of the animal. **Figure 3** compares the corresponding insulin-family peptides between *B. mori* and humans.

Despite striking functional similarities between insulin and bombyxin and between IGF and BIGFLP, it is unlikely that the ancestral insulin and IGF had already existed even prior to the divergence of protostomes and deuterostomes, because BIGFLP is much more similar to bombyxin than IGFs in its amino acid sequence. Both classes of insulin-family peptides may have evolved independently in vertebrates and insects (even in different groups of insects) from a single ancestral insulin/IGF peptide, as discussed by Okamoto et al. (2009b). In insects, this ancestral peptide may have been produced by various tissues including the brain and fat body but differentially regulated at the level of gene expression and/or of secretion in a tissue specific manner. We surmise that in the course of evolution of each insect group, gene duplications followed by divergence may have led to the brain-specific expression of an insulin-like gene(s) and to the fat body-specific expression of an IGF-like gene(s).

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Functional implications of *Drosophila* insulin-like peptides in metabolism, aging, and dietary restriction

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The neuroendocrine architecture and insulin/insulin-like signaling (IIS) events in *Drosophila* are remarkably conserved. As IIS pathway governs growth and development, metabolism, reproduction, stress response, and longevity; temporal, spatial, and nutrient regulation of *dilp*s encoding *Drosophila* insulin-like peptides (DILPs) provides potential mechanisms in modulating IIS. Of eight DILPs (DILP1–8) identified, recent studies have furthered our understanding of physiological roles of DILP2, DILP3, DILP5, and DILP6 in metabolism, aging, and responses to dietary restriction (DR), which will be the focus of this review. While the DILP producing IPCs of the brain secrete DILP2, 3, and 5, fat body produces DILP6. Identification of factors that influence *dilp* expression and DILP secretion has provided insight into the intricate regulatory mechanisms underlying transcriptional regulation of those genes and the activity of each peptide. Studies involving loss-of-function *dilp* mutations have defined the roles of DILP2 and DILP6 in carbohydrate and lipid metabolism, respectively. While DILP3 has been implicated to modulate lipid metabolism, a metabolic role for DILP5 is yet to be determined. Loss of *dilp2* or adult fat body specific expression of *dilp6* has been shown to extend lifespan, establishing their roles in longevity regulation. The exact role of DILP3 in aging awaits further clarification. While DILP5 has been shown associated with DR-mediated lifespan extension, contradictory evidence that precludes a direct involvement of DILP5 in DR exists. This review highlights recent findings on the importance of conserved DILPs in metabolic homeostasis, DR, and aging, providing strong evidence for the use of DILPs in modeling metabolic disorders such as diabetes and hyperinsulinemia in the fly that could further our understanding of the underlying processes and identify therapeutic strategies to treat them.

Keywords: *Drosophila* insulin-like peptides, insulin-like peptide producing cells, lifespan, metabolism, dietary restriction, dFOXO, dSir2

INTRODUCTION

Evolutionarily conserved insulin/insulin-like growth factor signaling (IIS) pathway governs growth and development, metabolism, reproduction, stress response, and longevity. In *Drosophila*, eight insulin-like peptides (DILPs) and one insulin receptor (DInR) are found. DILPs 1–8 have been identified mostly through their sequence homology to the mammalian insulin and the typical B-C-A domain structure as observed in mammalian insulin (Gronke and Partridge, 2010). Early biochemical studies confirmed tyrosine phosphorylation of the DInR stimulated by DILP2- or DILP5-containing conditioned media (Rulifson et al., 2002). Functional conservation of DILP5 was recently revealed where DILP5 binds to and activates the human insulin receptor in lowering circulating glucose levels (Sajid et al., 2011). Furthermore, altered expression of genes encoding DILP2, 3, 5, and 6 results in modulated IIS and profound metabolic and longevity consequences (Broughton et al., 2005, 2008; Gronke et al., 2010; Bai et al., 2012). In this review, we will discuss recent progress on our understanding of the diverse biological roles of DILPs in metabolic control, dietary restriction (DR), and lifespan, with a focus on DILPs 2, 3, 5, and 6 given available emerging research findings. Consistent with the broad and diverse physiological consequences of IIS, specific temporal, and spatial expression patterns of individual *dilps* suggest potentially specialized interactions between each DILP and the DInR. Furthermore, we will discuss the regulation, functional diversity, and redundancy of the DILPs as circulating peptides and the physiology of the tissues producing them. Recent discoveries of the involvement of the nutrient sensing fat body in controlling DILP secretion from insulin-like peptide producing cells (IPCs) in the brain has provided a physiological link between those two major tissues governing nutrient sensing, metabolism, and aging (Geminard et al., 2009; Bai et al., 2012; Rajan and Perrimon, 2012). Finally, we will discuss how DILPs are modulated under DR and how such regulation affects the lifespan of the organism.

NUTRIENT, TEMPORAL, AND SPATIAL REGULATION OF *dilp* EXPRESSION AND DILP SECRETION

More than a decade ago, the search for the extracellular ligands for the DInR led to the identification of seven *Drosophila insulin-like peptide* genes *(dilp*1–7) with diverse temporal and spatial specific

expression patterns (Brogiolo et al., 2001; Ikeya et al., 2002; Rulifson et al., 2002). The newest member *dilp8*, has recently been added to the family (Colombani et al., 2012; Garelli et al., 2012). During development, while *dilp2*, *dilp4*, and *dilp7* transcripts are detected in midgut and mesoderm during late-stage embryogenesis, transcripts of *dilp3*, *dilp5*, or *dilp6* are not detectable until larval stages (Brogiolo et al., 2001). In larvae, low levels of *dilp2* expression are detected in the imaginal discs whereas a high signal is measured in seven IPCs of each brain hemisphere and in salivary glands (Brogiolo et al., 2001). Similarly, *dilp5* transcripts, turned on in the second instar and *dilp3* transcripts measured in the mid to late third instar are both detected in the brain IPCs (Ikeya et al., 2002). Recent reports have revealed that *dilp5* is a transcriptional target of a synergistic interaction between Eyeless and Dachshund (Clements et al., 2008; Okamoto et al., 2012). *dilp6* is highly expressed in the larval fat body whereas low levels of its expression are detected in gut and brain (Slaidina et al., 2009). Recent reports demonstrated *dilp8* expression detected in larval imaginal discs (Colombani et al., 2012; Garelli et al., 2012). In the adult stage, expression of *dilps2*, *3,* and *5* but not *dilp1* is detected in IPCs (Broughton et al., 2005). In addition to its expression in IPCs, *dilp5* transcripts are also detected in follicle cells of stage 10 oocytes (Ikeya et al., 2002) and *dilp3* mRNA found in muscle cells of the midgut (Veenstra et al., 2008). Adult expression of *dilp4* is not known (Gronke and Partridge, 2010). Transcripts of *dilp6* are measured most abundantly in the adult fat body and at lower levels in head carcass and brain (Bai et al., 2012). Finally, transcripts of *dilp7* are detected in specific neurons of the ventral cord (dMP2) and several neurons in the brain (Miguel-Aliaga et al., 2008).

The critical roles of DILPs in animal development and energy homeostasis are evidenced by the fact that their expression is not only regulated temporally and spatially during development but also by nutrient status. As DInR activity is reduced following starvation, it was posited that this could be due to lack of DILPs under low nutrient availability (Britton et al., 2002). Indeed, upon starvation, expression levels of *dilp3* and *dilp5*, but not *dilp2* are reduced (Ikeya et al., 2002). A recent study demonstrated a role of dSir2, the *Drosophila* homolog of mammalian histone deacetylase SIRT1 in regulating the expression of *dilp2* and *dilp5* where systemic knockdown of *dSir2* up-regulates those two *dilps* (Banerjee et al., 2012). In addition, fat body-specific knockdown of *dSir2* is sufficient to up-regulate *dilp2* and *dilp5* expression with changes in *dilp3* transcript levels in those flies not reported (Banerjee et al., 2012, 2013). Finally, dSir2-mediated regulation of these two *dilps* is shown to act independently of dFOXO, a forkhead box-O transcription factor. Transcript levels of *dilp2* and *dilp5* were up-regulated in flies expressing both *dSir2 RNAi* and *dFOXO-TM* (constitutively active dFOXO) constructs in their fat body similar to the levels observed in *dSir2 RNAi* flies (Banerjee et al., 2013).

Investigation into the mechanism whereby the *Drosophila* ortholog for the mammalian neuropeptide Y (NPY), short neuropeptide F (sNPF) modulates metabolism and lifespan revealed an up-regulation of *dilp1* and *dilp2* mRNA as the result of sNPF overexpression accompanied by increased IIS in the periphery (Lee et al., 2008). As mammalian NPY positively regulates food intake, those results provide additional evidence linking nutrient status and *dilp* levels. A recent study by Yu et al. demonstrated that dCbl (Casitas B-lineage lymphoma), a member of *Drosophila* E3 ubiquitin ligases and adaptor proteins, negatively regulates the *expression of* brain *dilps*. Neuronal and IPC-specific knockdown of *dcbl* results in up-regulation of *dilps 2, 3, 5* whereas the Epidermal growth factor receptor (EGFR) signaling pathway mediates this regulatory effect of dCbl only on *dilps 2* and *3*. Thus, a likelihood of other mediators for *dilp5* is speculated (Yu et al., 2012).

Interestingly, unlike *dilp3* and *dilp5* whose expression levels are suppressed upon starvation, *dilp6* transcript levels are induced under nutrient deprivation and dFOXO is shown to modulate this response in larvae (Slaidina et al., 2009). During late larval and pupal stages when animals cease to feed, *dilp6* expression is strongly induced (Okamoto et al., 2009; Slaidina et al., 2009). As this high level of *dilp6* expression during the larval-pupal transition coincides with a surge of the hormone ecdysone, Slaidina et al. indeed demonstrated that *dilp6* transcription is induced by high levels of ecdysone in the fat body and is required for growth prior to metamorphosis (Slaidina et al., 2009). Fat body specific expression of dFOXO down-regulates *dilp2* which is mediated by DILP6 (Bai et al., 2012). Although basal levels of ecdysone regulates growth through dFOXO during larval development (Colombani et al., 2005), late stage larval expression of *dilp6* could be induced by ecdysone in *dFOXO RNAi* larvae, indicating that regulation of *dilp6* expression by ecdysone acts independently of dFOXO (Slaidina et al., 2009). In addition, *dilp6* also regulates the expression of *dilp5*, when over-expressed in the adult fat body (Bai et al., 2012).

MicroRNAs (miRNAs) play a prominent role in regulating insulin secretion in β-pancreatic cells (Poy et al., 2004). One such miRNA, miR-14 expressed in *Drosophila* IPCs systemically regulates fat levels. Using a reverse genetic approach, Varghese et al. detected reduced *dilp3* and *dilp5* mRNA levels in miR-14 mutant flies, which accompanied increased triglyceride levels (Varghese et al., 2010). Interestingly, the hyperlipidemic defect seen in miR-14 mutants was rescued by overexpressing *dilp3* implying that miR-14 regulates lipid metabolism through modulation of *dilp3* and also outlines a role for *dilp3* in this regard (Varghese et al., 2010). Another miRNA found in the fat body, miR-278 acts to improve insulin sensitivity. *miRNA-278* knockout flies had elevated transcript levels of *dilps2, 3, 5* and also had higher circulating levels of trehalose indicating a condition akin to insulin resistance (Teleman et al., 2006). The involvement of miRNAs in regulating insulin response in the fat body as well as *dilp* expression in IPCs provide exciting evidence for the complexity of selective *dilp* regulation that warrants further investigation.

There is a marked distinction between regulation of *dilp* expression and DILP secretion, as regulatory mechanisms exist in controlling the release of the DILPs. For example, while initial characterization of *dilp* expression pattern affected by diet conditions showed down regulation of *dilp3* and *dilp5* expression but not *dilp2* under starvation (Ikeya et al., 2002), recent availabilities of DILP antibodies made it possible to detect the accumulation of DILPs in IPCs as an indirect measure of DILP secretion. Interestingly, increased accumulation of DILP2 and DILP5 was measured in IPCs as the result of poor nutrient diet

or starvation despite unchanged *dilp2* mRNA levels (Geminard et al., 2009). Thus, understanding regulatory mechanisms that modulate DILP secretion should provide more physiological relevance of DILP action. Indeed, cell non-autonomous control of DILP secretion from the IPCs has been identified. NS3, a *Drosophila* nucleostemin family GTPase in the serotonergic neurons is shown to regulate DILP2 levels in the neighboring IPCs, establishing a possible communication between the two neuronal systems (Kaplan et al., 2008) and that an increased accumulation of DILP2 in IPCs and decreased peripheral insulin signaling measured in *ns3* mutants strongly suggested impairment in DILP2 secretion (Kaplan et al., 2008). Soon after this report, Geminard et al. demonstrated a distinct mode of long distance control network of DILP secretion between the fat body and IPCs (Geminard et al., 2009). In this study, it was elegantly demonstrated through *ex vivo* tissue co-culture experiments that the abdominal fat body, functionally homologous to mammalian liver and white adipose tissue and acting as a nutrient sensor, relays this information to brain IPCs by a hormonal signal that involves target of rapamycin (TOR) signaling (Geminard et al., 2009). This hormonal signal emanating from the larval fat body regulates the secretion of DILP2 and DILP5 from the brain IPCs according to the nutrition state (Geminard et al., 2009). Consistent with the notion that fat body relays this secretory signal to the IPCs through a hormone it releases, Unpaired 2 (Upd2), a cytokine produced by the fat body was recently shown to fulfill this role (Rajan and Perrimon, 2012). Upd2 senses the fed state and regulates DILP2 and DILP5 secretion from brain IPCs where under a fed state, there is less DILP accumulation in the IPCs indicating increased DILP secretion. As expected, flies with *upd2* knockdown exhibited increased DILP accumulation under a fed state, illustrating an inability of IPCs to respond to insulin demands (Rajan and Perrimon, 2012). Thus, Upd2 appears to be an important factor regulating DILP secretion from the IPCs.

A recent study by Bai et al. identified that DILP6, produced by the fat body could act as another regulatory factor directly or modulate other factors to affect levels of circulating DILP2 (Bai et al., 2012). Both *dilp2* transcripts in IPCs and circulating DILP2 peptides are reduced in flies overexpressing *dilp6* in the abdominal fat body. This effect appears to be specific to DILP2 as little change in circulating DILP5 levels was observed. Therefore, DILP6 cell non-autonomously decreases IIS by presumably serving as an adipokine or potentially regulating a downstream adipokine that represses *dilp2* expression in the brain IPCs and its secretion (Bai et al., 2012). This study has provided another important piece of evidence of how the abdominal fat body may influence systemic IIS by controlling DILP2 secretion from the IPCs.

Taken together, current studies suggest that temporal and spatial transcriptional regulation of major *dilps* is controlled by NS3, sNPF, ecdysone, and dFOXO in larval stages (**Figure 1**) whereas miRNAs, dFOXO, dSir2, Upd2, and dCbl are regulatory molecules involved in *dilp* transcriptional control in adult (**Figure 2**). Additional influences of nutrient status are likely to further contribute to differential regulation of *dilp2, 3, 5,* and *6*, which predicts diverse functionality of each DILP in mediating IIS under diverse physiological environments.

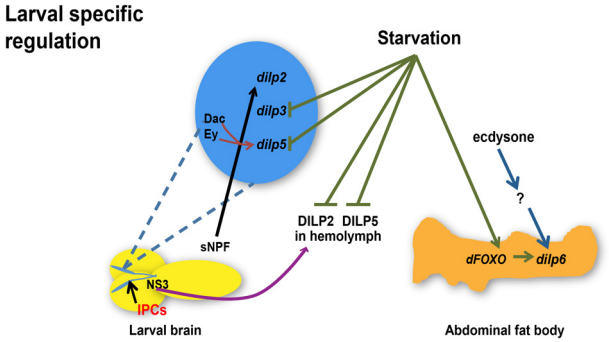

FIGURE 1 | Regulation of *dilp* expression and circulating DILP levels in response to nutrient status, transcriptional factors, hormone, and a neuropeptide during larval development. Starvation represses *dilp3* and *dilp5* expression and induces *dilp6* expression through dFOXO in the abdominal fat body whereas circulating DILP2 and DILP5 levels in the hemolymph are diminished. Ecdysone regulates *dilp6* during larval-pupal transition through unknown effectors. sNPF peptide secreted from sNPFnergic neurons located adjacent to IPCs increases *dilp2* expression. NS3 in serotonergic neurons positively regulates DILP2 secretion. Transcriptional factors Dachshund (Dac) and Eyeless (Ey) synergistically promote the expression of *dilp5*. *dilps,* genes encoding *Drosophila* insulin-like peptides; DILPs, *Drosophila* insulin-like peptides; IPCs, Insulin-like peptide producing cells; NS3, a nucleostemin family GTPase; sNPF, short neuropeptide F. Arrows indicate positive regulation whereas blunt-ended lines indicate negative regulation.

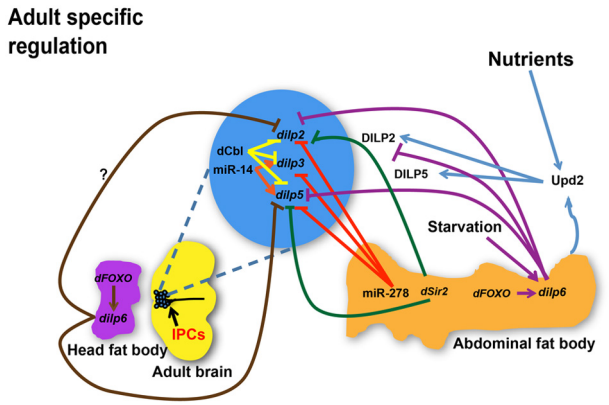

FIGURE 2 | Regulation of *dilp* expression and circulating DILP levels in response to nutrient status, micro RNAs (miRNAs), and transcriptional factors during adulthood. miR-278 down-regulates *dilps* expressed in the IPCs while miR-14 positively regulates *dilp3* and *dilp5* expression. dCbl in the IPCs down-regulates *dilps 2, 3,* and *5*. *dSir2* in the fat body represses expression of *dilp2* and *dilp5*. dFOXO acts through *dilp6* in the abdominal fat body to repress *dilp2* while it is not known if dFOXO in the head fat body regulates *dilp2* through a similar mechanism. *dilp6* in abdominal or head fat body tissues negatively regulates *dilp2* and *dilp5*. While *dilp6* only impairs hemolymph DILP2 levels, Upd2 senses the fed state and induces secretion of DILP2 and DILP5. *dilps,* genes encoding *Drosophila* insulin-like peptides; DILPs, *Drosophila* insulin-like peptides; IPCs, Insulin-like peptide producing cells; dCbl, a member of E3 ubiquitin ligases and adaptor proteins; *dSir2,* a histone deacetylase. Arrows indicate positive regulation whereas blunt-ended lines indicate negative regulation.

FUNCTIONS OF DILPs

Analogous to the opposing actions of insulin-secreting β-pancreatic cells and glucagon-secreting pancreatic islet α-cells in maintaining glucose homeostasis in mammals, IPCs and adipokinetic hormone (AKH)-producing corpora cardiaca (CC) cells are neurosecretory cells that function to regulate metabolic processes in the fly (Rulifson et al., 2002; Kim and Rulifson, 2004). Studies of neurosecretory network in larval brain have detected DILP2, but not *dilp2* mRNA in CC cells suggesting an undefined role of DILP2 in CC cells, away from the site of production, IPCs (Rulifson et al., 2002). Whether there is a similar movement of DILP3 or DILP5 from IPCs to CC cells remains to be determined. An interesting piece of evidence stems from the fact that adult IPCs respond to glucose or trehalose feeding as well as a K_{ATP} channel blocker with an increase in Ca^{2+} influx and membrane depolarization, which provides indirect evidence for the presence of ATP sensitive K^{+} channels in those neurosecretory cells similar to those found in β-pancreatic cells in mammals (Fridell et al., 2009).

The first set of compelling evidence demonstrating the functional extent of DILPs in controlling growth, development, and glucose homeostasis was generated by the destruction of IPCs. Ablation of IPCs during the early larval stage results in severe developmental delay with a reduction of both cell numbers and body size accompanied by an increased level of circulating sugars suggesting a diabetic-related phenotype (Rulifson et al., 2002). Importantly, a partial rescue of growth and circulating sugar phenotypes with *dilp2* overexpression strongly supported the notion that loss of DILP2 was responsible for the phenotypes (Rulifson et al., 2002). Ablation of IPCs in late larval stages results in a minor developmental delay and slightly decreased body size (Ikeya et al., 2002), reduced fecundity, higher energy stores of lipids and carbohydrates and an extended lifespan (Broughton et al., 2005). It was later demonstrated by Buch et al. that reduced fecundity was dissociated from the longevity effect, as flies with post-larval IPC ablation are long-lived on a protein-rich diet with normal fecundity (Buch et al., 2008). Similar to the larval effects on glucose homeostasis, adult-specific partial ablation of IPCs renders flies hyperglycemic and glucose intolerant but insulin sensitive as measured by peripheral glucose disposal upon insulin injection and serine phosphorylation of a key insulin-signaling molecule, Akt (Haselton et al., 2010). In addition, a significant increase in stored glycogen and triglyceride levels as well as an elevated level of circulating lipids was measured in adult IPC knockdown flies with an extended lifespan thus demonstrating that it is possible to modulate DILP action in adult flies to achieve lifespan extension without insulin resistance. With the development of an oral glucose tolerance test in the adult fly, this report documented that adult IPCs indeed are responsible for executing an acute glucose clearance response (Haselton et al., 2010). While this study clearly demonstrates profound metabolic and longevity phenotypes as the result of impaired DILP-producing IPCs in an adult-specific manner, it remains to be determined the specific involvement in metabolism and aging of each DILP produced in IPCs.

DISRUPTION OF DILPs IN LIFESPAN REGULATION

The role of IIS pathway in aging was first discovered when mutations of *daf-2*, a gene encoding the insulin receptor homolog in *C. elegans* nearly doubled the lifespan of the organism (Kenyon et al., 1993). Mutations disrupting IIS molecules such as DInR or the *Drosophila* homolog of the insulin receptor substrate CHICO similarly render cell non-autonomous effects in lifespan extension as the result of reduced IIS (Clancy et al., 2001; Tatar et al., 2001). Genetic manipulation of expression of additional IIS components such as overexpression of dFOXO in the abdominal or pericerebral fat body or dPTEN in the pericerebral fat body mimicking reduced IIS is sufficient to extend lifespan (Giannakou et al., 2004; Hwangbo et al., 2004). Likewise, partial ablation of IPCs, the production site of DILPs 2, 3, and 5 reduced IIS and recapitulated the longevity phenotype when starting in late larval stage (Broughton et al., 2005) or in an adult-specific manner (Haselton et al., 2010). However, the role of individual DILPs in controlling the aging process has proven difficult to ascertain due to functional redundancy and compensation among DILPs.

A significant amount of interest has been bestowed upon DILP2 as its transcript is most abundantly expressed among all *dilps* and DILP2 possesses the highest homology to the mammalian insulin with a 35% identity in protein sequence (Brogiolo et al., 2001). Down-regulation of *dilp2* is associated with lifespan extension under several conditions. First, activation of dFOXO in the pericerebral fat body extends lifespan with an accompanied reduction in *dilp2*, but not in *dilp3* or *dilp5* mRNA levels (Hwangbo et al., 2004). Second, upon JNK (Jun-N-terminal kinase) activation in the IPCs, dFOXO-dependent repression of *dilp2* is associated with the extension of lifespan observed in those flies (Wang et al., 2005). Third, expression of a dominant negative form of p53 in adult neurons extended lifespan and reduced *dilp2* transcript levels, again indicating that the reduction of *dilp2* expression was closely associated with extended longevity under those genetic conditions (Bauer et al., 2007). Although those results indicate a close association between decreased *dilp2* expression and increased lifespan, direct modulation of *dilp2* levels was needed to assess the causal relationship between *dilp2* expression and lifespan control. To this end, surprisingly, while causing a severe reduction of *dilp2* transcripts, targeted knockdown of *dilp2* in IPCs did not result in any lifespan extension (Broughton et al., 2008). But interestingly, an increase in *dilp3* and *dilp5* expression was observed in those flies raising the possibility that a compensatory mechanism exists to modulate overall *dilp* expression in the IPCs. However, this compensatory increase in *dilp3* and *dilp5* expression could not completely account for the lack of lifespan extension in *dilp2* knockdown flies as a similar increase of *dilp3* and *dilp5* transcripts was observed in long-lived *dilp2* null mutant flies and increased *dilp5* expression levels in long-lived *dilp2–3* mutants (Gronke et al., 2010). Thus, it remains possible that *dilp2* knockdown elicits additional genetic alterations neutralizing the effect on lifespan associated with reduced *dilp2* transcripts. The extended lifespan measured in *dilp2* null mutants, however, confirms a major role of DILP2 in longevity control. The absence of any change in lifespan in flies with a *dilp3* deletion is intriguing as both *dilp2* and *dilp5* transcript levels are lowered in those flies (Gronke et al., 2010). A lack of

consistent correlation between *dilp* transcript levels and lifespan effects in *dilp2, dilp2–3,* and *dilp3* null mutants requires further clarification with measurements of DILP peptide levels as possible compensatory mechanisms to modulate IIS and lifespan regulation. An involvement of DILP3 in longevity control is worth further investigation, however as *dilp3* transcript levels appeared to be specifically reduced in long-lived flies with increased mitochondrial uncoupling in adult IPCs (Fridell et al., 2009). While a *dilp5* null mutant appeared to have no effect on lifespan under standard diet (Gronke et al., 2010), *dilp5* levels are moderated in DR-mediated lifespan extension (Min et al., 2008) (discussed below). A *dilp6* loss-of-function mutation neither had any effect on adult *Drosophila* survival nor on any compensatory increase in the expression of other *dilps* (Gronke et al., 2010). On the other hand, Bai et al. recently showed that overexpressing *dilp6* in the adult abdominal fat body significantly extends lifespan in females in a diet-dependent manner and negatively affects expression of *dilp2* and *dilp5*, whereas a modest effect in survival is observed when *dilp6* is expressed in the pericerebral fat body. This study also shed light on the fact that the longevity effect of dFOXO when overexpressed in the pericerebral fat body requires *dilp6* (Bai et al., 2012). Taken together, creation of individual or combinatorial *dilp* mutants has confirmed lifespan extension as the result of *dilp2* deficiency suggesting a major role of DILP2 in modulating IIS. On the other hand, targeted expression of *dilp6* in the adult fat body results in profound longevity and metabolic consequences that underlies its role in lifespan regulation.

To aid a better understanding of the significance of DILP2 and DILP6, physiological alterations that accompanied lifespan extension in respective *dilp* mutants have paved the way. IPC ablated flies exhibit high levels of trehalose, lipid and glycogen stores, accompanied by increased stress resistance (Broughton et al., 2005). With respect to DILP2, the phenotypic changes as a result of its down-regulation were associated with higher trehalose storage levels and slight resistance to starvation (Broughton et al., 2008). Increased trehalose levels were also seen in a *dilp2* loss-of-function mutant with no change in lipid or glycogen levels (Gronke et al., 2010). Nevertheless, those findings imply a role for DILP2 in trehalose metabolism, which may explain a moderate starvation resistance in those flies. *dilp 1–4* loss of function mutants were starvation resistant recapitulating the role for *dilp2* in starvation resistance (Gronke et al., 2010). Surprisingly, neither *dilp2* null mutants nor *dilp 2–3, 5* deletion mutants, created by homologous recombination, were resistant to starvation (Gronke et al., 2010). The evidence that IPCs, independent of insulin signaling, mediate response to starvation (Mattaliano et al., 2007) could possibly account for the starvation resistance in IPC ablated flies (Broughton et al., 2005) and the lack of starvation response in *dilp2* null mutants. A putative role for DILP2 in response to oxidative stress was discovered in the context of JNK signaling upon oxidative stress where *dilp2* expression is repressed in IPCs (Wang et al., 2005). However, neither the *dilp2 RNAi* hypomorphs (Broughton et al., 2008) nor the *dilp2* loss-of-function mutants (Gronke et al., 2010) displayed any resistance to oxidative stress, excluding a direct role for DILP2 in response to oxidative stress. These studies thus, point to a role for DILP2 in trehalose metabolism, which could contribute to lifespan extension as the result of increased energy storage. While adult flies harboring *dilp6* over expression in the abdominal fat body exhibit metabolic phenotypes reminiscent of those seen as a consequence of reduced IIS (Bai et al., 2012), *dilp6* loss-of-function mutants only had high stored lipid levels revealing its specific role in lipid storage (Gronke et al., 2010). This is substantiated by the fact that DILP6 plays an important role in reallocating energy stores during the non-feeding pupal stage in preparation for metamorphosis (Slaidina et al., 2009).

Unlike DILP2 and DILP6, an involvement of DILP3 and DILP5 in any physiological feature that plays a part in lifespan regulation has not been identified. *dilp3* or *dilp5* single mutants were neither resistant to starvation or oxidative stress nor was there any change in their trehalose, glycogen, or lipid levels (Gronke et al., 2010), although *dilp3* overexpressors play a role in regulating triglyceride levels (Varghese et al., 2010).

MODULATION OF DILPs UNDER DIETARY RESTRICTION

Through dilution of nutrient content, DR is a robust intervention that has been shown to extend lifespan in *Drosophila*. While the exact molecular mechanisms behind DR-mediated lifespan extension are yet to be completely elucidated, several molecular pathways have emerged as important players involved in DR responses (Narasimhan et al., 2009). Within the scope of this review, we will discuss current understanding of the involvement of IIS cascades or DILPs in DR. Interestingly, in *C. elegans*, depending upon the methods of DR, lifespan extension associated with DR is largely independent of IIS (Kaeberlein et al., 2006; Lee et al., 2006; Bishop and Guarente, 2007; Smith et al., 2008). Similarly, IIS-dependent and IIS-independent mechanisms exist in DR-associated lifespan extension in *Drosophila* (Clancy et al., 2002; Min et al., 2008). While Clancy et al. demonstrated that long-lived *chico* mutants did not respond to optimal DR for additional lifespan extension indicating an overlap between IIS and DR (Clancy et al., 2002), Min et al. showed that *dFOXO* mutants remained sensitive to DR thus suggesting that DR acts independently of IIS (Min et al., 2008). A potential explanation for this discrepancy may be the different DR regimens used in those studies. An overall dilution in diet was used in (Clancy et al., 2002) whereas reducing yeast concentration to achieve DR was employed in Min et al. (2008). Thus, future studies should aim at standardizing DR conditions in *Drosophila* in order to reconcile discrepant findings as well as pinpoint a role of IIS in DR (Tatar, 2011).

There is emerging evidence on the role of IPCs in lifespan extension through DR, as those neurosecretory cells appear to respond to nutrient changes (Broughton et al., 2010). With regard to DILPs, DR conditions in *Drosophila* are shown to extend lifespan with changes in *dilp5* mRNA levels but not *dilp2* or *dilp3* levels (Min et al., 2008). Both *dilp5* mRNA and DILP5 protein levels are down-regulated in wild type flies under a yeast DR diet where only yeast is diluted while keeping carbohydrate levels constant (Broughton et al., 2010). Hence, DILP5 may serve as a central cue in understanding the molecular mechanisms behind DR.

Flies with *dilp5* knocked down by a UAS-*dilp3RNAi* construct that repressed expression of *dilps 2, 3,* and *5* and blocked

the nutrient-dependent expression of *dilp5*, exhibited a normal response to DR under a yeast DR regime implying that DR-mediated lifespan extension works independently of DILP5 (Min et al., 2008). Whereas overexpression of *dilp6* in the fat body lowers *dilp2* and *dilp5* expression as well as the respective hemolymph peptide levels, the lifespan of *dilp6* overexpressors was similar to the controls under a yeast restricted diet corroborating with the evidence that DR works independently of *dilp5* and DILP5 (Bai et al., 2012).

However, *dilp5* null mutant flies that displayed a normal DR response also exhibited a compensatory up-regulation of *dilp2* mRNA when raised on food with high yeast concentration while *dilp3* mRNA levels were up-regulated in these flies raised on food with relatively low yeast concentration (Gronke et al., 2010). Thus, it raises the possibility that compensatory transcriptional regulation could negate any change in lifespan in *dilp5* loss-of-function flies raised on yeast DR diet. Supporting evidence for the involvement of DILPs in DR, if not a direct role, was presented when *dilp 2–3, 5* deletion mutant flies (Gronke et al., 2010) or IPC ablated flies (Broughton et al., 2010) on yeast DR diet exhibit an atypical DR response. These results hint at a potential mechanism in DR involving DILPs as with dFOXO which is not required for DR *per se* but whose activity has shown to modulate DR response when over-expressed (Giannakou et al., 2008), a scenario for an indirect or a secondary role of DILP5 in DR remains possible. Alternatively, while *dilp5* is modulated under DR, this change in expression could simply be a response associated with dietary alterations but does not trigger the longevity effect of DR. Further clarification is required to definitively assign a physiological role of DILP5, if any, in DR response.

CONCLUSION AND OUTLOOK

DILPs are involved in a myriad of physiological processes ranging from growth (Brogiolo et al., 2001; Rulifson et al., 2002; Slaidina et al., 2009; Colombani et al., 2012; Garelli et al., 2012), metabolism (Broughton et al., 2008; Gronke et al., 2010; Bai et al., 2012), to lifespan (Broughton et al., 2005; Gronke et al., 2010; Bai et al., 2012) (**Figure 3**). This review that has focused on DILPs 2, 3, 5, and 6 has highlighted some of the regulatory mechanisms governing their expression and secretion, and their functions pertaining to lifespan regulation as well as the controversy surrounding the role of DILPs in DR. Compensatory transcriptional regulatory mechanisms and functional redundancy that exist among DILPs make it difficult to dissect out their individual roles. A similar functional redundancy is observed where the *Drosophila* homolog of IGF-binding protein, Imp-L2 is shown to bind to DILP2 (Honegger et al., 2008), although DILP6 is most similar in structure to vertebrate IGF (Okamoto et al., 2009). Nonetheless, genetic approaches that have targeted tissue specific expression or disruption of individual *dilp*s have confirmed that loss of *dilp2* and over-expression of *dilp6* is sufficient to extend lifespan (Gronke et al., 2010; Bai et al., 2012). Although a direct role for DILP5 in DR-mediated lifespan extension remains controversial, its involvement cannot yet be entirely excluded. In addition, information garnered from *Drosophila* as a model to study cross talk between the nutrient sensing fat body and the neurosecretory IPCs has shed significant insight into a systemic

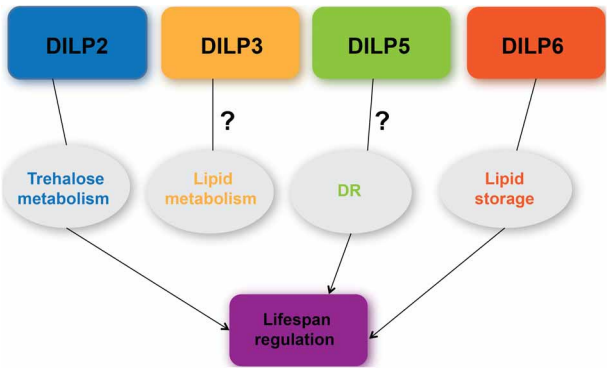

FIGURE 3 | A current understanding of the roles of DILPs 2, 3, 5, 6 in metabolism, DR response, and lifespan regulation.

control of DILP activities as the result of communication between those two tissues tightly associated with metabolism. The studies highlighted in this review have underscored the importance of measuring DILP levels in order to substantiate and validate their functional significance. Specifically, measuring circulating DILPs in the hemolymph should provide most relevant assessment on secreted DILP levels and their systemic effects (Bai et al., 2012).

Ablation of IPCs or deletion of *dilps 1–5* mimics phenotypes seen in type 1 diabetes (Rulifson et al., 2002; Zhang et al., 2009) while insulin resistance seen in flies fed a high sugar diet are associated with modulated *dilp* levels in modeling type 2 diabetes (Musselman et al., 2011; Morris et al., 2012). Thus, through genetic modulation of *dilps* in *Drosophila*, metabolic disorders such as diabetes, hyperinsulinemia, or those affecting glucose homeostasis can be modeled in this genetic organism. These approaches will likely further characterize the molecular mechanisms behind these disorders, discover drug targets, and screen potential therapeutic modes to treat these disorders. Apart from disease models, emerging research has revealed an involvement of DILPs in stem cell biology including stem cell proliferation (LaFever and Drummond-Barbosa, 2005; Sousa-Nunes et al., 2011), reactivation of neural stem cells from their quiescent stage (Chell and Brand, 2010) and germ-line stem cell maintenance (Hsu and Drummond-Barbosa, 2009). Recent findings on the differential expression of *dilp8* in tumor eye discs that responds to signals from peripheral tissues to mediate their growth and development has further strengthened *Drosophila* as a model for investigating mechanisms underlying inter-organ communication and demonstrated a role for DILP8 in cancer biology (Garelli et al., 2012). Overall, DILPs, as outlined in the review, contribute to growth and development, metabolic homeostasis, and longevity regulation in *Drosophila* through diverse mechanisms that are being unraveled.

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Insulin signaling pathways in lepidopteran ecdysone secretion

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Molting and metamorphosis are stimulated by the secretion of ecdysteroid hormones from the prothoracic glands. Insulin-like hormones have been found to enhance prothoracic gland activity, providing a mechanism to link molting to nutritional state. In silk moths (*Bombyx mori*), the prothoracic glands are directly stimulated by insulin and the insulin-like hormone bombyxin. Further, in *Bombyx*, the neuropeptide prothoracicotropic hormone (PTTH) appears to act at least in part through the insulin-signaling pathway. In the prothoracic glands of *Manduca sexta*, while insulin stimulates the phosphorylation of the insulin receptor and Akt, neither insulin nor bombyxin II stimulate ecdysone secretion. Involvement of the insulin-signaling pathway in *Manduca* prothoracic glands was explored using two inhibitors of phosphatidylinositol-3-kinase (PI3K), LY294002 and wortmannin. PI3K inhibitors block the phosphorylation of Akt and 4EBP but have no effect on ecdysone secretion, or on the phosphorylation of the MAPkinase, ERK. Inhibitors that block phosphorylation of ERK, including the MEK inhibitor U0126, and high doses of the RSK inhibitor SL0101, effectively inhibit ecdysone secretion. The results highlight differences between the two lepidopteran insects most commonly used to directly study ecdysteroid secretion. In *Bombyx*, the PTTH and insulin-signaling pathways intersect; both insulin and PTTH enhance the phosphorylation of Akt and stimulate ecdysteroid secretion, and inhibition of PI3K reduces ecdysteroid secretion. By contrast, in *Manduca*, the action of PTTH is distinct from insulin. The results highlight species differences in the roles of translational regulators such as 4EBP, and members of the MAPkinase pathway such as ERK and RSK, in the regulation of insect ecdysone secretion, and in the impact of nutritionally-sensitive hormones such as insulin in the control of ecdysone secretion and molting.

Keywords: insulin, ecdysteroids, *manduca sexta*, insects, prothoracic gland, prothoracicotropic hormone

INTRODUCTION

Secretion of the steroid hormone, ecdysone, from the insect prothoracic glands triggers molting and metamorphosis. Insect insulin-like hormones have been implicated in the regulation of prothoracic gland activity, linking nutritional state and ecdysis. As in vertebrates, insect insulin-like hormones activate a tyrosine-kinase-linked receptor, resulting in the activation of phosphatidylinositol kinase (PI3K), protein kinase B/Akt (Akt), and target-of-rapamycin (TOR). TOR enhances protein synthesis through the phosphorylation and inactivation of an inhibitory binding protein for initiation factor 4E (4EBP), and the phosphorylation and activation of ribosomal S6 kinase. In *Drosophila*, genetic manipulation of the insulin-signaling pathway strongly implicates insulin-like hormones in prothoracic gland activity. In *Drosophila*, overexpression of PI3K increases gland size and transcription of ecdysone target genes, as well as the steroidogenic genes *phantom* and *dib* (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005). Overexpression of PTEN, a lipid phosphatase that counteracts the effects of PI3K, or expression of dominant negative PI3K have the converse effect. Treatments that specifically increase glandular size through insulin signaling result in premature metamorphosis and small adults, while those that reduce glandular size lead to extended feeding and an increase adult body size, suggesting that the prothoracic glands, through response to insulin, serve as size-sensors in developing insects (Caldwell et al., 2005; Colombani et al., 2005; Mirth et al., 2005).

Insulin-like hormones have been found to increase ecdysone secretion by isolated prothoracic glands from the silk moth *Bombyx mori* (Kiriishi et al., 1992; Gu et al., 2009), and the bug, *Rhodnius prolixus* (Vafopoulou and Steel, 1997). However, ecdysone secretion by the prothoracic glands in *Manduca* is not

Abbreviations: 4EBP, binding protein for initiation factor 4E; Akt, protein kinase B/Akt; BE, brain extract; cAMP, cyclic adenosine monophosphate; DMSO, dimethyl sulfoxide; ERK, extracellular signal-regulated kinase; GRB2, growth factor receptor-bound protein protein 2; HRP, horseradish peroxidase; IGF, insulin-like growth factor; IR, insulin receptor; LY, LY294002, PI3K inhibitor; MAPK, mitogen-activated protein kinase; MEK, MAP kinase kinase; PAGE, polyacrylamide gel; PI3K, phosphatidylinositol-3-kinase; PLC, phospholipase C; PTEN, phosphatase and tensin homolog, a lipid phosphatase; PTTH, prothoracicotropic hormone; RAF, rapidly activated fibrosarcoma (MAP kinase kinase kinase); RAS, rat sarcoma protein; RSK, ribosomal S6 kinase; SDS, sodium dodecyl sulfate; SL0101, RSK inhibitor; SOS, son of sevenless protein; StAR, steroidogenic acute regulatory protein; START1, putative cholesterol transporter; TOR, target-of-rapamycin; TRS, Torso receptor substrate; U, U0126, MEK inhibitor.

stimulated by insulin (Walsh and Smith, 2011). Nonetheless, *Manduca* prothoracic glands appear to be insulin-responsive: insulin stimulates autophosphorylation of the insulin receptor (IR) and the phosphorylation of Akt, and prothoracic glands from nutritionally deprived *Manduca* show changes in the insulin-signaling pathway including upregulation of the IR (Walsh and Smith, 2011). Further, feeding of the TOR inhibitor rapamycin leads to smaller prothoracic glands, mimicking nutritional deprivation and delaying molting (Kemirembe et al., 2012).

In the present study, we further pursued the signaling pathways stimulated by insulin in *Manduca* to identify possible differences in the effects of insulin-like hormones on ecdysone secretion, focusing on probable sites of cross-talk between insulin-like hormones and PTTH. Like insulin, PTTH stimulates a tyrosine-kinase linked receptor. The PTTH receptor is known as Torso, first characterized in *Drosophila* embryos (Casanova and Struhl, 1989; Li, 2005; Rewitz et al., 2009). Unlike insulin, PTTH increases intracellular levels of cyclic AMP, through a PTTH-stimulated increase in intracellular calcium and the activation of a calcium-sensitive adenylyl cyclase (Smith et al., 1984, 1985; Meller et al., 1988; Dedos et al., 2005, 2007; Fellner et al., 2005). Increased intracellular calcium also results in activation of mitogen-activated protein kinases (MAPkinases) including MEK and ERK (Rybczynski and Gilbert, 2003). Inhibition of MEK effectively blocks PTTH-stimulated ecdysone secretion in *Bombyx* and *Manduca* (Rybczynski and Gilbert, 2003; Gu et al., 2010). In *Drosophila*, inactivation of the MAPkinase signaling pathway inhibits prothoracic gland activity and the action of Torso (Caldwell et al., 2005; Rewitz et al., 2009).

Protein synthesis is a key feature of prothoracic gland activation by PTTH. PTTH enhances translation, and inhibitors of translation block PTTH-stimulated ecdysone secretion (Keightley et al., 1990; Gilbert et al., 1997). In keeping with an effect of PTTH on translation, the phosphorylation of a 34 kDa protein characterized as ribosomal protein S6 is stimulated by PTTH, calcium, or cAMP analogs (Smith et al., 1986; Song and Gilbert, 1994, 1995). Similarly, in vertebrates, protein synthesis is required for ecdysone secretion (Keightley et al., 1990; Stocco and Clark, 1996). In vertebrates, a critical translation-dependent event is the synthesis of a short-lived protein known as steroidogenic acute regulatory protein (StAR). StAR activates cholesterol transfer from the outer to the inner mitochondrial membrane, enhancing steroid hormone synthesis (Stocco and Clark, 1996). Proteins with START1 domains, homologous to vertebrate StAR, have been found in insects, though their roles in ecdysone secretion are not clear (Roth et al., 2004; Sakudoh et al., 2005). It is likely that cholesterol mobilization of some type also underlies insect steroid hormone secretion (Lafont et al., 2011).

Studies by Gu et al. have characterized cross talk between the PTTH and insulin pathways in *Bombyx mori*. In this insect, PTTH stimulates the phosphorylation of Akt, suggestive of the activation of PI3K by PTTH (Gu et al., 2011b, 2012). Insulin, after 8 h exposure, also stimulates ecdysone secretion, particularly after the 4th day of the last larval instar. Inhibition of PI3K with wortmannin or LY294002 reduces basal and insulin- or PTTH-stimulated secretion. Inhibition of ecdysone secretion is associated with reduction in the phosphorylation of 4EBP, suggesting reduced protein synthesis. Further, the inhibition of ecdysone secretion by PI3K inhibitors occurs without a reduction in the phosphorylation of ERK, indicating that PTTH in *Bombyx* calls into play two required signaling pathways (Gu et al., 2011a).

The present study was undertaken to delineate points of intersection in *Manduca* prothoracic glands between PTTH and insulin. The results highlight a critical role for MAPkinases in Manduca in ecdysone secretion by a pathway distinctly independent from that stimulated by insulin.

MATERIALS AND METHODS

ANIMALS

M. *sexta* eggs were obtained from Carolina Biological Supply (Burlington, NC) or from adults raised from this stock. Larvae were reared on an artificial diet (Bell, 1976) at 25°C under a photoperiod of 16 h-light/8 h-dark. Feeding fifth instar larvae were used in experiments (4–7 g, days 2–3).

REAGENTS AND HORMONES

Grace's insect culture medium was obtained from Invitrogen. LY294002 and wortmannin, obtained from BioMol, U0126 from Calbiochem, and SL0101 from Toronto Research Chemicals, were prepared as stock solutions in DMSO and diluted in Grace's for use in experiments. Phosphopeptide and secondary antibodies were obtained from Cell Signaling Technology (phosphoAkt *Drosophila* Ser505; phosphoIGF-1receptor Tyr1135/1136; phosphoRSK; phospho4EBP; HRP-labeled anti-rabbit or anti-mouse secondary antibody), or Santa Cruz Biotechnology (phospho-ERK). The location of these proteins in the insulin- and PTTH-signaling pathways is diagrammed in **Figure 9**.

Recombinant PTTH and synthetic bombyxin II were generous gifts of Drs. Hiroshi Kataoka and Dr. Shinji Nagata (University of Tokyo). Human recombinant insulin solution was used (Sigma, 10 mg/ml), diluted into Grace's medium. *Manduca* brain extracts were prepared in our lab, using frozen day 0 pupal brains. Because we were using a crude extract, brains were initially homogenized in acetone to remove compounds that might interfere with the radioimmunoassay. This is the same first step used in preparing *Bombyx* brains for subsequent purification of bombyxin (Nagasawa et al., 1984). The homogenate was briefly centrifuged at 1000 × g and the pellet was then subjected to extraction by homogenizing in cold 2% NaCl, cooling on ice, centrifuging for 10 min at 10,000 × g, again, similar to the initial preparation of *Bombyx* bombyxins. The pellet was extracted one additional time in 2% NaCl, and the combined supernatants stored at −20°C. Insulin-like hormones can multimerize (see for example, Pandyarajan and Weiss, 2012), so to retain as broad a spectrum of insulin-like proteins as possible, we chose not to subject the homogenate to further filtration, as would be done to isolate PTTH (Walsh and Smith, 2011). Instead centrifugation was used to clarify the extract, as was done for preparations of bombyxin-containing brain extracts by Nijhout et al. (2007).

PROTHORACIC GLAND INCUBATIONS AND ECDYSONE RADIOIMMUNOASSAYS

Larval prothoracic glands were dissected into lepidopteran saline and maintained in Grace's medium for periods of less than 1 h

prior to experimentation. Individual glands were pre-incubated in standing droplets of culture medium, with or without hormones or inhibitors, for indicated periods of time. Experiments were terminated by placing glands directly into 2X SDS-sample buffer for Western blotting, described below. Medium was removed and stored at −20°C for ecdysone RIAs, conducted as previously described (Warren et al., 1984). The ecdysone antibody was produced in rabbits against an ecdysone-22-succinyl thyroglobulin synthesized by Dr. D.H.S. Horn (C.S.I.R.O., Canberra, Australia).

WESTERN BLOTS

Larval prothoracic glands were incubated in test or control solutions for the designated incubation periods, placed into SDS-sample buffer, and boiled for 3–5 min. Details of Western blot procedures are the same as those described previously (Smith et al., 2003). Samples were run on 8 or 13.5% SDS-PAGE gels to separate proteins. The samples were then transferred from gels to nitrocellulose membranes at 4°C for 75–90 min. The membranes were incubated with primary antibody overnight, then rinsed and treated with appropriate secondary antibody for 75 min, rinsed again, and bands visualized with Western blotting luminol reagents (Pierce ECL Western blotting substrate). The blots were exposed on chemiluminescence film (Marsh BioProducts Blue Film, carried by AbGene) and developed (Kodak GBX fixer and developer).

STATISTICS

Statistical analysis was performed using a One-Way ANOVA with a Tukey-Kramer *post-hoc* test to compare specific differences. For the insulin and bombyxin stimulation experiments, a paired *t*-test was used. In all cases, $p < 0.05$ was used to determine significance.

RESULTS

We have shown in previous experiments that *Bombyx* bombyxin II and *Manduca* brain extract stimulate the phosphorylation of signaling proteins in the insulin pathway in developing wing discs, including the phosphorylation of IR and Akt (Nijhout et al., 2007; Walsh and Smith, 2011). In the present study, we compared phosphoproteins stimulated by insulin with those stimulated by recombinant PTTH in the prothoracic glands. We used as our experimental tissue prothoracic glands removed from day 2–3 of the fifth instar, when the glands are sensitive to PTTH but not yet secreting high levels of steroid. As shown in **Figure 1**, insulin and PTTH elicit distinctly different patterns of phosphoprotein activation. PTTH enhances the phosphorylation of RSK, ERK, and 4EBP. By contrast, insulin enhances the phosphorylation of the IR and Akt. Ecdysteroid assays indicate that insulin does not stimulate ecdysteroid secretion on its own, at doses ranging from 6 nM to 6 uM (**Figure 2**), nor does it augment the effects of PTTH (**Figure 1**). Previous experiments had shown that insulin does not stimulate glands removed from day 5 (wandering) larvae (Walsh and Smith, 2011).

Prothoracic glands were also tested with *Bombyx* bombyxin II (generously provided by Drs. Hiroshi Kataoka and Shinji Nagata).

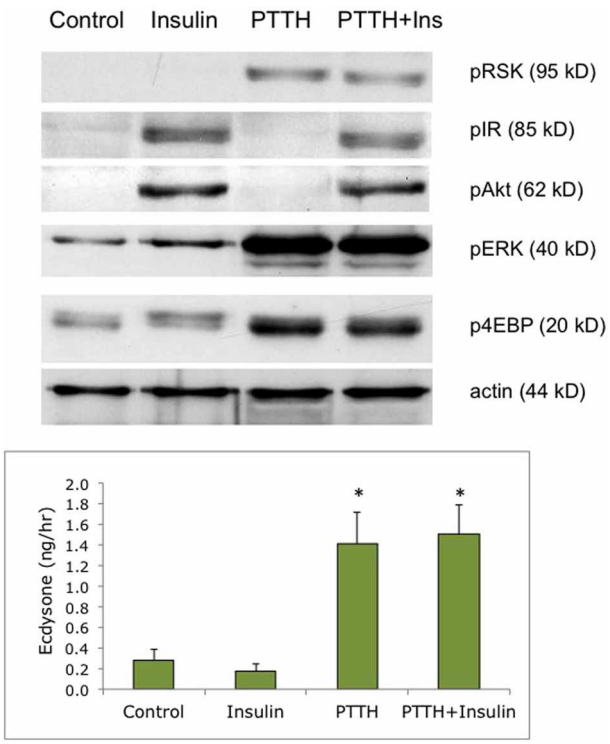

FIGURE 1 | Effects of PTTH and insulin on signaling phosphoproteins. Prothoracic glands from day 2 fifth instar larvae were incubated for 20 min medium with or without insulin (6 uM), followed by 80 min in one of the following treatments: no hormone (Control), insulin (6 uM), PTTH (7 nM), or insulin + PTTH. (TOP) Western blots of cellular lysates, probed for phosphoproteins; (BOTTOM) Ecdysone secretion determined by radioimmunoassay of culture medium at the end of the 80 incubation period. Asterisks denote statistically significant ecdysone secretion relative to no-hormone controls ($n = 5$ glands per group). Insulin did not enhance secretion, alone or in conjunction with PTTH (ANOVA, Tukey–Kramer *post-hoc* test, significance $p > 0.05$).

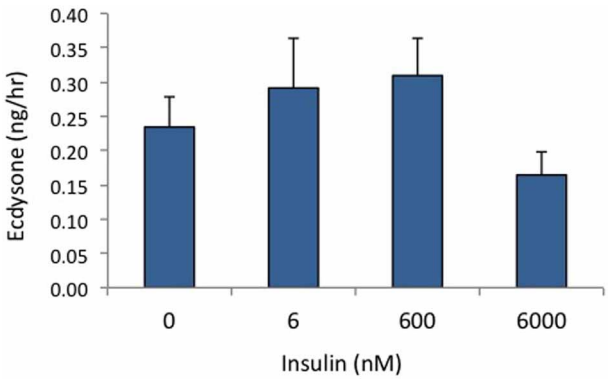

FIGURE 2 | Effects of insulin on ecdysone secretion. Prothoracic glands from day 2 fifth instar larvae were incubated for 80 min in designated concentrations of insulin, and culture medium was assayed for ecdysone ($n = 9$–14 glands per group). None of the insulin-treated groups differed from controls (ANOVA, Tukey–Kramer *post-hoc* test, $p > 0.05$).

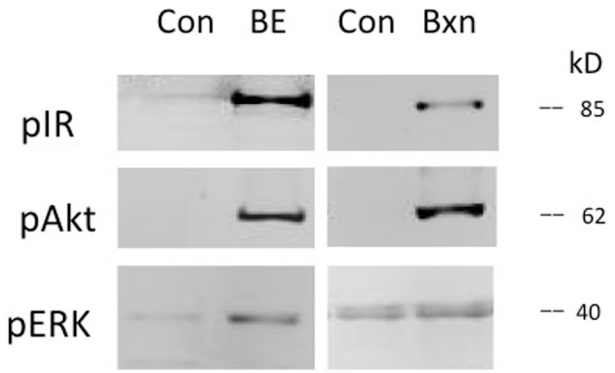

FIGURE 3 | Effects of *Manduca* brain extract and *Bombyx* bombyxin II on signaling phosphoproteins. Prothoracic glands from day 2 fifth instar larvae were incubated for 30 min in Grace's medium (Control), medium containing crude *Manduca* brain extract (BE, 0.5 brain equivalents/30 ul) with both PTTH and insulin-like activities, or medium containing synthetic *Bombyx* bombyxin II (Bxn, 160 nM). At the end of the incubation period cellular lysates were probed for phosphoproteins via Western blots.

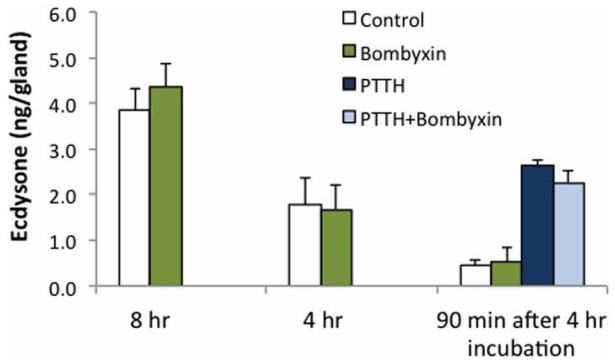

FIGURE 4 | Effects of *Bombyx* bombyxin II on ecdysone secretion. Prothoracic glands from day 3 fifth instar larvae were incubated for 4 or 8 h in medium containing no hormone (Control) or *Bombyx* bombyxin II (160 nM) ($n = 6$ and 11 glands per group, respectively). In separate experiments, glands were incubated for 4 h in no hormone or 160 nM bombyxin, and then challenged for 90 min in medium with no hormone, bombyxin, PTTH (7 nM) or PTTH + bombyxin ($n = 16$ glands per group). Culture medium was assayed for ecdysone by radioimmunoassay. None of the bombyxin-treated groups differed from their respective controls (ANOVA, Tukey–Kramer *post-hoc* test, $p > 0.05$).

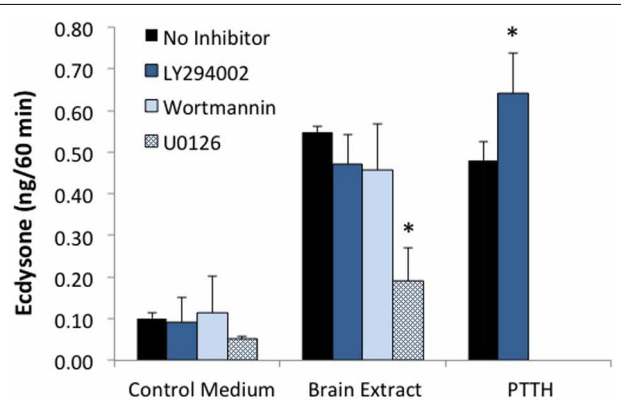

FIGURE 5 | Effects of PI3K inhibitors (LY294002 and wortmannin), and MEK inhibitor (U0126) on ecdysone secretion. Prothoracic glands from day 3 fifth instar larvae were incubated for 20 min in medium with or without the designated inhibitor, followed by 60 min in one of the following treatments: No Inhibitor (Grace's medium); LY294002 (60 uM); Wortmannin (10 uM); U0126 (10 uM); Brain Extract (0.5 brain equivalents/30 ul); Brain Extract + LY294002; Brain Extract + Wortmannin; Brain Extract + U0126; PTTH (7 nM); or PTTH + LY294002. At the end of the 60 min incubation period, culture medium was assayed for ecdysone by radioimmunoassay. Ecdysone secretion by groups treated with U0126 was significantly lower than by groups in the respective no-inhibitor controls (asterisks) (ANOVA, Tukey–Kramer *post-hoc* test, significance $p < 0.05$). Sample sizes were as follows (glands per group): Control = 51; BE = 38; LY294002 = 36; BE + LY294002 = 27; U0126 = 4; BE + U0126 = 5; Wortmannin = 10; BE + Wortmannin = 12; PTTH = 19; PTTH + LY = 19.

Bombyxin II is 41% identical to *Manduca* bombyxin (GenBank AAY84557.1, UniProt Q4JJX8_MANSE). Purified or recombinant forms of *Manduca* bombyxin are not currently available. Over 30 bombyxin genes have been cloned in *Bombyx* (Mizoguchi and Okamoto, 2013), hence it is likely that *Manduca* also possesses more than one type of bombyxin. We tested a crude *Manduca* brain extract, casting a wide net for *Manduca* endogenous insulin-like hormones. As shown in **Figure 3**, bombyxin II and *Manduca* brain extract, like insulin, stimulate phosphorylation of the IR and Akt. For this reason, we believe that the brain extract contains insulin-like activity. Because brain extract also contains PTTH, ERK is phosphorylated in response to brain extract (**Figure 3**), and brain extract stimulates ecdysone secretion (**Figure 5**). Bombyxin does not stimulate ERK phosphorylation (**Figure 3**), nor does it stimulate ecdysone secretion, even following prolonged exposure (**Figure 4**). Further, we do not observe that bombyxin increases responsiveness to PTTH (**Figure 4**).

A critical step in the action of insulin in both insects and vertebrates is the activation of PI3K. This kinase is inhibited by LY294002 or wortmannin. As seen in **Figure 5**, PTTH- and brain-extract-stimulated ecdysteroid secretion are unaffected by PI3K inhibitors. The efficacy of the PI3kinase inhibitors was confirmed by the absence of Akt phosphorylation (**Figure 6**). Hence, while brain extract does contain insulin-like ligands (evidenced by phosphorylation of the IR), if any of these ligands stimulate ecdysone secretion, neither they, nor PTTH, do so through PI3K. By contrast, the MEK (MAP kinase kinase) inhibitor U0126 inhibits basal and hormone-stimulated ecdysteroid secretion.

The effects of inhibitors on protein phosphorylation were examined in brain extract-treated samples. Because the extract contains both PTTH and insulin-like hormones, multiple phosphoproteins could be visualized including pRSK, pERK, and p4EBP (stimulated by PTTH in the extract) and pAkt (stimulated by bombyxin in the extract) (**Figure 6**). The MEK inhibitor U0126 blocks the phosphorylation of ERK and RSK, has no significant effect on Akt, and on its own slightly stimulates the phosphorylation of 4EBP. By contrast, the PI3K inhibitor LY294002 has no effect on the phosphorylation of ERK or RSK, but completely blocks the phosphorylation of Akt. LY294002 also blocks basal and hormone-stimulated phosphorylation of 4EBP.

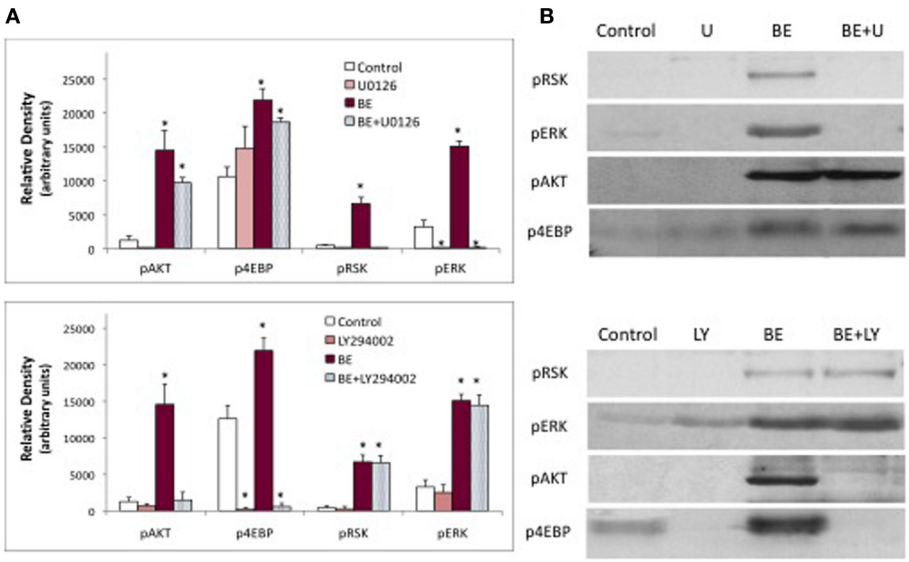

FIGURE 6 | Effects of PI3K inhibitor (LY294002) and MEK inhibitor (U0126) on protein phosphorylation. Western blots were prepared from prothoracic glands described in **Figure 5**, and probed for phosphoproteins. Treatments were as follows: no hormone (Control), 10 uM U0126 (U), 60 uM LY294002 (LY), brain extract (BE, 0.5 brain equivalents/30 ul), brain extract + U0126 (BE + U) or brain extract + LY294002 (BE + LY). **(A)** Results of a typical experiment. **(B)** Quantification of Western blots. Asterisks indicate groups that were statistically different than Controls (ANOVA, Tukey–Kramer *post-hoc* test, $p < 0.05$). $n = 4$–16 samples per group.

The inhibition of ecdysone secretion by a MEK inhibitor, but not by a PI3K inhibitor, indicates that in *Manduca*, the MAPkinase pathway is a more critical mediator of hormone-stimulated ecdysone secretion than PI3K. An important downstream enzyme in the MAPkinase pathway is RSK (Romeo et al., 2012), hence a RSK inhibitor, SL0101, was tested for its effects on ecdysteroid secretion. SL0101 targets the kinase domain of RSK (Smith et al., 2005). The inhibitor would be expected to inhibit phosphorylation events downstream of RSK itself, which, unfortunately, we did not have a means of visualizing. We found that 10, 25, and 50 uM doses of SL0101 actually increased basal ecdysone secretion, and a 25 uM dose significantly enhanced hormone-stimulated secretion (**Figure 7**). The reason for a stimulatory effect of RSK inhibition is not clear. By contrast, 100 and 200 uM doses of the RSK inhibitor significantly inhibited ecdysone secretion (**Figure 7**). However, as shown in **Figure 8**, 100 and 200 uM SL0101 also significantly reduced the phosphorylation of ERK, and 200 uM SL0101 reduced the phosphorylation of RSK, suggesting that the inhibitor at high doses may be acting non-specifically as a MEK inhibitor.

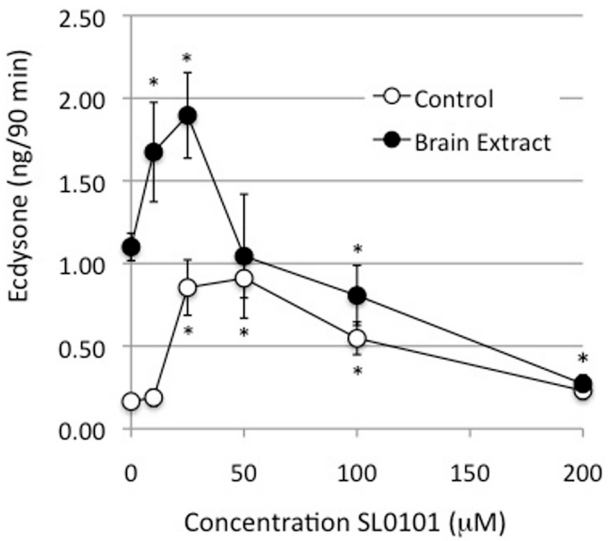

FIGURE 7 | Effects of RSK inhibitor (SL0101) on ecdysone secretion. Prothoracic glands from day 3 fifth instar larvae were incubated for 20 min in medium with or without the designated doses of SL0101, followed by 90 min in designated doses of SL0101 with or without brain extract (0.5 Brain equivalents/30 μl). At the end of the incubation period culture medium was assayed for ecdysone by radioimmunoassay. Ecdysone secretion by groups treated with 10, 25, or 50 μM SL0101 was statistically higher than groups incubated in the absence of inhibitor. Ecdysone secretion by groups treated with brain extract, and 100 or 200 uM SL0101, was significantly lower than groups treated with brain extract in the absence of inhibitor. Asterisks = significant differences from groups incubated in the absence of inhibitor (ANOVA, Tukey-Kramer *post-hoc* test, $p < 0.05$). $n = 8$–21 glands per SL0101-treated group; Controls = 53 glands per group; BE = 53 glands per group.

DISCUSSION

The present study confirms a lack of direct stimulation of insulin-like hormones on *Manduca* ecdysone secretion, as seen in an earlier study (Walsh and Smith, 2011). Our results do not rule out a role for insulin in regulating *Manduca* prothoracic gland function. We have previously shown that glands from nutritionally starved larvae exhibit increased transcription of IR and cellular content of 4EBP, in keeping with a resulting enhanced sensitivity to insulin. Indeed, injection of insulin reduces total 4EBP levels in the prothoracic glands (Walsh and Smith, 2011). However, insulin injection does not increase ecdysone secretion (Walsh and

Smith, 2011), and neither insulin nor bombyxin augment prothoracic gland sensitivity to PTTH. It is possible, however, that a bombyxin isoform not tested in the present study has a stimulatory effect or potentiating effect on *Manduca* prothoracic gland secretory activity. Such a factor would need to activate the prothoracic glands in some manner beyond the IR and Akt, which do not alone appear to stimulate ecdysone secretion by *Manduca* prothoracic glands.

In *Bombyx*, insulin does directly stimulate ecdysone secretion, as do high doses of bombyxin (Kiriishi et al., 1992; Gu et al., 2009). Further, unlike *Manduca*, both PTTH and insulin stimulate the phosphorylation of Akt in *Bombyx* prothoracic glands, and the activation of PI3K is necessary for ecdysone secretion (Gu et al., 2011a, 2012). Like *Manduca*, MAPkinases are activated by *Bombyx* PTTH, and MEK inhibitors reduce PTTH-stimulated ecdysone secretion (Gu et al., 2010). However, inhibition of PI3K blocks ecdysone secretion even when ERK is activated. Further, insulin can activate the prothoracic glands of *Bombyx* in the absence of ERK phosphorylation (Gu et al., 2009). We have outlined presumptive signaling pathways for PTTH and insulin in lepidopteran prothoracic glands in **Figure 9**. It appears that in *Bombyx*, unlike *Manduca*, stimulation of PI3K and TOR, through the action of either PTTH or insulin, are requisite steps in ecdysone secretion.

Our results point strongly to an essential role in *Manduca* for the MAPkinase pathway in ecdysone secretion. This is schematized in **Figure 9**, in which only ERK activation directly stimulates *Manduca* ecdysone secretion, and activation of PI3K and Akt are not required for ecdysone secretion. In *Bombyx*, in which insulin stimulates ecdysone secretion in an apparent absence of ERK activation, it appears that the insulin signaling pathway, including PI3K activation, can stimulate ecdysone secretion without a requisite role for ERK.

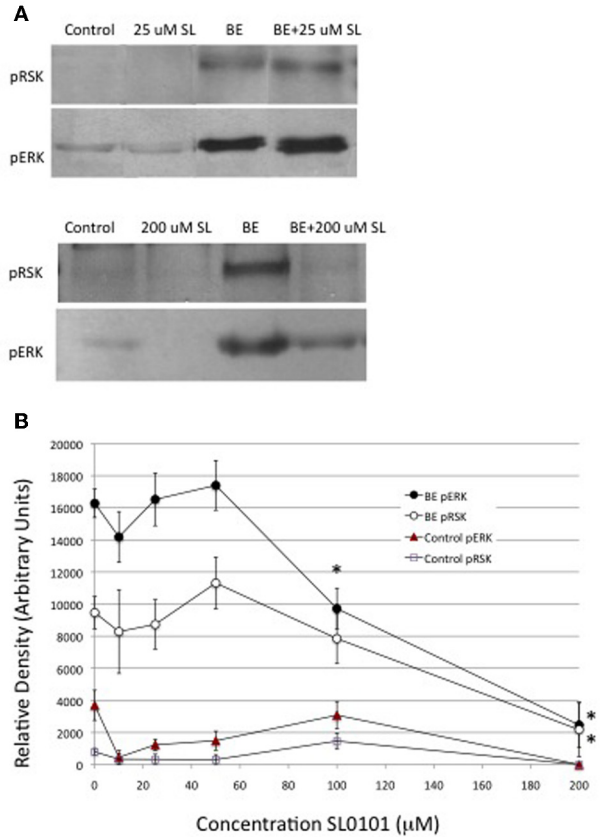

FIGURE 8 | Effects of RSK inhibitor (SL0101) on protein phosphorylation. Western blots were prepared from prothoracic glands described in **Figure 7**. Cellular lysates were probed for phosphoproteins via Western blots. **(A)** Results of a typical experiment. **(B)** Quantification of Western blots. Asterisks indicate groups that were statistically greater than their respective no-hormone controls (ANOVA, Tukey–Kramer *post-hoc* test). Sample sizes were as follows (replicates per group): Control = 28; BE = 28; 10 uM SL = 2; 25 uM SL = 7; 50 uM SL = 3; 100 uM SL = 15; 200 uM SL = 2; BE + 10 uM SL = 2; BE + 25 uM SL = 10; BE + 50 uM SL = 6; BE + 100 uM SL = 13; BE +200 uM SL = 6.

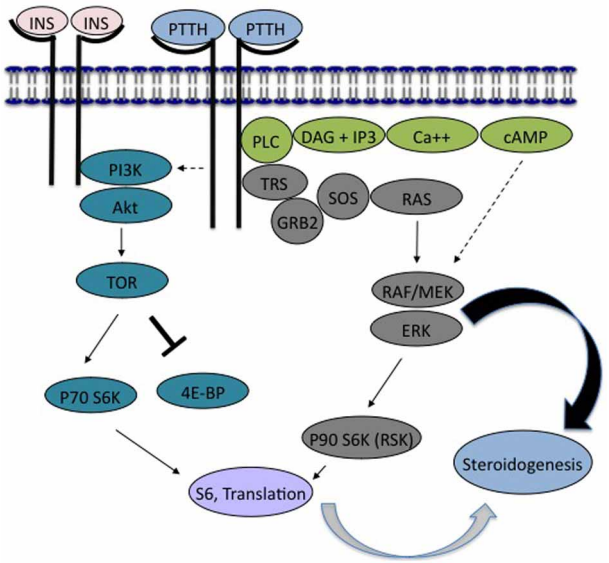

FIGURE 9 | PTTH and insulin signaling pathways in lepidopteran ecdysone secretion. PTTH: In both *Bombyx* and *Manduca*, PTTH has been shown to stimulate tyrosine kinase activity. We suggest that this leads to the phosphorylation of a postulated receptor substrate (modeled as Torso Receptor Substrate, TRS), capable of interacting with PLC (phospholipase C). PTTH is known to increase intracellular calcium, activate calcium-sensitive adenylyl cyclase (not shown), and to elevate cyclic AMP. TRS is postulated to recruit the adapter protein GRB2 and SOS, enhancing the activity of RAS and RAF, leading to known activation of MEK, ERK, and RSK. Cyclic AMP is also suggested to act via an effect on the MAPkinase pathway (dotted line). Insulin/Bombyxin: In both Bombyx and Manduca, insulin or bombyxin activate PI3K and Akt, which is presumed to increase the activity of TOR (target of rapamycin) through proteins not shown. TOR enhances translation by suppressing the translation inhibitor 4E-binding protein (4EBP), and activating p70 S6 kinase, which targets ribosomal protein S6. The primary difference seen between the prothoracic glands of Manduca and Bombyx is the ability of both insulin and PTTH to activate the insulin signaling pathway in Bombyx (PI3K, Akt, TOR, 4EBP) and by this pathway to stimulate ecdysone secretion (Gray curved arrow). By contrast, in Manduca, although PTTH stimulates the phosphorylation of 4EBP suggesting activation of PI3K (dotted line), the insulin signaling pathway is neither sufficient, nor required, for ecdysone synthesis. Instead, secretion is mediated by PTTH-stimulated activation of MAPkinases (Black curved arrow).

In *Manduca*, PTTH enhances the phosphorylation of 4EBP, which is strong evidence for PTTH-stimulated activation of TOR. Previous studies have shown that *Manduca* PTTH-stimulated ecdysone secretion is sensitive to the TOR inhibitor rapamycin and that PTTH enhances the phosphorylation of ribosomal S6 kinase (Song and Gilbert, 1994, 1995). In the present study, phosphorylation of 4EBP was one of the few PI3K-sensitive effects of PTTH (blocked by LY294002). Hence, it appears that PTTH activates TOR through PI3K, yet we did not observe concomitant phosphorylation Akt which would be expected to occur upstream of TOR activation. Also, the phosphorylation of 4EBP could be completely inhibited without affecting ecdysone secretion, indicating that this particular downstream effect of TOR is not essential for acute ecdysone secretion. PTTH enhances the phosphorylation of RSK, and enhanced activation of TOR by RSK has been found in several systems, an effect resulting from its effects on upstream proteins in the TOR pathway (Romeo et al., 2012). However, because 4EBP is phosphorylated in the prothoracic glands even following MEK inhibition, which blocks the phosphorylation of RSK, it is unlikely that RSK serves as the sole TOR activator in *Manduca* prothoracic glands. Hence the mechanism by which *Manduca* PTTH activates TOR remains to be determined.

The reasons for direct insulin stimulation of ecdysone secretion in the prothoracic glands of *Bombyx,* vs. the relative insulin-refractoriness of *Manduca*, are unclear. *Manduca* larvae, like other lepidopteran larvae, are active feeders. Hence there is no a priori reason to expect relative insensitivity of *Manduca* ecdysone secretion to insulin. The difference relative to *Bombyx* may arise in the timing of prothoracic gland growth, which in *Bombyx* occurs relatively late in the last instar (Gu and Chow, 2005). It may be that *Manduca*, in which prothoracic glands double in size daily during the first 4 days of the last instar as larvae feed (Smith, 1995), may dissociate insulin-stimulated glandular growth from secretion to prevent premature metamorphosis. The dissociation of insulin-stimulated glandular growth from acute ecdysone secretion would ensure the proper timing of metamorphosis-inducing ecdysone secretion.

The results of this study are, overall, a reminder that developmental signaling pathways, while fundamentally similar, vary in detail among model insect species. Differences, for example, in the relative impact of PI3K and ERK on ecdysone secretion have presumably evolved to permit different species to optimize the timing of key developmental changes with a conserved toolbox of environmentally and nutritionally sensitive hormonal cues.

AUTHOR CONTRIBUTIONS

Wendy A. Smith conducted experiments, analyzed data, wrote the manuscript. Anthony Lamattina assisted with experiments, manuscript preparation, and data analysis. McKensie Collins assisted with experiments and data analysis.

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Independent signaling by *Drosophila* insulin receptor for axon guidance and growth

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The *Drosophila* insulin receptor (DInR) regulates a diverse array of biological processes including growth, axon guidance, and sugar homeostasis. Growth regulation by DInR is mediated by Chico, the *Drosophila* homolog of vertebrate insulin receptor substrate proteins IRS1–4. In contrast, DInR regulation of photoreceptor axon guidance in the developing visual system is mediated by the SH2-SH3 domain adaptor protein Dreadlocks (Dock). *In vitro* studies by others identified five NPXY motifs, one in the juxtamembrane region and four in the signaling C-terminal tail (C-tail), important for interaction with Chico. Here we used yeast two-hybrid assays to identify regions in the DInR C-tail that interact with Dock. These Dock binding sites were in separate portions of the C-tail from the previously identified Chico binding sites. To test whether these sites are required for growth or axon guidance in whole animals, a panel of DInR proteins, in which the putative Chico and Dock interaction sites had been mutated individually or in combination, were tested for their ability to rescue viability, growth and axon guidance defects of *dinr* mutant flies. Sites required for viability were identified. Unexpectedly, mutation of both putative Dock binding sites, either individually or in combination, did not lead to defects in photoreceptor axon guidance. Thus, either sites also required for viability are necessary for DInR function in axon guidance and/or there is redundancy built into the DInR/Dock interaction such that Dock is able to interact with multiple regions of DInR. We also found that simultaneous mutation of all five NPXY motifs implicated in Chico interaction drastically decreased growth in both male and female adult flies. These animals resembled *chico* mutants, supporting the notion that DInR interacts directly with Chico *in vivo* to control body size. Mutation of these five NPXY motifs did not affect photoreceptor axon guidance, segregating the roles of DInR in the processes of growth and axon guidance.

Keywords: *Drosophila*, insulin receptor, Chico, Dock, RTK

INTRODUCTION

Receptor tyrosine kinases (RTKs) play diverse roles in development, differentiation, homeostasis and disease [reviewed in Lemmon and Schlessinger (2010)]. This diversity in biological function is exemplified by Insulin/Insulin-like signaling (IIS) pathways which have been implicated in a broad range of biological processes and diseases including diabetes, obesity, and cancer [reviewed in Nakae et al. (2001); Baserga et al. (2003); Kahn et al. (2005); Taguchi and White (2008)]. IIS impacts virtually all basic cellular processes, including transcription, translation, and cell growth, with specific effects on mitogenesis, glycogen synthesis, lipolysis, cell survival, and glucose uptake. How the different biological functions of single RTKs are executed—in different cell types, at different life cycle stages, or in response to different environmental cues—is not well understood.

Key components required to segregate the biological activities of RTKs are adapter proteins that link RTKs to discrete downstream pathways [reviewed in Kholodenko (2006); Murphy and Blenis (2006); Pawson (2007)]. Signaling is activated by ligand binding which induces a conformational change in the insulin receptor (IR), activating its kinase activity, resulting in autophosphorylation, substrate phosphorylation and the binding of adapter proteins to phosphorylated tyrosines [reviewed in Pessin and Frattali (1993); White (1998); Fisher and White (2004); Hou and Pessin (2007); Kohanski (2007); Taguchi and White (2008); Hubbard (2013)]. The insulin receptor substrates (IRS1–4) are key adapter proteins that mediate many IR downstream functions [reviewed in Taguchi and White (2008); Copps and White (2012)]. These substrates amplify receptor signals by interacting with a range of additional adapter proteins and enzymes. In addition to the IRS proteins, other receptor substrates have been identified including CBL, involved in glucose uptake (Hou and Pessin, 2007), SHC, involved in mitogenesis, and CEACAM1, involved in insulin internalization and degradation (Poy et al., 2002). Further complicating an understanding of the link between receptor activation and specific biological outcomes is the observation that different RTKs utilize many of these same adapter proteins to cause different biological "readouts" [reviewed in Lemmon and Schlessinger (2010)].

Here, we have made use of *Drosophila melanogaster* as a relatively simple but powerful genetic model system to investigate how a single RTK functions pleiotropically to cause distinct biological outcomes. In contrast to mammals, *Drosophila* harbor only one IIS-family receptor, the *Drosophila* insulin receptor

(DInR) (Petruzzelli et al., 1986a,b) [reviewed in Garofalo (2002)]. DInR is similar in protein sequence to both human IR and IGF-1R (∼26% and ∼27% identity respectively and ∼39% similarity) with highest similarity in the intracellular kinase domain, but was shown to autophosphorylate only in response to insulin (Fernandez-Almonacid and Rosen, 1987). Loss-of-function *dinr* mutations cause recessive lethality, demonstrating that DInR is essential for viability. Animals transheterozygous for combinations of *dinr* hypomorphic alleles are viable, but growth delayed and small (Fernandez et al., 1995; Chen et al., 1996; Bateman and McNeill, 2004; Colombani et al., 2005; Shingleton et al., 2005). DInR controls growth in a cell autonomous fashion: overexpression of DInR in the eye resulted in eye overgrowth due to increases in both cell size and cell number (Coelho and Leevers, 2000; Brogiolo et al., 2001; Leevers, 2001; Goberdhan and Wilson, 2003). These effects on growth are also seen for Chico, the *Drosophila* IRS1–4 homolog that acts downstream of DInR (Bohni et al., 1999). DInR and other IIS pathway members have been implicated in other biological processes including, but not limited to, regulation of lifespan and aging (Tatar et al., 2001; Hwangbo et al., 2004; Tatar, 2004; Mair et al., 2005; Piper et al., 2005, 2007; Piper and Partridge, 2007; Giannakou et al., 2008; Min et al., 2008; Partridge, 2008), locomotor activity (Belgacem and Martin, 2006), eating behavior (Wu et al., 2005; Lingo et al., 2007), oogenesis (Tatar et al., 2001; LaFever and Drummond-Barbosa, 2005; Hsu et al., 2008), heart function (Ocorr et al., 2007), nutrient sensing (Britton et al., 2002; Puig and Tjian, 2006), and metabolism, with surviving *dinr* transheterozygotes or heterozygotes displaying increased levels of whole body or circulating sugar (Shingleton et al., 2005; Belgacem and Martin, 2006).

DInR is highly expressed in the developing nervous system and we previously found that it is required for photoreceptor axon guidance (Song et al., 2003). In this previous study, we used a yeast two-hybrid screen to identify DInR intracellular partners. As bait for this screen, we used the intracellular portion of DInR, which autophosphorylated in yeast cells. This screen identified Dreadlocks (Dock) as a DInR partner. Dock had previously been shown to be required for photoreceptor axon guidance during development of the adult visual system in *Drosophila* (Garrity et al., 1996), suggesting that DInR might also play a role in this process. Our yeast two-hybrid assays showed that interaction with Dock requires DInR kinase activity and involves both the SH2 and the SH3 domains of Dock. This finding was consistent with *in vivo* rescue experiments showing that both SH2 and SH3 domains of Dock are required for photoreceptor axon guidance (Rao and Zipursky, 1998). Using the *eyFLP-FRT* system (Newsome et al., 2000) to generate homozygous *dinr* mutant tissue in a heterozygous background, we found that photoreceptor axon guidance was disrupted in these *dinr* mosaic animals. Whole animal *dinr* transheterozygotes showed similar, but more extreme defects, similar to defects seen in *dock* mutants. In contrast, animals carrying *chico* mitotic clones or whole animal *chico* mutants showed normal patterns of photoreceptor axon targeting. Since *chico* cells are small, similar to *dinr* clones, this result shows that the axon guidance defects seen for *dinr* mosaics are not a simple result of *dinr*-associated growth defects.

On the basis of these results, we previously proposed (Song et al., 2003) that the roles of DInR in growth and axon guidance are independent and mediated by different adapter proteins: binding to Dock regulates axon guidance while binding to Chico controls growth (**Figure 1A**). DInR interaction with either Dock

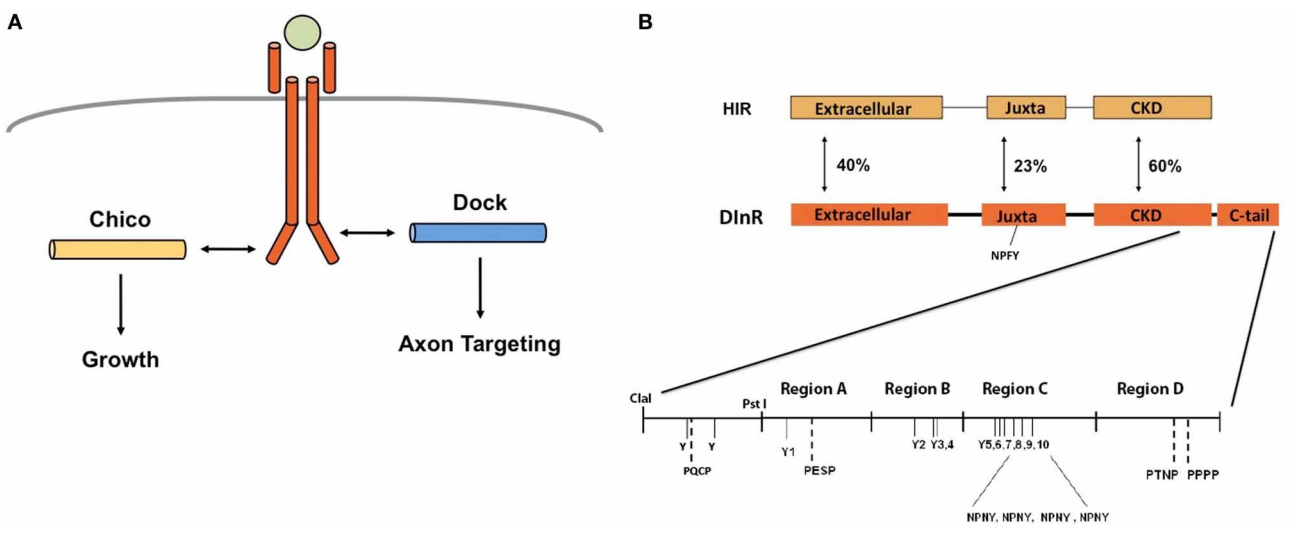

FIGURE 1 | DInR signals independently through Chico and Dock to control growth and axon guidance. (A) We and others proposed that DInR, after DILP binding, signals independently through Chico to control growth and Dock to control axon targeting. Panel modified from Dickson (2003). **(B)** Schematic of DInR sequence with candidate binding regions indicated. The C-tail of DInR, previously shown to be required for interaction with Dock (Song et al., 2003), was divided into four regions (Regions A–D) for analysis. Dock is expected to interact with tyrosine residues (Y) via its SH2 domain and PXXP residues via its SH3 domains. Four NPNY sites in the C-tail and the juxtamembrane NPFY (NPXY-1351) were required for interaction with Chico in cell-based assays (Poltilove et al., 2000). All tyrosine residues in the C-tail are indicated in the figure.

(Song et al., 2003) or Chico (Poltilove et al., 2000) *in vitro* requires the DInR C-terminal tail (C-tail), an extension absent in mammalian IR/IGF-1R (Fernandez et al., 1995; Ruan et al., 1995; Yenush et al., 1996). This C-tail contains multiple potential tyrosine phosphorylation sites and is required for DInR signaling in cell culture (Fernandez et al., 1995; Ruan et al., 1995; Yenush et al., 1996; Marin-Hincapie and Garofalo, 1999). The C-tail also contains YXXM motifs that mediate direct binding to PI3K in cell culture (Yenush et al., 1996), but rescue experiments in flies suggested that Chico is necessary to link DInR to PI3K for signaling and growth control *in vivo* (Oldham et al., 2002). Five NPXY motifs, one in the juxtamembrane region and four in the DInR C-tail, were shown to be important for interaction with Chico *in vitro* (Poltilove et al., 2000). Here, we used yeast two-hybrid assays to identify the regions of DInR that bind Dock. We found that the region of the DInR C-tail that binds Dock is distinct and separable from the region containing Chico interaction sites. We show that rescue of viability of *dinr* mutants requires kinase activity and one specific tyrosine residue in the C-tail. In contrast, a DInR protein carrying mutations in all Chico interaction sites rescued viability and axon guidance defects, but yielded growth defects similar to those seen in *chico* mutants. Finally, DInR proteins carrying mutations in identified Dock binding sites still rescued axon guidance defects, suggesting a high degree of redundancy for this function of DInR.

MATERIALS AND METHODS

CONSTRUCTION OF *dinr* cDNA

A full-length *dinr* cDNA was assembled from genomic fragments as follows: a 7.4 kb genomic EcoRI-NheI fragment spanning the entire *dinr* coding region and including 770 bp of 5′ UTR, 9 introns and 191 bp of 3′ UTR was isolated from BAC48I01 (BACPAC, Oakland, CA). Subsequently, this EcoRI-NheI fragment was inserted into *pSP-luc*$^{+}$*NF* (Promega, Madison, WI) to generate plasmid *pSPgdinr*. An EcoRI-KpnI fragment including exons 1–4 from *pSPgdinr* was inserted into *pSP72* (Promega, Madison, WI) generating plasmid *pSPgdinrEK* and introns 1, 2, and 3 were deleted sequentially by PCR (95°C for 1 min; 30 cycles of: 95°C for 30 s, 55°C for 30 s and 72°C for 6 min; 72°C for 10 min). PCR products were self-ligated after gel purification with GenElute agarose spin columns (Sigma, St. Louis, MO) to generate plasmid *pSPdinrEK*. Similarly, to remove introns 4–9, a KpnI-AflII fragment from *pSPgdinr* was inserted into *pSP72* to generate *pSPgdinrKAf*. Introns 4–7 were eliminated by replacing the KpnI-NsiI fragment of *pSPgdinrKAf* with a corresponding KpnI-NsiI cDNA fragment of *dinr* generated by RT-PCR using a SuperScript One-Step RT-PCR kit (Invitrogen, Carlsbad, CA). Total RNA for this reaction was isolated from 0 to 10 h w^{1118} fly embryos using Trizol reagent (GibcoBRL, Carlsbad, CA). Introns 8 and 9 were deleted from *pSPgdinrKAf* by PCR, as described above. Together, these steps generated plasmid *pSPdinrMid*, which contains the KpnI-AflII coding region, lacking all introns. To generate a full-length *dinr* cDNA, the KpnI site outside the *dinr* gene in *pSPgdinr* was deleted by digestion with NheI and AatII, followed by a fill-in reaction with Klenow. Genomic EcoRI-KpnI and KpnI-AflII fragments were replaced by cDNA fragments from *pSPdinrEK* and *pSPdinrMid*, respectively, to generate *pSPdinr*, the final construct. Sequencing of *pSPdinr* confirmed that it matched the BDGP sequence FBgn0013984. We note that plasmids containing partial or full-length *dinr* cDNA were extremely unstable and could only be maintained in One Shot® TOP10 cells (Invitrogen, Carlsbad, CA).

CONSTRUCTION OF MUTANT *dinr* cDNAs

To test the function of regions of DInR in intracellular signaling and rescue of *dinr* mutant phenotypes, cDNAs were generated that were truncated or that carried specific point mutations in candidate adapter binding sites. To generate deletions, the AflII-StuI fragment from *pSPdinr* was replaced by PCR amplification of *pSPdinr* of the fragment of interest. Borders of the C-tail and deletions are shown schematically in **Figure 1B**. The start of the C-tail is at position Q1672. Deletions were generated to target large regions including tyrosine and PXXP residues that are candidate protein interaction motifs in the C-tail. Deletions were generated by standard PCR reactions and removed the following regions: ΔAB removed amino acids D1611 to K1825; ΔCD removed S1860 to P2131; ΔD removed D2060 to P2131; ΔA removed D1611 to L1742; ΔBCD removed T1760 to P2131; ΔABC removed D1611 to S2042. Note that ΔA constructs removed a portion of the kinase domain, beginning at D1611, in addition to the A region. Also, for constructs missing the D region, the C-terminal end of DInR, from amino acids R2132 to A2144, is present upstream of the MYC3 tag. This region includes a PPPP sequence (P2133-P2136). The following point mutations were generated: DInR-KA (K1405→A) in the kinase domain; Y1F (Y1714→F) in the C-tail; Y2F (Y1776→F) in the C-tail; Y3,4F (Y1790→F, Y1793→F) in the C-tail; LESL (P1724→L, P1727→L) in the C-tail; Y7F (Y1965→F), Y8F (Y1989→F), Y9F (Y2005→F), Y10F (Y2026→F) and combinations thereof, in NPXY sites in the C-tail. To generate these point mutations, a PCR product of the *dinr* C-tail from plasmid *DInRCKD-PAS2-1* (Song et al., 2003) was subcloned into the vector *pSP72* at its ClaI/KpnI site to yield *pSPdinrMT*. Site-directed mutagenesis of *pSPdinrMT* was done by PCR (95°C for 2 min; 18 cycles of: 95°C for 30 s, 55°C for 1 min and 68°C for 8 min). The PCR product was digested with DpnI for 1 h at 37°C to destroy any unmutagenized template plasmid present, and was transformed into XL-1 competent cells. All mutations were confirmed by DNA sequencing. The ClaI-KpnI fragments with various *dinr* mutations were shuttled from *pSPdinrMT* into *pAS2-1OFCT* for yeast two-hybrid assays. Primer sequences available upon request.

To generate transgenic *Drosophila*, full-length, partial or point mutation-containing *dinr* cDNAs were inserted into *pUAST-dinrMYC3*, a P-element vector which includes a 102 bp region encoding a 3X Myc tag to generate in-frame C-terminal fusions. This vector was generated as follows. The AflII-NheI fragment in *pSPdinr* was replaced by the PCR fragment of *pSPdinr* amplified using two primers, P51 and Srf2, and digested with AflII/NheI to introduce an SrfI site so that the MYC3 tag could be inserted into the vector. The newly generated plasmid was named *pSPdinrM*. A MYC3 tag was excised from the plasmid *pSRL-hSNT MYC3* (a gift from Dr. Mitch Goldfarb, Hunter College) and subcloned into the SrfI/NotI site of *pSPdinrM*, generating *pSPdinrMYC3*. The *dinrMYC3* cDNA from *pSPdinrMYC3* was subcloned into the EcoRI/NotI site of *pUAST* to generate the

full-length *pUASTdinrMYC3* plasmid. *dinr* cDNAs carrying deletions (ΔABC, ΔAB, ΔCD) were inserted into *pUASTdinrMYC3* by replacing the BsiWI-NotI fragment of *pUASTdinrMYC3*. Point mutations in the C-tail of *dinr*, generated in *pSPdinrMT*, were moved into *pUASTdinrMYC3* by the replacement of the AflII-NotI fragment.

To test the one NPFY motif in the juxtamembrane region, *pUASTdinr(JM-NPFF)MYC3* was generated, in which the tyrosine in the juxtamembrane NPFY motif was changed to a phenylalanine (Y1354→F). Site-directed mutagenesis to change the TAT codon for tyrosine to the TTT codon for phenylalanine was carried out with standard methods using *pSPdinrMYC3* and Vent polymerase (NEB, Ipswich, MA). Then, a ∼7 kb fragment spanning the entire *dinrMYC3* coding region, and thus containing the mutated juxtamembrane NPFF site, was released from the mutated *pSPdinrMYC3* plasmid with NotI and EcoRI; this fragment was inserted into the NotI and EcoRI sites of *pUAST-dinr(Y7,8,9,10F)MYC3*, replacing the entire *dinr(Y7,8,9,10F)MYC3* coding region. *pUASTdinr(5NPXF)MYC3* was then made by excising a ∼4 kb fragment containing the 4 mutated NPXF sites in the C-tail from *pUASTdinr(Y7,8,9,10F)MYC3* using AflII and inserting it into the AflII site of *pUASTdinr(JM-NPFF)MYC3* to replace the AflII fragment. The orientation and sequence of each *dinr* variant was verified by sequencing.

YEAST TWO-HYBRID ASSAYS

Yeast two-hybrid assays were performed as described (Song et al., 2003) using *pAS2-1OFCT* carrying either wild type or mutant versions of DInR and *pACT-Dock* (Song et al., 2003). One hundred microliters of saturated culture was inoculated into 3 ml of fresh media and grown to mid-log phase. One milliliter of culture was spun down. The pellet was suspended completely in 200 μl of 10 mM Tris (pH 7.5)/0.05% Triton X-100. The samples were stored at −80°C. Frozen samples were allowed to thaw slowly on ice before analysis. One milliliter of ONPG solution was added and mixed by inverting several times. The reaction was carried out at 30°C. When the color changed to medium-dark yellow, the reaction was stopped by adding 500 μl 1 M Na_2CO_3 and OD_{420} was measured. The β-galactosidase activity was calculated with the formula: β-Gal units = [OD_{420}(absorbance by reaction product) × 1000]/[OD_{600}(sample cell density) × (1 ml) × t(time of reaction)]. Assays were repeated at least three independent times using at least 3 samples for each point in each assay.

GENETICS AND PHENOTYPIC ANALYSIS

Transgenic flies were generated by Rainbow Transgenic Flies, Inc. (Camarillo, CA) by standard P-element mediated transformation. Multiple independent transgenic lines were generated for each construct whenever possible. Transgenic lines carrying insertions on chromosome II were used for rescue experiments. Transgenic lines for rescue experiments expressed DInR proteins at similar levels, determined using a modification of the method of Ronshaugen et al. (2002).

Genetic crossing schemes used to generate stocks for the *dinr* rescue experiments are available upon request. For the following experimental crosses, parental flies were removed as necessary to prevent overcrowding of the progeny to be used for analysis. For the lethality rescue analysis, *arm-GAL4/arm-GAL4;FRT82Bdinr^{273}/TM3Sb,armGFP* virgin females were crossed to *UAS-X/(UAS-X* or *CyO);dinr^{ex15}/TM3Sb,armGFP* males. Adult progeny that had eclosed were scored for their bristle phenotype: either *Sb* or non-*Sb*. In the case that the *UAS-X* construct to be tested was homozygous lethal and had to be used in crosses with a *CyO* balancer, only non-*CyO* eclosed adult progeny were scored for their bristle phenotype.

For the growth defect rescue analysis, *arm-GAL4/arm-GAL4;FRT82Bdinr^{273}/TM3Sb,armGFP* virgin females were crossed to *UAS-X/(UAS-X* or *CyO);dinr^{ex15}/TM3Sb,armGFP* males. Eclosed non-Sb, non-CyO adult male or female progeny were collected separately in fresh food vials and were individually weighed in an ATI Cahn C-33 microbalance approximately 3–18 days after eclosion.

For the photoreceptor axon guidance rescue analysis, *arm-GAL4/arm-GAL4;FRT82Bdinr^{273}/TM6BTb,GFP* virgin females were crossed to *UAS-X/UAS-X; dinr^{ex15}/TM6BTb,GFP* males. Non-Tubby progeny at the wandering third instar larval or white prepupal stages were analyzed.

For SEM studies, adult flies were decapitated. Heads were fixed in 2.5% glutaraldehyde overnight at 4°C, washed 3 × 30 min. with 0.1 M PBS, dehydrated in ascending acetone grades and then critical point dried. They were then mounted on studs in the desired orientation under a stereo binocular microscope and coated with gold (thickness 30–35 nm). Scanning was done on SEM mode in an AMRAY 1820D electron microscope at 15 kV.

PHOTORECEPTOR AXON GUIDANCE ANALYSIS

Eye-brain complexes were dissected from third instar larvae or white prepupae in phosphate-buffered saline (PBS). A standard protocol kindly provided by C. H. Lee was generally followed for the staining of eye-brain complexes with *m*onoclonal *anti*body 24B10 (MAb24B10): eye-brain complexes were fixed in 2% paraformaldehyde in a lysine-phosphate buffer containing 0.25% sodium m-periodate, washed in 0.5% Triton-X-100 in PBS (PBT), blocked in 10% normal goat serum (NGS) in PBT, incubated in 1:200 MAb24B10 in 10% NGS in PBT at 4°C overnight or longer, washed in PBT, incubated in 1:200 HRP-conjugated goat anti-mouse antibody in 10% NGS in PBT at room temperature for at least 2 h, washed in PBT, incubated in DAB, washed in PBS, and cleared and mounted in 70% glycerol in PBS. MAb24B10 specifically stains the cell bodies and axonal membranes of differentiated photoreceptors in *Drosophila melanogaster* and was originally generated by Zipursky et al. (1984). MAb24B10 used in our experiments was purchased from the Developmental Studies Hybridoma Bank at The University of Iowa.

RESULTS

THE DInR C-TAIL HARBORS SEPARATE BINDING SITES FOR DOCK AND CHICO

As described above, we proposed that DInR signals independently through Dock and Chico to regulate axon guidance and growth, respectively (**Figure 1A**). To test this, yeast two-hybrid assays (Y2H) were used to identify potential Dock interaction sites in DInR. Because Dock interaction with DInR required the C-tail (Song et al., 2003), a series of small deletions and

point mutations in DInR was generated in this portion of DInR (**Figure 1B**; Materials and Methods). For the deletion series, the C-terminal portion of DInR was divided arbitrarily into 4 regions (Regions A-D, **Figure 1B**) which were fused to the rest of the intracellular domain of DInR to allow for autophosphorylation in yeast (see Song et al., 2003). Region A includes a portion of the highly conserved kinase domain (between the ClaI and PstI sites indicated in **Figure 1B**), as well as the N-terminal portion of the C-tail that harbors two potential Dock interaction sites, Y1714 and a PESP motif at position 1724. Region B harbors three tyrosines (Y1776,1790,1793) that could potentially interact with Dock. Region C includes 6 tyrosines, the last four of which are embedded within NPXY consensus sequences previously shown to be involved in Chico interaction (Poltilove et al., 2000). Finally, region D contains one PXXP sequence, potentially able to bind Dock's SH3 domains. These tyrosine residues, all indicated in **Figure 1B**, are the only tyrosine residues present in the DInR C-tail.

As shown in **Figure 2**, the full-length DInR intracellular domain interacted strongly with Dock. DInR-ΔD, which lacks the D region, and DInR-ΔCD, which lacks both C and D regions, interacted as strongly with Dock as full-length DInR. This result suggests that regions A and B are sufficient for the DInR/Dock interaction. Consistent with this, proteins lacking the A (DInR-ΔA) or A and B (DInR-ΔAB) regions did not interact detectably with Dock. However, the A region alone was not sufficient for interaction, as DInR-ΔBCD did not interact detectably with Dock. Note that the deletion of the A (DInR-ΔA) region alone suggests that the B region is also not sufficient for Dock interaction; however, as conserved regions of the kinase domain were removed in DInR-ΔA, we cannot make a firm conclusion about the C-tail requirements in this case.

To further investigate the sequence motifs necessary for DInR/Dock interaction, point mutations were generated in tyrosine residues in candidate adapter protein binding sites in the C-tail. As shown in **Figure 2**, mutation of Y1714 to F (DInR-Y1F) in region A did not significantly decrease interaction with Dock. In contrast, mutations of Y1776 in region B (DInR-Y2F) greatly decreased Dock binding. Mutations of the other tyrosine residues in the B region (double mutation of Y1790 and Y1793; DInR-Y3,4F) did not alter Dock interaction. Finally, point mutations of the tyrosine residues in the C region had modest or no effect on Dock interaction, shown here for Y2005F (DInR-Y9F). Together, these results suggested that DInR interaction with Dock requires the B region, in particular, Y1776 in this portion of DInR. Since the A region was also required for Dock interaction, but the tyrosine in this region was not, and since Dock function *in vivo* involves both SH2 and SH3 domains (Rao and Zipursky, 1998), these results suggested that the PXXP motif in the A region also interacts with Dock.

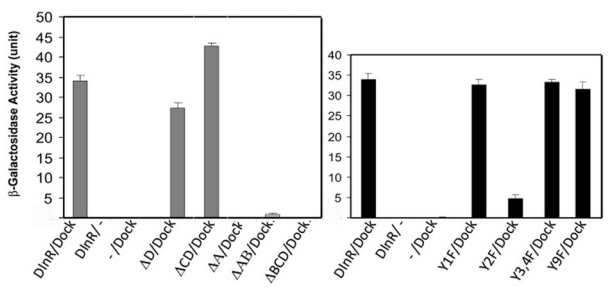

FIGURE 2 | Yeast two-hybrid assays identify candidate Dock interaction sites in the DInR C-tail. pACT-Dock was tested for interaction with pAS2.1 containing the DInR intracellular domain or variants thereof, as indicated. β-galactosidase activity is shown. Negative controls: pAS2.1-DInR with empty pACT (–); empty pAS2.1 (–) with pACT-Dock. Error bars indicate standard deviation.

FULL-LENGTH Myc-TAGGED DInR PROMOTES GROWTH AND RESCUES VIABILITY

Based upon the findings summarized above, we generated a series of transgenes carrying mutations or deletions of sites critical for kinase activity or adapter protein binding in the context of the full-length *dinr* coding region (**Figure 1B**, **Table 1**). A C-terminal 3xMyc tag was added to each *dinr* variant for antibody detection and each *dinr* cDNA was inserted into a *pUAST* vector for expression with the *GAL4/UAS* system (Brand et al., 1994). After P-element mediated transformation, multiple independent transformant lines were obtained for each transgene and expression levels were quantitated. To verify the function of full-length DInR in tissue growth, and to test whether the 3xMyc tag interferes with DInR function, DInR-MYC3 was expressed in the developing eye using a moderate *ey-GAL4* driver. This resulted in cell autonomous overgrowth of the eye (**Figures 3A–F**). The increased eye volume resulted in eyes occupying larger volumes with increased depth, not always clearly depicted in two-dimensional images of whole eyes. This eye size difference appeared to be due to an increase in both cell size and cell number. In contrast, and as expected, a DInR transgene carrying a point mutation in the ATP binding site ("kinase-dead" mutation; DInR-K1405A) appeared to function as a dominant negative in these experiments, resulting in smaller eyes (**Figures 3G–I**).

Table 1 | Rescue of adult lethality by DInR transgenes.

Transgene expressed	Rescued *dinr*$^{ex15/273}$ adults (% non-Sb)	*dinr*^{ex15}/+ and *dinr*^{273}/+ adults (% Sb)	Total no. of flies scored
UAS-lacZ	0	100	237
UAS-dinr	37	63	785
UAS-dinr-KA	0	100	34
UAS-dinr-ΔCD	27	73	271
UAS-dinr-ΔAB	0	100	242
UAS-dinr-Y1F	0	100	245
UAS-dinr-Y2F	8	92	153
UAS-dinr-LESL	42	58	177
UAS-dinr-LESL, Y2F	11	89	254
UAS-dinr-JM-NPFF	24	76	213
UAS-dinr-Y9F	16	84	145
UAS-dinr-Y7,8,9,10F	11	89	235
UAS-dinr-5NPXF	21	79	246

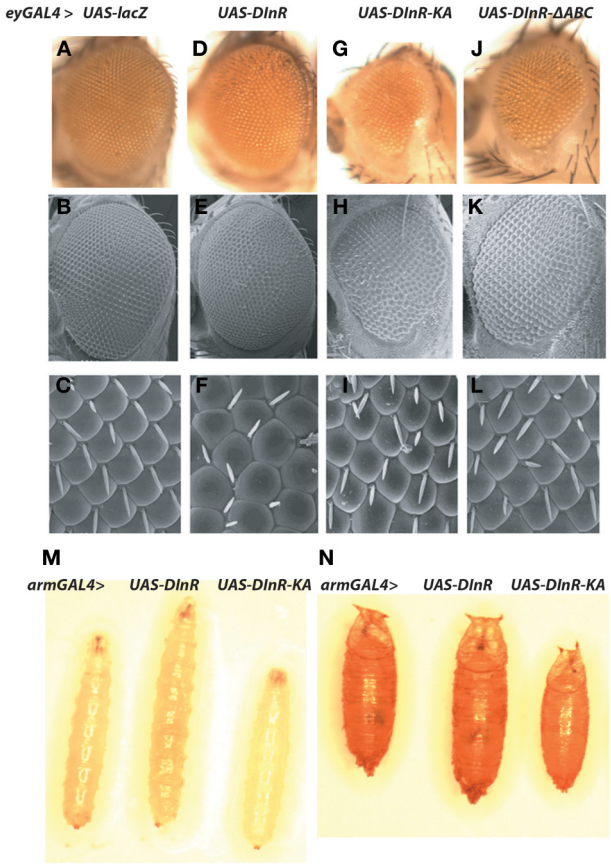

FIGURE 3 | Effects of ectopic expression of DInR on tissue-specific and whole animal growth. Effects of ectopic expression of full-length Myc-tagged DInR or variants was tested using the *UAS/GAL4* system. **(A–L)** Expression driven by an *eyeless-GAL4* driver. **(A–C)** Control, *UAS-lacZ*; **(D–F)** *UAS-DInR*; **(G–I)** *UAS-DInR-KA*; **(J–L)** *UAS-DInR-ΔABC*. Top row **(A,D,G,J)**, photographs of eyes with light microscopy. Note that expression of DInR results in increased eye volume such that the eyes now have increased depth. These eyes project out from the 2D image, occupying a larger 3D space. Middle row **(B,E,H,K)**, SEM. Bottom row **(C,F,I,L)**, high power SEM images show rough eyes induced by ectopic DInR expression. **(M,N)** Moderate expression using an *arm-GAL4* driver. **(M)** Third instar larvae: control, *UAS-DInR*, *UAS-DInR-KA*; **(N)** pupae: control, *UAS-DInR*, *UAS-DInR-KA*.

Similarly, expression of DInR-ΔABC resulted in smaller eyes (**Figures 3J–L**).

Overexpression of full-length DInR in a wild type background with a moderate ubiquitous driver, *arm-GAL4*, caused whole animal overgrowth evident at larval and pupal stages (**Figures 3M,N**). Expression of DInR-KA acted as a dominant negative on whole animal growth.

To test the ability of DInR transgenes to complement wild type functions of DInR, transgenes were expressed in a $dinr^{ex15/273}$ mutant background under the control of an *arm-GAL4* driver. These $dinr^{ex15/273}$ transheterozygotes carry one copy of the $dinr^{ex15}$ null allele and one copy of the $dinr^{273}$ weak hypomorphic allele. To test for rescue, *arm-GAL4/arm-GAL4; FRT82B*$dinr^{273}$*/TM3Sb,armGFP* virgin females were crossed to *UAS-X/(UAS-X* or *CyO);*$dinr^{ex15}$*/TM3Sb,armGFP* males and rescue of lethality was scored by counting non-Sb adults. As shown in **Table 1**, a negative control protein, β-galactosidase, encoded by the *UAS-lacZ* transgene, failed to rescue viability, as expected. However, viability to adulthood was fully rescued by wild type DInRMYC3 (*UAS-dinr*). *UAS-dinr-KA*, encoding a "kinase-dead" receptor, failed to rescue viability, consistent with the expectation that kinase activity is necessary for DInR function *in vivo*.

RESCUE OF *dinr*-ASSOCIATED LETHALITY BY DInR MUTANT TRANSGENES

To test the importance of the Dock and Chico binding sites identified *in vitro* (**Figure 2** and (Poltilove et al., 2000)) for DInR function *in vivo*, the rescue approach described above was used. Mutations were introduced into full-length *pUASTDInRMYC3*, as described in the Materials and Methods. The mutant proteins were designed to test *in vivo* requirements for different DInR sequences: (1) DInR-KA, mutation of K1405 to A in the ATP binding site ("kinase-dead"). (2) DInR-Δ*CD*, deletion of the C and most of the D regions of the C-tail. (3) DInR-Δ*AB*, deletion of the A and B regions of the C-tail and part of the adjoining kinase domain, N-terminal of the C-tail. (4) DInR-Y1F, mutation of Y1714 in the A region of the C-tail. (5) DInR-Y2F, mutation of Y1776 in the B region of the C-tail. (6) DInR-LESL was designed to test the role of the potential SH3 binding PXXP motif in region A of the DInR C-tail. (7) DInR-LESL, Y2F, mutation of the PXXP in the A region and of Y1776 in the B region. (8) DInR-JM-NPFF, mutation to F of Y1354, embedded in an NPFY motif in the juxtamembrane region, previously shown to be required for Chico interaction (Poltilove et al., 2000). (9) DInR-Y9F, mutation of one of four Chico binding sites in the C region of the C-tail. (10) DInR-Y7,8,9,10F, simultaneous mutation of all four Chico binding sites in the C region of the C-tail. (11) DInR-5NPXF (JM-NPFF,Y7,8,9,10F), simultaneous mutation of the juxtamembrane NPFY and the four Chico binding sites in region C of the C-tail. *dinr* cDNAs encoding the DInR proteins described above were inserted into the P-element vector *pUAST*. Multiple independent transformant lines were generated for each and insertions on chromosome II were selected for rescue experiments. DInR proteins were expressed with the *GAL4/UAS* system, using a moderate, ubiquitous *arm-GAL4* driver.

As shown in **Table 1**, the CD region of the C-tail was not required for rescue to adulthood, as *UAS-dinr-*Δ*CD* expression rescued viability. In contrast, the AB region, containing the N-terminal half of the C-tail and a small portion of the conserved kinase domain, was required to support viability, as no adults were observed when *UAS-dinr-*Δ*AB* was expressed in the $dinr^{ex15/273}$mutant background. Interestingly, DInR-Y1F completely failed to rescue adult lethality of $dinr^{ex15/273}$ transheterozygotes, indicating that tyrosine 1714, within the A region of the C-tail, is crucial for adult viability. Expression of *UAS-dinr-Y2F* rescued a small number of animals, suggesting that this residue contributes to but is not absolutely required for viability. The PESP motif in the A region of the C-tail did not appear to be required for viability, as indicated by rescue with *UAS-dinr-LESL* and with *UAS-dinr-LESL,Y2F*, the latter showing

similar rescue potential to the Y2 mutation alone. Mutation of the juxtamembrane NPFY tyrosine alone had little effect on viability as DInR-JM-NPFF rescued lethality substantially. Mutations in individual or multiple candidate Chico binding sites, DInR-Y9F and DInR-Y7,8,9,10F rescued lethality to a lesser degree. Finally, DInR-5NPXF, a compound variant containing mutations of the juxtamembrane NPFY tyrosine and all candidate Chico binding sites in the C-tail rescued viability. In sum, most DInR proteins carrying small deletions and specific mutations retained the ability to complement loss of DInR function, rescuing mutants to adulthood. The exceptions to this were DInR-ΔAB and DInR-Y1F. Since the latter targets only one tyrosine residue in the A region of the C-tail and was required for viability, this probably accounts for the failure of DInR-ΔAB to rescue lethality.

CHICO INTERACTION SITES ARE REQUIRED FOR DInR'S GROWTH FUNCTION

We next investigated the size of animals rescued by different DInR variants, with the expectation that animals carrying mutations in Dock binding sites would be similar in size to animals rescued by full-length DInR, while those lacking Chico interaction sites would be similar to small *chico* mutants. As shown in **Figure 4A**, the size of male transheterozygotes expressing full-length DInR was similar to that of sibling heterozygote controls expressing full-length DInR (*dinr, Sb*). Mutations in the putative Dock binding sites did not have detrimental effects on growth; DInR-LESL and DInR-Y2F rescued adult male growth, although DInR-LESL,Y2F animals were somewhat smaller. Mutation of the juxtamembrane NPFY tyrosine (DInR-JM-NPFF) led to an incomplete rescue of growth (note that an average of two independent lines is shown for this transgene; for all other transgenes, results are shown for a single transformant line). Mutation of one or more Chico binding sites in the C-tail did not have detrimental effects on growth: DInR-Y9F, DInR-Y10F and DInR-Y7,8,9,10F fully rescued growth defects, as did DInR-ΔCD, which lacks the C-terminal half of the C-tail containing Y7, 8, 9, and 10. However, there was a 50% decrease in the mean mass of adult males rescued by DInR-5NPXF compared to those rescued by the DInR control. This decrease is very similar to the 55% decrease in body weight seen in *chico* mutant males (Bohni et al., 1999). Thus, it seems likely that these five NPXY sites are responsible for most, if not all, of the control of growth by DInR that is mediated by Chico, and that there is considerable redundancy among these NPXY sites.

In females, there was a 52% decrease in the mean mass of adult females rescued by DInR-5NPXF compared to those rescued by the control DInR protein (**Figures 4B,C**). This was noticeably less than the 65% decrease in body weight measured in *chico* mutant females (Bohni et al., 1999). Interestingly, although the DInR-ΔCD protein, which lacks the four NPXY sites in the C-tail in addition to two other tyrosines and one PXXP site, rescued the mean mass of adult males to the same level as the control DInR protein, this did not occur for adult females; instead, there was a 23% decrease in mean mass in females rescued by the DInR-ΔCD protein. Thus, it is possible that another interaction site(s) in regions C and D, other than the four NPXY sites, may also be responsible for growth control through Chico in females. Since DInR is also involved in controlling female fertility [reviewed in Garofalo (2002)], an interplay between fertility and growth may be at work.

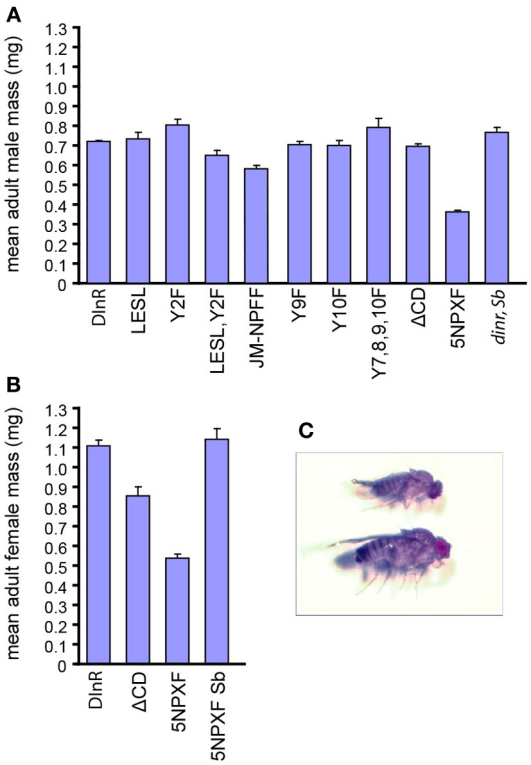

FIGURE 4 | Chico interaction sites are required for whole animal growth. (A,B) Mean mass (mg) of male **(A)** and female **(B)** adult *dinr*$^{ex15/273}$ flies expressing different UAS-DInR variants. Error bars represent standard error. Sample numbers for each rescue protein tested in males were: DInR control, 85; DInR-LESL, 10; DInR-Y2F, 5; DInR-LESL,Y2F, 4; DInR-JM-NPFF, 33; DInR-Y9F, 10; DInR-Y10F, 5; DInR-Y7,8,9,10F, 2; DInR-ΔCD, 27; DInR-5NPXF, 24; DInR control expressed in *dinr* mutant heterozygote siblings, 4. Sample numbers for each rescue protein tested in females were: DInR control, 32; DInR-ΔCD, 8; DInR-5NPXF, 9; DInR-5NPXF expressed in *dinr* mutant heterozygote siblings, 3. **(C)** Photograph showing an example of a small *dinr* mutant adult fly expressing DInR-5NPXF (top) compared to heterozygous sibling control expressing DInR-5NPXF (bottom).

MUTATION OF CHICO INTERACTION SITES DID NOT ABOLISH RESCUE OF *dinr*-ASSOCIATED AXON GUIDANCE DEFECTS

As shown in **Figure 5**, severe axon guidance defects were observed in *dinr*$^{ex15/273}$ transheterozygotes, which display gross disorganization of photoreceptor axon targeting in both the lamina and medulla (compare wild type eye-brain complexes, **Figures 5A,B**, to *dinr*$^{ex15/273}$ eye-brain complexes, **Figures 5C,D**). Clumps of axons were present above and in the lamina, and in the medulla. In general, the phenotypes observed in the *dinr*$^{ex15/273}$ eye-brain complexes were more severe than those of *dinr*$^{353/273}$ (Song et al., 2003). This is consistent with *dinr*^{ex15}, a null allele (Song et al., 2003), being a stronger loss-of-function allele than *dinr*^{353}. Importantly, these axon guidance defects were largely rescued

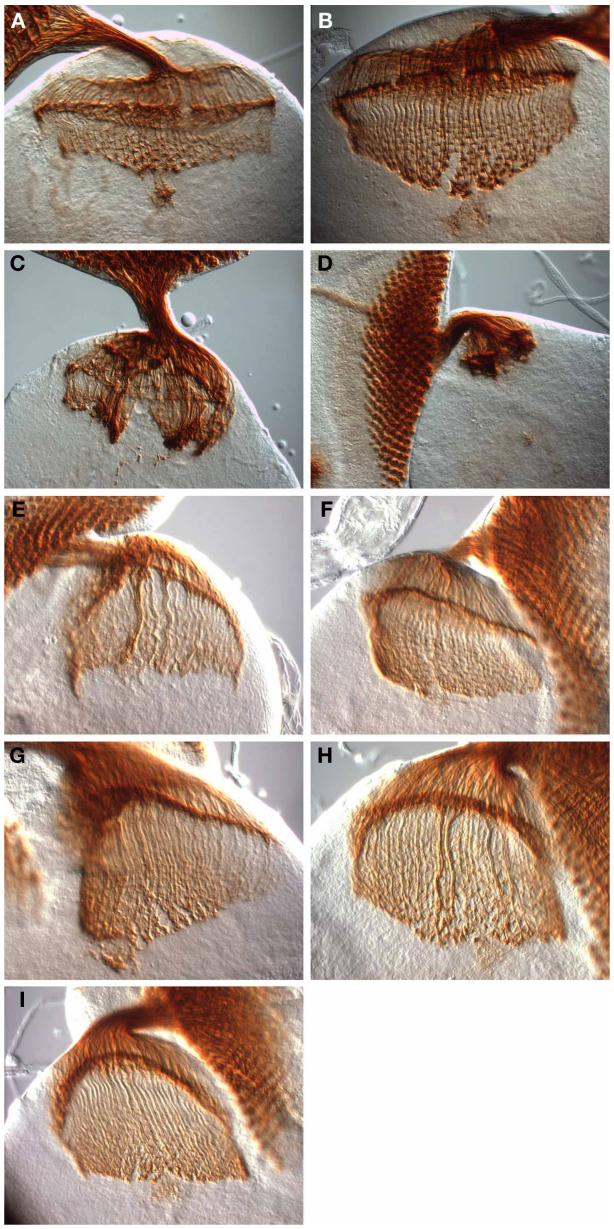

FIGURE 5 | Rescue of *dinr*-associated axon guidance defects by full-length DInR and variants. Third instar larval eye-brain complexes were stained with MAb24B10 to visualize differentiated photoreceptors and their axons. Imaging was done with DIC optics and a 40× objective. **(A,B)** Control eye-brain complexes of the genotype w^{1118}. The specimen shown in **(B)** is at a later stage of development than that shown in **(A)**. The rip in the medulla in **(B)** resulted during sample preparation. **(C,D)** Axon guidance was severely disrupted in eye-brain complexes from whole animal $dinr^{ex15/273}$ transheterozygotes. **(E)** Axon guidance defects were rescued with full-length DInR. **(F–I)** Axon guidance defects were rescued by expression of DInR variants: **(F)** DInR-LESL; **(G)** DInR-Y2F; **(H)** DInR-LESL, Y2F; **(I)** DInR-5NPXF.

by expression of full-length DInR (**Figure 5E**). This is the first demonstration that axon guidance defects are rescued by DInR, ruling out the possibility that defects seen in *dinr* mutants result from the genetic background of the *dinr* mutants. Surprisingly, the DInR variants carrying mutations in candidate Dock binding sites also rescued axon guidance defects. These include DInR-LESL (**Figure 5F**), in which one of the putative Dock binding sites was mutated, DInR-Y2F (**Figure 5G**), in which the second putative Dock binding site was mutated, and DInR-LESL, Y2F (**Figure 5H**), in which both putative Dock binding sites were mutated. Notably, DInR-5NPXF, which did not rescue growth defects (see above, **Figure 4**), did restore normal photoreceptor axon guidance (**Figure 5I**). This rescue by DInR-5NPXF demonstrates that Chico interaction sites are not required for axon guidance functions of DInR. Furthermore, this shows that *dinr*-associated axon guidance defects are not merely a secondary effect of *dinr*-associated growth defects.

CONCLUSIONS

Like other RTKs, DInR regulates multiple processes, including growth and axon guidance. Here we have tested the hypothesis that DInR interacts differentially *in vivo* with different adapter proteins to mediate different biological functions. In cell-based assays, different regions of DInR interact with Chico and Dock, adapter proteins implicated in growth and axon guidance, respectively. Using *in vivo* rescue experiments, we found that mutations in DInR's Chico interaction sites rescued viability and axon guidance defects, but, as expected, animals were small, similar to *chico* mutants. This finding supports the notion that DInR interacts directly with Chico to control growth during *Drosophila* development. In contrast, expression of both wild type and mutant DInR proteins rescued axon guidance defects of *dinr* mutants. Rescue by wild type DInR confirmed its role in photoreceptor axon targeting. However, it was not expected that DInR variants with mutations in Dock binding sites (Y2, LESL) would rescue axon guidance. At first glance, these results appear to suggest that Dock interaction with DInR is not required for DInR function in axon guidance. However, several other explanations are possible. For example, the single tyrosine identified in our studies that is required for viability (Y1) may also be required for DInR's role in axon guidance. Alternatively, Dock may be capable of binding to multiple sites in DInR *in vivo*, possibly including the many candidate tyrosines and PXXPs in the C-tail (**Figure 1B**). This would lead to functional redundancy *in vivo*, providing a high degree of buffering to ensure interaction between the two proteins and proper axon targeting. In sum, our studies demonstrate that DInR utilizes different protein domains, and likely different adapter proteins, to segregate signaling in axon guidance and growth.

AUTHOR CONTRIBUTIONS

Caroline R. Li and Dongyu Guo designed and carried out experiments and analyzed data. Leslie Pick designed experiments and oversaw experiments. Leslie Pick and Caroline R. Li wrote the paper.

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Regulation of insect behavior via the insulin-signaling pathway

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The insulin/insulin-like growth factor signaling (IIS) pathway is well-established as a critical regulator of growth and metabolic homeostasis across the animal kingdom. Insulin-like peptides (ILPs), the functional analogs of mammalian insulin, were initially discovered in the silkmoth Bombyx mori and subsequently identified in many other insect species. Initial research focused on the role of insulin signaling in metabolism, cell proliferation, development, reproduction and aging. More recently however, increasing attention has been given to the role of insulin in the regulation of neuronal function and behavior. Here we review the role of insulin signaling in two specific insect behaviors: feeding and locomotion.

Keywords: insulin signaling, *Drosophila melanogaster*, behavior, feeding, locomotion

INTRODUCTION

In both vertebrates and invertebrates, insulin is a key metabolic hormone that modulates carbohydrate and lipid metabolism in response to an organisms' nutritional state. Dysregulation of insulin production, release, or downstream signaling leads to metabolic disease, including diabetes and obesity (Baker and Thummel, 2007; Hoffmann et al., 2013). The role of insulin in metabolic homeostasis as well as development, fertility and lifespan is well-established, whereas the function of insulin in the brain and behavior is not as well-understood (Britton et al., 2002; Ikeya et al., 2002; Rulifson et al., 2002; Broughton and Partridge, 2009). In mammals, insulin is known to act on the brain to modulate behaviors relating to reproduction, feeding, and memory (Gerozissis and Kyriaki, 2003). However, given the complexities inherent to the study of mammals, the use of simpler organisms, such as insects, provides an excellent opportunity to elucidate the role of insulin signaling in neuronal function and behavior (Teleman, 2010).

Insulin-like peptides (ILPs) were originally discovered in the silkmoth *Bombyx mori* and subsequently identified in migratory locusts, mosquitos, and scarab beetles among other insects (Lagueux et al., 1990; Riehle et al., 2006; Lavine et al., 2013). The study of insulin signaling in insects was significantly advanced by the identification of eight insulin-like genes in the genome of the fruit fly, *Drosophila melanogaster* (Brogiolo et al., 2001; Grönke et al., 2010; Colombani et al., 2012). In mammals, insulin and insulin-like growth factors signal through several different receptors whereas in *Drosophila* all ILPs signal through a single insulin receptor (InR) (Fernandez et al., 1995; Yamaguchi et al., 1995; Brogiolo et al., 2001). In the adult fly, three of these ILPs (2, 3, and 5), are expressed in the dorso-medial protocerebrum by two clusters of neurosecretory cells known as the Insulin-Producing Cells (IPCs). The IPCs are located in the pars intercerabalis, a brain region analogous to the mammalian hypothalamus, and their axons project to other regions in the brain and to the fly heart (Rulifson et al., 2002). Secreted ILPs can activate insulin signaling in the brain or head or enter the fly circulatory system to activate systemic insulin signaling in peripheral tissues. The regulation of ILP production and release has recently been reviewed by Nässel et al. (2013).

In *Drosophila*, the field of behavioral genetics began with the identification of the *period* gene as a critical regulator of rest:activity rhythms in the fly (Konopka and Benzer, 1971). Since then the fruit fly has been used extensively to identify genes and pathways underlying many complex behaviors. This review will focus primarily on *Drosophila* largely because of its simple and well-studied neuroanatomy as well as its genetically tractable nature, both of which facilitate the study of insulin's role in the brain and complex behavior.

THE IPCs AND INSULIN SIGNALING PATHWAY INFLUENCE FEEDING BEHAVIOR

Maintaining adequate energy stores is critical for animal survival and reproduction. As a result, neural and molecular mechanisms have evolved to modulate feeding behavior in response to an animal's internal physiological state as well as changes in food availability in the environment. Feeding begins with the motivation to eat, followed by a search for a suitable food source, which can be influenced by smell and taste among other factors. Feeding is terminated upon reaching a satiated state. In mammals it has long been known that insulin signaling suppresses food intake (Woods et al., 1979; Williams and Elmquist, 2012). Similarly, insulin has also been shown to play an important role at several stages of feeding behavior in insects, including *Drosophila melanogaster*. Studies in insects have also identified neuropeptides, many of which have mammalian orthologs, involved in the regulation of physiology and behavior, including feeding behavior (Nässel and Winther, 2010). Many of these other molecules and

mechanisms regulating feeding behavior in fruit flies are reviewed by Itskov and Ribeiro (2013). Here we will focus solely only on instances in which insulin-signaling modulates *Drosophila* feeding behavior.

Hunger or deprivation due to limited food availability provokes animals to acquire food. Initiation of motivated foraging is crucial for survival so animals have developed mechanisms to ensure that this response occurs under appropriate conditions. The insulin signaling pathway links the fly's internal metabolic state with the initiation of feeding behavior. In larval IPCs, constitutive activation of a downstream effector of the InR, p70/S6 kinase (dS6K), reduces foraging motivation and food acquisition (Oldham and Hafen, 2003; Wu et al., 2005a). This attenuated feeding response may be mediated by increased ILP release since pan-neuronal overexpression of *ilp2* or *ilp4* also reduced motivated foraging (Wu et al., 2005a). Overall these data indicate that hunger normally downregulates S6K activity in the IPCs to reduce insulin release and drive deprived animals to search for and acquire food. Starvation-induced food acquisition is also modulated by insulin signaling in mushroom body neurons known as Kenyon cells (Zhao and Campos, 2012). Inhibition of insulin signaling in Kenyon cells throughout development reduces food intake following starvation. Interestingly, temporary suppression of synaptic transmission by these neurons also partially reduces food intake. Thus, insulin signaling in Kenyon cells during development may modulate the synaptic activity of these neurons to ultimately regulate food acquisition.

After assuming motivated foraging, animals must utilize their sense of smell in order to find an adequate food source. Starvation decreases the amount of time a fly takes to find food by heightening their sense of smell, or in more technical terms, by enhancing odor representation in neurons that process olfactory input, known as odorant receptor neurons (ORNs) (Root et al., 2011). Enhancement of odor representation is facilitated by the expression of short neuropeptide F (sNPF) and its receptor (sNPFR) in specific ORNs (Carlsson et al., 2010; Root et al., 2011). Both sNPF and its mammalian orthologue, Neuropeptide Y (NPY), are known to promote feeding behavior (Lee et al., 2004; Kageyama et al., 2012). Intriguingly, Root and colleagues found that insulin interacts with the sNPF pathway by acting as a satiety signal to decrease sNPFR expression in ORNs and in turn decrease motivated feeding (Root et al., 2011). Hence during starvation when insulin signaling is low, sNPFR levels are high to mediate increased ORN sensitivity to odors and to encourage food acquisition.

The gustatory system also plays a role in feeding behavior primarily by influencing food choice. When determining what to eat, animals must assess both the nutritional content and palatability of a food source. A hungry fly initially decides what to eat based on taste; choosing the sweeter option over the more calorie dense option. However, over time, this preference shifts toward the more calorie rich option (Stafford et al., 2012). This change in preference is at least partially mediated by insulin. *Ilp2* and *ilp3* mutants and decreased insulin signaling throughout the brain all show an increased initial preference for the more caloric food source instead of the sweeter option (Stafford et al., 2012). This suggests that insulin contributes to calorie sensing and food source preference by acting directly on neurons in feeding circuits.

In the absence of a preferred food source, animals will feed on less palatable food sources. Drosophila larvae prefer to feed on rich liquid food as opposed to solid food. This preference is partially mediated by insulin signaling in cells that promote food intake through signaling downstream of the receptor for NPF, a distinct peptide from sNPF but also a fly orthologue of NPY. Downregulating insulin signaling in NPFR neurons causes fed larvae to be hungry and also increases their consumption of the less preferred solid food (Wu et al., 2005b). Conversely, upregulation of the insulin pathway in NPFR neurons of deprived animals elicits attenuated feeding of solid food (Wu et al., 2005b). In states of starvation, animals adapt by undertaking risky behaviors including searching for food under less than optimal conditions and consuming normally aversive noxious food. Under unfavorable conditions like cold temperature, the NPF/IIS signaling cascade enables starving *Drosophila* larvae to adapt and continue searching for food in order to survive (Lingo et al., 2007). This pathway also regulates risk-sensitive food acquisition with respect to noxious or bitter compounds. Overexpression of NPFR as well as down regulation of insulin signaling in NPFR neurons both increase consumption of noxious or bitter compounds in non-deprived larvae (Wu et al., 2005b). These results indicate that the NPF and ILP signaling mediate foraging responses under adverse conditions presumably to promote survival.

After determining what to eat, animals must also determine how much they will eat. Under very poor nutrient conditions flies will compensate by eating more. Interestingly this change in feeding behavior does not occur in IPC ablated flies (Broughton et al., 2010). Similarly, inhibition of IPCs also results in attenuation of food intake under poor nutrient conditions (Cognigni et al., 2011). However, given that IPC ablation or inhibition presumably affects the expression levels of ILPs as well as other peptides expressed in these cells; alterations in feeding behavior may not simply be due to changes in insulin signaling. In addition to ILPs, the IPCs also express the cholecystokinin-like peptide, drosulfakinin (DSK) (Park et al., 2008; Söderberg et al., 2012). IPC specific reduction of *dsk* increases food consumption and alters the ability of the fly to discriminate between food choices. However, this effect is not independent of insulin because reduction of *dsk* also alters *ilp* transcript levels (Söderberg et al., 2012). Nonetheless, DSK likely modulates the amount of food a fly consumes by acting in conjunction with DILPs to convey fullness to the animal.

In addition to feeding behavior, *Drosophila* has also been used to investigate the mechanisms underlying physiological and behavioral responses to alcohol consumption (Devineni and Heberlein, 2013). The IPCs and insulin signaling in the brain are implicated in ethanol sensitivity and the behavioral response to this addictive drug. Inhibiting protein kinase A (PKA) activity in the IPCs increases ethanol sensitivity as does decreasing insulin receptor signaling suggesting that reduced PKA activity in IPCs results in decreased insulin production and/or release which ultimately causes increased ethanol sensitivity (Corl et al., 2005). On the other hand, exposure to ethanol can affect insulin signaling. Developmental ethanol exposure in the fly, as in many

other organisms, has been shown to have detrimental effects. Flies that experienced developmental ethanol exposure are less viable, developmentally delayed, smaller in body size, and respond differently to alcohol as adults. These phenotypes are all due to reduced insulin signaling in exposed animals (McClure et al., 2011).

Some of the mechanisms involved in the regulation of feeding behavior discussed above have also been found to play a role in feeding behavior regulation in other insects not discussed here such as the African malaria mosquito *Anopheles gambia* and honey bees (Arsic and Guerin, 2008; Wang et al., 2010). Generally insulin conveys the organism's nutritional state, whether starved or fed, and accordingly drives feeding behavior in response to that state.

EFFECTS OF INSULIN SIGNALING ON LOCOMOTOR ACTIVITY

Many insects, including *Drosophila*, are crepuscular; they are primarily active at twilight or dawn and dusk. An internal clock entrained to the external environment largely through light and temperature cues, tightly regulates the timing of locomotor activity [Reviewed in Allada and Chung (2010)]. This well-conserved molecular oscillator is comprised of interlocked transcriptional/translational feedback loops, in which clock proteins negatively regulate their own transcription to create an endogenous rhythm of roughly 24 h [Reviewed by Zheng and Sehgal (2012)]. Briefly, in *Drosophila*, the core molecular oscillator consists of two transcription factors, CLOCK (CLK) and CYCLE (CYC), which drive the expression of the clock genes *period* (*per*) and *timeless* (*tim*). Eventually PER and TIM proteins re-enter the nucleus and inhibit the activity of CLK/CYC, thus preventing their own transcription. Ultimately the inhibition of CLK/CYC is relieved following the degradation of PER and TIM, allowing the cycle to begin again.

The anatomical and functional organization of the roughly 150 clock neurons in each hemisphere of the adult fly brain is well-established (Allada and Chung, 2010). Clock neurons located within the ventral lateral regions of the brain, known as the small ventral lateral neurons (sLNvs), are sufficient for driving locomotor rhythms under conditions where no external cues are present (constant darkness and temperature). The sLNvs produce the neuropeptide pigment dispersing factor (PDF) which rhythmically accumulates in the dorsal projections of the sLNvs. These axonal projections terminate near the PI, the aforementioned neuroendocrine center that encompasses the IPCs among other neurosecretory cells. The PI is implicated in the control of circadian rest:activity rhythms; however, neither the IPCs or the ILPs produced by these cells have been reported to directly regulate rest:activity rhythms (Helfrich-Forster et al., 2000). Nevertheless, there is data implicating insulin signaling in modulating the sensitivity of the circadian clock to oxidative stress, as measured in response to the redox cycling agent paraquat (Zheng et al., 2007). Activation of the insulin receptor initiates a signaling cascade that results in the retention of the forkhead transcription factor FOXO in the cytoplasm (Puig et al., 2003). *Drosophila* FOXO mutants and, by extension, perhaps elevated insulin signaling (which leads to the deactivation of FOXO), display increased sensitivity to oxidative stress. Thus, the metabolic state of the fly can influence the clock to affect behavioral rhythms.

In contrast to mammals, circadian regulation of the endocrine system has not been as extensively investigated in insects (Bloch et al., 2013). Neurohormones are excellent candidates for conveying temporal information from neuronal pacemakers to peripheral tissues because they are released from the brain and circulate in the blood allowing them to reach distal tissues and cells. ILPs are one such potential time-conveying neurohormone, but whether they are indeed regulated by the circadian system has yet to be definitively established in insects. In the blood feeding bug, *Rhodnius prolixus*, brains have been found to release ilps with a 24 h rhythm *in vitro* (Vafopoulou and Steel, 2002). Additionally, IPC projections in *Rhodnius* are described as closely associating with the axonal projections of PDF positive neurons in the circadian system, but whether these associations are indicative of functional synapses has not yet been determined (Vafopoulou and Steel, 2012). In *Drosophila*, microarray studies suggest the insulin signaling pathway may be regulated by the circadian system through the circadian expression of *susi*, a negative regulator of Phosphatidylinositol-3 kinase (PI3K) activity (Claridge-Chang et al., 2001; McDonald and Rosbash, 2001; Ueda et al., 2002; Wittwer et al., 2005). Additionally, slowpoke binding protein (SLOB) is expressed in a circadian manner in the IPCs and *slob* mutants have altered insulin-signaling, however a function for SLOB in behavior is unclear (Jaramillo et al., 2004; Sheldon et al., 2011).

In addition to being under circadian control, rest:activity behavior is also under the control of the homeostatic system which regulates rest duration and quality. Short sleep duration and poor sleep quality are associated with metabolic dysfunction (Spiegel et al., 2009). In *Drosophila*, octopamine, the insect equivalent of norepinephrine, promotes wakefulness by binding to octopamine receptors on the cell membranes of IPCs (Crocker and Sehgal, 2008; Crocker et al., 2010). Given that the major output of the IPCs is ILPs, we asked whether octopamine signaling in the IPCs promotes wakefulness by modulating the insulin signaling pathway. We found that this is not the case as increasing octopaminergic signaling in a *ilp2-3* mutant background still results in decreased nighttime sleep similar to increasing octopaminergic signaling in a wildtype background (Erion et al., 2013). In addition, manipulations that either decreased or increased insulin signaling in the brain or fat body did not decrease sleep (Erion et al., 2013). These data do not rule out the possibility that sleep homeostasis is modulated by insulin signaling but does strongly suggest that it is not responsible for relaying the wake-promoting effects of octopamine.

Aspects of locomotor activity are sexually dimorphic in *Drosophila*. Males move more steadily compared to females as indicated by fewer activity/inactivity periods (or start/stop bouts), while the total distance traveled is similar for both sexes (Gatti et al., 2000; Belgacem and Martin, 2002). Restricted expression of the sex-determination transcription factor, *transformer* (tra), identified a subset of neurons in the PI, henceforth called feminizing cells (FCs), capable of feminizing the locomotor activity pattern of male flies (Gatti et al., 2000). The axonal projections of both FCs and IPCs terminate at the corpus cardiacum/corpus allatum (cc/ca) (Gatti et al., 2000; Ikeya et al., 2002). This gland synthesizes juvenile hormone (JH) which regulates many

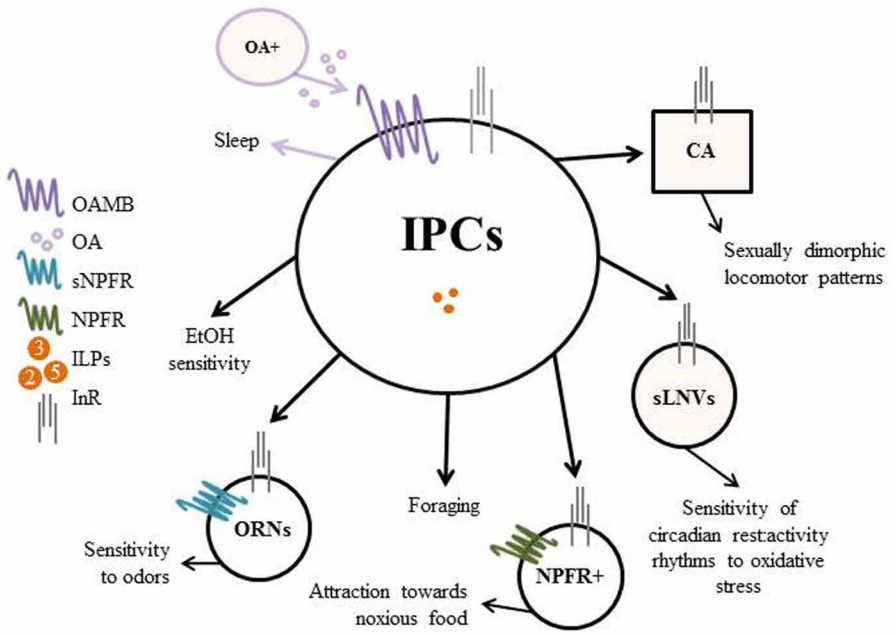

FIGURE 1 | Regulation of behavior by the Insulin-Producing Cells (IPCs) in Drosophila. The IPCs modulate feeding and locomotor (shaded) behavior through the insulin pathway. Decreased insulin production/release from the brain IPCs results in decreased downstream insulin signaling and leads to increased ethanol (EtOH) sensitivity and motivated foraging. Decreased insulin signaling in sNPFR (short Neuropeptide F Receptor) expressing odor receptor neurons (ORNs) and in NPFR (Neuropeptide F Receptor) expressing neurons, increases the sensitivity of ORNs to odors and increases the attraction of flies towards normally aversive or noxious food sources, respectively. With respect to locomotion, octopaminergic neurons signal through the IPCs to promote wake; however, this effect is independent of insulin signaling. Insulin signaling in the corpus allatum (CA), a non-neuronal endocrine gland (indicated by square), drives sexual dimorphism of locomotor patterns. Lastly, insulin signaling in the circadian small ventrolateral neurons (sLNVs) modifies the sensitivity of rest: Lactivity rhythms to oxidative stress.

important processes in insects including metamorphosis, reproduction, and aging (Tu et al., 2005). JH and insulin have both been implicated in the sexual dimorphism of locomotor behavior. Feeding male flies an inhibitor of 3-Hydroxy-3-Methylgluaryl CoA Reductase (HMGCR), a key JH biosynthesis enzyme, feminizes their locomotor activity (Belgacem and Martin, 2002). HMGCR mutants and targeted reduction of HMGCR in the ca both abolish sexual dimorphism. Similarly, sexual dimorphism in locomotor activity is eliminated in insulin receptor mutants and flies with ablated IPCs. Interestingly, the insulin receptor is expressed in the ca and insulin receptor mutants have altered JH levels suggesting that there is a link between the insulin and JH pathways (Tatar et al., 2001; Belgacem and Martin, 2006). Furthermore, targeted reduction of the insulin receptor in the ca suppresses HMGCR expression in this tissue (Belgacem and Martin, 2007). Lastly, male mutants of *takeout*, a putative JH binding protein and known circadian output gene, also display feminization of their locomotor activity (Meunier et al., 2007). Thus, it appears that insulin, JH, and takeout may interact to regulate sexual dimorphism of locomotor behavior in flies; however their exact relationship remains unclear.

CONCLUSION

Given that behavior is so intricate, model organisms like *Drosophila melanogaster* as well as other insect species provide researchers with a framework to begin to teasing apart the many different pathways and molecules that contribute to behavior. In this review we have described the progress that has been made with respect to the regulation of feeding and locomotion by insulin, however many questions still remain (**Figure 1**). Future investigations will hopefully give us additional insight into the interactions between insulin and neural circuits responsible for regulating behavior.

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Four-way regulation of mosquito yolk protein precursor genes by juvenile hormone-, ecdysone-, nutrient-, and insulin-like peptide signaling pathways

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Anautogenous mosquito females require a meal of vertebrate blood in order to initiate the production of yolk protein precursors by the fat body. Yolk protein precursor gene expression is tightly repressed in a state-of-arrest before blood meal-related signals activate it and expression levels rise rapidly. The best understood example of yolk protein precursor gene regulation is the *vitellogenin-A* gene (*vg*) of the yellow fever mosquito *Aedes aegypti*. *Vg-A* is regulated by (1) juvenile hormone signaling, (2) the ecdysone-signaling cascade, (3) the nutrient sensitive target-of-rapamycin signaling pathway, and (4) the insulin-like peptide (ILP) signaling pathway. A plethora of new studies have refined our understanding of the regulation of yolk protein precursor genes since the last review on this topic in 2005 (Attardo et al., 2005). This review summarizes the role of these four signaling pathways in the regulation of *vg-A* and focuses upon new findings regarding the interplay between them on an organismal level.

Keywords: mosquito, vitellogenesis, insulin, juvenile hormone, ecdysone, target of rapamycin, yolk proteins

INTRODUCTION

MOSQUITO SPECIES USE TWO DIVERGENT REPRODUCTIVE STRATEGIES

Mosquito species can be divided into two groups according to their reproductive strategy. Autogenous mosquitoes do not require an initial blood meal and produce their first batch of eggs relying solely on nutrients accumulated during their larval phase. Vitellogenesis, the production of yolk by the fat body for deposition in developing oocytes, begins a few hours after eclosion of autogenous adult females. Subsequent egg batches are dependent upon the energy and nutritional building blocks derived from vertebrate blood.

In contrast, anautogenous mosquitoes require an obligatory blood meal to produce their first batch of eggs. In these species, vitellogenesis is tightly repressed until blood meal-associated signals activate signaling cascades in involved organs and tissues. Most mosquito species fall into one of these two categories. However, there are exceptions to this rule. For example, *Aedes atropalpus* has both autogenous and anautogenous strains. Most of the human disease-transmitting species fall into the anautogenic category. The underlying reason for this is that the necessity for blood by anautogenous mosquitoes drives increased interaction between vector and host making them inherently better disease vectors. In addition, the fact that they feed earlier gives the parasites more time to finish the extrinsic part of their life cycle and become infective within the vector. Autogeny vs. anautogeny is discussed in more detail in Attardo et al. (2005).

YOLK PROTEIN PRECURSOR PROTEINS ARE ESSENTIAL FOR MOSQUITO REPRODUCTION

Insect oocytes are loaded with yolk proteins during the process of vitellogenesis. Yolk proteins provide the essential nutrients required for embryonic development. Mosquitoes produce yolk protein precursors (YPPs) exclusively in the fat body, the insect analog of the vertebrate liver. The YPPs are secreted into the hemolymph and taken up by the developing oocytes via receptor-mediated endocytosis (Sappington et al., 1996). Several yolk protein genes from *Ae. aegypti* have been independently cloned and characterized (Deitsch and Raikhel, 1993; Cho et al., 1999; Sun et al., 2000). The publication of the annotated *Ae. aegypti* genome sequence in 2007 (Nene et al., 2007) facilitated the identification of all potential yolk protein genes *in silico*. A detailed RNA-Seq comparison of genes differentially expressed by the *Ae. aegypti* fat body before and after a blood meal revealed that two genes encoding vitellogenic cathepsin B, three genes encoding vitellogenins (vitellogenin A, B, C), and three genes encoding vitellogenic carboxy-peptidases are upregulated several hundredfold in the female fat body 24 h after a blood meal (Price et al., 2011). Together with three lesser expressed vitellin membrane proteins, yolk proteins account for more than a third of all messenger RNAs at this time point which represents the pinnacle of vitellogenesis. The abundance of YPP associated gene transcripts reflects the massive scale of protein synthesis the fat body performs during this process.

SIGNALING PATHWAYS IN THE FAT BODY

Several signaling pathways regulate the transition of the fat body from the previtellogenic state-of-arrest to vitellogenic YPP synthesis. Our understanding of the interplay between these pathways has grown significantly in the last few years (see **Figure 1**). In this review we summarize the latest findings on each of the following topics: juvenile hormone, ecdysone, nutrient, and insulin-like peptide signaling pathways in mosquitoes, the roles they play in regulating vitellogenic gene expression in fat body trophocytes and the crosstalk that occurs between these signaling pathways on an organismal level.

JUVENILE HORMONE

WHAT IS IT?

Juvenile hormone (JH) is a sesquiterpenoid that regulates insect development, reproduction and other processes in basically all insect species. Different groups of insects use different forms of JH, for instance mosquitoes use JH III (Clements, 1992).

WHERE IS IT MADE?

JH is synthesized by the corpora allata, a pair of specialized glands that are attached to the brain through the nervus corpora cardiaca and are in close proximity to the aorta.

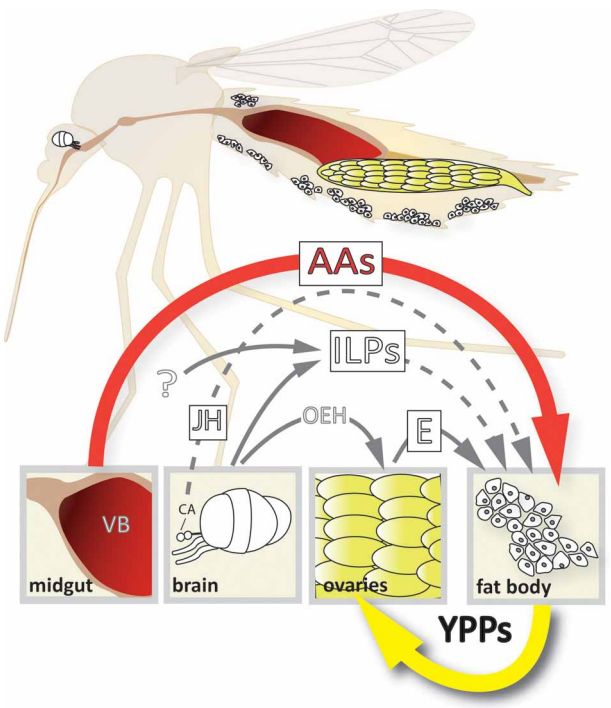

FIGURE 1 | Model of the 4-way regulation of YPP gene expression in *Ae. aegypti*. The upper part of the scheme depicts a longitudinal section showing the relative position of the different organs involved. Organ and cell size is not up to scale! The lower part shows the different signals involved and their origins. AAs, amino acids; CA, corpora allata; E, ecdysone; ILPs, insulin-like peptides; JH, juvenile hormone; OEH, ovary ecdysteroidogenic hormone; VB, vertebrate blood; YPPs, yolk protein precursors.

HOW IS JH SYNTHESIS REGULATED?

JH levels in the hemolymph and target tissues are thought to be regulated primarily by the rate of synthesis in the corpora allata. JH synthesis is controlled by peptide hormones that reach the glands through the hemolymph or by direct neural connections. Peptide hormones that stimulate JH synthesis are called allatotropins, those that inhibit JH synthesis are termed allatostatins (Gade et al., 1997). Signaling activity of JH is also regulated by proteins that facilitate its transport or breakdown in the hemolymph. Proteins promoting JH hydrolysis include JH esterase and JH epoxide hydrolases (Lassiter et al., 1994, 1995, 1996; Edgar et al., 2000; Bai et al., 2007), while JH-binding proteins facilitate the transport of this lipophilic molecule in the hemolymph (Prestwich et al., 1996). The rate of JH synthesis in newly eclosed female mosquitoes is in close correlation with their nutritional status (Noriega, 2004).

MODE-OF-ACTION

See **Figure 2A**—For several decades, efforts to identify a JH-receptor were unsuccessful. However, in recent years the understanding of JH's mode-of-action has grown significantly (Riddiford, 2012; Jindra et al., 2013). While there are still many open questions, there is growing evidence that the Methoprene-tolerant (Met) protein (and in *Drosophila* also its paralog germ-cell-expressed) acts as intracellular JH-receptor. In complex with other proteins these receptors form active complexes that bind JH-response elements in the DNA and regulate gene transcription (Li et al., 2011; Zou et al., 2013).

HOW DOES JH REGULATE MOSQUITO YPP EXPRESSION?

As its name suggests, one of the functions of JH during early postembryonic development is to prevent insect larvae from entering metamorphosis during the earlier larval molts. Another important role of JH is the regulation of insect reproduction. JH plays a central role in the regulation of *YPP* genes in mosquito females (Zou et al., 2013). After eclosion, females of anautogenous mosquito species enter a 3 day maturation period during which they do not blood-feed. Once this period is complete, the now "competent" females enter a state-of-arrest during which vitellogenic activity by the fat body is halted. The state-of-arrest is only broken when a blood meal is taken. Autogenous mosquitoes express *YPP* genes in the fat body earlier and without a blood meal stimulus; for example the vitellogenin-1 gene of *Culex tarsalis* is strongly expressed 24 h after eclosion (Provost-Javier et al., 2010). The ability or inability of an individual anautogenous mosquito to overcome the state-of-arrest and activate YPP gene expression is pre-established by its individual JH exposure history which in turn is correlated with the level of nutrients acquired during the larval phase (Noriega, 2004). Groups of *Ae. aegypti* larvae that are raised under severe nutritional restriction and overcrowded conditions give rise to significantly smaller adults compared to well-nourished under-crowded groups. Such small-sized *Ae. aegypti* females require more than one blood meal to complete vitellogenesis and develop a first batch of eggs (Shiao et al., 2008). This phenotype is due to the altered endocrinology of small mosquitoes; specifically these mosquitoes have lower JH levels during the 3 day maturation period which results in

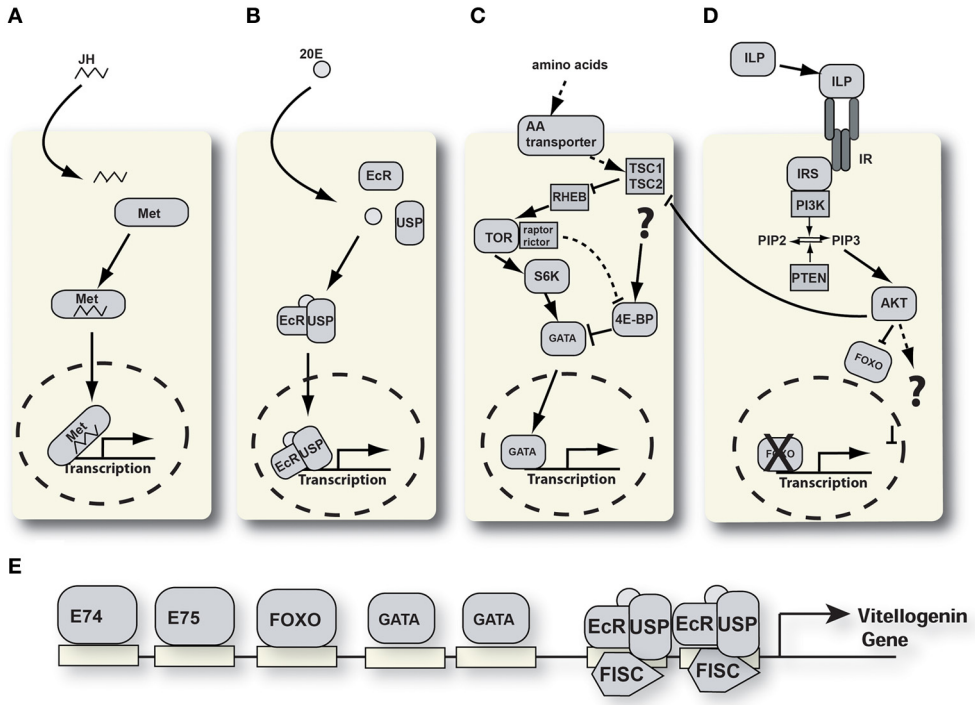

FIGURE 2 | Signaling pathways involved in YPP regulation. (A) Juvenile hormone signaling pathway. JH, juvenile hormone; Met, methoprene tolerant. **(B)** Ecdysone signaling pathway. 20E, 20 hydroxyecdysone; EcR, ecdysone receptor; USP, ultraspiracle. **(C)** Nutrient signaling pathway. 4E-BP, 4E-binding protein; TSC, tuberous sclerosis complex; RHEB, RAS homologue enriched in brain; S6K, S6 kinase; TOR, target of rapamycin. **(D)** Insulin-like peptide signaling pathway. AKT, protein kinase B; FOXO, forkhead box protein O; ILP, insulin-like peptide; IRS, insulin receptor substrate; PI3K, phosphatidylinositide 3-kinase; PIP2, phosphatidylinositol 4,5-bisphosphate; PIP3, phosphatidylinositol (3,4,5)-triphosphate; PTEN, phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase. **(E)** Schematic of the vg-A promoter with transcription factor binding sites.

the delayed onset of vitellogenesis (Noriega, 2004). Treatment of small mosquitoes with JH immediately after eclosion results in recovery of the nutrient-signaling machinery (see Nutrient signaling) in the fat body, timely vitellogenesis and the successful development of a batch of eggs after only one blood meal.

Therefore, the role that JH plays in the regulation of YPP expression in the fat body is most likely a preparatory one. Managing JH levels is critical for mosquitoes to regulate and coordinate their nutrient reserves with their reproductive cycle. This hormonally mediated balance avoids nutrient shortages and optimizes egg production.

It must be emphasized that the effects of JH on vitellogenesis and egg development are in no way restricted to the mosquito fat body. For example, a study by Clifton and Noriega showed that JH levels determine the fate of individual ovarian follicles in *Ae. aegypti* thereby determining the final egg numbers that are produced in a given gonotropic cycle (Clifton and Noriega, 2012).

ECDYSONE

WHAT IS IT?

Ecdysteroids are insect steroid hormones which, like JH, are key regulators of molting as well as reproduction in all holometabolous insects. Mosquitoes use the ecdysteroid ecdysone which is thought to be converted to its more bioactive form, 20-hydroxyecdysone (20E), in the fat body and other peripheral tissues, as described in *Drosophila* (Raikhel et al., 2002; Gilbert, 2004; Rewitz et al., 2006).

WHERE IS ECDYSONE MADE?

During larval development ecdysone is synthesized by the prothoracic glands. However, these glands degenerate and disappear rapidly after adult emergence (Clements, 1992). In adult female mosquitoes ecdysone is produced by the epithelial cells of the ovarian follicle in response to blood meal-derived signals (Hagedorn, 1982, 1989; Raikhel et al., 1999).

HOW IS ECDYSONE SYNTHESIS REGULATED?

During larval development a peptide hormone, prothoracicotropic hormone (PTTH), is released from the brain and stimulates ecdysone synthesis in the prothoracic gland by activating the RAS/ERK pathway (Gilbert et al., 2002; Rewitz et al., 2009). In adult females a different peptide hormone, the ovary ecdysteroidogenic hormone (OEH), substitutes the role of PTTH. OEH is produced in neurosecretory brain cells and stored in the corpora cardiaca (Brown and Cao, 2001). Upon blood feeding, stretch receptors within the midgut wall trigger a signal to the brain and OEH is released into the hemolymph of the mosquito (Hagedorn et al., 1975). OEH stimulates the follicle cells of the ovaries to synthesize and release ecdysone (Dhara et al., 2013). Four hours post-blood meal (PBM) ecdysone hemolymph titers

are slightly elevated. Levels continue to rise reaching a peak at 18–24 h PBM and decline afterwards (Hagedorn et al., 1975). OEH is a prominent regulator of ecdysone synthesis, but is by no means the only factor involved. Insulin-like peptide signaling also plays a role (see below).

MODE-OF-ACTION

See **Figure 2B**, 20E activates transcription of target genes by binding to a cytoplasmic receptor. The ecdysone receptor is a heterodimeric complex consisting of two proteins, the ecdysone receptor (EcR) and ultraspiracle (USP), a retinoid X receptor (RXR) homolog (Kapitskaya et al., 1996; Hall and Thummel, 1998; Wang et al., 2000a, 2002). Upon binding 20E, the EcR/USP/20E receptor complex binds to ecdysone-response elements in the promoter region of target genes and activates transcription (Tran et al., 2001; Marquardt, 2005). Functional ecdysone-response elements are critical constituents of *Ae. aegypti YPP* gene promoters (Martin et al., 2001a). The ecdysone signal is subsequently amplified by a cascade of primary and secondary response genes that are activated by rising ecdysone levels (Spindler et al., 2009).

HOW DOES 20E REGULATE YPP GENES?—PREVITELLOGENIC PHASE

During the previtellogenic state-of-arrest *YPP* gene expression is tightly repressed by multiple mechanisms, several of them affecting the reception of ecdysone signals. Both, EcR, and USP proteins are expressed during this stage in the *Ae. aegypti* female fat body (Yao et al., 1992; Wang et al., 2000b). However, these proteins do not form a heterodimer capable of binding to ecdysone response elements during this period. One key factor inhibiting the ecdysone hormonal cascade is the competitive binding of the orphan nuclear receptor AHR38 to USP during previtellogenesis (Zhu et al., 2000; Marquardt, 2005). After a blood meal and in the presence of 20E, EcR displaces AHR38 and forms a heterodimer with USP. Sevenup (Svp), a mosquito homolog of chicken ovalbumin upstream transcription factor, acts as a transcriptional repressor by binding directly to AGGTCA repeats within the ecdysone response elements of the vg-1 promoter, thereby inhibiting the ecdysone responsiveness of these promoters (Miura et al., 2002).

HOW DOES 20E REGULATE YPP GENES?—VITELLOGENESIS

During the vitellogenic period, ecdysone is converted to 20E by hydroxylation within the cytoplasm of the fat body cells. It activates gene transcription within trophocytes directly by binding to the EcR/USP heterodimer. This complex then translocates into the nucleus and binds to ecdysone receptor response elements (**Figure 2A**) (Hall and Thummel, 1998; Wang et al., 1998). *Aedes aegypti* has two characterized EcR and USP isoforms (*Aa*EcRa and *Aa*EcRb AaUSPa and AaUSPb) (Kapitskaya et al., 1996; Wang et al., 2000a, 2002).

An orphan nuclear factor, βFTZ-F1, mediates fat body competence for 20E responsiveness. βFTZ-F1 is upregulated pre-and post-vitellogenesis and interacts with a p160/SRC type coactivator, FISC, to directly recruit EcR/USP/FISC to 20E promoters at the onset of the 20E signaling (Li et al., 2000; Raikhel et al., 2002; Zhu et al., 2006; Ou and King-Jones, 2013). Transcripts for another nuclear receptor, HR3, increase over the 24 h after a blood meal suggesting it also plays a role in 20E responsiveness (Kapitskaya et al., 2000). 20E activation of EcR/USP induces the early response genes, *E74*, *E75*, and *Broad*, which in turn, activate transcription of late target genes like *vg-1* to amplify the hormonal signal (Raikhel et al., 2002; Sun et al., 2004; Chen et al., 2005).

Interestingly, 20E is also found in the male accessory glands of *Anopheles gambiae* mosquitoes and large amounts are transferred to females during mating. This suggests that 20E plays a role in modulating post-mating effects in females. However, a role for male derived 20E in *YPP* gene regulation has not yet been demonstrated in this mosquito (Pondeville et al., 2008).

NUTRIENT SIGNALING

WHAT IS THE SIGNAL?

Mosquitoes ingest vertebrate blood which functions as a rich source of amino acids. Amino acids function as building blocks for yolk protein precursor synthesis and are also used for energy production (Marquardt, 2004). The bulk of the protein contained within vertebrate blood is hemoglobin which is digested into free amino acids. The amino acids are transported via the hemolymph to the tissues of the female mosquito. After a blood meal, free amino acid levels in the mosquito hemolymph rise sharply as hemoglobin digestion in the mosquito midgut proceeds (Marquardt, 2004).

MODE-OF-ACTION

See **Figure 2C** The fat body monitors hemolymph amino acid levels via the target of rapamycin (TOR) signaling pathway which conducts the amino acid mediated signal to regulate *YPP* gene expression (Hansen et al., 2004). The key enzyme of this pathway, the TOR kinase, is a highly conserved protein that regulates protein translation in eukaryotic cells.

HOW DOES THE TOR SIGNALING PATHWAY REGULATE YPPs?

The exact mechanism eukaryotic cells utilize to sense hemolymph amino acid levels remains unknown. Amino acids are taken up by fat body trophocytes through a collection of specific amino acid transporter proteins that have different substrate specificities and may also work as receptors. The SLC7 family of cationic amino acid transporters play an important role in this process and are at the top of the nutrient signaling cascade in the fat body (Carpenter et al., 2012). RNAi-mediated gene knockdown of several of the SLC7-type transporters resulted in diminished TOR-signaling, *YPP* expression, and egg production (Attardo et al., 2006; Carpenter et al., 2012). The small GTPase Rheb functions upstream of TOR and is an indispensable part of this pathway in the fat body (Roy and Raikhel, 2011). Several downstream components of the TOR pathway are also associated with nutrient signaling in the fat body (**Figure 2B**). A well characterized downstream player in the TOR pathways is the S6 Kinase. S6 kinase is directly phosphorylated by TOR (Hansen et al., 2005) and in turn activates the translation of a GATA transcription factor in the fat body (AaGATAa). During the state-of-arrest, *YPP* gene transcription is repressed by another GATA transcription factor (*Aa*GATAr) that recognizes and binds to GATA-binding motifs upstream of the *YPP* gene region. Reception of a nutritional signal

results in GATAr being displaced by GATAa upon the vg1 promoter. GATAa acts as a transcriptional activator which enhances *YPP* gene expression (Martin et al., 2001b; Attardo et al., 2003; Park et al., 2006). 4E-BP, a repressor of translation, is also a downstream target of TOR (Hay and Sonenberg, 2004). After a blood meal 4E-BP is hyper-phosphorylated in the mosquito fat body. However, treatment with the TOR inhibitor rapamycin does not block 4E-BP hyper-phosphorylation which indicates the involvement of other signaling pathways (Roy and Raikhel, 2012).

INSULIN-LIKE PEPTIDE SIGNALING

WHAT IS IT?

Insulin-like peptides (ILPs) are an evolutionary conserved group of peptide hormones which are characterized by a conserved disulfide bond structure (De Meyts, 2004). ILP signaling plays a crucial role in immunity, reproduction and longevity of mosquitoes (Luckhart and Riehle, 2007). There are eight ILPs and one insulin receptor described in *Ae. aegypti* (Graf et al., 1997; Riehle et al., 2006).

WHERE ARE ILPs EXPRESSED?

ILPs and the insulin receptor are expressed in a variety of tissues in *Ae. aegypti* (Riehle and Brown, 2002; Riehle et al., 2006). Five ILPs are expressed in the brain and two are expressed in the head, thorax and abdomen of all life stages. These peptides assume a variety of possible roles including regulation of metabolic processes, cellular growth, lipid and glycogen processing, reproduction, and aging (Wu and Brown, 2006). Of the eight ILPs, ILP1, ILP3, and ILP 8 retain extremely conserved structures that are homologous to human insulin and relaxin 2 (Brown et al., 2008).

ILPs RECEPTOR

All known insulin receptors have tyrosine kinase activity. The first arthropod insulin receptor was characterized in D. *melanogaster* (Wu and Brown, 2006). The one known mosquito ILP receptor bears high homology to vertebrate insulin receptors and is comprised of two subunits, alpha and beta (Graf et al., 1997; Riehle and Brown, 1999). The alpha subunit contains the insulin binding domain while the beta subunit contains a tyrosine kinase domain which is responsible for the phosphorylation of downstream targets. Administration of human and porcine insulin to mosquitoes is capable of activating ILP signaling via their endogenous insulin receptor (Roy et al., 2007; Pakpour et al., 2012).

MODE OF ACTION

See **Figure 2D** Binding of the insulin receptor by ILPs activates a conserved signaling pathway as illustrated in **Figure 2D** (Wu and Brown, 2006). The phosphorylated insulin receptor substrate activates signaling via PI3K and other pathways. Downstream kinases phosphorylate effector proteins that regulate gene expression and other cellular processes.

HOW DOES INSULIN REGULATE YPPs GENES?

ILPs regulate *YPP* gene expression in the fat body both directly and indirectly. *In vitro* fat body culture experiments show that *YPP* expression is directly induced by the combination of the 20E and ILP signaling pathways acting together to activate gene expression. Roy et al., demonstrated the synergistic relationship of 20E and insulin by incubating isolated mosquito fat bodies for 3 h in different mixtures of porcine insulin and 20E (Roy et al., 2007). These experiments show that neither 20E nor insulin has an effect on expression individually; however a strong synergistic effect occurs when presented together.

ILPs also regulate *YPP* expression indirectly through the regulation of ecdysone synthesis. A blood meal stimulates the secretion of ILPs amongst other neuropeptides, namely OEH, that regulate ecdysteroid production (Riehle and Brown, 1999; Roy et al., 2007; Brown et al., 2008). In *Ae. aegypti,* ILP3 is characterized as the ILP responsible for initiating egg development (Brown et al., 2008). An experiment by Brown et al. demonstrates that ILP3, bovine, and porcine insulin stimulate the ovaries to produce ecdysteroids in decapitated females (Brown et al., 2008).

Manipulations of the ILP-signaling pathway in mosquitoes have resulted in profound changes in egg production. RNAi-mediated knockdown of the phosphatidylinositol-3,4,5-trisphosphate 3-phosphatase PTEN6, a suppressor of ILP signaling resulted in a significant increase in viable eggs produced by females (Arik et al., 2009).

One downstream effector gene of the ILP-signaling pathway has been characterized in mosquitoes. The forkhead-box transcription factor FOXO is a major target of ILP signaling in insects and vertebrates (Hwangbo et al., 2004). FOXO levels rise in the mosquito fat body after a blood meal and RNAi-mediated knockdown of this factor resulted in abolishment of *vg1* gene expression in isolated fat bodies that were stimulated with amino acids. Also, FOXO knockdown mosquitoes produced smaller egg numbers after a blood meal (Hansen et al., 2007). It is not known if this is due to direct or indirect effects.

CROSSTALK BETWEEN PATHWAYS

The interplay between the different signaling pathways regulating YPP gene expression is complex and only partially understood. However, significant levels of crosstalk appear to occur between the aforementioned signaling pathways in the different organs and tissues of adult female mosquitos. Several studies address these complex interactions.

JH/TOR

JH and nutrient signaling are connected in mosquitoes in two distinct ways. A recent study shows that JH biosynthesis is in part regulated by nutrient signaling via the TOR pathway (Perez-Hedo et al., 2013). Nutritional information impacts JH levels by regulating the expression of genes associated with the JH synthesis pathway in the corpora allata. A similar regulatory interplay between TOR signaling and JH expression was also found in the German cockroach, *Blatella germanica* (Maestro et al., 2009). JH in turn, regulates the expression of several genes coding for proteins associated with the TOR-signaling machinery in the mosquito fat body (Roy et al., 2007). Presence or absence of TOR pathway components profoundly influences the interpretation of nutritional signals by the fat body after a blood meal and decide if a blood meal results in immediate or delayed YPP synthesis.

ILP3/TOR, ILP3/ECDYSONE, ILP/ECDYSONE/JH

ILP3 has emerged as a key regulator of vitellogenesis via multiple mechanisms. After a blood meal, ILP3 activates the expression of

trypsin proteases in the midgut of mosquitoes thereby enhancing blood digestion and indirectly affecting TOR-mediated nutrient signaling (Gulia-Nuss et al., 2011). Three studies have demonstrated the effect of ILP signaling on ecdysone synthesis. The first study showed that unfed mosquitoes receiving ectopic treatment with bovine and porcine insulin results in activation of ecdysteroid secretion (Graf et al., 1997). The second study finds that ILP3 stimulates ecdysone synthesis and YPP uptake in female mosquitoes (Brown et al., 2008). The third study shows that insulin only stimulates ecdysteroid production when the mosquitoes are pre-treated with the JH analog methoprene (Borovsky et al., 1986).

A study in the red flour beetle, *Triboleum castaneum*, found that JH regulates *vg* gene expression through ILP signaling (Sheng et al., 2011). To our knowledge this has not been tested in mosquitoes, yet.

SYNOPSIS AND OUTLOOK

Figure 2E shows a schematic of the organization of transcription factor binding sites in the *vg1* promoter of *Ae. aegypti*. Several signaling pathways converge in the regulation of these and other YPP genes. In recent years our understanding of the signaling pathways involved and the interplay between them has undergone rapid growth. We know that at least four distinct pathways are involved: juvenile hormone, ecdysone, insulin, and the TOR nutritional signaling pathway. The challenge for the future is to understand the crosstalk between these pathways in different organs and during different physiological stages in adult female mosquitoes. Based upon the current understanding of signaling in mosquito reproduction we feel that the ILPs may play a key regulatory role in the determination of autogenous vs. anautogenous life histories in mosquitoes.

In addition, while ecdysone- and juvenile hormone analogs are already used as insect growth regulators, a challenge for the future is to use the novel and detailed understanding of nutrient and insulin-signaling pathways to develop new strategies for mosquito control.

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Overexpression of *dilp2* causes nutrient-dependent semi-lethality in *Drosophila*

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Insulin/insulin-like growth factor (IGF) plays an important role as a systemic regulator of metabolism in multicellular organisms. Hyperinsulinemia, a high level of blood insulin, is often associated with impaired physiological conditions such as hypoglycemia, insulin resistance, and diabetes. However, due to the complex pathophysiology of hyperinsulinemia, the causative role of excess insulin/IGF signaling has remained elusive. To investigate the biological effects of a high level of insulin in metabolic homeostasis and physiology, we generated flies overexpressing *Drosophila insulin-like peptide 2* (*Dilp2*), which has the highest potential of promoting tissue growth among the *Ilp* genes in *Drosophila*. In this model, a *UAS-Dilp2* transgene was overexpressed under control of *sd-Gal4* that drives expression predominantly in developing imaginal wing discs. Overexpression of *Dilp2* caused semi-lethality, which was partially suppressed by mutations in the insulin receptor (*InR*) or *Akt1*, suggesting that *dilp2*-induced semi-lethality is mediated by the PI3K/Akt1 signaling. We found that *dilp2*-overexpressing flies exhibited intensive autophagy in fat body cells. Interestingly, the *dilp2*-induced autophagy as well as the semi-lethality was partially rescued by increasing the protein content relative to glucose in the media. Our results suggest that excess insulin/IGF signaling impairs the physiology of animals, which can be ameliorated by controlling the nutritional balance between proteins and carbohydrates, at least in flies.

Keywords: *Drosophila* insulin-like peptides, insulin-like growth factor signaling, hyperinsulinemia, growth regulation, autophagy, protein-to-carbohydrate ratio

INTRODUCTION

In mammals, the peptide hormone insulin promotes glucose uptake in muscles and adipose tissues, induces cell growth and proliferation, and stimulates glyconeogenesis, lipogenesis, and protein synthesis (Saltiel and Kahn, 2001). The insulin/insulin-like growth factor (IGF) signal is evolutionally conserved throughout multicelluar organisms (Skorokhod et al., 1999). In insects, *Drosophila* has been extensively used as a model system to study insulin signaling, which plays an important role in regulating organ growth and the final size of the organism.

Drosophila possesses eight insulin-like peptides (*Dilps*), which can activate the *Drosophila* insulin receptor, InR (Brogiolo et al., 2001). Among the *Drosophila* insulin-like peptides (*Ilps*), *dilp2* is the most highly expressed and it has the highest potential for promoting tissue growth (Ikeya et al., 2002; Rulifson et al., 2002; Broughton et al., 2005). It has been demonstrated that reduction of *dilp2* increases the content of the insect blood sugar, trehalose, in adult flies, suggesting that *dilp2* regulates glucose homeostasis in *Drosophila* as it also does in mammals (Broughton et al., 2008). Furthermore, reduction of *dilp2* expression has been shown to increase lifespan, indicating that *dilp2* plays an important role in lifespan determination (Broughton et al., 2008).

On the other hand, excess activation of insulin signaling could impair the physiology of organisms. In humans, it has been proposed that increased levels of insulin in the blood is a primary cause of Type 2 diabetes associated with hypertension and cancers (Novosyadlyy and LeRoith, 2010). In fact, hyperinsulinemia, which is an excessive level of insulin in the blood, is often seen in several metabolic diseases, such as Type 2 diabetes mellitus (Samuel and Shulman, 2012). However, the coexistence of hyperglycemia, insulin resistance, and other hormonal and metabolic changes in patients with Type 2 diabetes makes it difficult to understand the causative role of excess insulin signaling in the pathophysiology of hyperinsulinemia (Corkey, 2012). Several animal models for hyperinsulinemia have been developed by overexpressing InR or IGFR in some tissues, by the short-time administration of insulin, or by feeding animals a high-sugar diet (Musselman et al., 2011). Although these models have contributed to elucidating the molecular mechanisms that regulate insulin/IGF signaling, how hyperinsulinemia affects animal physiology has remained elusive.

It has been demonstrated that dietary composition also affects physiology and lifespan of individuals. In *Drosophila*, the balance of protein to carbohydrate intake is one of the critical determinants for lifespan and fecundity (Lee et al., 2008; Skorupa et al., 2008; Lushchak et al., 2012). For example, flies maintained with glucose-rich/protein-poor food generally become obese with age and exhibited a shorten lifespan and vise versa. Although

insulin/IGF signaling plays crucial roles in regulation of glucose uptake, how the signal influences the dietary composition-dependent physiological changes is unclear.

To investigate how excess insulin affects insect physiology, we generated transgenic flies with a high level of *dilp2* and analyzed their phenotypes. Overexpression of *dilp2* increased the body size and caused semi-lethality. These phenotypes were partially suppressed by mutations in the insulin/IGF signaling pathway components, thereby suggesting that hyperactivation of the insulin/IGF signaling is toxic to flies. We found that *dilp2*-overexpressing flies exhibited intensive autophagy in fat body cells. Interestingly, increasing the protein content relative to glucose in the media partially rescued the *dilp2*-induced semi-lethality and autophagy. Our results suggest that excess insulin/IGF signaling impairs the physiology of animals, but it can be ameliorated by controlling the nutritional balance between proteins and carbohydrates, at least in flies.

MATERIALS AND METHODS

FLY STOCKS AND MEDIA

UAS-dilp2 (Brogiolo et al., 2001), $Akt1^{1}$ (Stocker et al., 2002), and InR^{304} (Brogiolo et al., 2001) were kindly provided by Dr. E. Hafen. $PTEN^{dj189}$ was a gift from Dr. D. Pan (Gao et al., 2000). Tor^{K17004}(Oldham et al., 2000), $S6K^{07064}$ (Montagne et al., 1999), and *M{3xP3-RFP.attP}ZH-51D* and *M{3xP3-RFP.attP}ZH-68E* (Bischof et al., 2007) were obtained from the Bloomington Stock Center. Flies were reared at 25°C on a standard cornmeal medium [3.6% neutralized yeast (Asahi Breweries, LTD. Y-4), 8.1% cornmeal, 10% glucose, and 0.7% agar] with propionic acid and *n*-butyl *p*-hydroxybenzoate as mold inhibitors, unless otherwise stated. We used different medium for the convenience of preparation. *Drosophila* Instant Medium (Formula 4-24, Carolina Biological. Supply, Burlington, NC) was used to as a basal medium to prepare media containing different concentration of yeast extracts: 2 g of *Drosophila* Instant Medium was mixed with 5 ml of Bacto™ Yeast Extract (Difco Laboratories, Detroit, MI, USA) dissolved in water at four different concentrations (0, 10, 20, and 40 g/L). Standard cornmeal agar medium was used to prepare media containing glucose at four different concentrations (0, 100, 200, and 300 g/L).

GENETIC INTERACTION EXPERIMENTS

To facilitate genetic interaction experiments, we generated a stock, *sd-Gal4/sd-Gal4; UAS-dilp2/TM6B, tub-Gal80*, in which GAL4-dependent expression of *dilp2* is repressed by GAL80. The stock is convenient to test the effects of mutations on the phenotype caused by overexpression of *dilp2*. To make an internal control, we made flies heterozygous for a mutation with a homologous chromosome marked with RFP: second chromosome-linked mutations (Tor^{K17004} and $PTEN^{dj189}$) and third chromosome-linked mutations ($Akt1^{1}$, InR^{304}, and $S6K^{07064}$) were crossed to *M{3xP3-RFP.attP}ZH-51D* and *M{3xP3-RFP.attP}ZH-68E*, respectively. The F1 progenies (mutations/*3xP3-RFP*) were crossed to *sd-Gal4/sd-Gal4; UAS-dilp2/ TM6B, tub-Gal80* flies. Numbers of resulting progenies (*sd-Gal4/+; UAS-dilp2/+* with mutations) and their sibling controls (*sd-Gal4/+; UAS-dilp2/+* with *3xP3-RFP*) were counted and calculated relative viabilities.

MEASUREMENT OF BODY WEIGHT AND WING SIZE

The adult flies were weighed using an Analytical Semi-Micro Balance (A&D Company, Tokyo, Japan). To measure wing size, the right wings of the adult flies were torn off by using forceps and mounted onto a microscopic slide using a drop of Fly Line Dressing (TIEMCO, Tokyo, Japan), a silicone grease with very low surface tension (Tsuda et al., 2010). The wings were photographed using a MZ APO stereomicroscope (Leica, Wetzlar, Germany) equipped with a DP50 digital camera (Olympus, Tokyo, Japan) at a constant magnification. The areas of the wings were measured by using ImageJ software (NIH).

QUANTITATIVE REAL-TIME PCR

Total RNA from the adult flies was extracted using TRIzol® (Qiagen, Valencia, CA, USA) and it was reverse-transcribed using ReverTra Ace® (Toyobo, Osaka, Japan). Quantitative-PCR reactions were carried out using SYBR® Premix Ex Taq™ (Takara Bio, Otsu, Japan).

WESTERN BLOT ANALYSIS

The adult flies were homogenized in SDS sample buffer (12.5 mM Tris (pH 6.8), 20% glycerol, 4% SDS, 2% 2-mercaptoethanol, and 0.001% bromophenol blue) and boiled for 10 min at 95°C. The samples were separated by 10% SDS-PAGE and transferred to PVDF membranes (GE Healthcare, Buckinghamshire, UK). After blocking with 5% bovine serum albumin (Sigma-Aldrich, St. Louis, MO, USA), the membranes were incubated with a primary antibody in Tris-buffered saline (TBS) containing Tween-20 (TBST) overnight at 4°C and then with a secondary antibody in TBST for 1 h at 25°C. The signals were detected with an ECL-plus kit (GE Healthcare). As primary antibodies, rabbit anti-phospho-Akt antibody (Cell Signaling Technology, Danvers, MA, USA), rabbit anti-phospho-p70 S6 kinase (Cell Signaling Technology), and mouse anti-α-tubulin (Sigma-Aldrich) were used at dilutions of 1:1000, 1:1000, and 1:5000, respectively. HRP-conjugated anti-rabbit IgG (Cell Signaling Technology) and HRP-conjugated anti-mouse IgG (GE Healthcare) were used as secondary antibodies at dilutions of 1:2000 and 1:1000, respectively.

LIQUID CHROMATOGRAPHY COUPLED TO TANDEM MASS SPECTROMETRY (LC-MS/MS)

LC-MS/MS was used to determine the concentrations of glucose, trehalose, glucose metabolites, and free amino acids. Ten female flies were weighted and homogenized with 75% acetonitrile on ice. The homogenates were centrifuged for 10 min at 2400 × g and the supernatant was re-centrifuged for 10 min at 1200 × g. The supernatant was evaporated and dissolved to mobile phase (10 mM DBAA, Tokyo Chemical Industry, Tokyo, Japan), in H_2O (pH 4.75). After centrifuging at 1200 × g to remove residue, the supernatants were collected and used for metabolome analysis with a Waters LC-QTofMS system composed of LC (Acquity UPLC) and MS (Xevo™ QTofMS) (Waters, Milford, MA, USA). The metabolites were separated on an Acquity® UPLC HSS T3 column (2.1 Å ∼ 100 mm, 1.8 μm; Waters). For the carbon metabolites, the columns were equilibrated with 10 mM

DBAA (Tokyo Chemical Industry) in H_2O (pH 4.75), and the compounds were eluted with an increasing gradient of acetonitrile. The total run time was 20 min. The MS system was equipped with a dual electrospray ionization probe and operated in the negative ion mode with the source temperature at 120°C. For the amino acids, the analytes were separated by a gradient of mobile phase ranging from water containing 0.05% acetic acid to methanol over a 15 min run. The capillary voltage and the cone voltage for electrospray ionization was maintained at 0.7 kV and 15 V for negative mode detection and at 0.7 kV and 13 V for positive mode detection, respectively. The source temperature and the desolvation temperature were set at 120 and 350°C, respectively. Nitrogen was used as both the cone gas (50 l/h) and the desolvation gas (600 l/h) and argon was used as the collision gas. For accurate mass measurement, the mass spectrometer was calibrated with sodium formate solution (range m/z 50–1000) and monitored by the intermittent injection of the lock mass leucine enkephalin ([M + H]+ = 556.2771 m/z and [M − H]− = 554.2615 m/z) in real time. The MS data were analyzed using QuanLynx™ (Waters). The compounds were identified based on their retention time, their m/z ratio, and the MS/MS spectrum of standard reference materials.

MEASUREMENT OF PROTEIN CONTENT

The concentration of soluble protein was measured using the Bio-Rad protein assay reagent. At least three trials were carried out for each genotype.

TRIGLYCERIDE MEASUREMENT

Ten adult flies were weighed and homogenized in 1% Triton-X. The homogenates were heated for 10 min at 70°C and stored at −80°C. After thawing on ice, the samples were centrifuged for 10 min at 14,700 × g at 25°C. The amount of triglycerides (TAG) was determined using a Serum Triglyceride Determination Kit (TR0100; Sigma–Aldrich, St. Louis, MO, USA). All data were normalized with soluble protein contents in homogenates. At least three samples were used to determine the average amount of triglycerides.

AUTOPHAGY STAINING

The fat bodies were dissected from the early third larvae in PBS and incubated with LysoTracker® Red DND-99 (Molecular Probes®, Life Technologies, Grand Island, NY, USA) at a 1:1000 dilution in PBS for 1 min at room temperature. After a brief wash in PBS, the sample was observed under a Nikon C1 laser scanning confocal microscope.

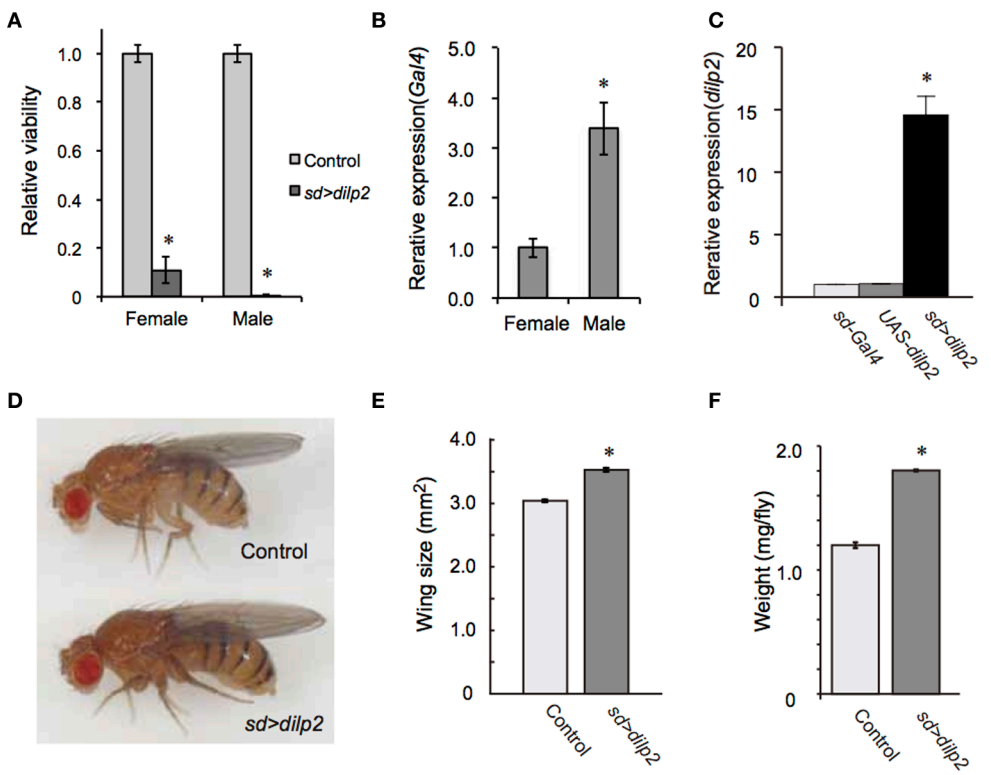

FIGURE 1 | Overexpression of *dilp2* increases body size and causes semi-lethality. Relative viability of *dilp2*-overexpressing flies **(A)**. Flies homozygous for *sd-Gal4* were crossed with *UAS-dilp2/3xP3-RFP* males, and the number of flies expressing RFP served as an internal control; those not expressing RFP are expressing *dilp2*. Expression level of Gal4 in *sd-Gal4* male is much higher than that of *sd-Gal4* female **(B)**. Overexpression of *dilp2* caused semi-lethality for both males and females. The relative expression level of *dilp2* mRNA in the *dilp2*-overexpressing flies (*sd* > *dilp2*) and the parental lines (*sd-Gal4* and *UAS-dilp2*) served as controls and was determined by real-time RT-PCR **(C)**. *dilp2*-overexpressing flies have increased body size **(D)**, increased wing area by 17% **(E)**, and increased weight by 50% **(F)**. Student *t*-test was performed to analyze statistical significance. $^*p < 0.01$.

RESULTS AND DISCUSSION

OVEREXPRESSION OF *dilp2* REDUCED THE EGG-TO-ADULT VIABILITY OF THE FLIES

To generate a transgenic fly model of hyperinsulinemia, we overexpressed *dilp2* which is known to promote tissue growth in *Drosophila*. We found that flies show high lethality when *dilp2* was overexpressed ubiquitously using *actin5c-Gal4*, suggesting that high levels of Dilp2 are toxic to flies. However, the reason for this lethality was not understood. An alternative driver, which gives a milder phenotype, would be useful to investigate the developmental toxicity associated with *dilp2* overexpression. We tested several *Gal4* lines, and found that *sd-Gal4*, which predominantly expresses *Gal4* in developing imaginal wing discs, was an appropriate driver. When *sd-Gal4* was crossed with *UAS-dilp2*,

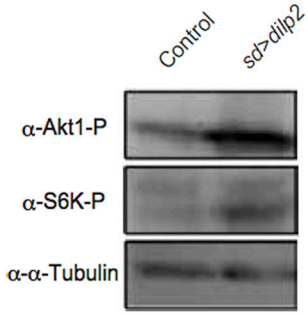

FIGURE 2 | *dilp2*-overexpression activates Akt1 and S6 kinase (S6K). The amount of phosphorylated Akt1 and S6K were analyzed by western blots. α-tubulin was used as a loading control.

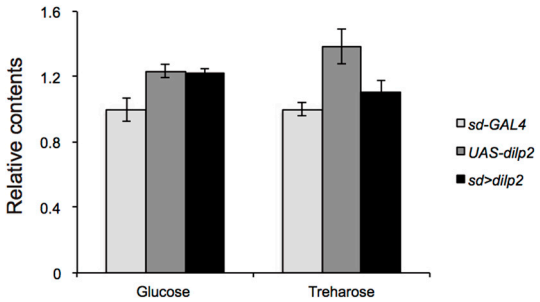

FIGURE 4 | Trehalose and glucose content in *dilp2*-overexpressing flies. Relative amount of glucose and trehalose in the *sd > dilp2* flies was calculated based on the values of a parental line (*sd-Gal4*) and indicated as the mean ± SE of at least three samples with a minimum of five flies per group. There was no significant difference in the amount of these sugars.

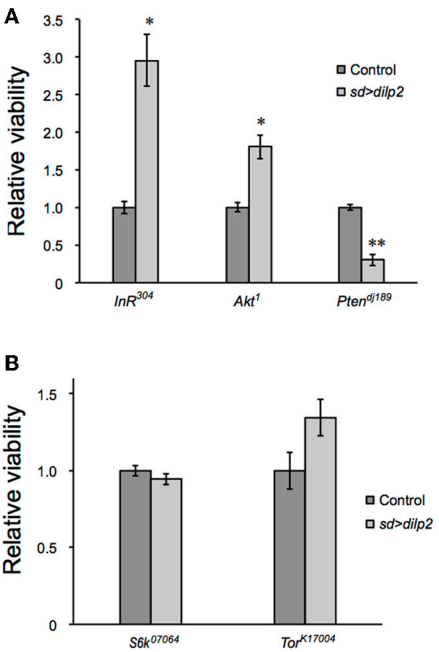

FIGURE 3 | Genetic manipulation of InR/Akt1/PI3K modifies the *dilp2*-induced lethality. Relative viability of the *sd > dilp2* flies was determined in combination with loss of function mutations in *InR*, *Akt1*, or *PI3K*, which are components of the insulin/IGF signaling pathway. *dilp2*-induced semi-lethality was markedly improved by reduction of *InR* or *Akt1*, but enhanced by reduction of *PTEN* **(A)**. Relative viability of the *sd > dilp2* flies in combination with loss-of-function mutations in *Tor* and *S6K* **(B)**. The Tor/S6K pathway may not contribute to mediating the semi- lethality of *dilp2*-overexpressing flies. Student *t*-test was performed to analyze statistical significance. $*p < 0.05$, $**p < 0.01$.

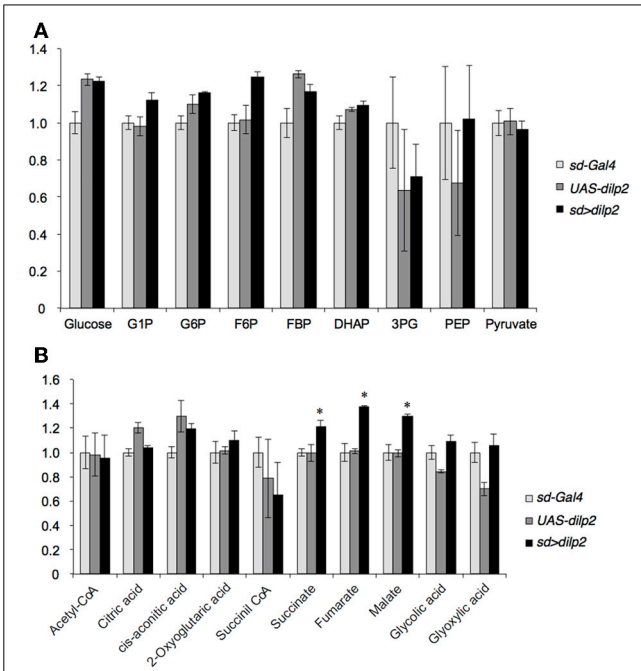

FIGURE 5 | Glucose metabolism in *dilp2*-overexpressing flies. Glycolysis **(A)** and TCA cycle **(B)** metabolites were analyzed by using LC-MS/MS in negative mode. Relative amounts of metabolites in the *sd > dilp2* flies were calculated based on the values of the parental lines (*sd- Gal4*) and shown as the mean ±S.E of at least three samples with a minimum of five flies per group. G6P: glucose 6-phosphate;F6P: fructose 6-phosphate; F1,6BP: fructose 1,6-bisphosphate; 2/3-PG: 2/3-phophoglycerate; PEP: phosphoenolpyruvate. Student *t*-test was performed to analyze statistical significance. $*p < 0.05$

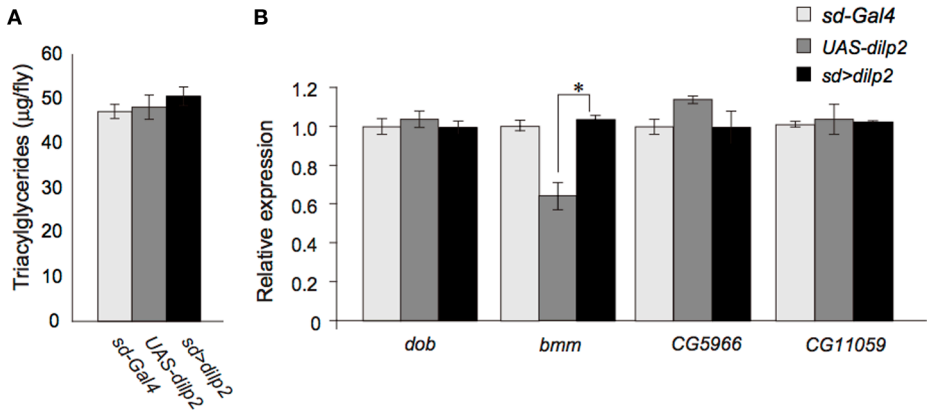

FIGURE 6 | Lipid metabolism in *dilp2*-overexpressing flies. Comparison of TAG levels between the *dilp2*-overexpressing flies and the parental lines as controls **(A)**. Relative mRNA levels of four major genes involved in lipid catabolism in *Drosophila*, *doppelganger von brummer* (*dob*), *brummer* (*bmm*), *CG5966*, and *CG11055* **(B)**. Results are shown as the mean ± SE of at least three experiments. ANOVA with Tukeys HSD was performed to analyze statistical significance. $^{*}p < 0.01$.

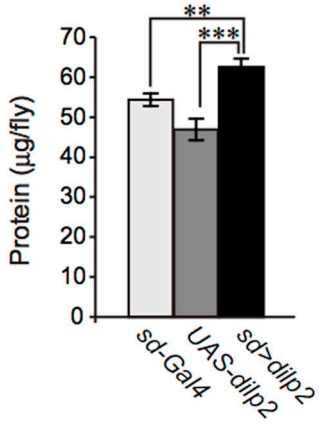

FIGURE 7 | Protein content in *dilp2*-overexpressing flies. The protein content was significantly increased in the *dilp2*- overexpressing flies (*sd* > *dilp2*) compared to the parental lines (*sd-Gal4* and *UAS-dilp2*) as controls. Results are shown as the mean ± SE of at least three experiments. Student *t*-test was performed to analyze statistical significance. $^{**}p < 0.01$; $^{***}p < 0.001$.

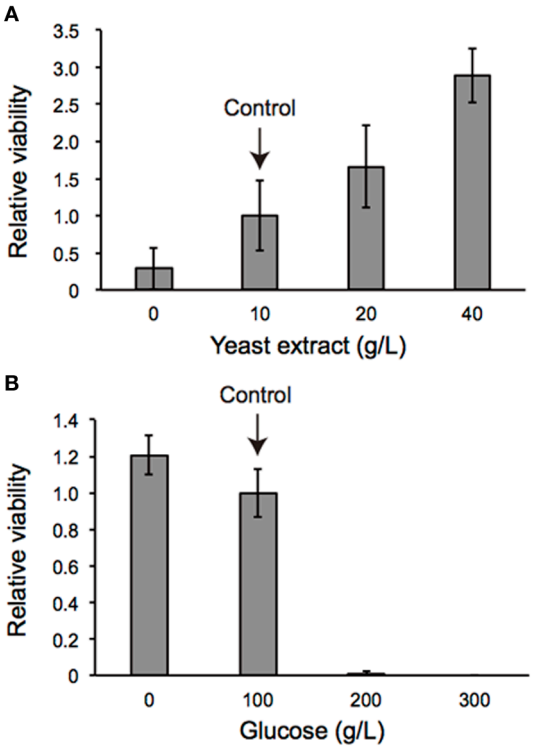

FIGURE 8 | Viability of *dilp2*-overexpressing flies depends on nutritional conditions. *Dilp2*-overexpressing flies (*sd* > *dilp2*) were reared on media supplemented with various concentrations of yeast extract **(A)** or glucose **(B)**. Relative viability of the *dilp2*-overexpressing flies (*sd* > *dilp2*) for each medium was calculated based on the number of control siblings.

the number of progenies from the cross was significantly less than the number of progenies obtained from the parental lines, thereby suggesting that overexpression of *dilp2* was toxic to flies. To more quantitatively determine the viability, the number of progenies were compared between the *dilp2*-overexpressing flies and their siblings bearing the *M{3xP3-RFP.attP}ZH-68E* chromosome expressing red fluorescence protein (RFP) under the control of an artificial *3xP3* promoter (Bischof et al., 2007). Female flies homozygous for *sd-Gal4* were crossed to *UAS-dilp2/M{{3xP3-RFP.attP}ZH-68E* heterozygous males and the number of adult flies was counted. In theory, one half of the progeny inherit and express *UAS-dilp2* under control of *sd-Gal4*, and the other half, serving as an internal control, carry an RFP-bearing chromosome. The number of *dilp2*-overexpressing flies was markedly reduced compared to that of the control flies (**Figure 1A**). Both sexes are semi-lethal, but the effects were more severe in the males than in the females. This is likely due to the dosage compensation mechanism, since *sd-Gal4* is an X-linked transgene,

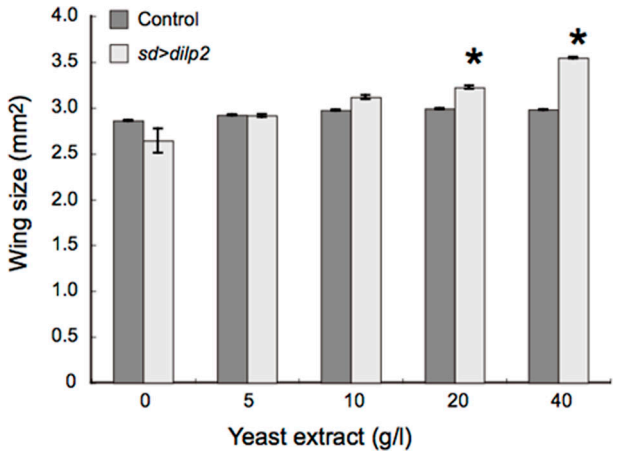

FIGURE 9 | Effects of protein-rich media on the wing size of *dilp2*-overexpressing flies. *dilp2*-overexpressing flies (*sd* > *dilp2*) were reared on media supplemented with various concentrations of yeast extract and their wing sizes were compared with those of the internal control flies. Mean wing area was positively correlated with the concentration of yeast extract in the media. Student *t*-test was performed to analyze statistical significance. $^*p < 0.05$.

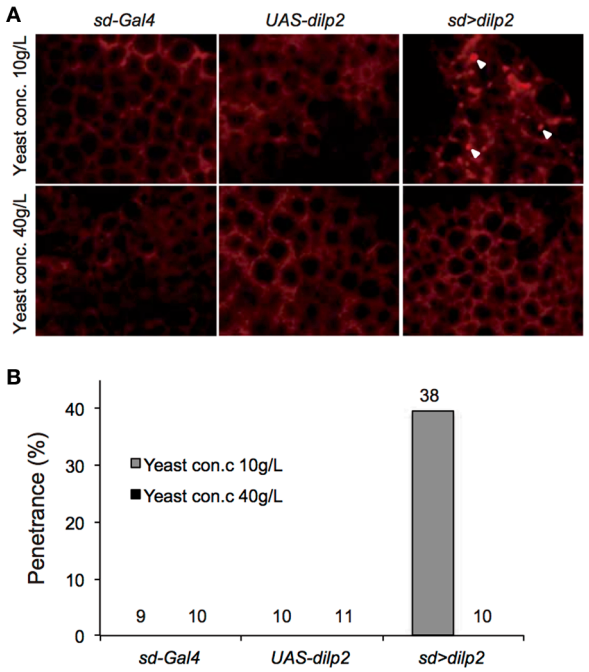

FIGURE 10 | Intensive autophagy in *dilp2*-overexpressing flies. Fat bodies were dissected from early third instar larvae overexpressing *dilp2* (*sd* > *dilp2*) and stained with LysoTracker Red DND-99 **(A)**. When the flies were reared on standard media containing 10 g yeast/L, strong punctate signals (arrowheads), corresponding to autophagy were observed in the *sd* > *dilp2* flies (upper right panel) but not in the parental lines (*sd-Gal4* or *UAS-dilp2*) as the controls (upper left and middle panels). Autophagy was not detectable in the *sd* > *dilp2* flies when they were reared on protein-rich media containing 40 g yeast/L (lower panels). The penetrance (frequency) of ectopic autophagy in *sd* > *dilp2* flies **(B)**. Numbers of samples observed were indicated above each bar.

which drives expression of *dilp2* two-times higher in males (**Figure 1B**).

Using female flies that survived to adult stage, we determined the expression level of *dilp2* in females. Quantitative real-time PCR revealed that the levels of *dilp2* mRNA in the adult female increased 16-times higher than that of the control, suggesting that overexpression of *dilp2* occurred and is toxic to fly development (**Figure 1C**).

We also noticed that the body size of the *sd* > *dilp2* flies that survived to the adult stage was larger than that of the control (**Figure 1D**). To compare the body size more precisely, wing size was measured as described in the Materials and Methods section. The wing size of the *sd* > *dilp2* flies was increased by 17% compared to the wing size of the control flies (**Figure 1E**). In addition, the body weight of the *dilp2*-overexpressing flies increased by 50% compared to the control flies (**Figure 1F**). These results indicated that overexpression of *dilp2* caused semi-lethality, but promoted growth for the survivors.

THE PI3K/Akt1PATHWAY MEDIATES *dilp2*-INDUCED SEMI-LETHALITY

dilp2 stimulates the PI3K/Akt1 pathway through insulin receptor (InR) activation. Western blotting analysis revealed that the active form of Akt1 was significantly increased in the *dilp2*-overexpressing flies compared to the control, indicating that overexpression of *dilp2* indeed activates the PI3K/Akt1 signaling (**Figure 2** upper panel). To further confirm this, we investigated whether the mutations in the pathway components had an effect on *dilp2*-induced semi-lethality. We found that, for both males and females, the heterozygous flies that carry a loss-of-function mutation in InR or Akt1 showed higher levels of viability compared to the control flies (**Figure 3A**). On the other hand, a loss-of-function mutation in Ptne, a negative regulator of PI3K, further reduced the viability of *dilp2*-overexpressing flies (**Figure 3A**). These results suggest that *dilp2*-induced semi-lethality is mediated by the PI3K/Akt1 signaling. Overexpression of *IlpP2* also activated the Tor/S6K signaling, a downstream signal component of the PI3K/Akt1 signaling, since the level of phosphorylated S6K was increased (**Figure 2** middle panel). However, interestingly, the loss-of-function mutations in Tor or S6K had no effect on the reduced viability of the *dilp2*-overexpressing flies, suggesting that the Tor/S6K signaling is irrelevant to the semi-lethality or the single copies of mutations were not sufficient to suppress the *dilp2*-induced semi-lethality (**Figure 3B**).

GLUCOSE AND LIPID METABOLISM IN *dilp2* OVEREXPRESSING FLIES

Next, we examined whether overexpression of *dilp2* disrupts glucose homeostasis and metabolism. We first measured the concentration of glucose and trehalose, the major blood sugars in insects. There was no significant difference in the concentration of these sugars between the *dilp2*-overexpressing flies (*sd* > *dilp2*) and the parental lines (*sd-Gal4* or *UAS-dilp2*) as the controls (**Figure 4**). We also measured the amounts of glycolysis and TCA cycle metabolites using liquid chromatography coupled to tandem mass spectrometry (LC-MS/MS) with ion-pair reagents (**Figures 5A,B**). There was no significant difference

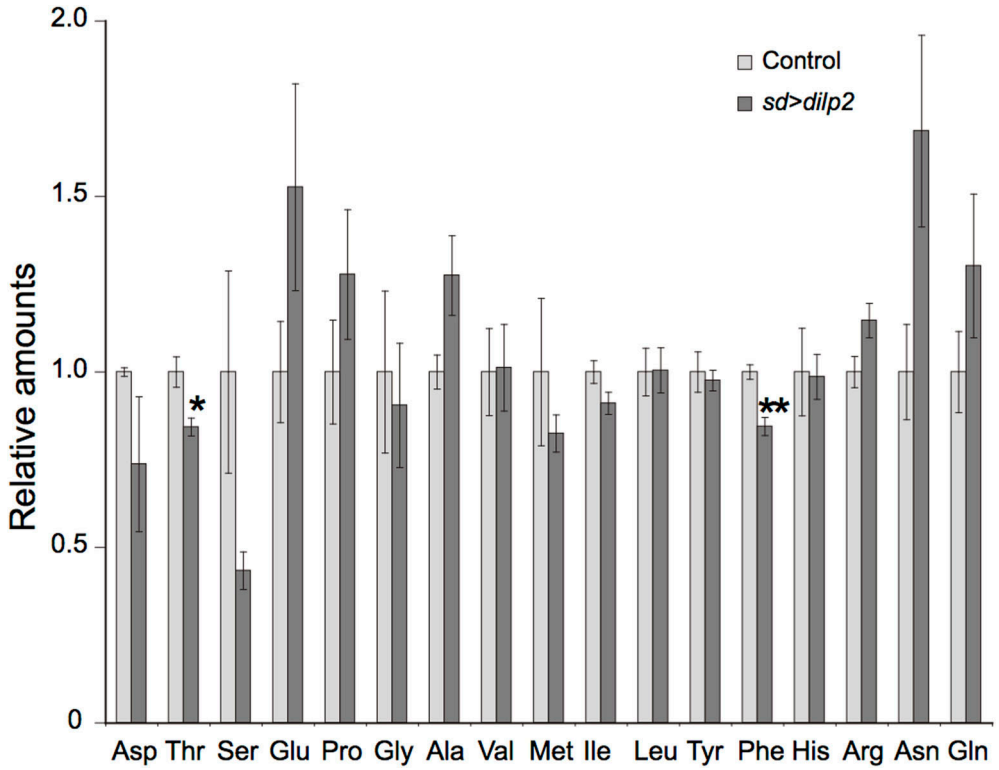

FIGURE 11 | Amino acid content in *dilp2*-overexpressing flies. A free amino acids in the adult flies were quantified by using an LC-MS/MS in positive mode. Relative amounts of each amino acid in *sd > dilp2* flies were calculated based on those in the *sd > RFP* flies as a control, and are shown as the mean ± SE of at least three experiments with a minimum of five flies per group. Student *t*-test was performed to analyze statistical significance. $^{*}p < 0.05$, $^{**}p < 0.01$.

in the amounts of glycolytic metabolites between the *dilp2*-overexpressing flies (*sd > dilp2*) and the parental lines (*sd-Gal4* or *UAS-dilp2*) as the controls. These results suggest that glucose homeostasis itself was unaffected by overexpression of *dilp2*. For the TCA cycle metabolites, the amount of succinate, fumarate, and malate was slightly increased in the *dilp2*-overexpressing flies. Since fumarate and malate have been shown to extend lifespan in *Caenorhabditis elegans* (Edwards et al., 2013), overexpression of *dilp2* might have affected the energy metabolism associated with lifespan determination.

Insulin/IGF signaling might down-regulate lipid catabolism via regulation of lipases (Xu et al., 2012). Thus, we next measured the lipid content in the *dilp2*-overexpressing flies and the control parental lines. There was no significant difference in the level of triacylglycerol, the most abundant of the storage lipids (**Figure 6A**). In addition, quantitative real-time PCR analysis revealed that the expression levels of four major *Drosophila* lipases, *doppelganger von brummer* (*dob*), *brummer* (*bmm*), *CG5966*, and *CG11055* (Gronke et al., 2005) were not altered in the *dilp2*-overexpressing flies (**Figure 6B**). Expression level of *bmm* in the parental line, *UAS-dilp2* was significantly lower than those of another parental line *sd-Gal4* and of their progeny *sd > dilp2*. There might be unknown mechanisms that downregulate *bmm* expression in the background of *UAS-dilp2* line. We also compared the expression levels of *bmm* between *sd > dilp2* and *sd-Gal4*, *3xP3-RFP* flies, and found that there was no significant change in *bmm* expression level when *dilp2* was overexpressed (data not shown).

These results suggested that overexpression of *dilp2* does not affect the lipid storage and catabolism in flies.

NUTRIENT DEPENDENT EFFECTS OF *dilp2*-OVEREXPRESSION

We also examined the protein content per fly and found that the *dilp2*-overexpressing flies contain more protein than the parental control lines (*sd-Gal4* and *UAS-dilp2*), thereby suggesting that protein synthesis is elevated in *sd > dilp2* flies (**Figure 7**). Interestingly, the viability of the *dilp2*-overexpressing flies increased depending on the concentration of the yeast extracts in the media (**Figure 8A**; Spearman's rank correlation coefficient = 0.79, $p < 0.0003$). The relative viability of the *dilp2*-overexpressing female flies was only 2% in the medium without yeast extract, whereas it was 16.6% in the medium containing 40 g/L yeast extract. In addition, increasing protein content in the media significantly increased the mean wing area of the *dilp2*-overexpressing flies, while it did not affect the wing size of the control flies (**Figure 9**). These results indicated that to support their development the *dilp2*-overexpressing flies require more protein as a nutrient than the control flies. It is possible that a shortage of protein sources occurs in the *dilp2*-overexpressing flies. To test this hypothesis, we examined whether autophagy

FIGURE 12 | *dilp2*-overexpressing flies develop faster in media with reduced glucose. Overexpression of *dilp2* retarded development of flies when they were reared on the standard media containing 100 g/L glucose (left). However, flies develop faster when kept in glucose-deprived media (right). The mean (±SE) durations of egg-to-early pupa was 106.3 ± 1.3 and 82.6 ± 1.3 h, with control media and glucose-deprived media, respectively (Student *t*-test: $p < 0.001$).

occurs in the fat bodies dissected from the early third instar larvae. A large number of autophagy positive-cells were observed in the fat bodies of the *dilp2*-overexpressing flies While no autophagy positive-cell was found in the fat bodies of the control flies, (**Figure 10**). Increasing protein content in the media significantly suppressed the *dilp2*-mediated autophagy, suggesting that the *dilp2*-overexpressing flies were suffering from an insufficiency of protein sources. Namely, the shortage of protein sources could be one of the reasons for *dilp2*-induced semi-lethality.

Overexpression of *dilp2* might cause chronic deficiency of amino acids. To examine this possibility, we quantified free amino acids in the *sd* > *dilp2* and the *sd* > *RFP* flies using an LC-MS/MS. Although the relative amount of threonine and phenylalanine were significantly different between the two groups, all changes were subtle and are unlikely to affect the viability (**Figure 11**). These results suggested that survivors of the *dilp2*-overexpressing flies were maintaining normal amino acid homeostasis probably though inducing ectopic autophagy. However, considering the semi-lethality of the animals, those failed to maintain amino acid homeostasis might have died earlier during development.

A PROTEIN-TO-CARBOHYDRATE RATIO IS CRITICAL FOR THE SURVIVAL OF *dilp2*-OVEREXPRESSING FLIES

The occurrence of autophagy in *dilp2*-overexpressing flies suggested that the animals were suffered from the shortage of amino acids. It has been demonstrated that a protein-to-carbohydrate ratio can affect the lifespan and the fecundity of flies (Lee et al., 2008). It is possible that overexpression of *dilp2* affected the optimal ratio of these nutrients for the flies' development. Thus, we examined whether the glucose content of the fly food could modify the *dilp2*-induced semi-lethality. Increasing glucose concentration in the media significantly reduced the viability of the *dilp2*-overexpressing flies, indicating that overexpression of *dilp2* enhances the susceptibility to glucose. On the other hand, decreasing glucose content in the media significantly improved the viability of the *dilp2*-overexpressing flies (**Figure 8B** Spearman's rank correlation coefficient $= -0.86$, $p < 0.0001$). Interestingly, the *dilp2*-overexpressing flies kept in a glucose-reduced condition grew significantly faster than those reared on standard media (**Figure 12**). The mean (±SE) durations of egg-to-early pupa was 106.3 ± 1.3 and 82.6 ± 1.3 h, with control media and glucose-deprived media, respectively (Student *t*-test: $p < 0.001$). These results strongly suggested that a high-protein low-carbohydrate diet is optimal for the viability of *dilp2*-overexpressing flies.

In this study, we demonstrated that overexpression of *dilp2* severely decreases the egg-to-adult viability of flies and induced a high frequency of ectopic autophagy in fat bodies of early third instar larvae. As in mammalian cells, nutrient starvation induces autophagy through inhibition of Tor activity in *Drosophila* (Scott et al., 2004). However, the *dilp2*-overexpression-dependent autophagy does not seem to be regulated by the down-regulation of Tor, since S6K, a downstream target of Tor, was strongly activated (**Figure 2**). Activated S6K may execute autophagy, since expression of activated S6K increases starvation-induced autophagy in the absence of Tor in *Drosophila* (Scott et al., 2004). It is possible that a hyperactivation of S6K in the *dilp2*-overexpressing flies might contribute to promoting autophagy. In isolated rat hepatocytes, some amino acids inhibit induction of autophagy in an mTor (mammalian Tor) independent manner (Kanazawa et al., 2004). Therefore, a shortage of protein sources may induce autophagy directly. The *dilp2*-overexpression-dependent autophagy was reverted by a high-protein diet, suggesting that the nutritional condition is critical for survival of *dilp2*-overexpressing flies. It has been demonstrated that flies cultured on nutrient-rich food contain a significantly high level of secreted *dilp2* compared to flies cultured on nutrient-deprived food, indicating that flies can sense the nutrient availability and modulate their insulin secretion accordingly (Geminard et al., 2009). Our results suggest that excess insulin/IGF signaling impairs the physiology of animals, which can be ameliorated by controlling the nutritional balance between proteins and carbohydrates, at least in flies.

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