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Endocrine regulation of ageing and reproduction in *Drosophila*

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Abstract

Hormonal signals can modulate lifespan and reproductive capacity across the animal kingdom. The use of model organisms such as worms, flies and mice has been fundamentally important for ageing research in the discovery of genetic alterations that can extend healthy lifespan. The effects of mutations in the insulin and insulin-like growth-factor-like signalling (IIS) pathways are evolutionarily conserved in that they can increase lifespan in all three animal models. Additionally, steroids and other lipophilic signalling molecules modulate lifespan in diverse organisms. Here we shall review how major hormonal pathways in the fruit fly *Drosophila melanogaster* interact to influence reproductive capacity and ageing.

1. Introduction

In many multicellular organisms, coordination of the activities of spatially separated organs is achieved by diffusible messengers, hormones, produced within endocrine tissues or specialized cells of the body. Hormones are secreted and circulated in the body, and trigger intracellular events in the target tissues, through the ligand binding domain of the cognate hormone receptors. Upon hormonal exposure, cellular function is modified by activation of cytoplasmic signaling cascades (e.g. receptor tyrosine kinases, such as the insulin receptor) or by direct alteration of

transcriptional activity of the receptor (e.g. nuclear receptors). The outcome of hormone binding can be modified by other intracellular signaling pathways that interact with downstream components of the primary signal, by co-regulators that activate or repress the receptor, and by feedback signals from secondary hormones. Such integrated systems between organs allow orchestration of growth and development and maintenance of metabolic homeostasis in response to environmental changes, such as in nutrient availability. Furthermore, hormonal signals modulate lifespan and reproduction (Tatar et al., 2003, Taguchi and White, 2008, Russell and Kahn, 2007, Piper et al., 2008).

1.1 Drosophila as a model for endocrinology

Insect endocrinology is one of the oldest branches of insect physiology (Nijhout, 1994, Nation, 2002). *Drosophila* has brought to this topic the power of molecular genetics and genomics. Much of our understanding of genetic transmission is based on early experiments with the fruit fly *Drosophila melanogaster* (Kohler, 1994, Sturtevant, 2001). Because of its small size, it is not an ideal organism for endocrinological studies using approaches such as microsurgery or tissue transplantation, for which larger species such as some moths, locusts or cockroaches are more convenient. However, the plethora of genetic techniques available (Dow, 2007) allows other kinds of precise manipulation of endocrinological systems. For example, genetic ablation of specific neurosecretory glands or neuroendocrine cell populations has provided valuable insights into endocrinology of metabolism and longevity (Rulifson et al., 2002, Lee and Park, 2004, Broughton et al., 2005, Grönke et al., 2007, McBrayer et al., 2007). *Drosophila* also has tissues that more strongly resemble those of mammals than do those of the nematode *Caenorhabditis elegans*, the other major multicellular invertebrate model organism (Partridge and Tower, 2008). Functional genetic analysis in flies is facilitated by a vast and constantly growing collection of

mutations and mis-expression lines, such as RNAi lines, available from public stock centers (e.g. Bloomington *Drosophila* Stock Center, Indiana, U.S.A (<http://flystocks.bio.indiana.edu/>), Vienna *Drosophila* RNAi Center, Austria (Dietzl et al., 2007, <http://stockcenter.vdrc.at/control/main>), National Institute of Genetics, Japan (<http://www.shigen.nig.ac.jp/fly/nigfly/index.jsp>)) and by sequenced genomes for several related species (Crosby et al., 2007, Clark et al., 2007). A large fraction of human genes, including those implicated in ageing and ageing-related disease, have orthologues in flies (Bernards and Hariharan, 2001) and, importantly, complications in the analysis of gene function can often be avoided because the fly genome (Adams et al., 2000) carries a smaller number of gene paralogues than do those of mammals or worms, and is hence less redundant. Additionally, most of the basic metabolic functions are conserved between *Drosophila* and mammals (Baker and Thummel, 2007) making flies ideal organism for genetic probing of metabolic homeostasis, endocrinology and ageing (Partridge and Tower, 2008). However, the life history of *Drosophila* involves a radical metamorphosis, in which larval tissues are replaced or remodeled to give a morphologically quite different adult fly. Most work on *Drosophila* endocrinology, and indeed most other aspects of *Drosophila* biology, has been done in embryos and larvae, mainly through work on control of growth and development. It is clear from systematic studies of RNA expression (Chintapalli et al., 2007) that genes involved in endocrinological regulation of pre-adult events are also expressed in the adult fly. However, their functions in the adult, and hence any role in regulation of ageing and reproduction, largely await elucidation.

1.2 Ageing and endocrinology in *Drosophila*

Like mammals, *Drosophila* has discrete endocrine organs and cells (Figure 1). Glandular tissues, such as the prothoracic gland (PG), corpus allatum (CA) and corpus cardiacum (CC), produce

hormones that regulate developmental timing, metamorphosis, metabolism and reproduction (Leopold and Perrimon, 2007, Nijhout, 1994, Tatar et al., 2003, Flatt et al., 2005, Grönke et al., 2007, Mirth and Riddiford, 2007, Colombani et al., 2005, Belgacem and Martin, 2007). In larvae, these three endocrine tissues constitute the ring gland, a master endocrine organ located dorsally between the two hemispheres of the brain. The ring gland undergoes a dramatic change during metamorphosis (Dai and Gilbert, 1991). The PG degenerates and the CA/CC complex migrates to its distinctive location above the junction between the crop (food storage organ) and the midgut (stomach). Larval ecdysteroids (steroids with molt-promoting activity) are produced in the PG. After PG-degeneration, in adult females the ovarian follicle cells are thought to take over this function (Riddiford, 1993). Little is known about ecdysteroid synthesis in other tissues in *Drosophila*. Ecdysteroids are present in adult males, where they play role in control of fertility and reproductive behaviour (Wismar et al., 2000, Ganter et al., 2007). In both larvae and adults, the CA are the source of the sesquiterpenoid juvenile hormone (JH), while the lipid-mobilizing adipokinetic hormone (AKH) is produced in the CC. Some of the *Drosophila* insulin-like peptides (DILPs) are produced in large, specialized neurons of the central nervous system, the DILP-producing median neurosecretory cells (hereafter called MNCs) (Cao and Brown, 2001, Ikeya et al., 2002). Products of other distinct populations of neurosecretory cells (not shown in Figure 1, for clarity) include biogenic amines such as dopamine and serotonin, which also work as neurotransmitters and neuromodulators (Monastirioti, 1999).

This review focuses on endocrinological systems that have been demonstrated or proposed to influence adult lifespan in *Drosophila*, and hormones with relevant functions have been listed in Table 1. Developmental phenotypes controlled by endocrine signalling have been well reviewed elsewhere (Truman and Riddiford, 2002, Mirth and Riddiford, 2007). In *Drosophila*, representatives of peptide hormones, lipophilic hormones and bioactive amines have been shown

to modulate lifespan and reproduction, by manipulations that directly decrease hormone production (Broughton et al., 2005), through inactivating mutations in hormone receptors or their downstream targets (Tatar et al., 2001, Clancy et al., 2001, Simon et al., 2003) or by polymorphic alterations in the genes required for hormone biosynthesis (De Luca et al., 2003, Carbone et al., 2006). Many of these systems also affect reproduction, and we shall discuss this connection. We shall also discuss other phenotypes that commonly associate with longevity, such as changes in lipid and carbohydrate homeostasis, and resistance to starvation or oxidative stress. Of special interest here will be how these major hormonal pathways interact with each other to influence both reproductive capacity and ageing. Although recent work has made important strides, undoubtedly much information about the endocrinology of adult *Drosophila* remains to be discovered.

2. Insulin / IGF-like signaling (IIS) in *Drosophila*

In mammals, insulin and insulin-like growth factor (IGF) signaling control blood glucose metabolism, growth, stress resistance, reproduction and ageing (Tatar et al., 2003, Taguchi and White, 2008, Piper et al., 2008). In *Drosophila*, the functions of these two signaling pathways are united as insulin/insulin-like growth factor-like signaling (IIS) (Tatar et al., 2003, Taguchi and White, 2008, Piper et al., 2008, Wu and Brown, 2006, Giannakou and Partridge, 2007, Baker and Thummel, 2007). Five types of dimeric insulin/IGF receptors are produced in mammals by combinations of monomers of a single insulin receptor and two IGF-receptor subtypes (Taguchi and White, 2008). Flies only have a single gene for the insulin receptor (dINR) which is regulated both transcriptionally (Casas-Tinto et al., 2007) and post-translationally by proteolytic processing (Fernandez et al., 1995). However, the fly genome encodes seven insulin-like peptides, DILP1-7, each of which can promote larval growth, suggesting that they function as dINR agonists

(Brogiolo et al., 2001, Ikeya et al., 2002). All DILPs display a higher degree of overall amino-acid identity with human insulin than with human IGFs, DILP2 being most closely related with 35% identity to human preproinsulin (Brogiolo et al., 2001). Differentiation of function of different DILPs may contribute to the multiple functions of IIS, through differential timing or tissue-specificity of expression, by inducibility in response to environmental stimuli or by differences in their action on dINR. In larvae and adults, DILP2, DILP3 and DILP5 are synthesized in the MNCs (Brogiolo et al., 2001, Ikeya et al., 2002, Broughton et al., 2005). Whereas DILP2 and DILP3 seem to be MNC-specific, DILP5 is also expressed in the ovarian follicle cells (Ikeya et al., 2002), where its function remains unknown. Expression of *dilp3* and *dilp5* in the larval MNCs is regulated by food availability (Ikeya et al., 2002), and *dilp3* mRNA in adults is down-regulated in long-lived flies with reduced olfactory function (Libert et al., 2007), consistent with a role in nutrient-sensing (Drummond-Barbosa and Spradling, 2001, Gershman et al., 2007). In larvae and adults, expression of *dilp7* is restricted to the ventral nerve cord (also called ventral ganglia in the adults) (Brogiolo et al., 2001, Chintapalli et al., 2007). In agreement with this, DILP7 protein is present in a few postmitotic neurons of the ventral ganglia that send projections to the intestine (Miguel-Aliaga et al., 2008), to distinct positions in the ventral ganglia and the brain, and to the female reproductive tract (Yang et al., 2008). Interestingly, reduced function of DILP7-expressing neurons (by hyperpolarization) results in sterility due to lack of oviposition, and DILP7 itself might participate in the decision-making process that leads to the proper execution of egg-laying under different nutritional conditions (Yang et al., 2008). The functions of DILP1, DILP4 and DILP6 are currently unknown.

The patterns of gene expression in cell lineages that give rise to the *Drosophila* DILP-producing cells and AKH-producing cells show remarkable similarity to those giving rise to mammalian pancreatic beta and alpha cells, respectively (Wang et al., 2007). Survival and differentiation of

DILP7-producing neurons in *Drosophila* requires insulinergic differentiation factors orthologous to those involved in differentiation of mammalian pancreatic beta cells (Miguel-Aliaga et al., 2008). There are also remarkable parallels with the development of the mammalian anterior pituitary and hypothalamic axis. Both MNCs and CC cells originate from a single pair of neural stem cells in the anterior neuroectoderm, a region that expresses genes orthologous to those expressed in the vertebrate adenohypophyseal placode, which gives rise to the endocrine anterior pituitary and neurosecretory hypothalamic cells (Wang et al., 2007). Both the development of the brain endocrine axis and its role in modulation of lifespan (see below) are thus evolutionarily conserved. A considerable body of work on ageing in *Drosophila* has focused on endocrinology of IIS, and on cell-autonomous signaling that interacts with IIS (e.g. TOR, JNK, recently reviewed in Giannakou and Partridge, 2007).

2.1 Reduced IIS can extend lifespan

At least one of the DILPs expressed in the MNCs is likely to contribute to the endocrine regulation of longevity, since partial genetic ablation of MNCs both reduces the expression of the three MNC-specific *dilp*-RNAs and leads to increased lifespan in both females and males (Broughton et al., 2005). The MNC-ablated flies are resistant to oxidative stress and starvation and show an increase in circulating glucose, whole body trehalose (disaccharide glucose), glycogen and lipids. The females also show reduced fecundity. These changes in metabolism and life history traits are recapitulated by mutations that inactivate components of IIS downstream of DILPs, such as dINR (Tatar et al., 2001) and the fly orthologue of the mammalian insulin receptor substrates, CHICO (Clancy et al., 2001, Tu et al., 2002a). The key downstream transcription factor of IIS in flies is dFOXO (Jünger et al., 2003, Kramer et al., 2003, Puig et al., 2003), the single orthologue of *C. elegans* DAF-16 and of the highly conserved mammalian

forkhead transcription factors, which regulate cell cycle, apoptosis, DNA repair, metabolism and oxidative stress resistance (Puig et al., 2003, Jünger et al., 2003, Gershman et al., 2007, van der Horst and Burgering, 2007, Partridge and Brüning, 2008). The IIS pathway (Figure 2), through a conserved kinase cascade involving PI-3 kinase (PI3K) and protein kinase B (PKB), negatively regulates dFOXO by phosphorylation, which prevents its nuclear translocation. When IIS is blocked, the unphosphorylated dFOXO enters the nucleus and regulates the transcription of genes (including oxidative stress genes) that subsequently enhance longevity and stress resistance. Additionally, under low nutrient availability (low IIS) nuclear dFOXO "sensitizes" cells for changing nutritional conditions through a transcriptional upregulation of the *dInr* (Puig et al., 2003) and, interestingly, analogous feedback mechanism occurs in mammals (Puig and Tijan, 2005). A recent study implicated dFOXO as a major factor in organizing the transcriptional response to nutrients, and suggested that mitochondrial biogenesis associated with increased nutrition is linked to IIS via loss of dFOXO-mediated repression of *Drosophila* peroxisome proliferator- γ coactivator-1 (PGC-1) homologue (Gershman et al., 2007). IIS also interacts with at least two intracellular signaling cascades: the amino-acid-sensing Target Of Rapamycin (TOR) pathway and the stress-sensing Jun NH₂-terminal kinase (JNK) pathway, both shown to contribute to the control of ageing in worms and flies (Giannakou and Partridge, 2007).

2.2 Pleiotropic effects of reduced IIS on reproduction

Reduced fecundity is the most common trade-off observed in *Drosophila* populations experimentally selected for long life (Rose, 1989, Zwaan, 1999, Sgro and Partridge, 1999, Stearns et al., 2000), and lowered systemic insulin signaling can lead to pleiotropic effects that decrease the fitness of an organism, the most common associated effect being reduced fecundity (Clancy et al., 2001, Tatar et al., 2001, Gems et al., 1998). There is strong evidence, however,

that the beneficial effects on lifespan can be uncoupled from the negative ones by spatiotemporal control (tissue-specific and/or adult-specific interventions) over the inhibition of the IIS. In worms, knockdown DAF-2 (*C. elegans* orthologue of the dINR) expression by RNA-mediated interference (RNAi) during development alone is sufficient to decrease fecundity, whereas the adult-specific loss of DAF-2 increases lifespan without affecting reproduction (Dillin et al., 2002). In *Drosophila*, the long-lived heteroallelic combinations of dINR and CHICO homozygotes are small and female-sterile, as are some severe *short-lived* heteroallelic combinations of dINR (Clancy et al., 2001, Tatar et al., 2001). Additionally, long-lived CHICO heterozygotes have normal body size and are fertile (Clancy et al., 2001), as are long-lived flies overexpressing dFOXO in the adult fat body (below) (Hwangbo et al., 2004, Giannakou et al., 2004, Giannakou et al., 2007). Therefore, there is no necessary association between increased lifespan and reduced fertility by altered IIS in *Drosophila*. This largely resembles the situation in nematodes, where variable pleiotropic effects are observed in classical DAF-2 mutants (Gems et al., 1998), and in mice where some (Bartke and Brown-Borg, 2004) but not all (Holzenberger et al., 2003) IIS-related, long-lived mutants show reduced fecundity. However, in contrast to flies and worms, evidence for adult-specific effects of reduced IIS on lifespan in mammals is still missing.

Reproductive tissues have been directly implicated in lifespan determination. In *C. elegans* removal of the whole gonad (germline and somatic tissues) abolishes egg production but does not affect lifespan, but removal of the germ line alone results in robust lifespan extension that is dependent on the downstream IIS effector, DAF-16 (Hsin and Kenyon, 1999). Although gonadal signals also appear to affect lifespan in mice (Cargill et al., 2003), this mechanism may not be evolutionarily conserved because removal of the germ line by mutations does not increase lifespan in flies (Barnes et al., 2006). However, as opposed to worms, where the somatic gonad

does not proliferate after development, both the germ line and somatic follicle cells of the ovaries actively proliferate in adult females. Their proliferation rate is also responsive to nutritional signals mediated by IIS (Drummond-Barbosa and Spradling, 2001, LaFever and Drummond-Barbosa, 2005) and, interestingly, ovarian somatic tissue shows more than 3-fold over-proliferation in germ-line ablated flies compared with wild type ovaries (Barnes et al., 2006). Therefore, somatic reproductive tissues seem to respond to removal of the germ line and it remains possible that this tissue could modulate longevity. Understanding exactly how IIS can affect lifespan and fecundity both separately and co-ordinately is an interesting challenge for future research.

2.3 Fat tissue signals long life in IIS mutants

Drosophila fat body, which resembles mammalian liver and white adipose tissue, consists of masses and sheets of tissue, and in larvae functions as a sensor of nutritional status that controls organismal development and growth (Britton and Edgar, 1998, Colombani et al., 2003). During metamorphosis, the larval fat body undergoes histolysis and finally, in young flies, is replaced by the adult fat body that arises from distinct pool of progenitor cells and surrounds all the major internal organs. Over-expression of wild type dFOXO or a constitutively active dFOXO mutant in the adult fat body is sufficient to extend lifespan, without concomitant effects on fertility (Hwangbo et al., 2004, Giannakou et al., 2004, Giannakou et al., 2007). A discrepancy exists as to whether the extension of lifespan is mediated by a systemic signal from the fat body that results in decreased levels of *dilp2* in the MNCs (Hwangbo et al., 2004) or by some other mechanism (Giannakou et al., 2007). Inactivation of the insulin receptor specifically in white adipose tissue is sufficient to increase lifespan in mice (Bluher et al., 2003), and in worms activation of DAF-16 specifically in the intestine (also a fat tissue) largely accounts for the long-

lived phenotype of the DAF-2 mutants (Libina et al., 2003). Whether inactivation of dINR specifically in the *Drosophila* fat body can lead to increased lifespan is unknown, but is likely since fat-body-specific up-regulation of dPTEN, a negative regulator of IIS (Figure 2), also extends life (Hwangbo et al., 2004). Therefore, although its downstream effectors are currently unknown, the life-promoting signal of decreased fat-specific IIS seems to be evolutionarily conserved (Kloting and Bluher, 2005).

2.4 DILPs and AKH in carbohydrate and fat metabolism

Much like insulin and glucagon in mammals (Vroemen et al., 1998) DILPs and AKH have antagonistic effects on circulating carbohydrates. In both larvae and adult flies, AKH is produced exclusively in the cells of corpus cardiacum (CC) (Schaffer et al., 1990, Noyes et al., 1995, Lee and Park, 2004, Isabel et al., 2005), processes of which innervate the larval heart, where they make extensive contacts with the axons of MNCs, as well as with the ecdysone-synthesizing PG of the larval ring gland (Kim and Rulifson, 2004, Lee and Park, 2004). In adults, these processes can be traced proximate to the esophagus foramen, where they are likely to enter the brain, and to the crop (Lee and Park, 2004). AKH mobilizes stored energy by increasing the breakdown of fat and by stimulating the production of trehalose (disaccharide glucose) by the fat body. Fat-body-targeted over-expression of AKH or its receptor (AKHR) reduces levels of triglyceride, the main storage form of lipids (Lee and Park, 2004, Grönke et al., 2007). Hemolymph sugar in flies is mainly composed of trehalose and, to some extent, monomeric glucose. Genetic ablation of CC cells in larvae and adults leads to decreased total hemolymph trehalose and starvation resistance (Lee and Park, 2004, Kim and Rulifson, 2004, Isabel et al., 2005). Consistently, *AKH*-mutants are also starvation resistant, as are mutants for *Brummer* (*bmm*), the homologue of mammalian adipose triglyceride lipase (Grönke et al., 2007). The mechanism by which reduced AKH or *bmm*

activity contributes to starvation resistance is possibly through a decreased rate of mobilization of energy reserves, which enables these flies to live longer under poor nutrition. Interestingly, *AKHR bmm* double mutants, although extremely fat, are starvation-sensitive (Grönke et al., 2007), indicating that total incapability to mobilize lipids is detrimental during nutritional stress.

MNC-ablation also leads to starvation resistance and increased total lipids, but to *increased* circulating carbohydrates (Broughton et al., 2005). It is possible that increased hemolymph carbohydrates inhibit AKH-release from adult CC, as they do *in vitro* in larvae (Kim and Rulifson, 2004). This could, in principle, lead to lipid accumulation in the MNC-ablated flies. Because AKH null mutants have normal lifespan (Grönke et al., 2007), increased lipid alone is not sufficient for lifespan extension. Therefore, it would be of interest to examine the role of increased trehalose content (which is only up in IIS mutants) in regulation of longevity. Both trehalose itself and levels of enzymes that catalyze trehalose biosynthesis are upregulated in worm IIS mutants (Murphy et al., 2003, McElwee et al., 2006). Trehalose has cytoprotective properties during hypertonic stress in worms (Lamitina and Strange, 2005) as well as hypoxic and anoxic injury in flies (Chen and Haddad, 2004). Cellular mechanisms that could explain the protective effect of trehalose include decreased protein denaturation through direct protein-trehalose interactions (Singer and Lindquist, 1998) and trehalose-mediated TOR-dependent induction of autophagy (Sarkar et al., 2007). Both IIS-dependent chaperone action (Hsu et al., 2003, Morrow et al., 2004, Walker and Lithgow, 2003) and increased protein turnover by autophagy (Juhasz et al., 2007, Hars et al., 2007, Melendez et al., 2003, Simonsen et al., 2007) contribute to lifespan-extension in worms and flies. Although an increased trehalose level is a common phenotype in IIS mutants, it remains to be seen if trehalose *per se* promotes longevity in *Drosophila*.

3. Lipophilic signaling

Drosophila has two primary lipophilic signalling molecules, ecdysteroids and juvenile hormone (JH). In adult females, both ecdysone and JH regulate oogenesis in a complex manner that is also dependent on functional IIS (Bownes, 1982, Buszczak et al., 1999, Carney and Bender, 2000, Drummond-Barbosa and Spradling, 2001, LaFever and Drummond-Barbosa, 2005, Soller et al., 1999, Terashima et al., 2005). Virtually nothing is known about the roles of ecdysone or JH in males, although ecdysone signaling is required for spermatogenesis (Wisnar et al., 2000), JH stimulates protein synthesis in male accessory glands (Wilson et al., 2003) and both seem to regulate at least some aspects of courtship behaviour (Ringo et al., 1992, Ganter et al., 2007). Larval growth and metamorphic pathways require extensive crosstalk between IIS and lipophilic hormones (Colombani et al., 2005, McBrayer et al., 2007, Mirth and Riddiford, 2007). Because it has been suggested that the longevity effects of reduced IIS might at least partially be mediated through secondary signalling via JH or ecdysone (Tatar et al., 2001, Tu et al., 2002b, Tatar et al., 2003), it is important to consider how these signals might interact with IIS in adult flies.

3.1 Ecdysone

Ecdysteroids are the only class of steroid hormones in *D. melanogaster*, and trigger both general and cell-specific transcriptional responses (Thummel, 2002, Riddiford et al., 2000). Ecdysone is the immediate precursor for the major insect moulting hormone 20-hydroxyecdysone (20E). In larvae, ecdysone is synthesized in the PG, from where it is released to initiate major developmental transitions and maturation in response to neuropeptide signaling involving prothoracicotropic hormone (PTTH) (McBrayer et al., 2007) and IIS (Caldwell et al., 2005, Colombani et al., 2005, Mirth, 2005). Recent work indicates that, although PTTH is not essential

for molting and metamorphosis, it controls developmental timing and final body size together with growth-regulating IIS (McBrayer et al., 2007). Ecdysone is converted to 20E in the peripheral tissues, such as the epidermis, the gut, the Malpighian tubules (fly 'kidney') and the fat body, by the 20-monooxygenase enzyme encoded by *shade* (Petryk et al., 2003). In adult females, the ovaries are thought to be the main source of ecdysteroids (Riddiford, 1993), a view that is strongly supported by the preferential expression of cytochrome P450 genes involved in ecdysone biosynthesis in the ovarian follicle and/or nurse cells (Chavez et al., 2000, Niwa et al., 2004, Warren et al., 2002, Warren et al., 2004). *shade* is also expressed in the ovaries, which therefore have the capacity to both synthesize ecdysone and convert it to 20E (Petryk et al., 2003). However, exact details of how different cell types of the ovary contribute to the ecdysone synthesis and its conversion to 20E are largely unknown (Kozlova and Thummel, 2000). Sources of ecdysone in adult males also remain to be identified.

In the target tissues, 20E stabilizes the heterodimeric nuclear receptor complex comprised of the *ecdysone receptor* (EcR) and *ultraspiracle* (USP) (Koelle et al., 1991, Baker et al., 2000, Yao et al., 1993), the orthologues of the vertebrate farnesoid X receptor (FXR) or liver X receptor (LXR), and retinoid X receptor (RXR), respectively (King-Jones and Thummel, 2005). USP has been also proposed to function as a JH receptor (below) (Jones and Sharp, 1997, Jones et al., 2006), although *in vivo* evidence for this is still lacking. The classical function of the EcR/USP dimer is transcriptional activation in response to an ecdysone pulse, which results in larval or pupal molt in the presence or absence of JH, respectively. However, due to the bipartite role of the receptor complex, and the fact that several ligands possibly exist for both EcR and USP (Palanker et al., 2006, Beckstead et al., 2007), interplay between the ligands is likely to be extremely complex.

3.1.1 Ecdysone signaling modulates lifespan

Flies heterozygous for inactivating mutations in the ligand binding domain or DNA binding domain of EcR show robust (typically 20-50%) lifespan-extension (Simon et al., 2003). Lifespan is extended in both sexes and in several genetic backgrounds, and is associated with resistance to oxidative stress, heat and starvation. An ecdysteroid deficient, temperature-sensitive mutant DTS-3 (the molecular identity of which remains to be discovered) is also long-lived at the restrictive temperature, at which females show low ecdysone titre. Lifespan of DTS-3 mutant is rescued by 20E provided in the food medium supporting a direct role of ecdysone in lifespan extension. However, a genomic rescue of the effects of the EcR-mutants has yet to be described, and the specific tissue(s) that mediate the lifespan-extending effects of EcR hypomorphy are unknown. Conditional gene expression systems, where the transgene is induced by feeding the flies with drugs such as RU486 (Osterwalder et al., 2001, Roman et al., 2001) or doxycycline (Bello et al., 1998, Bieschke et al., 1998), should prove useful in investigating critical tissues for lifespan modulation by EcR inactivation or, conversely, where over-expression of EcR can mitigate the longevity of traditional EcR mutants. The power of inducible systems is that they can fully eliminate confounding developmental effects and, most importantly, enable the use of genetically identical control strains where EcR function is not affected. Control of genetic background is of utmost importance when working with outbreeding diploid organisms such as *Drosophila* (Spencer et al., 2003, Martin and Grotewiel, 2006, Toivonen et al., 2007). Surprisingly, a recent study (V. Monnier, personal communication) indicates that ubiquitous, adult-specific inactivation of EcR using RNAi does not extend female lifespan, but can extend that of males up to 25% in conditions where EcR is mildly reduced. Stronger reduction in EcR levels leads to deleterious effects and *shortens* lifespan in both sexes. This demonstrates that adult-specific manipulation of ecdysone signalling components can indeed modify longevity, but the discrepancies with the

earlier work also suggests that the level of EcR activity is likely to be an important factor in the effects on lifespan and that the effects may well be sex-specific.

3.1.2 IIS, ecdysone and ageing

Reduction in both IIS and ecdysone signalling in flies can extend lifespan and reduce fertility (Clancy et al., 2001, Tatar et al., 2001, Simon et al., 2003, Carney and Bender, 2000), suggesting that the effects of one of these pathways could be mediated by the other. The ovary is the main site of ecdysone synthesis in adult females, and both production and reception of ecdysone within developing follicles is essential for yolk protein production and uptake (vitellogenesis) (Gilbert et al., 1998, Buszczak et al., 1999, Carney and Bender, 2000). A requirement for IIS for ovarian ecdysteroid production has been reported in *Drosophila* (Tu et al., 2002b) and other Diptera (Maniere et al., 2004), suggesting that reduced IIS could increase lifespan by reducing ovarian ecdysone production. However, reduced ecdysone does not directly correlate with lifespan in IIS mutants, since both long-lived and normal-lived dINR mutants have decreased ecdysone levels (Tu et al., 2002b). Long-lived CHICO mutants show no defect in ovarian ecdysone release or total hemolymph ecdysteroids and, in fact, CHICO homozygotes show *increased* hemolymph ecdysteroids when normalized by body size (Richard et al., 2005). Critically, wild type ovaries transplanted into homozygous CHICO mutant females are vitellogenic, whereas the mutant ovaries stay immature in the wild type host (Richard et al., 2005). This indicates that systemic factors required for oocyte maturation (including ecdysteroids and JH) are present in CHICO homozygotes, and that the effect of reduced IIS on fertility is ovary-autonomous. In clonal ovarian cell lineages, CHICO is required for maintenance of follicle cell proliferation rate in response to nutrition and egg chamber development through pre-vitellogenic stages (Drummond-Barbosa and Spradling, 2001). Additionally, dINR is required cell autonomously for normal

germline cyst development (LaFever and Drummond-Barbosa, 2005) implying a direct role for insulin signalling in the ovary. Adult-specific manipulations of ecdysone, for example by manipulation of genes involved in its synthesis, are required to determine if reduced IIS and ecdysone/JH signaling affect lifespan by overlapping or distinct mechanisms.

3.2 Juvenile hormone

Juvenile hormones (collectively abbreviated as JH) are acyclic, sesquiterpenoid lipids that function as major developmental regulators in insects and, by their actions, are probably the most versatile hormones in the animal kingdom (Nijhout, 1994, Flatt et al., 2005). In *Drosophila*, three subtypes of JH (JH III, JHB3 and methyl farnesoate) are produced by the CA, although how these differ in their action is poorly understood (Flatt et al., 2005, Richard et al., 1989). In adult females, JH stimulates vitellogenesis by the developing oocytes and, together with 20E and IIS, controls the nutrient-sensitive checkpoint in oogenesis (Soller et al., 1999, Gilbert et al., 1998, Bownes, 1982, Drummond-Barbosa and Spradling, 2001, Terashima et al., 2005). Molecular components of JH signalling are largely unknown and mutations directly affecting JH production are limited. Two candidates for a JH receptor have been proposed: a nuclear transcriptional regulator *Methoprene-tolerant* (Wilson and Fabian, 1986, Ashok et al., 1998) and USP (Jones and Sharp, 1997, Jones et al., 2006), a nuclear receptor and an essential binding partner of the EcR. However, rapid non-transcriptionally mediated pathways for JH action have also been suggested, and it remains possible that JH participates in both cell surface signal transduction and direct transcriptional regulation (Wheeler and Nijhout, 2003).

3.2.1 JH, reproduction and ageing

In response to changes in photoperiod or temperature, insects, including *Drosophila*, can enter reproductive diapause, which is associated with increased stress resistance and enhanced survival (Tatar and Yin, 2001, Tatar, 2004). This mechanism is reminiscent of nematode *dauer* diapause (Hu, 2007), a non-feeding, stress-resistant stage of negligible senescence induced by adverse environmental conditions and commonly promoted in long-lived IIS mutant worms. Diapause entry in *Drosophila* is apparently under the control of the endocrine system, which disrupts the synthesis of JH and/or ecdysone (Saunders et al., 1990, Gilbert et al., 1998). JH synthesis by isolated CA and ovarian ecdysteroid synthesis are low during diapause and, although ecdysteroids have been proposed to play more important role in regulation of vitellogenesis (Richard et al, 2001), application of both JH and 20E can stimulate exit from diapause as measured by restored vitellogenesis. It has been proposed that JH could be an important proximal mediator underlying trade-offs between reproduction and survival (Tu et al., 2005, Flatt et al., 2005). Developmental exposure of wild-type *Drosophila* larvae to a JH analog (JHa) dramatically reduces adult female survivorship (even in the absence of JHa in the adult stage), and this is accompanied by increased early life fecundity (Flatt and Kawecki, 2007). Long-term developmental selection by continuous JHa- feeding eliminated its adverse effects on adult lifespan, indicating JHa-resistance in the selected lines. Interestingly, selection also resulted in small, but significant, lifespan-extension in flies in the absence of JHa, suggesting that the JHa-resistant lines had evolved compensatory changes that, by an unknown mechanism, reduced JH metabolism or otherwise antagonized JH-signaling. However, because early fecundity was not affected in the JHa-resistant flies, increased lifespan did not result from an evolutionary trade-off with reproductive capacity. As discussed by Flatt and Kawecki (2007), alternative explanations such as increased stress resistance (Salmon et al., 2001) or enhanced immune function (Rolff and Siva-Jothy, 2002, Rantala et al., 2003) are possible modulators of lifespan in the JH-selected lines.

3.2.1 Does IIS control JH levels?

In *Drosophila*, dINR is present in both the larval and adult CA (Belgacem and Martin, 2006) and IIS therefore has the potential to regulate JH production throughout life. Low JH titres have been reported in some flies with defective IIS (Tatar et al., 2001, Tu et al., 2005) and RNAi-knockdown of dINR specifically in the CA mimics IIS mutants, and results in small flies and abolition of the sexually dimorphic locomotor behaviour typical of wild-type flies (Belgacem and Martin, 2007). This behaviour is also disrupted in MNC-ablated flies (Belgacem and Martin, 2006). Furthermore, CA-specific dINR-RNAi simultaneously down-regulates expression of 3-hydroxy-3-Methylglutaryl CoA Reductase (HMGCR) (Belgacem and Martin, 2007), an enzyme that in mammalian liver participates in cholesterol biosynthesis and that in insects (which do not synthesize cholesterol *de novo*) is a key step in JH biosynthesis (Belles et al., 2005). Although direct evidence of altered JH levels is yet to be produced, the identical phenotypic outcome of CA-specific knockdown of dINR and HMGCR suggests that IIS could positively regulate JH production by transcriptional upregulation of HMGCR, possibly through an evolutionarily conserved mechanism involving *Drosophila* Sterol Response Element Binding Protein, dSREBP (Belgacem and Martin, 2007). If JH production in these flies is indeed reduced, this model could be valuable in dissecting the complex interactions of IIS and JH signaling for reproduction.

Much like dwarfism or ecdysteroid levels, low capacity for JH biosynthesis is not consistently linked with longevity in the IIS mutants (Partridge et al., 2005). Although various heteroallelic mutant combinations of dINR all decrease JH synthesis *in vitro* (Tatar et al., 2001), this decrease is present in both long-lived and short-lived allelic combinations. Additionally, CHICO homozygotes show actually increased JH biosynthesis when normalized by body size (Richard et

al., 2005). The restoration of vitellogenesis by exogenous hormone replacement (JHa-treatment) is also dependent on the type of dINR alleles (Tatar et al., 2001) and the vitellogenic block cannot be rescued by JHa treatment in CHICO homozygotes (Richard et al., 2005). Again, adult-specific, direct manipulations of JH production (for example by inhibition of IIS in the adult CA) are required to determine the effects of decreased JH signaling on lifespan in an otherwise wild-type IIS context.

5. Biogenic amines

Dopamine (DA) and serotonin (5-hydroxytryptamine, 5-HT) are monoamine neurotransmitters that also function as neurohormones and regulate physiology and behaviour in both invertebrates and vertebrates. They are synthesised from the amino acids tyrosine and tryptophan, respectively, and aromatic amino acid hydroxylases involved in production of these hormones are conserved from nematodes to mammals (Zhu and Juorio, 1995, Livingstone and Tempel, 1983, Coleman and Neckameyer, 2005). DA and 5-HT are involved in diverse processes regulated by the brain including mating behaviour, circadian rhythms, learning and memory (Monastirioti, 1999, Neckameyer et al., 2000). Evidence for an involvement of these biogenic amines in ageing in *Drosophila* has come both from population-genetic association studies and experimental manipulation.

5.1 Dopamine (DA)

The first, rate limiting reaction in DA synthesis is catalysed by tyrosine hydroxylase (TH), and polymorphisms in the gene encoding TH are associated with variation in human longevity (De Luca et al., 2001, Tan et al., 2002). In *Drosophila*, polymorphisms in the gene *Catecholamines*

up (*Catsup*), a negative regulator of TH (Stathakis et al., 1999), are associated with variation in lifespan and stress resistance (Carbone et al., 2006). The last step in the biosynthesis of both DA and 5-HT is catalyzed by Dopa decarboxylase (*Ddc*), several single nucleotide polymorphisms of which are associated with natural variation in *Drosophila* longevity, one of these being located in an exon that is translated only in the CNS (De Luca et al., 2003). These association studies encourage direct attempts to manipulate biogenic amine levels *in vivo* to investigate their role in ageing.

Pharmacological or mutational alterations in biogenic amines frequently associate with reduced fecundity or sterility in *Drosophila* (Monastiriotti et al., 1996, Neckameyer, 1996, Stathakis et al., 1999), and DA and some other biogenic amines could affect ageing by regulation of systemic gonadotropin levels. Increased DA levels through feeding of a DA precursor produced concomitant increases in both 20E and JH levels in young females (Rauschenbach et al., 2007). Conversely, 20E feeding upregulated DA (Gruntenko et al., 2005), possibly as a direct result of decreased activity of biogenic amine catabolism (Rauschenbach et al., 2007). As discussed above, the balance between 20E and JH is important in the control of *Drosophila* oogenesis (Soller et al., 1999) and both hormones have been suggested to be important in the longevity of some fly mutants (Tatar et al., 2001, Tu et al., 2005).

5.2 Serotonin (5-HT)

In *C. elegans*, deletion mutants of the first, and rate-limiting, enzyme in 5-HT synthesis, tryptophan hydroxylase (TPH), show decreases in egg laying and food ingestion, and increases in fat storage, dauer diapause and reproductive lifespan (Sze et al., 2000). Like DAF-2 mutants, TPH mutants exhibit increased DAF-16 nuclear accumulation and are resistant to heat stress

(Liang et al., 2006). Inactivation of 5-HT signaling at the receptor level can delay age-related decline in locomotor activity in worms (Murakami et al., 2007) and also increase lifespan in an IIS-dependent manner (Murakami and Murakami, 2007). However, various worm 5-HT receptors seem to modulate longevity in an antagonistic manner, and deletions of some can actually decrease lifespan (Murakami and Murakami, 2007, Sibille et al., 2007). Therefore, lifespan-modulating effects of the serotonergic system are likely to be complex. Interestingly, 5-HT can directly stimulate insulin release from mammalian pancreatic islets *in vitro* (Peschke et al., 1997) and therefore has the potential to influence IIS, at least in mammals. The two TPH genes of *Drosophila* have been characterized (Neckameyer et al., 2007), providing tools to study effect of the serotonergic system on life-history traits. Recently, 5-HT has also been shown to modulate aggression (Dierick and Greenspan, 2007) and promote baseline sleep in flies (Yuan et al., 2006), providing evidence that physiological processes controlled by the serotonergic system can be strikingly similar in flies and humans.

6. Outlook

Recent years have established that ageing is under endocrinological control across broad groups of species (Tatar et al., 2003, Taguchi and White, 2008, Russell and Kahn, 2007, Piper et al., 2008). Model organisms such as worms, flies and mice are central tools in dissecting these mechanisms, especially in the case of evolutionarily conserved signaling pathways that mediate longevity. IIS clearly is one of these pathways, but several important challenges remain. For example, the biochemical nature of the processes modified by altered IIS is largely unknown. Although several proximal mechanisms have been suggested (McElwee et al., 2006, Kenyon, 2005), it still needs to be resolved if these are also conserved across species. In addition to its broad, systemic effect on lifespan, IIS in specific organs can reduce tissue and whole organism

ageing (Bluher et al., 2003, Wessells et al., 2004, Taguchi et al., 2007). Further characterization of tissues that deteriorate during senescence and the nature of damage they accumulate will be informative when carried out in wild-type flies in parallel with long-lived models.

In *Drosophila*, several specific questions regarding hormonal control of ageing and reproduction await elucidation: Which DILPs are important in regulation of lifespan and which for reproduction? What is the mechanism of DILP secretion? What is the nature of signals that have been suggested to regulate DILP expression and/or secretion in long-lived models? How does IIS regulate lipophilic hormone production in adult CA and in the ovary? Conversely, can ecdysteroids and JH regulate DILP-production in the adult MNCs? Do reduced IIS and ecdysone signaling affect lifespan by overlapping or distinct mechanisms? Finally, as novel bile-acid -like signals mediate diapause, reproduction and ageing in worms (Motola et al., 2006, Held et al., 2006, Gerisch et al., 2007), and similar molecules are upregulated in some long-lived mice (Amador-Noguez et al., 2007) it will be of great interest to investigate if other lipophilic signaling molecules underlie ageing and reproduction in flies.

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Figure texts

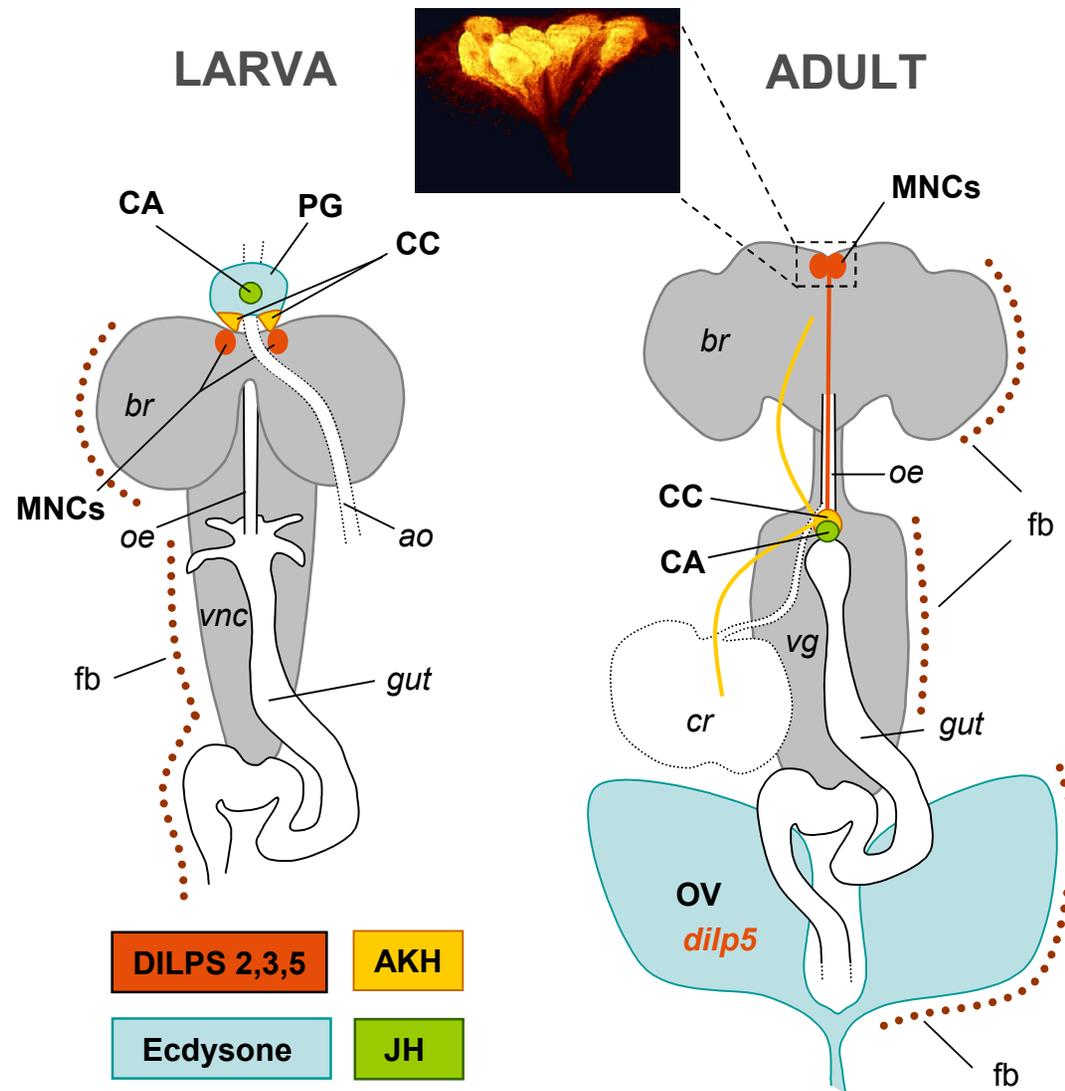
Figure 1. Schematic representation of neuroendocrine tissues in *Drosophila*. Larval (left) and adult (right) cell types or tissues involved in production of DILPs (red), AKH (yellow), ecdysone (blue) and JH (green). Larval master

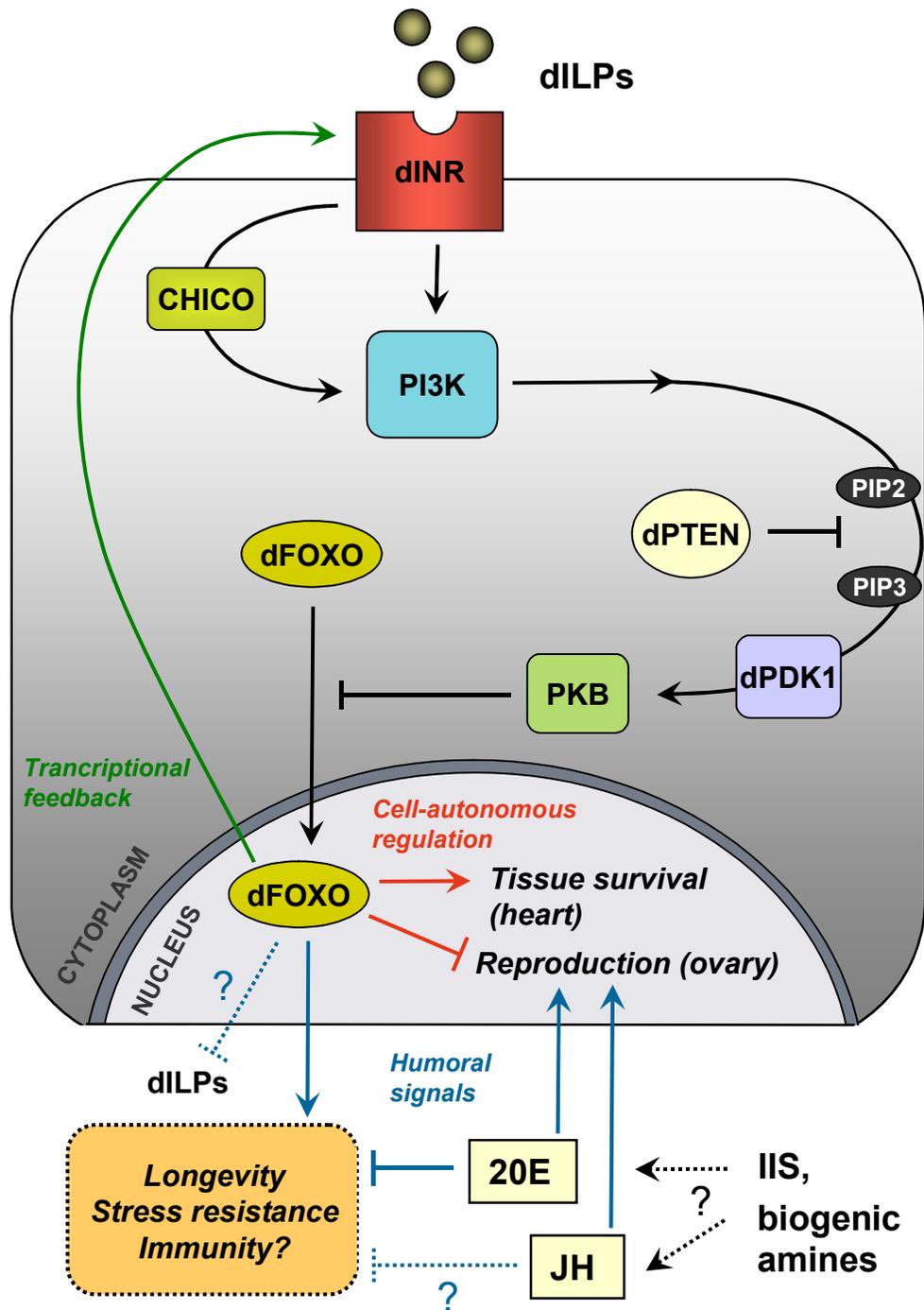
neuroendocrine organ, the ring gland, is located between the two hemispheres of the larval brain and consists of PG, CA and CC. The *dilp2*, *3* and *5* genes are expressed in bilaterally clustered MNCs in the pars intercerebralis of the larval brain (Ikeya et al, 2002). CC cells and have projections (not shown for clarity) to the PG, and to the aorta where they make extensive contacts with the axons of the MNCs (Rulifson et al, 2002, Kim and Rulifson, 2004). In adults, PG has degraded and the ovary is thought to be the primary site of the ecdysone synthesis. CC/CA complex in the adults is located in above the oesophagus, in the junction of the crop and the gut, and makes contacts with the axonal nerve tracts from the MNCs (red line). Projections from the CC cells have been tracked at least to the brain and to the crop (yellow lines, Lee and Park, 2004). Also adult MNCs express *dilp2*, *3* and *5* genes (Broughton et al, 2005) and, additionally, *dilp5* transcripts have been observed in the ovary (Ikeya et al, 2002). The fat body tissue, that surrounds all major organs, is presented only schematically (brown dotted lines). Cell clusters producing DILP7, PTH and biogenic amines are omitted for clarity (see Miguel-Aliaga et al., 2008, McBaryer et al., 2008, Monastrioti, 1999). Inset: Fluorescent *in situ* hybridization showing *dilp3* transcript in the adult MNC-clusters (image kindly provided by Jake Jacobson and Susan Broughton). Abbreviations: CA, corpus allatum; CC, corpus cardiacum; MNCs, median neurosecretory cells; PG, prothoracic gland; OV, ovaries; br, brain; vg, ventral ganglia; vnc, venral nerve cord; oe, oesophagus; ao, aorta, cr, crop; fb, fat body.

Figure 2. Overview of endocrine control of ageing and reproduction in flies. *Drosophila* insulin-like peptides (dILPs) produced by MNCs (and other tissues) activate *Drosophila* insulin receptor (dINR) which transduces the signal, either directly or through insulin receptor substrate (CHICO), to the PI3-kinase (PI3K). PI3K converts phosphatidylinositol (4,5)-bisphosphate (PIP2) to second messenger phosphatidylinositol (1,4,5)-trisphosphate (PIP3) resulting in activation of kinases dPDK1 and PKB, latter of which phosphorylates dFOXO and prevents its nuclear translocation. Downregulation of IIS by mutations in dINR or CHICO (or by overexpression of IIS antagonist dPTEN) results in increased nuclear dFOXO localization. dFOXO regulates transcription of genes that increase longevity and stress resistance. Additionally, nuclear dFOXO can transcriptionally upregulate *dInr* (green arrow) providing a feedback loop that can "prime" cells to respond rapidly to changing nutritional conditions (Puig et al., 2003). Both cell autonomous effects (red) and humoral signals (blue) can contribute to regulation of lifespan and reproductive capacity. Examples of cell-autonomous (or tissue-autonomous) regulation of decreased IIS are increased heart performance (Wessels et al, 2004) and ovary-autonomous requirement for oogenesis (Drummond-Barbosa et al, 2001; Richard et al, 2005; LaFever et al, 2005). Fat body has been implicated in production of humoral signal that can increase longevity and stress resistance, among other things. The nature of this signal is currently unknown, although reduced dILP production has been reported in some studies (Hwangbo et al, 2004) but not in others (Giannkou et al, 2007). Lipophilic signaling molecules ecdysone (here abbreviated 20E) and juvenile hormone (JH) are essential for reproduction, and reduced ecdysone signalling has been implicated in regulation of lifespan and stress resistance (Simon et al, 2003). JH has been suggested to function as a secondary downstream hormone under IIS to influence lifespan but direct evidence for its role is still missing. Although JH production could potentially be affected by IIS in adult flies (Belgacem and Martin, 2007), decreased JH biosynthetic capacity is not consistently associated with longevity in IIS mutants (Tatar et al, 2001; Tu et al, 2005; Richard et al, 2005). Increased IIS can upregulate 20E production in larval PG (Colombani et al, 2005), but this yet needs to be demonstrated in *Drosophila* ovaries. Additionally, biogenic amines have been implicated in regulation of lipophilic hormone titres in adult flies. Solid arrows indicate activation, dashed arrows potential activation. Bars indicate inhibition, dashed bars potential inhibition. Blue arrows and bars represent humoral signaling, and red ones cell-autonomous regulation.

Peptides	Gene ID	Larval expression (tissue)	Adult expression (tissue)	Major hormonal actions / processes involved
DILP-1	CG14173	MNCs ³	?	dINR agonist ²
DILP-2	CG8167	MNCs ¹⁻²	MNCs ⁴	dINR agonist ¹⁻² , carbohydrate homeostasis ^{*3-4} , stress response ^{*4} , fecundity ^{*4} , aging ^{*4-5}
DILP-3	CG14167	MNCs ¹⁻²	MNCs ⁴ , midgut ⁶	dINR agonist ² , carbohydrate homeostasis ^{*3-4} , stress response ^{*4} , fecundity ^{*4} , aging ^{*4}
DILP-4	CG6736	Larval midgut ¹	?	dINR agonist ²
DILP-5	CG32051	MNCs ¹⁻² , gut ^{*1}	MNCs ⁴ , ovaries ²	dINR agonist ² , carbohydrate homeostasis ^{*3-4} , stress response ^{*4} , fecundity ^{*4} , aging ^{*4}
DILP-6	CG14049	FB ⁶ , gut ^{*1}	CNS ⁶ , others ^{*6}	dINR agonist ²
DILP-7	CG13317	Several neurons in the VNC ^{1,7}	Several neurons in the VG ^{7-8, 6}	dINR agonist ² , egg laying ^{*8}
AKH	CG1171	CC ⁹⁻¹²	CC ¹⁰⁻¹¹	Lipid and/or carbohydrate metabolism ¹⁰⁻¹³ , starvation response ^{11,13}
Lipohilic Hormones		Larval synthesis (tissue)	Adult synthesis (tissue)	Major processes involved
Ecdysteroids		PG ¹⁴	The ovary ¹⁴ , others [*]	Molting ¹⁴ , growth ¹⁵ , metamorphosis ¹⁴ , reproduction ¹⁶ , aging ¹⁷
JH		CA ¹⁸	CA ¹⁹	Reproduction ^{20,21} , aging ^{*21-23}
Biogenic Amines		Larval synthesis (tissue)	Adult synthesis (tissue)	Major processes involved
Dopamine		Cell clusters (brain / VNC) ²⁴	Cell clusters (brain / VG) ²⁴	Learning, memory ²⁴ , ageing and stress resistance ^{*25} , 20E/JH metabolism ^{*26}
5-HT		Distinct neurons (brain / VNC) ²⁴	Distinct neurons (brain / VG) ²⁴	Locomotion, feeding ^{24,27} , sleep ²⁸ , aggression ²⁹ , ageing and stress resistance ^{*25}

Table 1 Major hormones implicated in regulation of lifespan and reproduction in *Drosophila*. Unknown tissue is indicated by a question mark (?) and putative function or indirect evidence by an asterisk (*). Abbreviations: DILP, *Drosophila* insulin-like peptide; dINR, *Drosophila* insulin receptor; MNCs, median neurosecretory cells; FB, fat body; CNS, central nervous system; VG, ventral ganglia; VNC, ventral nerve cord, CC, corpus cardiacum; PG, prothoracic gland; CA, corpus allatum. References: ¹Brogiolo et al., 2001, ²Ikeya et al., 2002, ³Rulifson et al., 2002, ⁴Broughton et al., 2005, ⁵Hwangbo et al., 2004, ⁶Chintapalli et al., 2007, ⁷Miguel-Aliaga et al., 2008, ⁸Yang et al., 2008, ⁹Noyes et al., 1995, ¹⁰Lee and Park, 2004, ¹¹Isabel et al., 2005, ¹²Kim and Rulifson, 2004, ¹³Grönke et al., 2007, ¹⁴Riddiford, 1993, ¹⁵Colombani et al., 2005, ¹⁶Carney and Bender, 2000, ¹⁷Simon et al., 2003, ¹⁸Richard et al., 1989, ¹⁹Dai and Gilbert, 1991, ²⁰Bownes, 1982, ²¹Flatt and Kawecki, 2007, ²²Tatar et al., 2001, ²³Tu et al., 2005, ²⁴Monastirioti, 1999, ²⁵De Luca et al., 2003, ²⁶Rauschenbach et al., 2007, ²⁷Neckameyer et al., 2007, ²⁸Dierick and Greenspan, 2007, ²⁹Yuan et al., 2006.





Toivonen and Partridge, Figure 2