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The identification and characterisation of novel peptide signalling pathways that function in the *Drosophila* prothoracic gland

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Abstract

Across many species, steroid hormones are typical regulators of animal development and growth. In order to fully appreciate animal development, it is important to understand the mechanisms that regulate steroid hormone production. This can be done using the fruit fly, *Drosophila melanogaster*, in which developmental transitions are primarily regulated by the steroid hormone, ecdysone. Several pulses of ecdysone are produced during development, each corresponding with a particular developmental transition such as the moults between larval stages. If ecdysone is incorrectly produced, the animal will suffer severe consequences such as developmental delays, changes in final body size, or even lethality. Therefore, regulation of ecdysone production is extremely important as it dictates both survival and fitness of the animal.

Ecdysone is produced in and secreted from a larval endocrine gland known as the prothoracic gland (PG). The PG responds to various environmental stimuli to tune the timing and quantity of ecdysone synthesised. These environmental signals are relayed to the PG via the action of several peptide signalling pathways, which function within the PG to control the production of ecdysone. There are two well characterised peptide signalling pathways which do this. The first is the prothoracicotropic hormone (PTTH) pathway, which controls ecdysone production in response to photoperiod and tissue damage. The second is the insulin signalling pathway, which controls ecdysone production in response to nutrition. While these two pathways have proven to be crucial in regulating the production of ecdysone in the PG, evidence to suggest that additional peptide signalling pathways carry out the same role has surfaced in recent years. To fully understand insect development, it is imperative that the spectrum of neuropeptide signalling pathways that control ecdysone are unravelled.

Therefore, the goal of this thesis was to identify and to characterise novel neuropeptide signalling pathways which function in the *Drosophila* PG. A PG-specific RNAi screen targeting neuropeptide receptors was carried out, and this led to the identification of eight neuropeptide receptors that potentially function in the PG. Preliminary characterisation of several of these hits helped to elucidate their function in the PG. Two neuropeptide receptors in particular, Neuropeptide F Receptor (NPFR) and Diuretic Hormone 44 Receptor 1 (Dh44-R1), were extensively investigated in this thesis. Both NPFR and Dh44-R1 were shown to

have novel roles in regulating developmental timing and body size by regulating the timing of ecdysone synthesis. Additionally, NPFR appears to control ecdysone production by negatively regulating the insulin signalling pathway in the PG – a new mechanism by which insulin signalling is regulated in this context. The results of this thesis demonstrate that more peptide signalling pathways function to control ecdysone production in the PG than previously thought. These results therefore provide insights into peptide regulation of steroid hormones in general by demonstrating their range and complexity.

Thesis including published works declaration

I hereby declare that this thesis contains no material which has been accepted for the award of any other degree or diploma at any university or equivalent institution and that, to the best of my knowledge and belief, this thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

This thesis contains one submitted publication and two unpublished chapters. The core theme of this thesis is the identification and characterisation of novel peptide signalling pathways in the *Drosophila* prothoracic gland. The ideas, development and writing up of all the papers in the thesis were the principal responsibility of myself, the student, working within the School of Biological Sciences under the primary supervision of Dr. Christen Mirth.

The inclusion of co-authors reflects the fact that the work came from active collaboration between researchers and acknowledges input into team-based research. For all three chapters, these were collaborative projects and the co-authors reflect my supervisors Christen Mirth, Coral Warr, Michelle Henstridge, and Linda Parsons. For Chapter 3, Shu Kondo generated a null mutant used in the study, as acknowledged.

In the case of Chapter 3, my contribution to the work involved the following:

Thesis Chapter	Publication Title	Status	Nature and % of student contribution	Co-author name(s) Nature and % of Co-author's contribution for student Co-authors only	Co-author(s), Monash student Y/N
3	Neuropeptide F Receptor acts in the *Drosophila* prothoracic gland to regulate growth and developmental timing	Submitted	90%. Concept, collecting data, and writing first draft	1. Michelle Henstridge, supervised, assisted with experiments N/A 2. Linda Parsons, supervised, provided input on experiments N/A 3. Shu Kondo, generated a reagent N/A 4. Christen Mirth, supervised, provided input on experiments, wrote manuscript N/A 5. Coral Warr, supervised, provided input on experiments, wrote manuscript N/A	No

I have renumbered sections of submitted papers in order to generate a consistent presentation within the thesis.

Student name: Jade Kannangara

Student signature:  **Date:** 4/12/2019

I hereby certify that the above declaration correctly reflects the nature and extent of the student's and co-authors' contributions to this work. In instances where I am not the responsible author I have consulted with the responsible author to agree on the respective contributions of the authors.

Main Supervisor name: Christen Mirth

Main Supervisor signature:  **Date:** 1/12/2019

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Abbreviations

20E – 20-hydroxyecdysone

AKH – Adipokinetic Hormone

Akt – Protein kinase B

AstA – Allatostatin A

AstA-R2 – Allatostatin A Receptor 2

BMS – Bommo-myosuppressin

BMSR - Bommo-myosuppressin Receptor

CA – Corpora Allata

cAMP- Cyclic adenosine monophosphate

CC – Corpora Cardiaca

CCKLR-17D1 - Cholecystokinin-like receptor at 17D1

CCKLR-17D3 – Cholecystokinin-like receptor at 17D1

CG – Protein coding gene

CNS – Central nervous system

CRF – Corticotropin releasing factor

crz – corazonin

dcrII – dicerII

Dh44 – Diuretic Hormone 44

Dh44-R1 – Diuretic Hormone 44 Receptor 1

Dh44-R2 – Diuretic Hormone 44 Receptor 2

dib – disembodied

Dilps – insulin like peptides

Dsk – Drosulfakinin

e74b - ecdysone-induced protein 74EF

ERK – Extra cellular signal-related kinases

EtOH – Ethanol

FoxO – Forkhead Box O

GFP – Green fluorescent protein

GPCR – G-protein coupled receptor

GS – Geneswitch

hAEL – hours after egg lay

hAL3E – hours after L3 ecdysis

hug – hugin

InR – insulin receptor

IPCs – insulin producing cells

JH – Juvenile Hormone

Lgr 1 - Leucine-rich repeat containing G-protein coupled receptor 1

Lgr3 – Leucine-rich repeat containing G-protein coupled receptor 3

Lgr4 - Leucine-rich repeat containing G-protein coupled receptor 4

MAPK - Mitogen-activated protein kinases

Ms - Myosuppressin

MsR1 – Myosuppressin receptor 1

MsR2 – Myosuppressin receptor 2

NPF – Neuropeptide F

NPFR – Neuropeptide F Receptor

nvd – neverland

Octβ3R - β3-octopamine receptor

pdf – pigment dispersing factor

PG – prothoracic gland

phm – phantom

PI3K - Phosphoinositide 3-kinase

PK2-R1 - Pyrokinin 2 Receptor 1

PLCβ- Phospholipase C *beta*

PTTH – Prothoracicotropic hormone

RU486 – Mifepristone

S6K – S6 kinase

sad – shadow

shd – shade

sNPF – Small neuropeptide F

sNPFR – Small neuropeptide F Receptor

spok – spookier

TOR – Target of rapamycin

Tor – torso

Chapter 1:

General Introduction

1.1. Control of developmental timing and body size by ecdysone

All animals need to regulate their development so that the correct processes are induced at the right time. Across a broad range of animals, developmental hormones are important regulators of developmental time. This is no different in insects, where the steroid hormone ecdysone acts to regulate when larvae will moult, initiate metamorphosis, and differentiate adult structures. By regulating developmental time, ecdysone also dictates final body size (Riddiford, 1993). Final body size is a product of both the rate and duration of the growth phase (Nijhout, 2003). This growth phase occurs either between the hatching of a nymph to the adult moult in hemimetabolous insects, or between the hatching of a larva to the onset of metamorphosis in holometabolous insects. Once insects have reached the adult stage, their rigid adult exoskeleton prevents further growth. For this reason, changing development time in the nymphal or larval stages can have profound effects on final adult size.

As the developmental processes that ecdysone regulates are fundamental to the survival and fitness of the animal, ecdysone production is consequently sensitive to a number of environmental conditions. This ensures that the animal only develops when the correct environmental conditions have been met. Studies in recent years have indicated that this sensitivity to environmental conditions is mediated by neuropeptides and peptide hormones. To date, researchers have yet to match the range of environmental conditions known to modify the timing and quantity of ecdysone synthesis with their peptide signals. If we hope to understand how insects adjust their development to produce a functional adult across environments, it is important to understand the molecular mechanisms that regulate ecdysone synthesis.

The fruit fly, *Drosophila melanogaste*r, is an excellent model animal in which to study ecdysone synthesis, given their advanced genetic toolkit. *Drosophila* undergo three larval moults and a pupal stage before initiating metamorphosis and finally emerging as adults. The duration and rate of the larval stages, as well as the onset of pupariation and metamorphosis, are under the control of carefully timed pulses of ecdysone that are produced throughout development (Figure 1.1). More specifically, a pulse of ecdysone is produced just before each larval moult (for review, see Richards, 1981), and these pulses trigger the relevant gene cascades that permit the moult to occur.

Within the third larval instar, three additional pulses of ecdysone are also produced (Warren *et al.*, 2006). The first of these pulses correlates with a developmental checkpoint known as critical weight (Warren *et al.*, 2006, Koyama *et al.*, 2014). Once critical weight is reached, the animal no longer relies on nutrition to reach metamorphosis (Mirth *et al.*, 2005). A second small pulse of ecdysone initiates the onset of glue production (Warren *et al.*, 2006). This glue will later be used to stick the pupal case formed at pupariation to a given surface (Hansson and Lambertsson, 1983). Later in the third larval instar, a small pulse of ecdysone is produced that induces wandering behaviour, whereby the animal finds a suitable location in which to begin pupariation (Warren *et al.*, 2006). Finally, the last pulse of ecdysone produced controls the onset of pupariation, signalling the beginning of pupal development (Warren *et al.*, 2006). If a pulse of ecdysone is absent or incorrectly produced, the animal will suffer developmental defects such delays in development, changes in final body size, or even lethality (Mirth *et al.*, 2005, Colombani *et al.*, 2005, Caldwell *et al.*, 2005, McBrayer *et al.*, 2007, Gibbens *et al.*, 2011). This demonstrates the importance of this hormone in regulating these developmental transitions and for overall growth.

In addition to its role in regulating developmental timing and body size, ecdysone is also known to play other important roles throughout the life cycle. Firstly, embryos synthesise ecdysone during mid-late embryogenesis in *Drosophila* and other insects (Maróy *et al.*, 1988). Ecdysone synthesis at this time correlates with the morphogenetic changes associated with the development of the first instar larva (Kozlova and Thummel, 2003), as well as cuticle formation (Chavez *et al.*, 2000). Ecdysone also plays a role in the growth and patterning of adult tissues, like the wing imaginal discs and neuroblasts in the central nervous system in *Drosophila* (Lanet *et al.*, 2013, Champlin and Truman, 1998, Mirth and Riddiford, 2007, Mirth *et al.*, 2009) and in the tobacco hornworm, *Manduca sexta* (Nijhout and McKenna, 2018, Nijhout and Grunert, 2002), thereby dictating tissue and organ identity during development. During metamorphosis, ecdysone is required for the eversion and proliferation of cells of the imaginal discs, apoptosis of larval cells, and restructuring of the nervous system (for review, see Riddiford, 1993 and Cranna and Quinn, 2009). In the adult fly, ecdysone influences egg development in the female germline (Soller et al 1999, Buszczak et al 1999). While all the roles ecdysone plays throughout the life cycle are important, this thesis focuses on the role ecdysone plays in regulating developmental timing and body size, as much more is known about peptide control of ecdysone in this context.

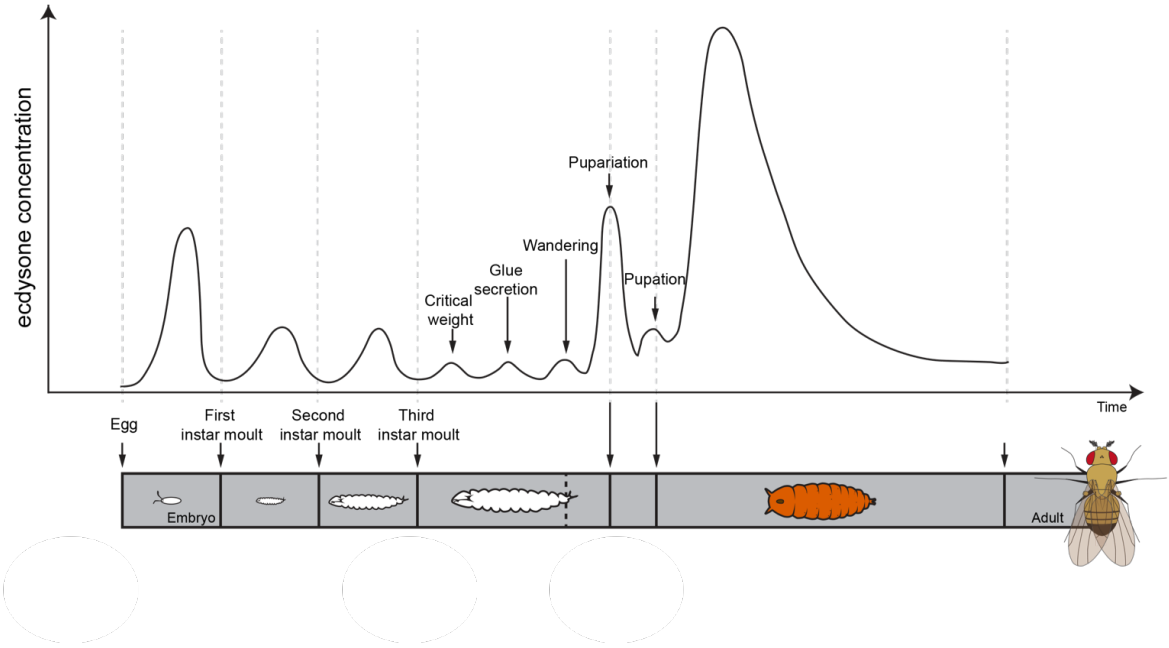


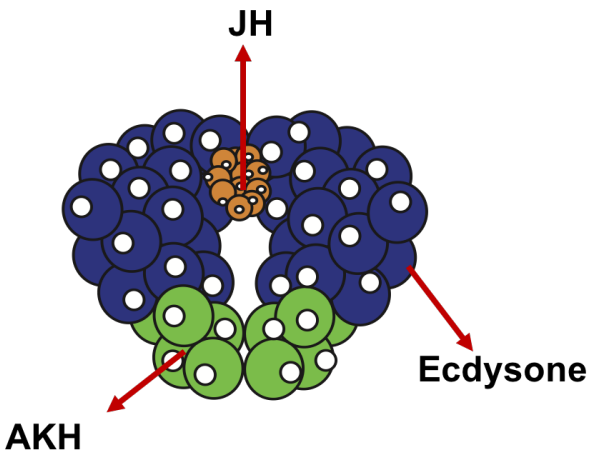
Figure 1.1: Several pulses of ecdysone are produced during *Drosophila* development

Several pulses of ecdysone are produced during development, and each pulse correlates with a particular developmental transition. These pulses of ecdysone can trigger larval moults, as well as pupariation and pupation (Riddiford, 1993). In addition, ecdysone can also activate other developmental checkpoints and behaviours such as critical weight, glue secretion and wandering behaviour (Riddiford, 1993, Warren *et al.*, 2006). Figure illustrated by Marisa Oliveria (2014).

1.2. Ecdysone production and secretion

In the larva, ecdysone is produced in and secreted from an endocrine gland known as the prothoracic gland (PG). The PG itself is part of a composite gland known as the ring gland, which also consists of the corpora allata (CA) and corpora cardiaca (CC). The CA and CC produce juvenile hormone and adipokinetic hormone, respectively (Figure 1.2). Ecdysone is synthesised in the PG from dietary cholesterol by a group of P450 enzymes collectively known as the "Halloween genes" (Figure 1.2; for review, see Rewitz et al., 2006). The Halloween genes act in a sequential manner, each converting cholesterol derivatives until ecdysone is produced. It is then secreted from the PG into the hemolymph (Yamanaka *et al.*, 2015) where it is converted to its active form, 20-hydroxyecdysone (20E), in other tissues by the enzyme, Shade (Petryk *et al.*, 2003). 20E then activates the Ecdysone Receptor in target tissues, triggering the relevant gene cascades that promote developmental transitions.

A


JH
Ecdysone
AKH

B

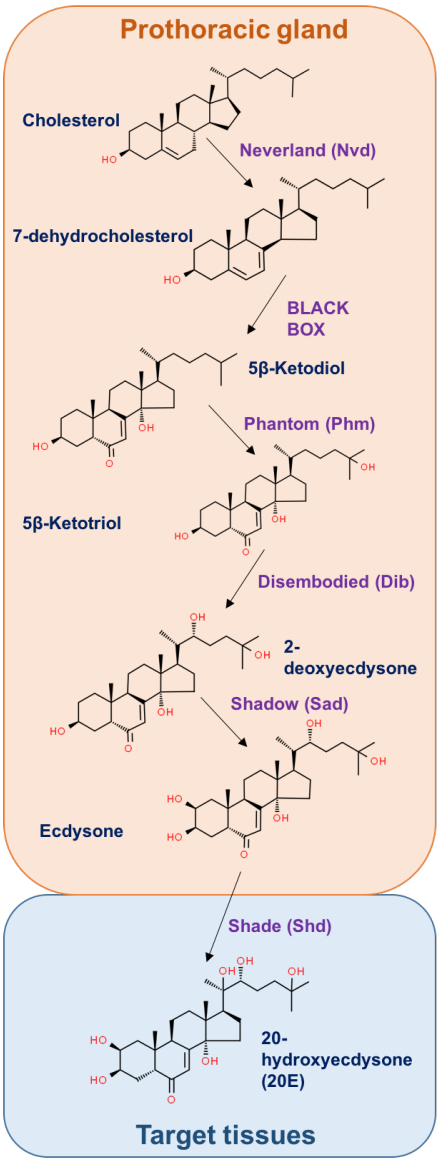

Prothoracic gland
Cholesterol
Neverland (Nvd)
7-dehydrocholesterol
BLACK BOX
5β-Ketodiol
Phantom (Phm)
5β-Ketotriol
Disembodied (Dib)
2-deoxyecdysone
Shadow (Sad)
Ecdysone
Shade (Shd)
20-hydroxyecdysone (20E)
Target tissues

Figure 1.2: The prothoracic gland produces and secretes ecdysone

(A) The ring gland is made up of the corpora allata (CA) (orange), which produces and secretes Juvenile Hormone (JH), the corpora cardiaca (CC) (green), which produces and secretes Adipokinetic hormone (AKH), and the prothoracic gland (PG) (purple), which produces and secretes pulses of ecdysone throughout development. **(B)** Ecdysone is synthesised from dietary cholesterol by several ecdysone biosynthetic enzymes, including *phantom, shade*, *disembodied, shadow and neverland* (for review, see (Rewitz *et al.*, 2006). The "Black box" genes are a group of genes that have not been characterised, but are predicted to be responsible for converting 7-dehydrocholesterol to 5β-ketodiol (Lafont *et al.*, 2012, Warren *et al.*, 2009). Ecdysone is then secreted from the PG and into the hemolymph and converted to its active form, 20E by Shade.

1.3. Peptide regulation of ecdysone production

The PG can be thought of as a sensor, responding to signals from the external environment to ensure that ecdysone is produced at the right time and in the right amount, given the specific environmental conditions. Ecdysone production by the PG has been shown to be circadian-gated, and to respond to inputs from nutrition and imaginal disc damage (McBrayer *et al.*, 2007, Jaszczak *et al.*, 2016, Mirth *et al.*, 2005). These inputs are relayed through the action of neuropeptides and peptide hormones that bind to receptors in the PG, triggering the relevant gene cascades that regulate the synthesis of ecdysone.

Much of our knowledge regarding peptide control of ecdysone production comes from studies of Lepidoptera. This is because these animals are typically larger, which facilitates easy culturing of their prothoracic glands, and makes biochemical assays straightforward. Almost 100 years ago, Kopeć (1922) described a brain-derived peptide responsible for regulating developmental transitions. After much work, this peptide, prothoraciotropic hormone (PTTH), was finally isolated from the silkworm, *Bombyx mori,* in the 1970s (for review, see Ishizaki, 2004). However, the molecular cloning of *Bombyx* PTTH was not conducted until the early 1990s (Kawakami *et al.*, 1990, Kataoka *et al.*, 1991). The release of PTTH from the brain in *Bombyx* correlated with an increase in ecdysone titres and developmental transitions (Sakurai, 1983, Sakurai, 1984), suggesting that PTTH stimulated the production of ecdysone.

Manduca is also an excellent model in which to study ecdysone production, as they have paired prothoracic glands which produce ecdysone derivatives at the same rate. This means that experimental manipulation can be conducted on one gland, with the other acting as an internal control (Bollenbacher *et al.*, 1979). Studies in *Manduca* also revealed the presence of PTTH, but in two different sizes; big PTTH and small PTTH (Gray *et al.*, 1993, Muehleisen *et al.*, 1993). Both of these peptide families have been shown to stimulate *Manduca* prothoracic glands *in vitro* to produce ecdysone (Bollenbacher *et al.*, 1984), demonstrating a conserved function with *Bombyx* PTTH. In *Manduca,* PTTH is produced in two lateral cells in the larval brain (Agui *et al.*, 1979), and is released from the CA (Agui *et al.*, 1980).

During efforts to biochemically purify PTTH from *Bombyx,* a different peptide was identified from *Bombyx* brains (Ichikawa and Ishizaki, 1963). Surprisingly, this peptide did not stimulate ecdysone production in *Bombyx*, but did so in the saturniid moth, *Samia Cynthia ricini* (Ishizaki

et al., 1983). It was later revealed that this peptide shared homology with vertebrate insulin, and was later termed Bombyxin (Nagasawa *et al.*, 1984, Nagasawa *et al.*, 1986, Akira *et al.*, 1987). This was the first study to demonstrate the presence of insulin-like peptides in invertebrates. Together, these studies on Lepidopteran PTTH and Bombyxin formed the basis for early investigations into peptide control of ecdysone synthesis.

While studies in Lepidopterans proved to be pivotal in identifying these key peptides involved in regulating ecdysone production, the advanced genetics techniques used in *Drosophila* studies provided the means for more detailed dissection of the potential range of peptides regulating ecdysone synthesis. Using the Gal4 enhancer trap system in *Drosophila*, researchers were able to identify that *Drosophila PTTH* is expressed in paired bilateral neurons that innervate the PG (Siegmund and Korge, 2001, McBrayer *et al.*, 2007). What is more interesting is they were also able to identify ten other groups of neurons that innervated the ring gland, five of which directly innervated the PG (Siegmund and Korge, 2001). This suggests that several other neuropeptides could act to regulate ecdysone synthesis.

While neurons that directly innervate the *Drosophila* PG serve as a source of neuropeptides for this gland, neurons that innervate the CC and CA are also speculated to secrete peptides onto the PG (Niwa and Niwa, 2014). In support of this, neurons that express a subset of the *Drosophila* insulin-like peptides (Dilps) innervate the CC, which borders the PG (Rulifson *et al.*, 2002). The Dilps are known to regulate ecdysone production in *Drosophila* (Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005), so it is possible that these Dilps are secreted from CC-innervating neurons onto the PG. Neurons that express other neuropeptides, such as *short neuropeptide f* (*sNPF*), *corazonin* (*Crz*), and *hugin* (*hug*), have also been shown to innervate the CC (Kapan *et al.*, 2012, Melcher and Pankratz, 2005, Bader *et al.*, 2007). It is possible they are also secreted onto the PG to regulate ecdysone, however, more work is needed to determine if this is the case.

In addition to neuronal innervations of the ring gland, peptide hormones secreted from elsewhere in the animal travel in the hemolymph and have the potential to bind to receptors on the PG, influencing the production of ecdysone. These circulating peptide hormones and the neuropeptides produced in neurons that innervate the ring gland suggest there might be a rich and complex array of peptide regulators for ecdysone synthesis in *Drosophila* (Figure 1.3).

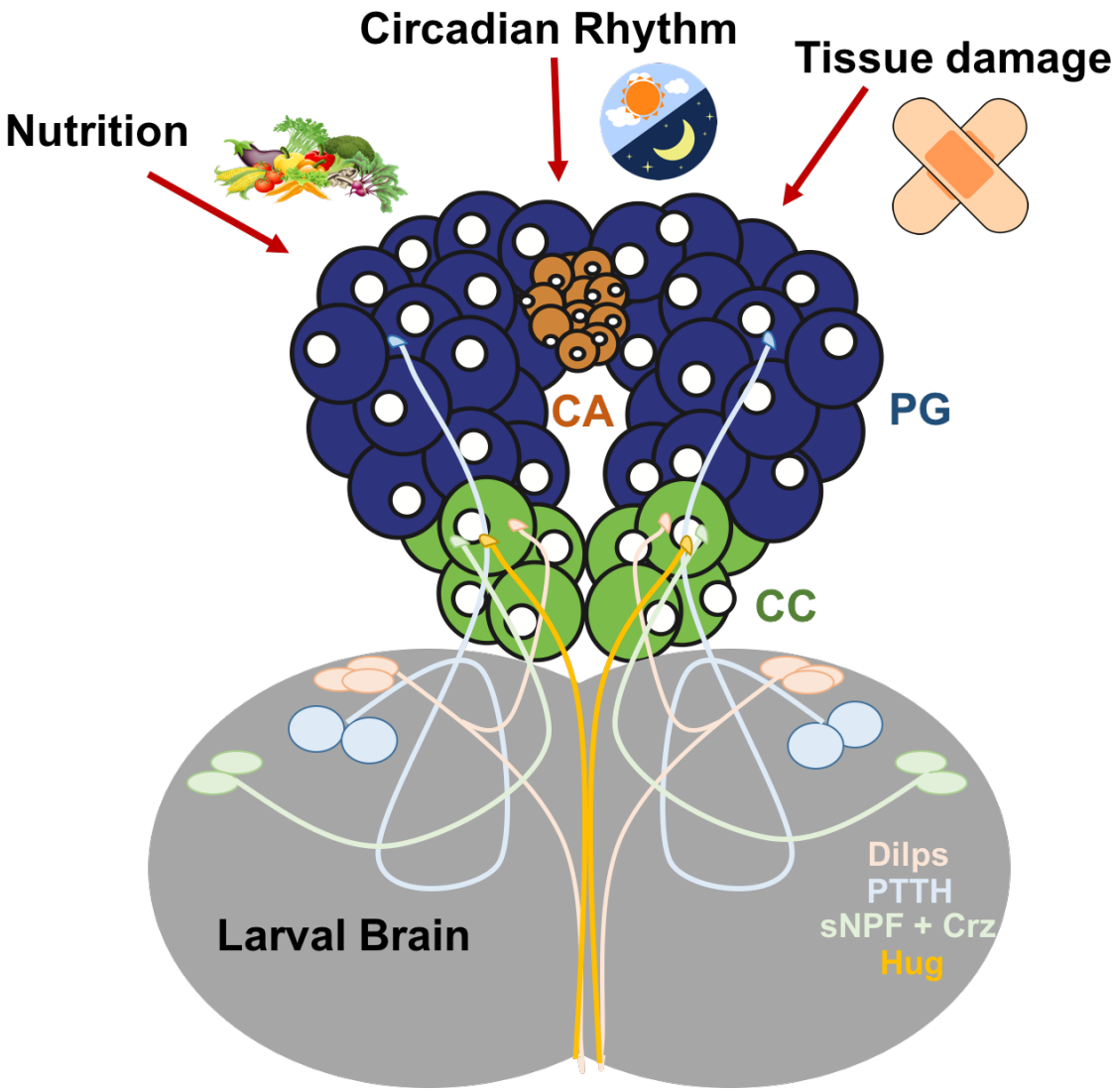


Figure 1.3: Examples of known innervations of the *Drosophila* ring gland

The PG produces and secretes ecdysone in response to environmental and physiological stimuli. These environmental signals then trigger the secretion of peptides onto the PG. Sources of such peptides include the PTTH-producing neurons, which innervate the PG. Additionally, the insulin producing cells secrete the Dilps, and Dilp-producing neurons innervate the CC (Rulifson et al., 2002). Furthermore, *short neuropeptide f* (*sNPF*)-, *corazonin* (*Crz)*- and *hugin* (*Hug*)- expressing neurons also innervate the CC (Kapan *et al.*, 2012, Melcher and Pankratz, 2005, Bader *et al.*, 2007), although whether these function in the PG is unknown.

1.4. Prothoracicotropic Hormone and the insulin-like peptides regulate ecdysone production in *Drosophila*

The two best characterised peptides involved in regulating ecdysone synthesis are PTTH and the Dilps. Given the plethora of work done on these peptides in Lepidopterans, it was logical for them to be the first peptides to be further explored in this context in *Drosophila*. Studies in *Drosophila* have contributed greatly into ascertaining the molecular mechanisms by which these peptides act, giving us a deeper understanding into how ecdysone production is regulated in the PG.

1.4.1. Prothoracicotropic Hormone function in the Drosophila PG

Through Lepidopteran studies, PTTH had been shown to be crucial in regulating ecdysone production in the PG. While these insects were powerful systems for describing how PTTH acted to control moulting and metamorphosis, the paucity of genetic tools made further characterisations of the signalling pathways through which PTTH acted difficult. Using *Drosophila,* McBrayer *et al* (2007) showed that genetically ablating PTTH-producing neurons resulted in animals with a significant developmental delay and increased final body size. Additionally, these phenotypes were found to be due to reduced expression levels of ecdysone biosynthesis genes and low ecdysone titres found in these animals (McBrayer *et al*., 2007). It was later shown that PTTH null mutants also have a significant developmental delay and increased adult size due to low ecdysone titres in these animals (Shimell *et al.*, 2018), further confirming that PTTH does indeed regulate developmental timing by controlling ecdysone production.

While Lepidopteran studies had shown that PTTH secretion was circadian-gated (Truman, 1972, Truman and Riddiford, 1974), genetic manipulations of circadian rhythm genes in *Drosophila* were able to confirm that this was indeed the case. In animals mutant for the clock gene, *pigment dispersing factor* (*pdf*), the transcriptional profile of PTTH becomes altered, indicating that it is under circadian-control (McBrayer *et al.*, 2007). Further to this, abolishing the activity of clock genes specifically in the PG results in developmental arrest due to a lack of ecdysone production, which is caused, in part, by lack of PTTH signalling

(Di Cara and King-Jones, 2016). These studies highlighted the importance of circadian rhythm in regulating PTTH secretion and consequently, ecdysone production.

Further studies in *Drosophila* have shown that PTTH production is also influenced by imaginal disc damage. When imaginal discs become damaged, PTTH expression becomes down regulated in an effort to slow down ecdysone synthesis in the PG (Halme *et al.*, 2010, Colombani *et al.*, 2012). This serves as a mechanism by which development is decelerated to provide the animal enough time for tissue repair. Thus, in addition to circadian cycles, a second environmental factor, imaginal disc damage, regulates PTTH secretion.

Studies in *Drosophila* also led to the discovery that the receptor for PTTH is a receptor tyrosine kinase called Torso (Tor) (Rewitz *et al.*, 2009). Upon binding to PTTH, Tor triggers Ras/Raf/ERK signalling to drive the production of ecdysone (Figure 1.4) (Rewitz *et al.*, 2009, McBrayer *et al.*, 2007). Knockdown of *tor* specifically in the PG results in a significant developmental delay, as well as an increase in final body size due to a prolonged third larval stage (Rewitz *et al.*, 2009), phenocopying the ablation of PTTH neurons and PTTH null mutants (McBrayer *et al.*, 2007, Shimell *et al.*, 2018). These studies in *Drosophila* were the first to demonstrate the phenotypic consequences of specifically removing PTTH/Tor.

The PTTH/Tor signalling pathway appears to regulate ecdysone biosynthesis at both the transcriptional and translational level. When PTTH-producing neurons are ablated in *Drosophila,* several ecdysone biosynthesis genes show reduced expression (McBrayer *et al.*, 2007). Furthermore, in *Manduca*, PTTH stimulation leads to a significant up-regulation of the Halloween gene, *spook* (Rewitz *et al.*, 2009). These experiments thus suggest that the PTTH/Tor pathway regulates transcription of ecdysone biosynthetic genes. In addition, the PTTH/Tor pathway also appears to up-regulate the translation of these enzymes (Rewitz *et al.*, 2009, Gibbens *et al.*, 2011). Precisely how the PTTH/Tor pathway regulates the transcription and translation of Halloween genes remains to be explored.

1.4.2. Insulin-like peptide function in the Drosophila PG

After the identification of Bombyxin, the invertebrate homolog of mammalian insulin, the Dilps were soon discovered and characterised (Brogiolo *et al.*, 2001). Eight Dilps have been described in *Drosophila*, and they have been shown to be both functionally diverse and differentially expressed (Grönke *et al.*, 2010). Dilps 1-7 bind to the Insulin Receptor (InR), while Dilp8 binds to a different receptor, and is more closely related to vertebrate relaxins (Garelli *et al.*, 2015, Garelli *et al.*, 2012, Vallejo *et al.*, 2015, Colombani *et al.*, 2012). Only *dilps 2, 3* and *5* are expressed in the insulin producing cells (IPCs) of the CNS, and these peptides are secreted from these cells in response to nutrition (Brogiolo et al., 2001). Given that Bombyxin was shown to regulate the production of ecdysone in *Samia* (Ishizaki *et al.*, 1983), it was soon found that the Dilps also play this same role in *Drosophila* (Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005). Reducing Dilp secretion by the IPCs prolongs development time and results in small adults, suggesting that Dilps 2, 3, and 5 are likely to play important roles in modulating ecdysone synthesis (Walkiewicz and Stern, 2009).

The Dilps bind to InR, which causes InR to phosphorylate itself (for review, see Stocker and Hafen, 2000). The insulin receptor substrate, Chico, then interacts with the phosphorylated receptor, recruiting Phosphoinositide 3-kinase (PI3K) as well as other proteins and downstream effectors. This in turn promotes the activation of Protein Kinase B (Akt), which stimulates protein synthesis by activating downstream kinases Target of Rapamycin (TOR) and S6 Kinase (S6K), and inhibits the activity of the transcription factor Forkhead Box O (FoxO) (for review, see Stocker and Hafen 2000; Figure 1.4). At least some of the effects of insulin signalling on ecdysone synthesis are mediated by the activity of FoxO (Koyama *et al.*, 2014).

During the third larval instar, the timing of the critical weight ecdysone pulse is known to be under the control of the insulin signalling pathway in the PG (Mirth *et al*., 2005 and Koyama *et al*., 2014). Up-regulating the expression of FoxO, the negative regulator of insulin signalling, significantly delays the timing of the critical weight ecdysone pulse (Koyama *et al.*, 2014), resulting in both an increase in body size and developmental delay (Caldwell *et al*., 2005 Colombani *et al*., 2005 and Mirth *et al*., 2005). Interestingly, FoxO exerts its effects, at least in part, by binding with a component of the ecdysone receptor, Ultraspiracle (Usp) (Koyama *et al.*, 2014).

The insulin signalling pathway has also been shown to affect endocycling in the PG cells at critical weight (Ohhara et al., 2017). These endocycles are thought to enable the production of the pulse of ecdysone that induces the critical weight transition (Ohhara *et al.*, 2017). Whether endocycling is induced through the FoxO/Usp complex is unknown.

Interestingly, the insulin signalling pathway plays different roles both pre- and post- critical weight. In pre-critical weight larvae, the insulin signalling pathway promotes the production of ecdysone (Shingleton *et al.*, 2005, Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005). In contrast, in post-critical weight animals, insulin signalling is thought to repress ecdysone production as low insulin signalling during starvation results in accelerated development (Stieper *et al.*, 2008, Shingleton *et al.*, 2005). This suggests that pre-critical weight, insulin signalling promotes ecdysone production whereas post-critical weight, it has the opposite effect (Figure 1.5). How this same pathway is able to exert different effects throughout development remains to be elucidated.

One hint at a potential mechanism regulating these differences might arise from the way in which the insulin signalling pathway affects ecdysone synthesis genes. Early in the third instar, the insulin signalling pathway regulates the mRNA expression levels of ecdysone biosynthesis genes, including *phm*, *dib, nvd, spok,* and *sad* (Colombani *et al.*, 2005, Caldwell *et al.*, 2005, Koyama *et al.*, 2014, Gibbens *et al.*, 2011). However, later in the third larval instar, insulin signalling regulates translation of ecdysone biosynthesis genes (Gibbens et al., 2011), but not their transcription. More work into the downstream components of this pathway responsible for the transcription/translation of ecdysone biosynthesis genes is needed to tease out how a single pathway is able to exert multiple and, in some cases, opposing effects on ecdysone production. Nevertheless, these studies demonstrate the complex array of roles the insulin signalling pathway plays in regulating ecdysone synthesis in the PG, highlighting the importance of this pathway in controlling overall developmental timing.

1.5. Other peptide signalling pathways known to regulate ecdysone production in the *Drosophila* PG

While the PTTH and Dilp-induced pathways are important in regulating the production of ecdysone in the PG, there is increasing evidence that other peptide signalling pathways also work in the PG to carry out the similar roles. The growth factor, activin, is able to control the competence of the PG to receive inputs from PTTH and the Dilps (Gibbens *et al.*, 2011). When components of the activin pathway are knocked down specifically in the PG, akin to down-regulating both PTTH and insulin signalling, animals are able to survive up until the last larval stage (Gibbens *et al.*, 2011). This indicates that additional factors, other than PTTH and the Dilps, are required for at least the first two larval moults. The following section will explore the recent advances in uncovering additional peptide signalling pathways that act on the *Drosophila* PG to regulate ecdysone.

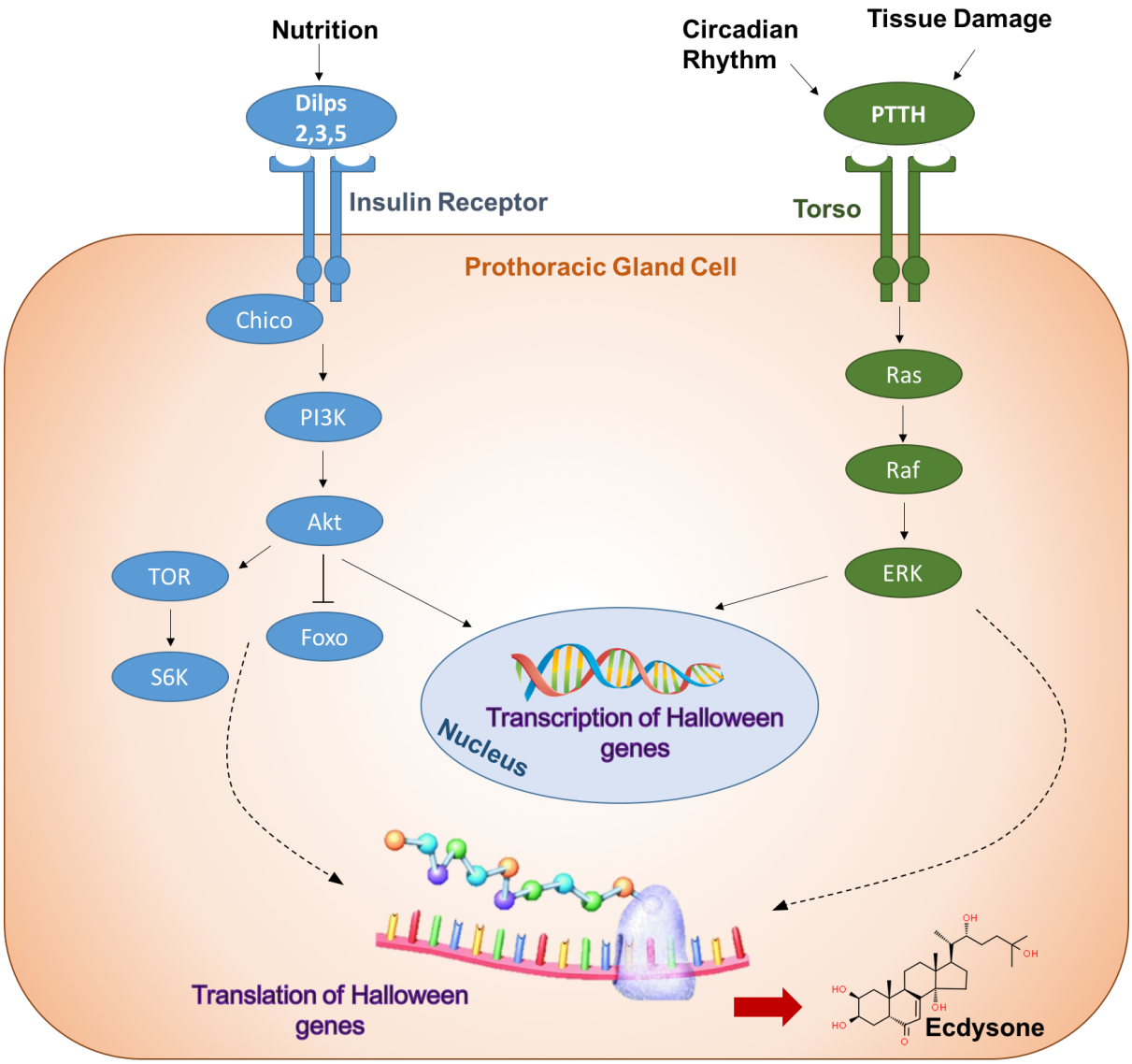


Figure 1.4: The Tor and InR pathways function in the *Drosophila* PG

Dilps 2, 3, and 5 are secreted in response to nutrition. They then bind to the Insulin Receptor (InR) which autophosphorylates. The insulin receptor substrate, Chico, then recruits Phosphoinositide 3-kinase (PI3K) as well as other proteins and downstream effectors. PI3K activates Protein Kinase B (Akt), which stimulates protein synthesis by activating downstream kinases Target of Rapamycin (TOR) and S6 Kinase (S6K), and by inhibiting the transcription factor Forkhead Box O (FoxO) activity. This signalling cascade leads to the transcription and translation of ecdysone biosynthesis genes. Prothoracicotropic Hormone (PTTH) binds to Torso to trigger as/Raf/ERK signalling to drive the production of ecdysone, via either the transcription or translation of ecdysone biosynthesis genes (for review, see Yamanaka *et al.*, 2013).

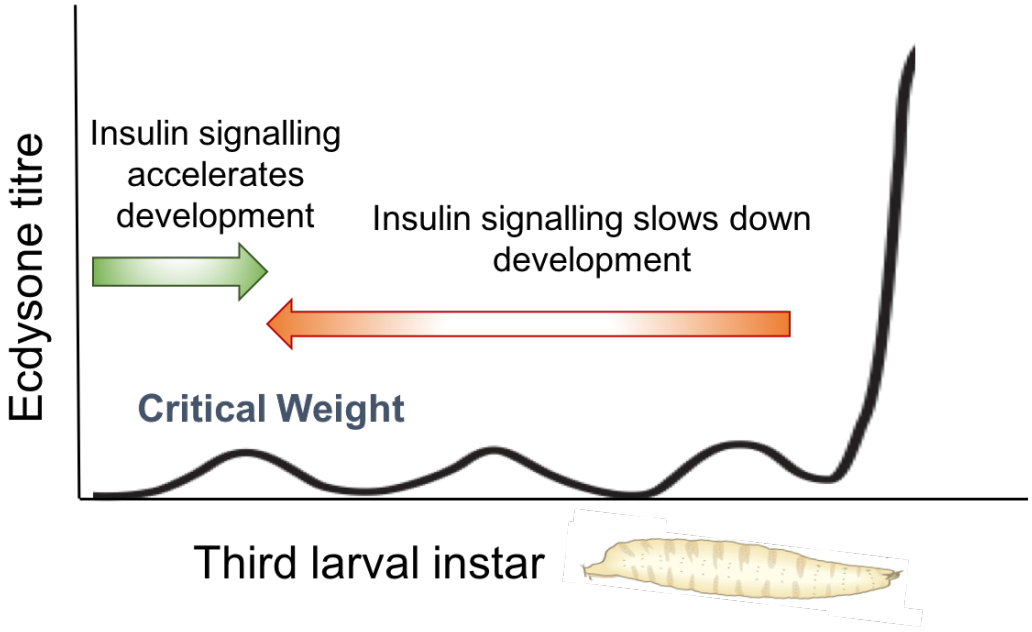


Figure 1.5: Insulin signalling plays different roles pre- and post-critical weight

Before critical weight is reached in the third larval instar, insulin signalling promotes developmental timing by positively influencing the production of ecdysone (Shingleton *et al.*, 2005, Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005). However, after critical weight has been reached, insulin signalling is thought to delay development by negatively influencing the production of ecdysone (Stieper *et al.*, 2008, Shingleton *et al.*, 2005).

1.5.1. Leucine-rich repeat-containing G protein-coupled receptors 1 and 3

A neuropeptide receptor that has been shown to function in ecdysone production in the PG is Leucine-rich repeat containing G-protein coupled receptor 3 (Lgr3). Lgr3 is a relaxin receptor which belongs to the larger family of leucine-rich repeat containing G-protein coupled receptors (LGRs) (Barker *et al.*, 2013). Using a GFP-tagged protein, Lgr3 has been shown to be expressed in the PG (Jaszczak et al., 2016). It is thought that the ligand for Lgr3 is Dilp8. There are several lines of evidence to support this. For example, Dilp8 stimulates Lgr3 activity *in vitro* (Vallejo *et al.*, 2015) as well as *in vivo* (Garelli *et al.*, 2015, Vallejo *et al.*, 2015), suggesting that Dilp8 is the likely ligand for Lgr3.

Dilp8 is secreted by damaged imaginal discs during larval development to delay development and to slow down the growth in undamaged discs (Colombani *et al.*, 2012, Garelli *et al.*, 2015, Garelli *et al.*, 2012) This delay is important, as damaged imaginal discs need time to regenerate. The delay in whole organism growth is induced by inhibition of PTTH by Dilp8 in the CNS, which results in delays in the timing of ecdysone pulses (Halme *et al.*, 2010, Colombani *et al.*, 2012). However, the inhibition of growth in undamaged imaginal discs is carried out through a reduction in basal, between-pulse levels of ecdysone synthesis (Parker and Shingleton, 2011), mediated by Lgr3 signalling in the PG upon Dilp8 binding (Jaszczak *et al.*, 2015, Jaszczak *et al.*, 2016).

Lgr1, another member of the LGRs, has also been shown to influence developmental transitions. Ubiquitous knockdown of *Lgr1* results in suppression of puparium formation, reduced ecdysone levels, and reduced expression levels of the ecdysone biosynthesis genes, *shadow* and *spookier* (Vandersmissen *et al.*, 2014). However, it has not yet been elucidated if Lgr1 is expressed in the PG, nor what the Lgr1 ligand is.

1.5.2. β3-octopamine receptor signalling

Another signalling pathway found to be essential for ecdysone biosynthesis in the PG is the β3-octopamine receptor (Octβ3R) pathway. Rather than being activated by a traditional neuropeptide, Octβ3R is activated by the trace amine, tyramine (Maqueira *et al.*, 2005), which acts like a neurotransmitter. Both *tyramine* and *Octβ3R* are expressed in the PG, and

when *Octβ3R* is knocked down in this gland, larvae fail to continue to develop and cannot pupate (Ohhara et al., 2015). In addition, RNAi knockdown of *Octβ3R* in the PG resulted in reduced expression of ecdysone biosynthesis genes, a lower 20E titre, and reduced Dilp and PTTH signalling (Ohhara et al., 2015). These larval arrest phenotypes could be rescued by feeding these animals ecdysone (Ohhara et al., 2015). Given that tyramine is produced in the PG and binds to Octβ3R in the PG, the authors suggest that perhaps autocrine signalling is needed among PG cells to promote an increase in responsiveness to the Dilps and PTTH. This would ensure the coordinated synthesis of ecdysone from different PG cells following exposure to neuropeptides.

1.5.3. Evidence for more peptide/receptor signalling pathways in the Drosophila PG

As mentioned previously, several neuropeptide-expressing neurons innervate the *Drosophila* ring gland (Siegmund and Korge, 2001). For example, the neuropeptide *hugin* is expressed in neurons that innervate the CC (Melcher and Pankratz, 2005, Bader et al., 2007), and so it may be secreted onto the PG from these neurons. In support of this, when *hugin* is ubiquitously mis-expressed, larvae die in the second instar (Meng *et al.*, 2002). This is suggestive of a moulting defect, possibly due to a dysregulation of ecdysone. The receptor for Hugin is the G-protein coupled receptor, Pyrokinin 2 Receptor 1 (PK2-R1; Rosenkilde *et al.*, 2003), and a transcriptome analysis carried out on ring glands found *PK2-R1* to be significantly upregulated in the ring gland compared to a whole larval sample (Ou *et al.*, 2016). However, it is currently unknown if *PK2-R1* is specifically expressed in the PG.

In addition to this, antibodies generated against the *Manduca* neuropeptide, Diuretic Hormone 44 (Dh44), stain axons that innervate the *Drosophila* ring gland, indicating that *Drosophila* Dh44 may influence ecdysone synthesis (Zitnan *et al.*, 1993). Dh44 has two receptors, Dh44-R1 and Dh44-R2. Dh44-R2 was found to be enriched in the ring gland compared to a whole-larval sample (Ou *et al.*, 2016), adding further evidence that Dh44 signalling functions in the *Drosophila* ring gland. However, more experiments are needed to explore whether Dh44 actually functions in the PG to regulate ecdysone synthesis.

Finally, it has been shown that ecdysone is actively secreted from the PG via calcium-stimulated vesicles (Yamanaka *et al.*, 2015), as opposed to freely diffusing through the

membranes of PG cells. In this study, the authors predicted that there would be a G-protein coupled receptor facilitating this process in the PG, as it was found that a $G\alpha_q$ subunit and a PLCβ class enzyme, both GPCR signalling components, were both required for proper release of ecdysone. Given that GPCRs are well known to respond to neuropeptides, it is possible that GPCR control of ecdysone release functions in response to a neuropeptide.

We can also look at other insects for additional peptide signalling pathways that might function in the *Drosophila* PG. For example, in *Bombyx,* several other neuropeptides and receptors have been found to both positively and negatively influence ecdysone biosynthesis (Table 1.1). Furthermore, in the kissing bug, *Rhodnius prolixus*, several other neuropeptide receptors have found to be expressed in the PG, all of which have *Drosophila* homologs (Table 1.1). Given the commonalities observed so far across insects in the regulation of ecdysone, it is certainly possible that these peptides and receptors may play a role in the *Drosophila* PG.

Table 1.1: Examples of other insect peptide/receptor systems known to function in or be expressed in the PG

Receptor	**Ligand**	**Species**	***Drosophila* Receptor Homolog**	**Known function or expression pattern**	**Reference**
Bombyx neuropeptide GPCR-B2 (BNGR-R2)	Pigment Dispersing Factor (PDF)	*Bombyx mori*	PDFR	Stimulates ecdysone synthesis in vitro	Iga *et al*., 2014
Unknown	Orcokinins	*Bombyx mori*	Unknown	Stimulate ecdysone synthesis in vitro	Yamanaka *et al*., 2011
Diapause Hormone Receptor (DHR)	Diapause Hormone (DH)	*Bombyx mori*	None	Expressed in PG and regulates ecdysone	Watanabe *et al*., 2007
Neuropeptide Receptor A34	Unknown	*Bombyx mori*	CG30340	Enriched in PG	Nakaoka *et al*., 2017
Corazonin Receptor (CrzR)	Corazonin (Crz)	*Rhodnius prolixus*	CrzR	Expressed in PG	Hamoudi *et al*., 2016
Calcitonin-like diuretic hormone Receptor 1 (CT/DH-21)	Unknown	*Rhodnius prolixus*	Hec	Expressed in PG	Zandawala *et al*., 2013
Calcitonin-like diuretic hormone Receptor 1 (CT/DH-R1)	Diuretic Hormone 31 (Dh31)	*Rhodnius prolixus*	Dh31R	Expressed in PG	Zandawala *et al*., 2013
Bommo-Myosuppressin receptor (BMSR)	Bommo-Myosuppressin (BMS) and Bommo-FMRFamides (BRFas)	*Bombyx mori*	MsR1 and MsR2	Suppress ecdysone synthesis	Yamanaka *et al*., 2005 and 2006
Sex Peptide Receptor (SPR)	Prothoracicostatic Peptide (PTSP)	*Bombyx mori*	SPR	Suppress ecdysone synthesis	Yamanaka *et al*., 2010

1.6. Thesis aims and structure

The primary aim of this thesis was to identify novel neuropeptide signalling pathways that function in the PG to regulate ecdysone synthesis. Determining the various neuropeptide receptor pathways which function in the PG will not only provide an understanding of the complexity of ecdysone regulation, but can also provide insights into the various environmental factors that control this important developmental hormone.

The results of this thesis are separated into three main results chapters (Chapters 2-4):

- Chapter 2 sets out to determine if there were any additional neuropeptide signalling pathways that function in the *Drosophila* PG. This chapter also includes preliminary experiments on newly identified genes that function in the PG, other than those explored in proceeding chapters.

- Chapter 3 characterises in detail the role of Neuropeptide F Receptor (identified from the screen from Chapter 2) in the *Drosophila* PG. Its role in ecdysone production, and what other signalling pathways it interacts with in the PG, are explored. The results presented in this chapter have been submitted to *Current Biology*.

- Chapter 4 details the characterisation of Diuretic Hormone 44 Receptor 1 (identified from the screen in Chapter 2) in the PG. Its role in regulating ecdysone production is explored, and hypotheses as to how and why it may be functioning in the PG are formulated.

The final chapter of this thesis (Chapter 5) synthesises the main findings and provides a general discussion that includes ideas and suggestions for future research.

Chapter 2:

An RNAi screen to identify novel regulators of ecdysone synthesis in the *Drosophila* prothoracic gland

2.1. Introduction

As discussed in Chapter 1, developmental transitions in insects are under the control of the steroid hormone, ecdysone. Ecdysone is produced in and secreted from an endocrine gland known as the prothoracic gland (PG) in response to environmental stimuli. These external stimuli are typically communicated to the PG via the action of neuropeptides, small peptides that are produced in neurons, as well as peptide hormones, peptides that travel through the hemolymph (Nässel and Larhammar, 2013). The two peptide signalling pathways that have been extensively studied in the *Drosophila* PG are the Prothoraciotropic hormone (PTTH) pathway and the insulin signalling pathway, which signal in response to circadian rhythm, physiological cues, as well as nutrition, respectively (Halme *et al.*, 2010, Colombani *et al.*, 2012, McBrayer *et al.*, 2007, Colombani *et al.*, 2005, Caldwell *et al.*, 2005, Mirth *et al.*, 2005). If the activity of either of these pathways is perturbed in the PG, defects such as delays in development time and changes in final body size are observed, demonstrating the importance of these pathways in regulating ecdysone production and overall development.

Despite the importance of the PTTH and insulin signalling pathways, there is evidence to suggest that additional neuropeptides and peptide hormones also function in the PG to control ecdysone production. This is unsurprising as the production of ecdysone must be tightly regulated in accordance with various environmental cues, therefore it is likely that several peptide signalling pathways would be needed to achieve this. To fully appreciate how insect development is controlled, it is necessary to identify and characterise the spectrum of peptide signalling pathways responsible for regulating ecdysone in the PG.

Several studies provide evidence that additional peptide signalling pathways, other than those induced by PTTH and the Dilps, regulate ecdysone in the PG. For example, loss of PTTH or its receptor is not lethal, but rather leads to a delay in metamorphosis (McBrayer *et al.*, 2007, Shimell *et al.*, 2018, Rewitz *et al.*, 2009). Similarly, ablation of the insulin producing cells (IPCs), the main source of *Drosophila* insulin-like peptides (Dilps), in the developing larva, causes a developmental delay but is again not lethal (Rulifson *et al.*, 2002). Furthermore, it has been shown that animals that have both PTTH and insulin signalling reduced specifically in the PG survive until the last larval moult (Gibbens *et al.*, 2011), suggesting that that other signalling pathways must be controlling ecdysone production in at least the first two larval moults.

Furthermore, evidence suggests that additional neuropeptides may be present in PTTH-producing neurons; neurons already known to innervate the *Drosophila* PG. PTTH null mutants have less-severe developmental delays compared to animals with ablated PTTH-producing neurons (Shimell *et al.*, 2018, McBrayer *et al.*, 2007). This implies that factors other than PTTH could be produced from PTTH-producing neurons to regulate ecdysone production in the PG.

In addition to the PTTH and Dilp neurons, ten types of additional neurosecretory neurons directly innervate the ring gland (Siegmund and Korge, 2001), a composite gland which contains the PG. It is unknown which, if any, of these neurons project specifically to the PG. However, even if they innervate another tissue of the ring gland, the corpora cardiaca (CC), it is possible they could still signal to the PG (Niwa and Niwa, 2014).

In other insect species, several peptides that have *Drosophila* homologs have been shown to function in the PG to control ecdysone production (see Chapter 1; Table 1). For example, in the silkworm, *Bombyx mori*, Myosuppressin has been shown to function in the PG to inhibit ecdysone production (Yamanaka *et al.*, 2005). In addition to this, *Bombyx* Pigment Dispersing Factor (Pdf) has been found to stimulate ecdysone synthesis *in vitro* (Iga *et al.*, 2014). As both of these neuropeptides have *Drosophila* homologs, it is possible that they also function in the *Drosophila* PG.

Lastly, it has been shown that other environmental factors, such as oxygen levels and temperature, can influence developmental timing and body size (Callier *et al.*, 2013, French *et al.*, 1998, Texada *et al.*, 2019, Ghosh *et al.*, 2013). These environmental factors presumably exert their effects on developmental timing and body size by regulating ecdysone production in the PG. Whether the PG directly senses these stimuli or uses the secretion of additional peptides to receive this information is poorly understood. Therefore, it is possible that unidentified peptide signalling pathways function in the PG to link these environmental cues to ecdysone production.

Therefore, the overall aim of this Chapter was to identify novel peptide signalling pathways that function in the *Drosophila* PG to regulate ecdysone. To this end, an RNAi screen in the PG was performed to identify novel peptide receptors involved in ecdysone synthesis. The

results presented in this study indeed suggest that more peptide signalling pathways function in the *Drosophila* PG than previously thought. This work therefore provides a strong starting point for further investigations into peptide control of ecdysone production in *Drosophila.*

2.2. Results

2.2.1. An RNAi screen identifies novel peptide receptors that may regulate ecdysone production in the PG

To identify novel peptide signalling pathways that function in the PG, an RNAi screen was carried out specifically in this gland. This screen targeted 48 neuropeptide and peptide hormone receptors, all of which belong to the G-protein coupled receptor (GPCR) family - a common neuropeptide-activated receptor family (Hanlon and Andrew, 2015; Table 2.1). This candidate list of receptors was compiled using information from a review of all known *Drosophila* GPCRs (Caers *et al.*, 2012). Additionally, a query search on Flybase (Thurmond et al., 2019) using the terms “Neuropeptide hormone activity", "Neuropeptide receptor binding", "Neuropeptide receptor activity”, "GPCR Neuropeptide + protein hormone receptors" and "Neuropeptides, peptide + protein hormones” was carried out, and additional genes identified were added to the candidate list.

Each of these receptors was knocked down specifically in the PG using the PG-specific *phantom* (*phm*)-Gal4 driver. In conjunction with driving an RNAi construct for each gene of interest, *phm*-Gal4 was also used to co-express *dicerII* (*dcrII*), an enzyme involved in cleaving dsRNA (Tomari and Zamore, 2005), in order to maximise gene knockdown. The use of Dicer has shown to increase RNAi knockdown specifically in the PG (personal communications, Prof. O’Connor). To determine if any of these receptors plays a role in regulating ecdysone in the PG, time from egg lay to pupariation was measured for each genotype, and any developmental defects were observed.

Interestingly, knocking down eight of these receptors in the PG resulted in developmental defects (Figure 2.1). PG-specific knockdown of *Leucine-rich repeat-containing G protein-coupled receptor 3* (*Lgr3*) resulted in embryonic lethality. PG-specific knockdown of *Diuretic Hormone 44 Receptor 1* (*Dh44-R1*) and *Allatostatin A Receptor 2* (*AstA-R2*) resulted

in larval lethality at either the first or second larval instar. Knockdown of *Leucine-rich repeat-containing G protein-coupled receptor 4* (*Lgr4*), *Neuropeptide F receptor* (*NPFR*), *Cholecystokinin-like receptor at 17D1* (*CCKLR-17D1*), *Myosuppressin Receptor 2* (*MsR2*) and *Short neuropeptide F receptor* (*sNPFR*) resulted in significant delays to pupariation. These data suggest that these receptors may play a role in regulating ecdysone production in the PG, as developmental delays and larval lethality are phenotypes consistent with a dysregulation of ecdysone (Christesen *et al.*, 2017, McBrayer *et al.*, 2007, Colombani *et al.*, 2005).

An independent genome-wide RNAi screen targeting the PG identified 1,906 genes that have a potential role in regulating ecdysone production (Danielsen *et al.*, 2016). Two receptors identified in the screen performed in this thesis, NPFR and Dh44-R1, were also identified in the Danielsen et al. 2016 screen. In the published study, neither NPFR nor Dh44-R1 were further characterised. Given the phenotypes for these two genes were validated by an independent group, the effects of these two genes on ecdysone synthesis were extensively characterised and are explored in Chapters 3 and 4, respectively.

The remainder of this chapter will therefore focus on the other hits from the screen, for which preliminary characterisation studies were performed. As these preliminary experiments required animals that survive to adulthood, attempts to restore viability to lethal genotypes caused by *phm>Lgr3 RNAi, dcrII and phm>AstA-R2 RNAi, dcrII* were made. This involved using a combination of weaker Gal4 and RNAi lines, as well as rearing animals at lower temperatures. As these were not successful (data not shown), these genes were not further studied.

Gene abbreviation	CG number	Gene name	Stock number	Predicted off targets
AstA-R1	CG2872	Allatostatin A receptor 1	39222	0
AstA-R2	CG10001	Allatostatin A receptor 2	1327	0
AstC-R1	CG7285	Allatostatin C receptor 1	13560	2
AstC-R2	CG13702	Allatostatin C receptor 2	106146	0
CapaR	CG14575	Capability receptor	105556	0
CCAP-R	CG33344	Crustacean cardioactive peptide receptor	14768	0
CCHa1-R	CG30106	CCHamide-1 receptor	1678	0
CCHa2-R	CG14593	CCHamide-2 receptor	1658	0
CCKLR-17D1	CG42301	Cholecystokinin-like receptor at 17D1	100760	1
CCKLR-17D3	CG32540	Cholecystokinin-like receptor at 17D3	9154	0
CG12290	CG12290	-	1247	0
CG13229	CG13229	-	100433	0
CG13575	CG13575	-	9363	2
CG13995	CG13995	-	42525	0
CG30340	CG30340	-	100088	0
CG32547	CG32547	-	101062	0
CG33639	CG33639	-	108753	0
CG33696	CG33696	-	23223	0
CNMaR	CG33696	CNMamide Receptor	23223	0
CrzR	CG10698	Corazonin receptor	44310	0
Dh31-R	CG32843	Diuretic hormone 31 Receptor	8777	0
Dh44-R1	CG8422	Diuretic hormone 44 receptor 1	110708	1
Dh44-R2	CG12370	Diuretic hormone 44 receptor 2	43314	0
ETHR	CG5911	ETHR	101996	0
FMRFaR	CG2114	FMRFamide Receptor	9594	0
hec	CG4395	hector	7223	0
Lgr1	CG7665	Leucine-rich repeat-containing G protein-coupled receptor 1	13566	0
Lgr3	CG31096	Leucine-rich repeat-containing G protein-coupled receptor 3	B55910	0
Lgr-4	CG34411	Leucine-rich repeat-containing G protein-coupled receptor 4	102681	0
Lkr	CG10626	Leucokinin receptor	22845	0
moody	CG4322	Moody	1800	0
MsR1	CG8985	Myosuppressin receptor 1	9370	1
MsR2	CG43745	Myosuppressin receptor 2	50780	0
NPFR	CG1147	Neuropeptide F receptor	9605	0
PK1-R	CG9918	Pyrokinin 1 receptor	101115	0
PK2-R1	CG8784	Pyrokinin 2 receptor 1	15989	0
PK2-R2	CG8795	Pyrokinin 2 receptor 2	44871	0
Proc-R	CG6986	Proctolin receptor	7217	0
rk	CG8930	rickets	29932	0
RYa-R	CG5811	RYamide receptor	1259	0
SIFaR	CG10823	SIFamide receptor	1783	0
sNPF-R	CG7395	short neuropeptide F receptor	9379	0
SPR	CG12731	Sex Peptide Receptor	106804	0
TkR86C	CG6515	Tachykinin-like receptor at 86C	13392	0
TkR99D	CG7887	Tachykinin-like receptor at 99D	44369	0
Tre1	CG3171	Trapped in endoderm 1	7220	0
TrissinR	CG34381	Trissin receptor	7886	1

Table 2.1: List of neuropeptide receptors tested in the PG-specific RNAi screen

Gene names, abbreviations, CG numbers, predicted off targets and RNAi stock numbers used for initial screening are included. All RNAi lines were purchased from the Vienna *Drosophila* Resource Centre (VDRC), unless stock number begins with a "B", in which case were purchased from the Bloomington *Drosophila* Stock Centre (BDSC).

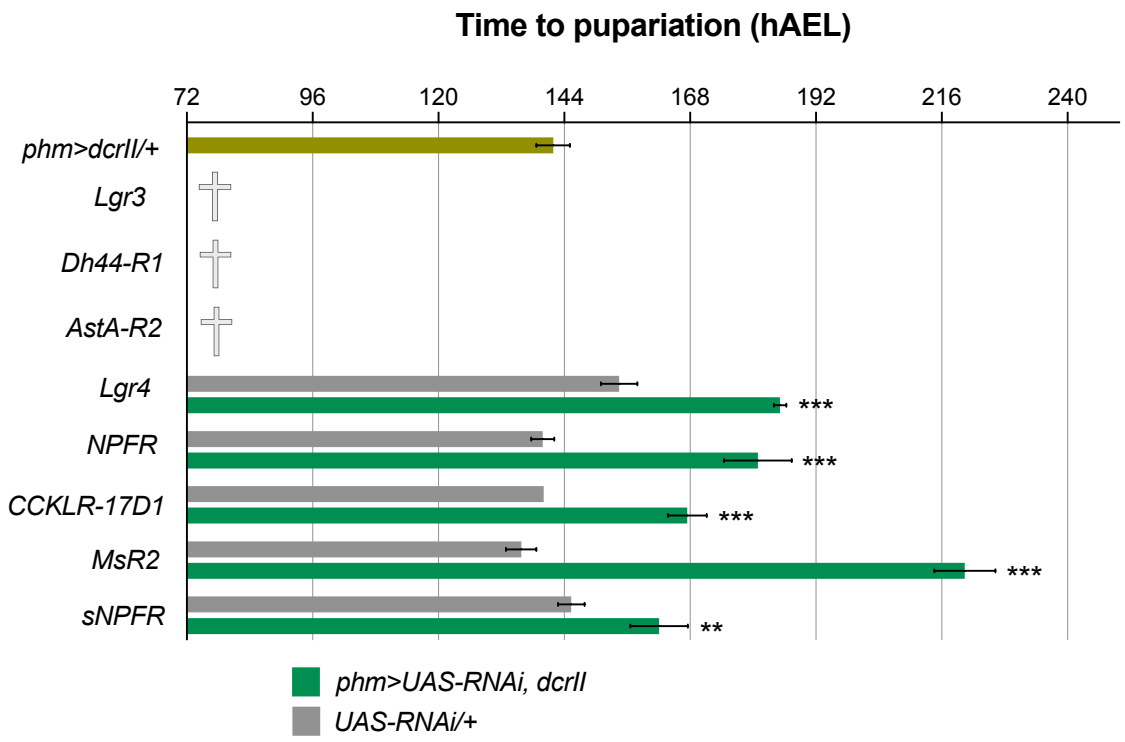


Figure 2.1: PG-specific knockdown of eight neuropeptide receptors results in developmental defects

The brown bar represents the *phm*-Gal parental control (*phm>dcrII/+*), grey bars represent the respective UAS-RNAi parental control (*UAS-RNAi/+*), while green bars represent PG-specific knockdown of the gene (*phm>UAS-RNAi, dcrII*). PG-specific knockdown (using *phm*-Gal4) of *Lgr3* resulted in embryonic lethality. PG-specific knockdown of *Dh44-R1* and *AstA-R2* results in larval lethality at either the first or second larval instar. PG-specific knockdown of *Lgr4, NPFR, CCKLR-17D1*, *MsR2* and *sNPFR* all resulted in significant developmental delays. hAEL= hours after egg lay. Error bars represent ±1 SEM. ** = $p<0.01$, *** = <0.001 (ANOVA and pairwise *t* tests). Cross represents lethality. Ten biological replicates of 15-10 larvae were tested per genotype.

2.2.2. MsR2 and Lgr4 affect body size

When ecdysone synthesis is disrupted, final body size is frequently altered. For example, knocking down components of the PTTH pathway leads to increases in body size (McBrayer *et al.*, 2007, Rewitz *et al.*, 2009). Therefore, it was of interest to determine if individually knocking down the genes that resulted in a developmental delay (*Lgr4, MsR2, sNPFR or CCKLR-17D1*) specifically in the PG also results in an altered final body size.

When *Lgr4* was knocked down specifically in the PG, an increase in body size for both females and males was observed (Figures 2.2A, 2.2B). Interestingly, when *MsR2* was knocked down specifically in the PG, a significant increase in body size was observed for females (Figure. 2.2C), but not males (Figure. 2.2D). Additionally, this change in body size in *phm>MsR2 RNAi, dcrII* animals was not found to be due to an altered growth rate, or due to defects in the development of the PG itself, as PG-specific knockdown of *MsR2* does not alter PG size or morphology (see Supplementary materials, Figures 2.1 and 2.2). However, these experiments did not differentiate between males and females, so sex-specific differences could not be identified. Nevertheless, these results suggest that both Lgr4 and MsR2 are involved in regulating final body size in the PG, but MsR2 may play a sexually dimorphic role in doing so.

The other two genes (sNPFR and CCKLR-17D1) did not influence final body size when knocked down in the PG. When *sNPFR* was knocked down in the PG, no significant difference in body size was observed for both males and females (Figures 2.2E, 2.2F). When *CCLKLR-17D1* was knocked down specifically in the PG, it was found that females had a subtle increase in body size compared to the UAS-RNAi parental control, but not compared to the *phm*-Gal4 parental control (Figure. 2.2G). No difference in final body size was observed for male flies (Figure. 2.2H). Interestingly, knocking down *CCKLR-17D1* in the PG resulted in a smaller PG size compared to the control (see Supplementary materials, Figure 2.2), however this decrease in PG size does not seem to influence final body size. This suggests that PG-specific sNPFR and CCKLR-17D1 may regulate developmental timing, but have no impact on final body size. Alternatively, it is possible that subtle differences in body size exist when these genes are manipulated in the PG, but were not able to be captured by the methods used.

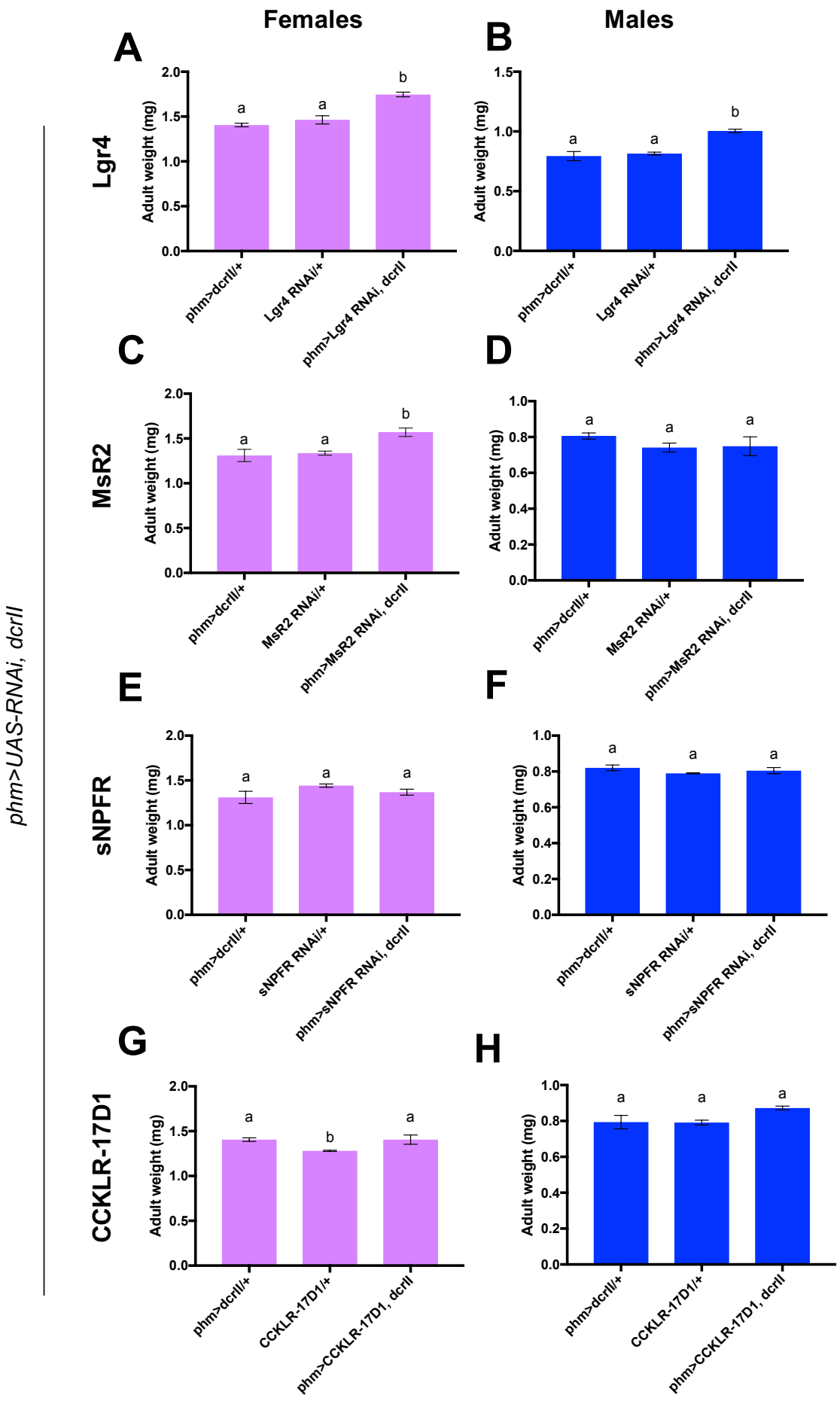

phm>UAS-RNAi, dcrII
Females
Males
A
B
C
D
E
F
G
H
Lgr4
MsR2
sNPFR
CCKLR-17D1
Adult weight (mg)
a
b
phm>dcrII/+
Lgr4 RNAi/+
phm>Lgr4 RNAi, dcrII
MsR2 RNAi/+
phm>MsR2 RNAi, dcrII
sNPFR RNAi/+
phm>sNPFR RNAi, dcrII
CCKLR-17D1/+
phm>CCKLR-17D1, dcrII

Figure 2.2: Effects of knocking down *Lgr4, CCKLR-17D1* and *MsR2* in the PG on final body size

Knocking down *Lgr4* specifically in the PG results in an increased adult body size for both **(A)** females **(B)** and males**.** Knockdown of *MsR2* specifically in the PG results in an increased body size for **(C)** females but not **(D)** males. **(E, F)** PG-specific knockdown of *sNPFR* does not alter final body size. **(G)** PG-specific knockdown of *CCKLR-17D1* results in an increased body size for females, however this increase is only significant compared to the UAS-RNAi control and not the *phm*-Gal4 control. **(H)** No significant differences were found for males. Error bars represent ±1 SEM. Genotypes sharing the same letter indicate that they are statistically indistinguishable from one another, while genotypes with different letters indicate that they are statistically different ($p < 0.05$, two-tailed *t* tests). 15-20 animals were tested per biological replicate, and 10 biological replicates were tested per genotype.

2.2.3. Knockdown of Ms and sNPF specifically in Ms and sNPF-expressing cells results in developmental defects

To provide further evidence that these receptors play a role in regulating developmental timing and body size, it was of interest to determine whether knocking down the ligands to these receptors generated similar effects. Therefore, the ligands for MsR2 (*Myosuppressin*; *Ms*) and sNPFR (*short neuropeptide F*; *sNPF*), were knocked down specifically in *Ms*-expressing cells (using *Ms*-Gal4) and sNPF-expressing cells (using *sNPF*-Gal4), respectively. The ligand for CCKLR-17D1 was not able to be tested due to reagents not being available, and the ligand for Lgr4 is currently unknown.

It was found that when both *Ms* and *sNPF* were knocked down with their respective Gal4 drivers, animals have a significant developmental delay (Figures 2.3A, 2.3B). In addition to this, these animals had an increased body size compared to controls, as measured by pupal length (Figures 2.3C, 2.3D). Taken together, these data add further evidence to suggest that both Ms/MsR2 and sNPF/sNPFR signalling play a role in regulating developmental timing and body size.

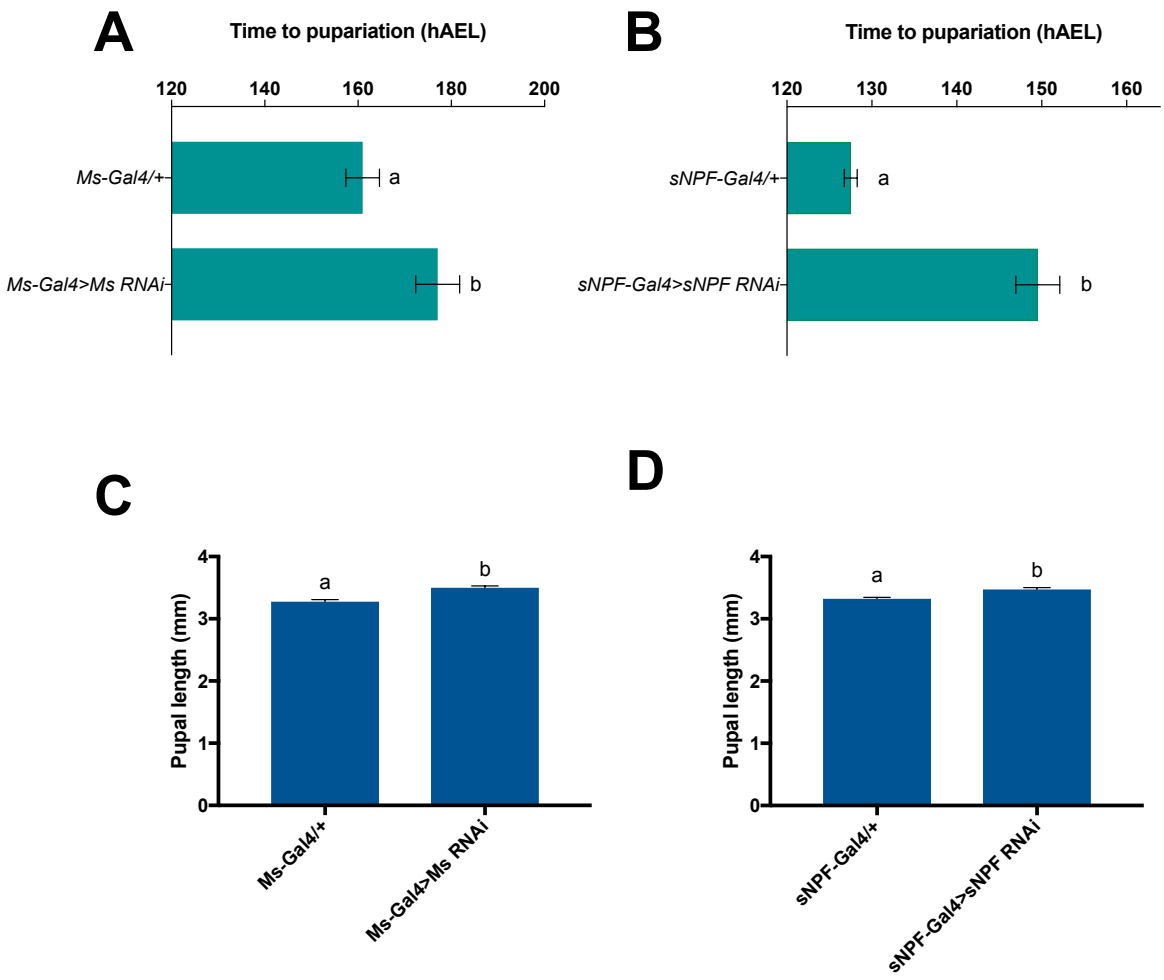


Figure 2.3: Knockdown of *Ms* and *sNPF* specifically in Ms and sNPF-producing cells results in developmental delays and increased body size

(A) Knocking down *Ms* specifically in *Ms*-expressing cells (using *Ms*-Gal4) causes a significant developmental delay. Similarly, **(B)** when *sNPF* is knocked down specifically in *sNPF*-expressing cells (using *sNPF*-Gal4), a significant developmental delay is observed. Both **(C)** *Ms>Ms RNAi* and **(D)** *sNPF>sNPF RNAi* animals have an increased final body size. hAEL= hours after egg lay. Error bars represent ±1 SEM for all graphs. Genotypes with different letters indicate that they are statistically different from each other ($p < 0.05$, two-tailed *t* tests). 15-20 animals were tested per biological replicate, and 10 biological replicates were tested per genotype.

2.3. Discussion

The aim of this study was to determine if there were unidentified peptide signalling pathways that function in the PG to regulate ecdysone. The results presented in this chapter have identified eight novel neuropeptide receptors with potential functions in the PG, supporting the idea that more peptide signalling pathways are likely to function in the PG than originally thought. However, the results presented from this screen, including additional characterisation of hits, are only preliminary, and more work is needed to confirm the roles of the identified genes.

It was somewhat surprising that around 15% of genes screened resulted in a hit, given that the regulation of ecdysone production in *Drosophila* has been extensively studied for the last two decades. An independent genome-wide screen targeting the PG identified 1,906 genes that have a potential role in regulating ecdysone production (Danielsen *et al.*, 2016). However, only two genes identified by this thesis, NPFR and Dh44-R1, were also identified in the published study. Furthermore, this thesis was able to detect five additional hits that were not detected by the published study. The reason that only NPFR and Dh44-R1 were mutually detected as hits across both screens may be a result of the differences in methodology used. In the screen presented in this chapter, time to pupariation was measured every eight hours after egg lay, whereas in the published study, animals were only scored 11-13 days after egg lay. Additionally, ten replicates of 15-20 animals were measured here whereas only two replicates were used for the genome-wide screen. Taken together, the methods used in this chapter provide greater resolution and allow the detection of subtler phenotypes than the Danielsen *et al*. study.

Another reason for the lack of overlap between studies could also be attributed to the RNAi lines used to drive gene knockdown. For example, the Danielsen *et al.* study used RNAi lines from the TRiP collection while this study used RNAi lines from the VDRC library to drive knockdown of *AstA-R2, Lgr3*, and *MsR2*. If the RNAi lines used to drive knockdown in the current study were more efficient, this would explain the difference in hits between this study and Danielsen *et al.* It is, however, worth mentioning that both studies only used one RNAi line per gene. Further experiments are needed to ensure that RNAi knockdown of these genes truly do result in specific knockdown of the gene of interest and not that the phenotype is not

due to an off-target. Of the hits from this screen, the four genes whose roles in the PG were further characterised will be discussed below.

2.3.1. Myosuppressin Receptor 2

The results presented here suggest that MsR2 plays a role in the *Drosophila* PG to regulate developmental timing and body size. Knocking down *MsR2* in the PG resulted in a significant developmental delay and altered adult body size, although the body size phenotype was only observed in females. As the developmental timing assays used in this study are unable to differentiate between males and females, it is not known if the developmental timing defects are also specific to one sex. Knockdown of *Ms* in *Ms*-expressing cells also resulted in a significant developmental delay and larger animals, providing additional evidence that MsR2 signalling does indeed regulate developmental timing and body size in the PG. In support of this, a transcriptome analysis carried out on *Drosophila* ring glands identified that *MsR2* is enriched in the ring gland compared to a whole-larval sample (Ou *et al.*, 2016). Whether this expression was specific to the PG, or to other parts of the ring gland, is unknown.

Ms binds to two GPCRs in *Drosophila*; Myosuppressin Receptor 1 (MsR1) and MsR2 (Egerod *et al.*, 2003). In this screen, knocking down *MsR1* specifically in the PG did not have any effect (data not shown). This may suggest that Ms regulates developmental timing and body size via activation of MsR2. However, it is also possible that the RNAi line used to drive *MsR1* knockdown was inefficient. Further validation, using a second RNAi line, is required to determine if MsR1 plays a role in the PG.

Little is known about the function of MsR2, however a few studies characterising its ligand, Ms, have been carried out. Ms is expressed primarily in the CNS from late embryonic stages to adulthood, with expression additionally seen in the larval and adult gut (McCormick and Nichols, 1993). In both larvae and adults, Ms has shown to play a role in muscle physiology, specifically by regulating gut and heart contractions (Dickerson *et al.*, 2012). In addition to this, activating neurons in which *Ms* is expressed results in precocious ecdysis (Ruf *et al.*, 2017), suggesting Ms plays a role in initiating eclosion.

While, no role for MsR2 signalling in regulating larval development in the *Drosophila* PG has previously been identified, interestingly PG-specific roles for Ms signalling have been described in other insects. For example, in *Bombyx* it has been shown that *bommo-myosuppressin receptor* (*bmsr*), the homolog of MsR1 and MsR2, is expressed in the PG of final instar larvae (Yamanaka *et al.*, 2008, Yamanaka *et al.*, 2005). Additionally, a ligand for BMSR, Bommo-myosuppressin (BMS), inhibits ecdysone synthesis (Yamanaka *et al.*, 2005). Interestingly, BMSR has been shown to bind to several different neuropeptides to exert its effects on the production of ecdysone (Yamanaka *et al.*, 2006). The neuropeptides that bind to BMSR are expressed in neurons that directly innervate the *Bombyx* PG, and are able to exert their effects through BMSR by being secreted on the surface of the PG (Yamanaka *et al.*, 2006). This suggests that BMSR plays a vital role in the regulation of ecdysone in *Bombyx*. Additionaly, in the cabbage moth, *Mamestra brassicae*, Ms supresses ecdysone levels by inhibiting PTTH-stimulated activity of the PG to prepare the animal for diapause (Yamada *et al.*, 2017). Given that Ms signalling plays a role in regulation ecdysone production in these other insects, it is certainly plausible that it also does so in *Drosophila.*

Interestingly, the data presented in this chapter suggest that MsR2 signalling plays the opposite role in regulating ecdysone in *Drosophila* compared to *Bombyx* and *Mamestra*. In the two Lepidopteran species BMSR represses ecdysone synthesis, whereas in *Drosophila* it appears to promote ecdysone synthesis. This is demonstrated by the fact that removal of *MsR2* in the PG, or of *Ms* neuronally, results in delays in developmental timing, indicative of low ecdysone levels (for review, see Gilbert *et al*., 2002). Given this possible opposing role on regulating ecdysone between these species of insects, it will be of great interest to further characterise the influence that Ms/MsR2 signalling has on ecdysone synthesis in the *Drosophila* PG.

2.3.2. Short neuropeptide F receptor

When *sNFPR* was knocked down specifically in the PG, a significant developmental delay was observed, suggesting sNPFR plays a role in regulating developmental timing in the PG. In support of this, knocking down *sNPF* in *sNPF*-expressing neurons also results in a significant developmental delay, and increased body size. These data therefore suggest a

potential role for sNPFR signalling in regulating developmental timing, likely due to a function in the PG.

If sNPFR signalling does play a role in the PG, how it functions can be hypothesised by examining the roles sNPFR signalling plays in other contexts in *Drosophila*. sNPF/sNPFR signalling has been shown to have a number of different roles in *Drosophila*. One of the most studied roles is regulation of feeding behaviour. Gain of function mutations in *sNPF* increase food intake, resulting in larger adults (Lee *et al.*, 2004). Conversely, loss of function *sNPF* mutants show decreased food intake (Lee *et al.*, 2004). This suggests that the sNPF signalling pathway affects adult body size by controlling food intake. The data presented here has shown that PG-specific knockdown of *sNPFR* does not alter final body size, but increases the duration of the growth period. This suggests that the role of sNPFR signalling in the PG is to regulate developmental timing but not body size. Alternatively, it is possible that PG-specific sNPFR does indeed regulate body size, but this phenotype was too subtle to be detected with the methods used in this study.

Interestingly, this study found that knockdown of *sNPF* in *sNPF*-expressing cells results in an increase in final body size, as well as a significant developmental delay. Animals who have a prolonged larval period often display an increased adult size as the growth period is extended, meaning the animals spend more time feeding. Therefore, the observation that *sNPF>sNPF RNAi* animals are larger in size fits in with what is known about the interaction between developmental timing and body size.

Curiously, the *sNPF* mutants used in the Lee *et al*., (2004) study displayed a significantly reduced body size, opposite to what this study observed with *sNPF>sNPF RNAi* animals. One way in which animals can have both an extended growth period and smaller body size is if they have a reduced growth rate (Mirth and Riddiford, 2007). Therefore, an explanation for this discrepancy in phenotypes is that the *sNPF* whole-animal mutants have a reduced growth rate, leading to a smaller body size, while the *sNPF>sNPF RNAi* animals have an unaltered growth rate but an extended growth period. At this point, it is hard to ascertain how knocking down *sNPF* in all *sNPF* expressing cells can result in a different growth rate compared to whole animal mutants. One possibility is that the *sNPF*-Gal4 driver used to knockdown *sNPF* does not fully capture the endogenous expression of *sNPF.* Therefore, it may have only knocked down *sNPF* in cells that are responsible for regulating developmental timing, but not

the other aspects of growth. Nevertheless, the results from this chapter support the hypothesis that *sNPF* signalling does in fact influence final body size.

Interestingly, sNPF has been shown to interact with the insulin signalling pathway by regulating expression of the Dilps (Lee *et al.*, 2008). sNPF activates extracellular signal-related kinases (ERK) in the IPCs of both the larval and adult brain (Lee *et al.*, 2008), which leads to increased *dilp* expression. This in turn releases Dilp2 into the haemolymph, which activates insulin signalling in the fat body, resulting in regulation of growth, metabolism, and lifespan (Lee *et al.*, 2008). In addition to this, when insulin signalling is low, *sNPFR* expression is increased to facilitate food-search behaviour (Root *et al.*, 2011). Furthermore, it was found that insulin signalling suppresses *sNPFR* expression (Root *et al.*, 2011, Hong *et al.*, 2012), suggesting a negative relationship between sNPFR and insulin signalling in this context. It is therefore possible that sNPF signalling acts similarly as a mechanism by which insulin signalling is fine-tuned in the PG.

In addition to its role in feeding behaviour, in developing animals sNPF has been found to act as an intermediate messenger between the central clock and the peripheral clock in the PG. Information from the central clock is communicated via sNPF to neurons that produce PTTH, which then act on the PG to produce ecdysone (Selcho *et al.*, 2017, McBrayer *et al.*, 2007). Given this interaction between sNPF and PTTH neurons, sNPF and PTTH may also interact in the PG itself to control the production of ecdysone. One hypothesis is that sNPF is produced in response to circadian rhythm, like PTTH, and binds to sNPFR on the PG, resulting in a signalling cascade that interacts with that of PTTH. Perhaps in this manner, sNPF signalling can be responsible for fine tuning PTTH signalling to ensure precise control of ecdysone production.

In support of the findings that sNPFR signalling functions in the PG, Ou *et al* (2016) found that both *sNPF* and *sNPFR* are enriched in the ring gland compared to a whole larval sample. In addition to this, a study that extensively characterised *sNPF* and *sNPFR* expression in the larval CNS found both *sNPF* and *sNPFR* expression in neurons that projected to the ring gland (Carlsson *et al.*, 2013). To date, it is unclear whether the mRNA enrichment or the neuronal projections are specific to the PG, or if they are specific to other tissues of the ring gland.

These studies have demonstrated that sNPFR signalling plays multiple roles in *Drosophila*, including regulating multiple aspects of feeding behaviour, body size, and circadian rhythm. Given this multitude of roles, it will likely be complicated to tease apart the role sNPFR plays specifically in the PG. Nevertheless, future experiments should firstly focus on further validating sNPFR function in the PG.

2.3.3. Cholecystokinin-like receptor at 17D1 and Leucine-rich repeat-containing G protein-coupled receptor 4

This study has found that PG-specific knockdown of *CCKLR-17D1* and *Lgr4* results in significant developmental delays, and increased body size for PG-specific knockdown of Lgr4, but not sNPFR. While these data indicate a potential role for CCKLR-17D1 and Lgr4 signalling in the PG, these genes were only tested with a single RNAi line and furthermore, the ligands to CCKLR-17D1 and Lgr4 were not able to be tested. Therefore, the data presented for these genes are very preliminary and it is certainly possible that the developmental timing and body size defects are due to off-target effects, rather than knockdown of the gene itself. Nevertheless, hints that CCKLR-17D1 and/or Lgr4 may play a role in the PG can be found by looking at roles for these genes in other contexts.

The ligand to CCKLR-17D1 is known to be Drosulfakinin (Dsk) (Chen *et al.*, 2012). *Dsk* has shown to be expressed in the IPCs in the larval brain, where it functions as a satiety signal. Reducing *Dsk* specifically in the IPCs results in a dysregulation of food intake and food choice (Söderberg *et al.*, 2012). Given this established interaction between Dsk and the Dilps in the IPCs, a possibility is that CCKLR-17D1 in the PG interacts with the insulin signalling pathway to control ecdysone production in the PG. In support of this, it was found that knocking down *CCKLR-17D1* in the PG resulted in smaller PG size compared to the control. It has previously been shown that when insulin signalling is down-regulated in the PG, smaller PGs are observed (Mirth *et al.*, 2005, Colombani *et al.*, 2005). Therefore, it is possible that CCKLR-17D1 signalling and the insulin signalling pathway may interact in the PG to control the production of ecdysone. However, this theory is highly speculative and more work needs to firstly focus on confirming CCKLR-17D1 function in the PG.

There is also some evidence to suggest that Lgr4 has an endogenous role in the PG. *Lgr4* is expressed at almost every stage of the *Drosophila* life cycle, with the highest expression seen in the first larval instar (Van Hiel *et al.*, 2015). This therefore suggests a developmental role. Furthermore, Ou *et al*. (2016) also found that *Lgr4* is enriched in the ring gland compared to a whole larval sample. However, it is unknown if this enrichment is specific to the PG vs other components of the ring gland. Knocking down another member of the Leucine-rich repeat-containing G protein coupled receptor (LGR) family, *Lgr1*, results in developmental defects due to low ecdysone levels (Vandersmissen *et al.*, 2014), suggesting Lgr1 has a role in regulating ecdysone production. It has also recently been shown that another member of the LGR family, Lgr3, plays a role in regulating ecdysone production in response to tissue damage (Garelli *et al.*, 2015, Jaszczak *et al.*, 2016, Vallejo *et al.*, 2015). Additionally, when *Lgr3* was knocked down in the PG as seen in this chapter, embryonic lethality was observed. Given that these other members of the LGR family play roles in regulating ecdysone production, it certainly plausible that Lgr4 does so too. However, similarly to CCKLR-17D1, much more work needs to be done to firstly elucidate if Lgr4 has a true function in the PG, or whether the results seen are consequences of off-target effects.

2.3.5. Future directions

This chapter has presented preliminary evidence to suggest that the regulation of ecdysone synthesis in the PG involves many more peptides than previously thought. However, though the screen conducted in this study revealed several hits, one caveat is that only one RNAi line was tested for each gene of interest. Therefore, it is possible that genes that do function in the PG were not detected in this screen as a result of inefficient RNAi lines used. Alternatively, for the genes that did result in a hit, further experiments are needed confirm that these RNAi lines are indeed knocking down the gene of interest and not an off target. Such experiments may include examining expression levels of individual genes upon RNAi knockdown, as well as measuring expression levels of off target genes to ensure they are not being knocked down. These experiments would be pivotal in fully ascertaining how many neuropeptide receptors have a true role in the PG.

The roles of sNPFR and MsR2 in regulating developmental timing were able to be further validated by developmental timing and body size experiments with their respective ligands.

However, the results seen with CCKLR-17D1 and Lgr4 were only conducted with a single RNAi line. Therefore, much more work needs to go into determining the validity of the results seen with these genes. Such experiments can include knocking down *CCKLR-17D1* and *Lgr4* in the PG with alternative RNAi lines, as well as characterising mutants of these genes. While the ligand for Lgr4 is unknown, developmental timing and body size experiments should be tested on the ligand for CCKLR-17D1 in order to additionally characterise its role in developmental timing. The suggested experiments will aid in elucidating if CCKLR-17D1 and Lgr4 do truly function in the PG.

For the other genes that were validated by experiments on their ligands (MsR2 and sNPFR), future experiments should involve exploring and characterising their role in regulating ecdysone in the PG. Such experiments can include quantifying ecdysone titres in animals where the gene of interest has been knocked down in the PG. Furthermore, carrying out qPCR on ecdysone synthesis genes in these same animals further validates the effects on ecdysone synthesis. Additionally, feeding such animals food supplemented with ecdysone and observing if the developmental defects are rescued is another way to further characterise their role in regulating ecdysone. These experiments are very useful in determining the role a gene plays in regulating ecdysone production, and would be essential to carry out for the genes discussed above.

If the above experiments yield positive results, it would be interesting to determine which pathways, if any, these newly identified peptide signalling pathways interact with in the PG. For example, there is evidence to suggest that both MsR2 and sNPFR signalling interact with the PTTH signalling pathway (Yamada et al., 2017, Selcho et al., 2017). Determining whether these pathways interact would involve quantifying the expression levels of components of the PTTH pathway when *MsR2/sNPFR* is knocked down in the PG, or conducting genetic interaction studies between PTTH signalling components and MsR2/sNPFR. In a similar manner, both sNPFR and CCKLR-17D1 signalling have been implicated in controlling the insulin signalling pathway to control feeding behaviour (Söderberg *et al.*, 2012, Lee *et al.*, 2004). It would be useful to determine whether sNPFR/CCKLR-17D1 interacts with the insulin pathway in the PG using similar approaches.

Finally, both MsR2 and CCKLR-17D1 seem to have sex-specific effects on body size. It would be worth examining whether these differences are caused by effects on growth rates or

developmental timing, which are processes known to differ between male and females (Testa *et al.*, 2013). This would provide further insight into sex-specific regulation of ecdysone pulses.

2.4. Conclusion

Animal development is a complex process that requires input from the external environment, which is typically communicated via the action of neuropeptides and their receptors. While some of these neuropeptide signalling pathways are known to function in the *Drosophila* PG, there is increasing evidence that there are more yet to be discovered. In this study, a screen was performed to identify novel genes that function in the PG to regulate ecdysone. In this screen, receptors to neuropeptides and peptide hormones were specifically targeted, and it was found that PG-specific knockdown of eight receptors resulted in developmental defects. Further characterisation of four of these genes added further evidence to suggest that these genes do play an endogenous role in the *Drosophila* PG to regulate ecdysone. However, additional validation of the hits identified in this screen is needed, and further work needs to be carried out to ascertain their true role in the PG. Nevertheless, these data have demonstrated that more neuropeptide-receptor signalling pathways are likely to function in the *Drosophila* PG than originally thought, highlighting the complexity of ecdysone regulation.

2.5. Materials and Methods

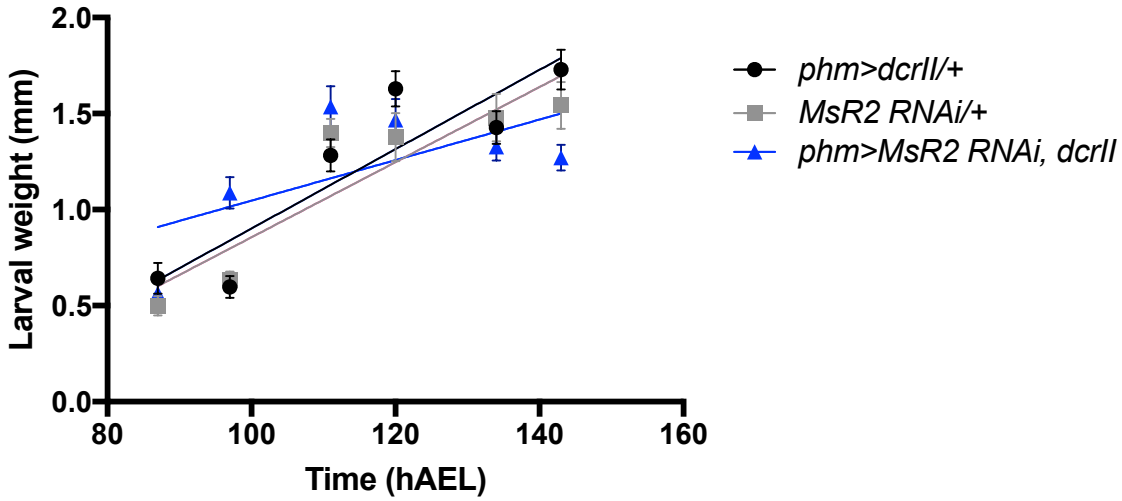
2.5.1. Drosophila stocks

The following stocks were used: w^{1118} (BL5905), UAS-*dicerII*; *phm*-Gal4-22, a gift from Michael O'Connor, University of Minnesota, Minneapolis (Ono *et al*., 2006), *phm*-Gal4-22, UAS-mCD8::GFP/TM6B, a gift from Takashi Koyama, *Ms*-Gal4 (B51986), UAS-*Ms* RNAi (v108760), *sNPF*-Gal4 (B51991), UAS-*sNPF* RNAi (B25567). For a list of stocks used in the initial RNAi screen, please refer Table 2.1 in the main text.

2.5.2. Developmental timing assays and body size analysis

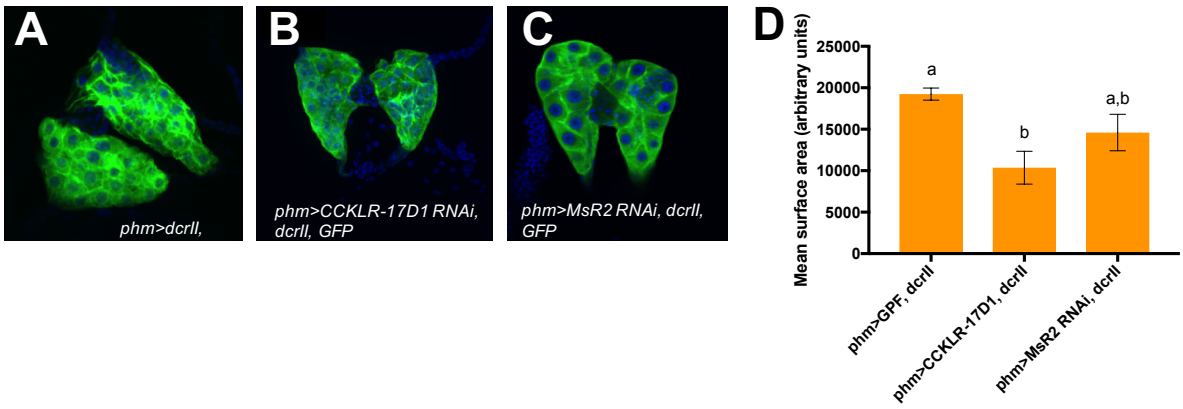
Parental flies (carrying either the Gal4 driver or the UAS-RNAi) were allowed to lay eggs on 25mm apple juice agar plates for 3-4 hours. Twenty-four hours later, 15-20 L1 larvae were picked into standard food vials. For initial screening, five biological replicates were used. To confirm hit phenotypes, ten biological replicates were used. Time to pupariation of the F1 offspring were scored every 8 hours and any developmental defects were observed. Larvae for all experiments were raised inside an insulated, moist chamber at 25 degrees in the dark. Each set of genetic crosses included a UAS-RNAi or Gal4 control crossed to w^{1118}; the genetic background for the RNAi library from the VDRC. To measure body size, weights of female and male flies that eclosed from the developmental timing assays were measured on an ultra-microbalance (Mettler Toledo). When measuring pupal length, photos of newly-formed pupal cases were taken using a compound light microscope, and length was measured using Fiji. Differences in developmental timing or body size were determined using a one-way ANOVA.

2.6. Supplementary Materials



Supplementary Figure 2.1: MsR2 signalling in the PG does not influence growth rate

Knocking down *MsR2* specifically in the PG does not significantly alter larval growth rate compared to parental controls. Each point represents 10-15 animals. Slopes were compared by linear regression analysis using GraphPad Prism ($p>0.05$).



Supplementary Figure 2.2: PG-specific knockdown of *CCKLR-17D1*, but not *MsR2*, results in reduced PG size.

(A, B) PG-specific knockdown of *CCKLR-17D1* (using *phm*-GFP-Gal4) results in normal PG morphology compared to the control, **(D)** but also results in smaller PG size ($p<0.05$). **(A, C)** Knocking down *MsR2* specifically in the PG results in no difference in both PG morphology, **(D)** as well as size. Four – six PGs were dissected per genotype. PGs were dissected from wandering third instar larvae and confocal microscopy was used to visualise PGs. DAPI was used to stain nuclei. Magnification = 20x.

Chapter 3: Neuropeptide F receptor acts in the *Drosophila* prothoracic gland to regulate growth and developmental timing

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Drosophila melanogaster

3.1. Abstract

As juvenile animals grow, their behaviour, physiology, and development need to be matched to environmental conditions to ensure they survive to adulthood. However, we know little about how behaviour and physiology are integrated with development to achieve this outcome. Neuropeptides are prime candidates for achieving this due to their well-known signalling functions in controlling many aspects of behaviour, physiology and development in response to environmental cues. In the growing *Drosophila* larva, while several neuropeptides have been shown to regulate feeding behaviour, and a handful to regulate growth, it is unclear if any of these play a global role in coordinating feeding behaviour with developmental programs. Here, we demonstrate that Neuropeptide F Receptor (NPFR), best studied as a conserved regulator of feeding behaviour from insects to mammals, also regulates development in *Drosophila*. Knocking down *NPFR* in the prothoracic gland, which produces the steroid hormone ecdysone, generates developmental delay and an extended feeding period, resulting in increased body size. We show that these effects are due to decreased ecdysone production, as these animals have reduced expression of ecdysone biosynthesis genes and lower ecdysone titres. Moreover, these phenotypes can be rescued by feeding larvae food supplemented with ecdysone. Further, we show that NPFR negatively regulates the insulin signalling pathway in the prothoracic gland to achieve these effects. Taken together, our data demonstrate that NPFR signalling plays a key role in regulating animal development and may thus play a global role in integrating feeding behaviour and development in *Drosophila*.

3.2. Introduction

When faced with variation in the quantity and quality of the diet, young animals must adjust their feeding behaviour, metabolism, growth, and developmental time. Failure to do so has profound consequences on their ability to survive to adulthood and to resist future stress . Extensive research into the regulation of food intake has uncovered a handful of neuropeptides that mediate changes in feeding behaviour in response to diet, including the highly conserved Neuropeptide F signalling pathway (Beck, 2001, Williams *et al.*, 2001, Wu *et al.*, 2003, Garczynski *et al.*, 2002). Developmental processes, such as growth and developmental time to adulthood, are controlled through the action of the conserved insulin and steroid hormone signalling pathways (Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005). However, we know little about the extent to which feeding behaviour and developmental processes are coordinated, and the molecular mechanisms necessary for this coordination.

The fruit fly, *Drosophila melanogaster*, provides an excellent model in which to study the molecular mechanisms that integrate feeding behaviour with developmental processes. *Drosophila* development proceeds through three larval stages (instars), after which the animal initiates pupariation and metamorphosis to become an adult. The timing of the transitions between these developmental stages is regulated by a series of precisely-timed pulses of the steroid hormone, ecdysone, produced and secreted by the prothoracic gland (PG; for review, see Yamanaka *et al.* 2013). Because these insects grow primarily during the larval stages, ecdysone dictates the length of the growth period, ceasing growth once metamorphosis begins (Caldwell *et al.*, 2005, Mirth *et al.*, 2005, Nijhout *et al.*, 2014). In this way, ecdysone determines final adult size.

The PG produces and secretes ecdysone in response to various environmental cues, such as the day-night cycle, nutrition, and tissue damage (McBrayer *et al.*, 2007, Jaszczak *et al.*, 2016, Mirth *et al.*, 2005, Koyama *et al.*, 2014). These external cues are communicated to the PG via the action of a number of secreted peptides. Nutritional signals are particularly important, and are communicated throughout the body via the insulin signalling pathway. When larvae are well fed, they secrete insulin-like peptides (Dilps) into the bloodstream (Brogiolo *et al.*, 2001). In the *Drosophila* PG the insulin receptor (InR) is activated by the Dilps, which in turn leads to the activation of ecdysone biosynthesis genes and therefore the

production of ecdysone (Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005). Starvation early in the third larval instar delays the onset of metamorphosis by delaying the timing of an early ecdysone pulse (Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005, Shingleton *et al.*, 2005). Later in the third larval instar starvation accelerates developmental timing (Mirth *et al.*, 2005, Stieper *et al.*, 2008), presumably by accelerating the production of at least one of the later ecdysone pulses. This highlights how the effects of nutrition change growth outcomes over developmental time.

As well as regulating development, the quantity and quality of nutrients in the diet also causes larvae to alter both the amount and the quality of foods they consume (Almeida de Carvalho and Mirth, 2017, Rodrigues *et al.*, 2015). Several peptide hormones and neuropeptides have been shown to regulate different aspects of feeding behaviour in the fly (Wang and Wang, 2019). Amongst these, Neuropeptide F (NPF) signalling increases feeding rates and affects food choice in response to poor food quality (Wu *et al.*, 2003, Wu *et al.*, 2005). The mammalian homologue of NPF, Neuropeptide Y, also regulates feeding behaviour in response to food quality (Brown *et al.*, 1999, Mercer *et al.*, 2011). To guarantee that the animal survives, these changes in feeding behaviour must be appropriate to the changes needed in the different stages of animal development. We therefore wondered if any of these neuropeptides act as global coordinators of development and feeding behaviour in response to nutritional signals.

Here, we show that the NPF receptor (NPFR) regulates development in *Drosophila* by regulating the production of ecdysone in the PG. We further show that NPFR signalling exerts its effects on developmental timing and body size by interacting with the insulin signalling pathway in the PG, revealing that it acts as a previously undescribed regulator of insulin signalling in this gland. Our data demonstrate that NPF signalling, well known for regulating feeding behaviour across species, also plays a key role in regulating animal development by affecting the production of developmental hormones.

3.3. Results and Discussion

3.3.1. NPFR signalling regulates developmental timing

Our aim was to determine whether any of the neuropeptides that control feeding behaviour also act to alter development in response to nutrition. Because the known effects of nutrition on developmental time are controlled by ecdysone production in the PG, we hypothesised that such neuropeptides would act on receptors on the PG cells. We therefore knocked down a set of receptors for neuropeptides known to regulate feeding behaviour in larvae (see supplementary materials, Table. 3.1) specifically in the PG. To do this we used the *phantom* (*phm*)-Gal4 driver to drive expression of RNAi constructs for the different receptors, as well as the expression of dicer II (*dcrII*) to enhance the RNAi knockdown (Tomari and Zamore, 2005).

We found that when we knocked down *NPFR* in the PG with the v9605 RNAi line, we observed a significant delay to pupariation of about 35 hours (Fig. 3.1A, $p>0.001$). With a second *NPFR* RNAi line (v107663), we only observed a significant delay when compared to the UAS-*NPFR* RNAi parental control, and not the *phm*-Gal4 parental control (Fig. 3.1B, $p = 0.01$). Therefore, to further verify that NPFR regulates developmental timing, we tested an *NPFR* loss of function mutant strain (*NPFR* ^{SK8}*;* Ameku *et al.*, 2018). The *NPFR*^{SK8} mutant larvae also displayed a significant delay in time to pupariation compared to a heterozygous control (~15 hours; Fig. 3.1C, $p = 0.008$).

NPFR could alter developmental timing because it regulates PG development. To test this, we dissected PGs from *phm-GFP>NPFR RNAi; dcrII* wandering larvae, measured their size, and examined their morphology. PG size was indistinguishable between *phm-GFP>NPFR RNAi; dcrII* and control larvae (see supplementary materials, Fig. 3.1; $p = 0.528$). Furthermore, the morphology of the PG itself appeared similar to that of the control. This data suggests that NPFR signalling does not control the development of the PG, but rather its function.

NPFR is a G-protein coupled receptor that is activated by neuropeptide NPF (Garczynski *et al.*, 2002). To further test the role of NPFR signalling in developmental timing, we knocked

down NPF specifically in the NPF-producing neurons using *NPF*-Gal4 (using UAS-*NFP* RNAi v108772). We found that these larvae exhibited a 10-hour delay in time to pupariation when compared to controls (Fig. 3.1D, $p = 0.029$). Together, these data therefore suggest that NPF acts on NPFR on the PG cells to regulate developmental timing.

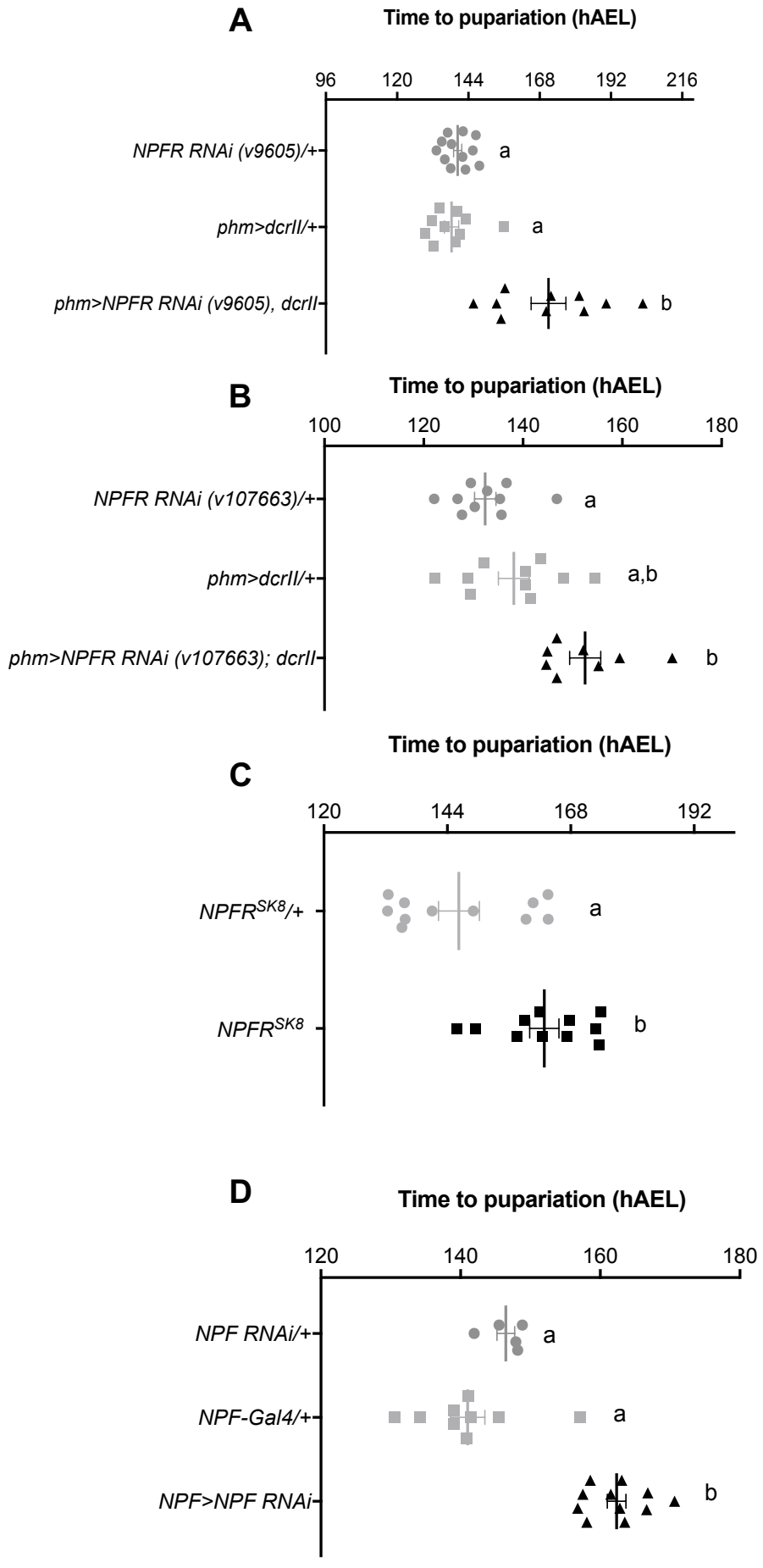

A
Time to pupariation (hAEL)
96
120
144
168
192
216
NPFR RNAi (v9605)/+
phm>dcrII/+
phm>NPFR RNAi (v9605), dcrII
a
a
b
B
Time to pupariation (hAEL)
100
120
140
160
180
NPFR RNAi (v107663)/+
phm>dcrII/+
phm>NPFR RNAi (v107663); dcrII
a
a,b
b
C
Time to pupariation (hAEL)
120
144
168
192
NPFR^SK8/+
NPFR^SK8
a
b
D
Time to pupariation (hAEL)
120
140
160
180
NPF RNAi/+
NPF-Gal4/+
NPF>NPF RNAi
a
a
b

Figure 3.1: NPFR regulates developmental timing

(A) Knockdown of *NFPR* (using UAS-*NPFR* RNAi v9605) specifically in the PG (using *phm*-Gal4>*dcrII*) results in a significant delay in time to pupariation compared to controls ($p<0.0001$). **(B)** Knockdown of *NPFR* specifically in the PG using a second, independent RNAi line (UAS-*NPFR* RNAi v107663) also results in a significant delay in time to pupariation ($p=0.0002$). **(C)** *NPFR* null mutants ($NPFR^{SK8}$) have a significant delay in time to pupariation ($p=0.008$). **(D)** Knockdown of *NPF* in *NPF*-expressing neurons (using *NPF*-Gal4) results in a significant developmental delay ($p=0.029$). hAEL= hours after egg lay. Error bars represent ±1 SEM for all graphs. In each experiment, genotypes sharing the same letter indicate that they are statistically indistinguishable from one another, while genotypes with contrasting letters indicate that they are statistically different (ANOVA and pairwise *t* tests). Each point represents a biological replicate of 15-20 animals.

3.3.2. NPFR regulates the production of ecdysone in the prothoracic gland

Given that NPFR does not seem to regulate PG development, we next tested whether NPFR signalling regulates the primary function of the PG – to produce ecdysone. We reasoned that if NPFR acts in the PG to regulate ecdysone production, then feeding *phm>NPFR RNAi; dcrII* larvae with ecdysone should rescue the developmental delay. Consistent with this prediction, supplying 20-hydroxyecdysone (20E), the active form of ecdysone, to *phm>NPFR RNAi; dcrII* larvae completely restored normal developmental timing (Fig 3.2A $p>0.0001$). Interestingly, we found that these animals pupariate even faster than controls (Fig 3.2A), suggesting that they may have an increased sensitivity to ecdysone. When we quantified the ecdysone titre, we found that *phm>NPFR RNAi; dcrII* animals produced significantly less ecdysone later in the third instar, between 32 and 56 hours after the third instar moult, when compared to controls (Fig. 3.2B).

This reduction in total ecdysone concentration could be due to a defect in either its biosynthesis or in its secretion. To distinguish between these two possibilities, we quantified the expression levels of two CYP450 ecdysone biosynthetic genes, *phm* and *disembodied* (*dib*). The mRNA expression levels of these two enzymes are well-established as reliable proxies for ecdysone biosynthesis (Colombani *et al.*, 2005, McBrayer *et al.*, 2007, Koyama *et al.*, 2014). Additionally, the expression of an ecdysone response gene, *ecdysone-induced protein 74EF (e74B)*, was quantified as a readout of ecdysone signalling activity. Furthermore, the internal reference genes, Rpl23, was chosen as it has previously been shown to be a reliable internal reference when investigating Halloween gene expression (McBrayer *et al.,* 2007, Koyama *et al*., 2014, Gibbens *et al.,* 2011). When *NPFR* was knocked down in the PG, there was an overall reduction in *phm* and *dib* between 32 and 56 hours after the third instar moult compared to controls (Fig. 3.2C, D). Further, *e74b* expression was reduced compared to controls (Fig. 3.2E), demonstrating lower levels of ecdysone signalling activity in larvae where *NPFR* was knocked down in the PG. These data suggest that NPFR signalling is involved in the regulation of ecdysone biosynthesis in the PG, although does not rule out that it could play additional roles in regulating ecdysone secretion.

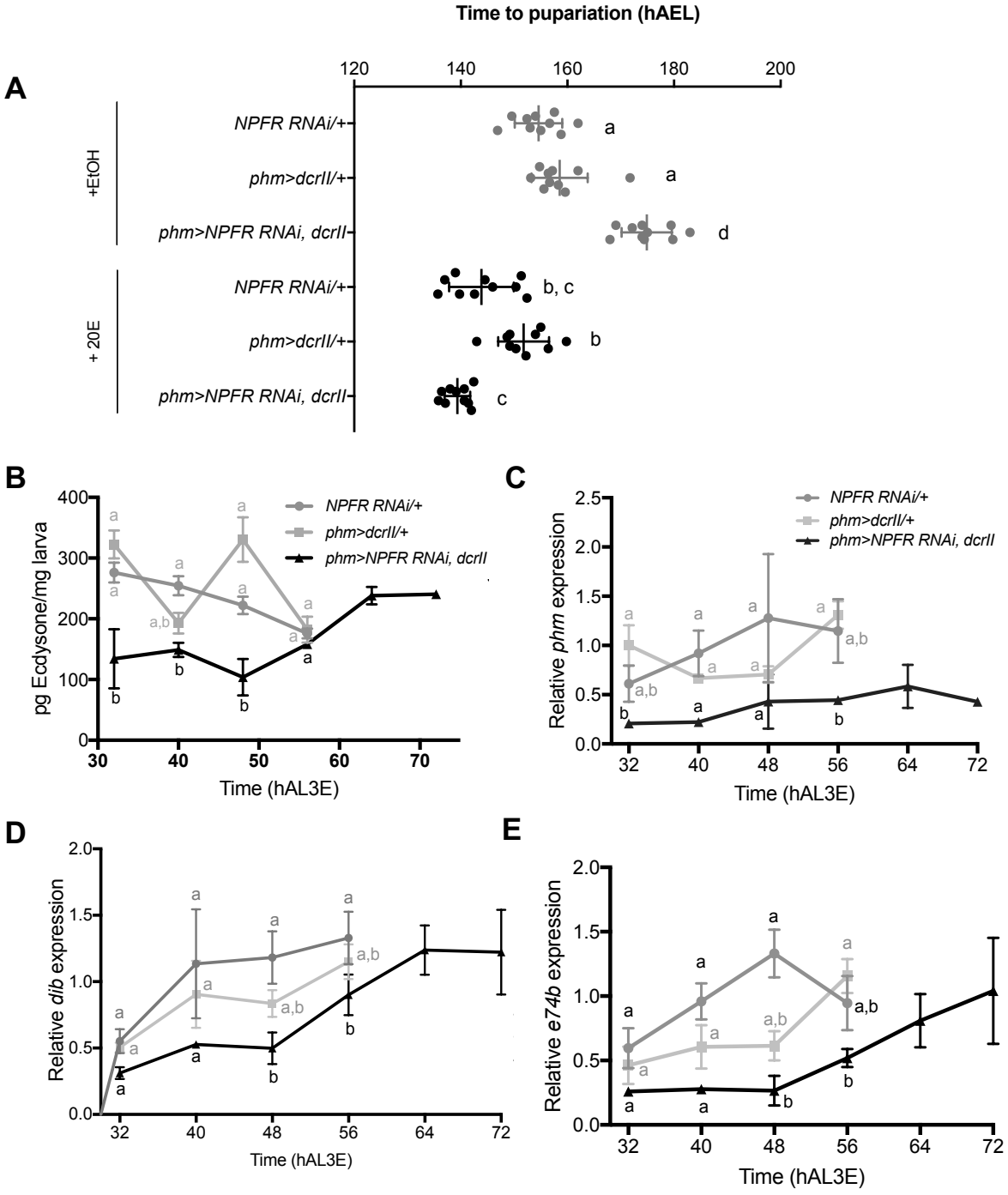

A
Time to pupariation (hAEL)
120
140
160
180
200
+EtOH
+ 20E
NPFR RNAi/+
phm>dcrII/+
phm>NPFR RNAi, dcrII
NPFR RNAi/+
phm>dcrII/+
phm>NPFR RNAi, dcrII
a
a
d
b, c
b
c
B
pg Ecdysone/mg larva
Time (hAL3E)
C
Relative phm expression
Time (hAL3E)
D
Relative dib expression
Time (hAL3E)
E
Relative e74b expression
Time (hAL3E)

Figure 3.2: NPFR regulates the production of ecdysone in the prothoracic gland

(A) Time to pupariation was measured for *phm>dcrII; NPFR RNAi* (v9605) larvae fed on either food supplemented with 96% EtOH (grey) or 20-hydroxyecdysone (20E) (black). Supplying *phm>NPFR RNAi; dcrII* larvae with 20E is able to completely rescue the developmental delay seen when *NPFR* is knocked down in the PG ($p<0.0001$). Each point represents a biological replicate of 15-20 animals. **(B)** *phm>NPFR RNAi; dcrII* animals have an overall reduction in ecdysone titre compared to parental controls during the late third instar. hAL3E = hours after L3 ecdysis. Five biologically independent replicates of 8-10 larvae were measured for each time point. Relative expression of **(C)** *phm,* **(D)** *dib* and **(E)** *e74B* in *phm>NPFR RNAi; dcrII* animals is overall reduced as determined by quantitative PCR. Values were normalised using an internal control, *Rpl23*. hAL3E = hours after L3 ecdysis. Expression level of each gene was standardised by fixing the values at 32hrs in *NPFR* RNAi (9605)/+ as 1 in all panels. Approximately 8-15 larvae were used for each sample, and five biologically independent samples for each time point. Error bars represent ±1 SEM for all experiments. In each experiment, genotypes sharing the same letter indicate that they are statistically indistinguishable from one another, while genotypes with contrasting letters indicate that they are statistically different (ANOVA and pairwise *t* tests).

3.3.3. Loss of NPFR signalling phenocopies loss of insulin signalling

Changes in insulin signalling also regulate development time by regulating the rate of ecdysone synthesis (Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005, Koyama *et al.*, 2014). This has been demonstrated in animals which are hypomorphic for loss of insulin signalling (complete loss causes early lethality; Fernandez *et al.*, 1995), such as flies that bear a heteroallelic combination of mutations in the *insulin receptor* (*InR*), or are homozygous for a loss of function mutation of the adaptor protein, *chico* (Shingleton *et al.*, 2005). These animals take longer to reach metamorphosis and have decreased adult body sizes (Shingleton *et al.*, 2005, Stocker and Hafen, 2000). As *NPFR* mutants are homozygous viable, like *chico* mutants, we hypothesised that they may be hypomorphic for loss of insulin signalling. In support of this, we found that in addition to being developmentally delayed, $NPFR^{SK8}$ mutants have smaller body sizes compared to controls (Fig. 3.3A, $p > 0.0001$). Similarly, *NPF >NPF RNAi* animals are smaller than controls (Fig. 3.3B, $p = 0.00437$). To check that the body size defect was not a result of decreased food intake due to altered feeding behaviour, we quantified food intake in $NPFR^{SK8}$ mutants on our standard fly food. This showed that under well fed conditions there were no significant differences in the amount of food consumed compared to controls (see supplementary materials, Fig. 3.2A, $p = 0.414$).

Whole-animal mutants of the insulin signalling pathway have smaller body sizes due to reduced growth rates (Böhni *et al.*, 1999). We therefore measured the growth rate of $NPFR^{SK8}$ mutants. This showed that $NPFR^{SK8}$ mutants have a significantly reduced growth rate compared to controls (Fig. 3.3C). Together, these data suggest that animal-wide loss of NPFR phenocopies animal-wide reduction in insulin signalling.

Given these similarities in phenotype, we hypothesised that NPFR interacts with the insulin signalling pathway to regulate ecdysone production. To test this idea, we quantified insulin signalling levels in *NPFR* mutants by measuring levels of phosphorylated protein kinase B (pAKT), a downstream component of the insulin signalling pathway. A significant reduction in pAKT level was observed in $NPFR^{SK8}$ mutants compared to controls (Fig. 3.3D-E, $p>0.001$). This demonstrates that whole animal loss of *NPFR* leads to an overall reduction in insulin signalling.

To further explore this, we looked at another well-known phenotype of loss of insulin signalling; response to nutrition. Wild-type animals that are fed on less-nutritious foods have a reduction in final body size (Robertson, 1963), presumably because of the resulting reduction in insulin signalling under poor nutritional conditions. When insulin signalling is suppressed in an organ, the organ loses its ability to adjust its size in response to nutrition (Tang *et al.*, 2011). If *NPFR* mutants have an overall reduction in insulin signalling, then they should also have decreased body size plasticity in response to less nutritious foods. We therefore fed $NPFR^{SK8}$ mutants diets of varied caloric concentration, and measured pupal weight as an indication of body size. This showed that these animals had the same sensitivity to nutrition as controls, with indistinguishable slopes between body size and the caloric concentration of the food between genotypes (Fig. 3.3F). Taken together, this data suggests that loss of *NPFR* reduces, but does not fully ablate, overall insulin signalling. The reduction in insulin signalling is sufficient to cause reduced body size and growth rate, but not enough to interfere with plasticity in body size in response to poor nutrition.

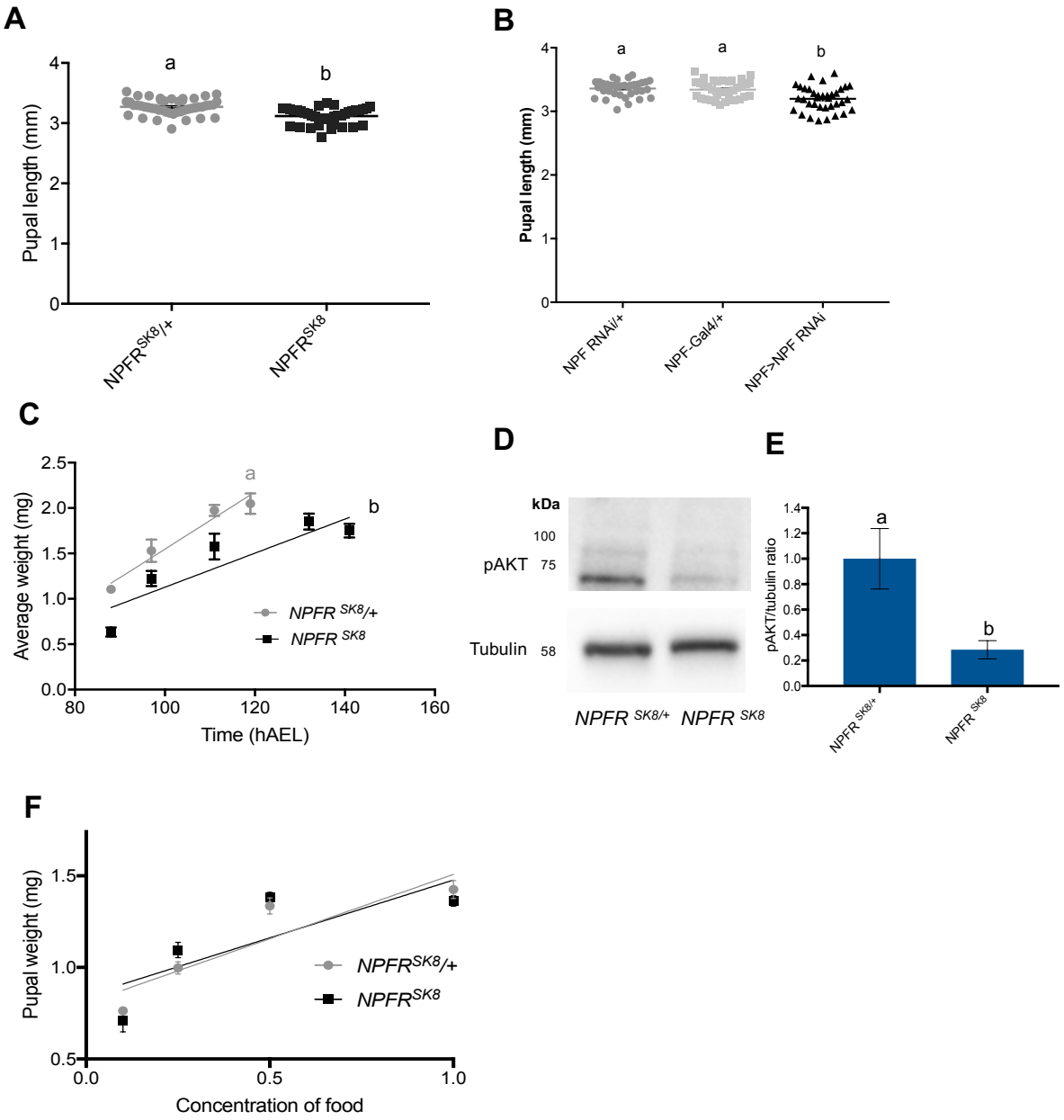

A
Pupal length (mm)
a
b
NPFR^SK8/+
NPFR^SK8
B
Pupal length (mm)
a
a
b
NPF RNAi/+
NPF-Gal4/+
NPF>NPF RNAi
C
Average weight (mg)
Time (hAEL)
a
b
NPFR SK8/+
NPFR SK8
D
kDa
100
pAKT 75
Tubulin 58
NPFR SK8/+
NPFR SK8
E
pAKT/tubulin ratio
a
b
NPFR SK8/+
NPFR SK8
F
Pupal weight (mg)
Concentration of food
NPFR^SK8/+
NPFR^SK8

Figure 3.3: *NPFR*^{SK8} mutants phenocopy loss of insulin signalling

(**A)** *NPFR*^{SK8} mutants and **(B)** *NPF>NPF-RNAi* animals have a smaller body size compared to controls, as measured by pupal length ($p<0.0001$, $p=0.00437$, respectively). Each point represents an individual pupa, and no less than 40 individuals were tested per genotype. **(C)** *NPFR*^{SK8} mutants have a reduced rate of growth compared to controls ($p<0.01$, linear regression analysis). hAEL = hours after egg lay. 10-15 animals were tested per time point for each genotype. **(D)** *NPFR*^{SK8} mutants have reduced levels of phosphorylated Akt (pAkt). **(E)** Quantification of pAkt/Tubulin densities were standardised by fixing the values of *NPFR*^{SK8}/+ to 1. Six biological replicates of 10 animals were used per genotype. **(F)** *NPFR*^{SK8} mutants fed on diets of decreasing caloric density do not adjust their body size differently to controls ($p>0.05$, linear regression analysis). 10 biological replicates of 10-15 animals were used per diet. For all graphs, genotypes sharing the same letter indicate that they are statistically indistinguishable from one another while genotypes with contrasting letters indicate that they are statistically different (ANOVA and pairwise *t* tests). Error bars represent ±1 SEM for all graphs.

3.3.4. NPFR negatively regulates insulin signalling in the prothoracic gland

Given that *NPFR*^{SK8} mutants have reduced insulin signalling, and that knocking down *NPFR* in the PG reduces ecdysone synthesis, we next wanted to determine if NPFR modifies insulin signalling specifically in this organ. Changes in insulin signalling in the PG could cause a developmental delay under one of two different scenarios: 1) if insulin signalling is increased in the PG early in third instar larvae, or 2) if insulin signalling is reduced in the PG in the mid to late third instar. This is because insulin signalling has different roles before and after an important developmental checkpoint in third instar larvae, known as "critical weight" (Nijhout, 2003, Mirth *et al.*, 2005, Shingleton *et al.*, 2005). Prior to critical weight, either starving animals or reducing insulin signalling results in a developmental delay (Beadle *et al.*, 1938, Mirth *et al.*, 2005, Shingleton *et al.*, 2005). This occurs because low levels of insulin signalling delay the timing of the ecdysone pulse that is necessary to trigger the critical weight checkpoint (Mirth *et al.*, 2005). After critical weight has been achieved, starving animals has the opposite effect and accelerates developmental timing (Mirth *et al.*, 2005, Koyama *et al.*, 2014, Stieper *et al.*, 2008). This has previously been described as the "bail out response", referring to the fact that under starvation conditions (when insulin signalling is low), the developmental program encourages the animal to pupariate (Hatem *et al.*, 2015, Nijhout *et al.*, 2014). Thus, an increase in insulin signalling in late third instar animals should cause a developmental delay.

We therefore set out to determine if loss of NPFR in the PG reduces or increases insulin signalling in this tissue, and if this changes over the third larval instar period. To do this we examined the localisation of the transcription factor, Forkhead Box class O (FoxO), a negative regulator of insulin signalling (Jünger *et al.*, 2003), in PG cells. When insulin signalling is high, FoxO is cytoplasmic, and when insulin signalling is low, FoxO is localised to the nucleus (Jünger *et al.*, 2003). We knocked down *NPFR* in the PG and determined FoxO localisation in both early and mid-late third instar animals. In both cases, PG cells in *phm>NPFR RNAi; dcrII* animals had significantly more cytoplasmic FoxO than controls (Fig 3.4A and 3.4B, $p > 0.01$). This suggests that insulin signalling activity is increased in the PG in both early and late third instar larvae when *NPFR* is knocked down specifically in this tissue, and thus that NPFR normally functions to repress insulin signalling in the PG in third instar larvae. The developmental delay that was observed by knocking down *NPFR* throughout the third instar could then be explained if the increase in insulin signalling pre-

critical weight is not sufficient to accelerate developmental timing, but the increase in insulin signalling during the late third instar is sufficient to cause a developmental delay.

We next measured body size when *NPFR* is knocked down in the PG, using pupal length as an indication of final body size. This was of interest as increasing insulin signalling before critical weight would be expected to cause a decrease in body size, whereas increasing insulin signalling after critical weight would be expected to cause an increase in body size. We found that *phm> NPFR RNAi; dcrII* animals are larger than controls (Fig. 3.4C, $p = 0.0239$). To ensure the body size alteration was not a result of increased food intake, we quantified food intake in the *phm> NPFR RNAi; dcrII* animals. This showed that the amount of food consumed was indistinguishable from controls (see supplementary materials, Fig. 3.2B; $p = 0.588$). Taken together, while the FoxO localisation data suggests NPFR is able to repress insulin signalling throughout L3, the combination of a developmental delay and increased body size observed when we knock down *NPFR* in the PG suggests that NPFR function in the PG is most important post critical weight.

Lastly, we looked at the response to nutrition. If knocking down *NPFR* in the PG causes increased insulin signalling in the gland, this could result in animals being more sensitive to nutrition (Tang *et al.*, 2011). To test this, we knocked down *NPFR* in the PG, and animals were fed on one of four concentrations of food. We then measured pupal weight as an indication of final body size (Fig. 3.4D). On a diet of standard caloric concentration (1x), *phm> NPFR RNAi; dcrII* animals were significantly larger than controls. As the calorie concentration in the food decreases, we observed a much steeper decrease in body size for *phm> NPFR RNA; dcrII* animals compared to controls. This demonstrates that body size is indeed more sensitive to nutrition in these animals, further supporting a negative regulatory role in insulin signalling.

Taken together, given that NPFR regulates insulin signalling in the PG, these data suggest that under nutritional stress NPF could be both acting on NPFR neurons in the brain to regulate feeding behaviour (Wu *et al.*, 2003, Wu *et al.*, 2005) while also signalling through NPFR in the PG to regulate developmental timing. In this way, NPF signalling could act as a nexus between feeding behaviour and development.

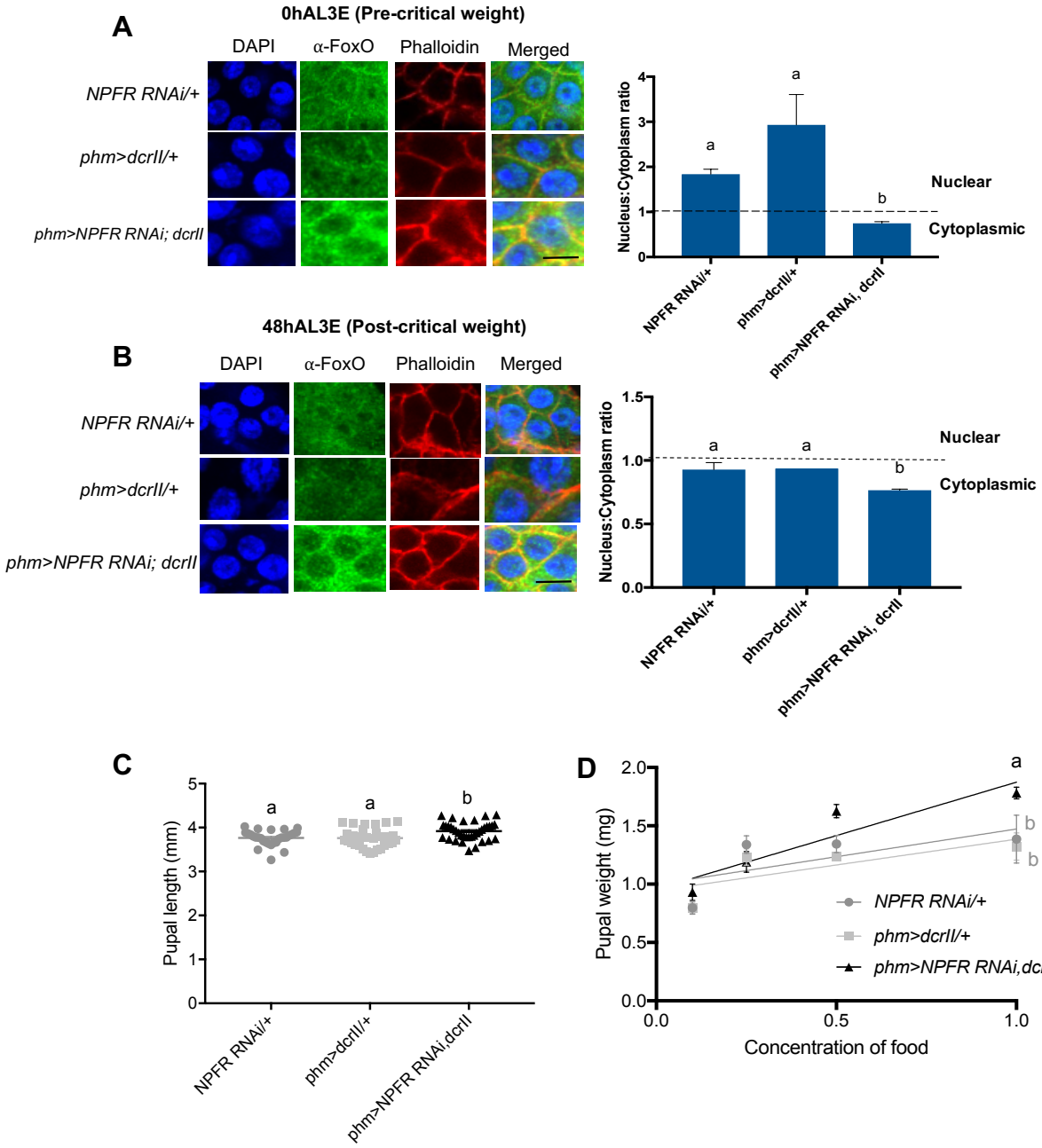

A
0hAL3E (Pre-critical weight)
DAPI
α-FoxO
Phalloidin
Merged
NPFR RNAi/+
phm>dcrII/+
phm>NPFR RNAi; dcrII
Nucleus:Cytoplasm ratio
Nuclear
Cytoplasmic
B
48hAL3E (Post-critical weight)
C
Pupal length (mm)
D
Pupal weight (mg)
Concentration of food
phm>NPFR RNAi,dcrII

Figure 3.4: NPFR negatively regulates insulin signalling in the prothoracic gland

(A) Knocking down *NPFR* specifically in the PG results in increased cytoplasmic FoxO accumulation in pre-critical weight larvae (0hAL3E) compared to controls (p=0.01). **(B)** Knocking down *NPFR* specifically in the PG results in primarily cytoplasmic FoxO in post-critical weight larvae (48hAL3E) compared to controls (p=0.0011). hAL3E = hours. after L3 ecdysis. Eight – ten PGs were analysed per genotype. Scale bar is 10μm. **(C)** Knocking *NPFR* down specifically in the PG results in an increase in final body size as measured by pupal length (p=0.0239). Each point represents an individual pupa and no less than 40 individuals were tested per genotype. For **(A) – (C),** error bars represent ±1 SEM. Genotypes sharing the same letter are statistically indistinguishable from one another, while genotypes with different letters are statistically different (ANOVA and pairwise *t* tests) **(D)** *phm>NPFR RNAi; dcrII* animals display significantly different changes in final body size compared to controls when fed on diets of decreasing caloric density ($p<0.01$, linear regression analysis). 10 biological replicates of 10-15 animals were assessed per diet.

3.3.5. NPFR regulates developmental timing by acting downstream of the insulin receptor in the prothoracic gland

Finally, we conducted genetic interaction experiments to determine where in the insulin signalling pathway NPFR acts in the PG. We first overexpressed a constitutively active and ligand-independent form of InR (InR^{CA}) in the PG. As expected (Walkiewicz and Stern, 2009), expression of InR^{CA} specifically in the PG significantly reduced the time to pupariation (Fig 3.5A, $p < 0.01$). When we knocked down *NPFR* while simultaneously expressing InR^{CA} in the PG, we observed a significant developmental delay, similar to that seen with PG-specific knockdown of *NPFR* alone (Fig. 3.5A, $p > 0.0001$). This suggests that NPFR functions downstream from InR.

We then asked if NPFR acts upstream of FoxO. Mutations in *foxo* do not affect body size in fed animals (Slack *et al.*, 2011), likely due to an unchanged development time. However, altering FoxO activity does impact developmental timing and size in starved larvae (Koyama *et al.*, 2014). This is presumably because reducing FoxO in the PG of fed animals is insufficient to further increase insulin signalling in this gland, and so animals pupariate at normal times. If NPFR functions upstream of FoxO, then the developmental delay seen when *NPFR* is knocked down in the PG should be rescued when *foxo* is simultaneously knocked down. Therefore, we knocked down both *NPFR* and *foxo* in the PG and found that this was able to partially rescue the delay seen when knocking down alone (Fig. 3.5B, $p > 0.0001$). These results therefore suggest that NPFR functions to regulate the insulin signalling pathway downstream of InR and upstream of FoxO.

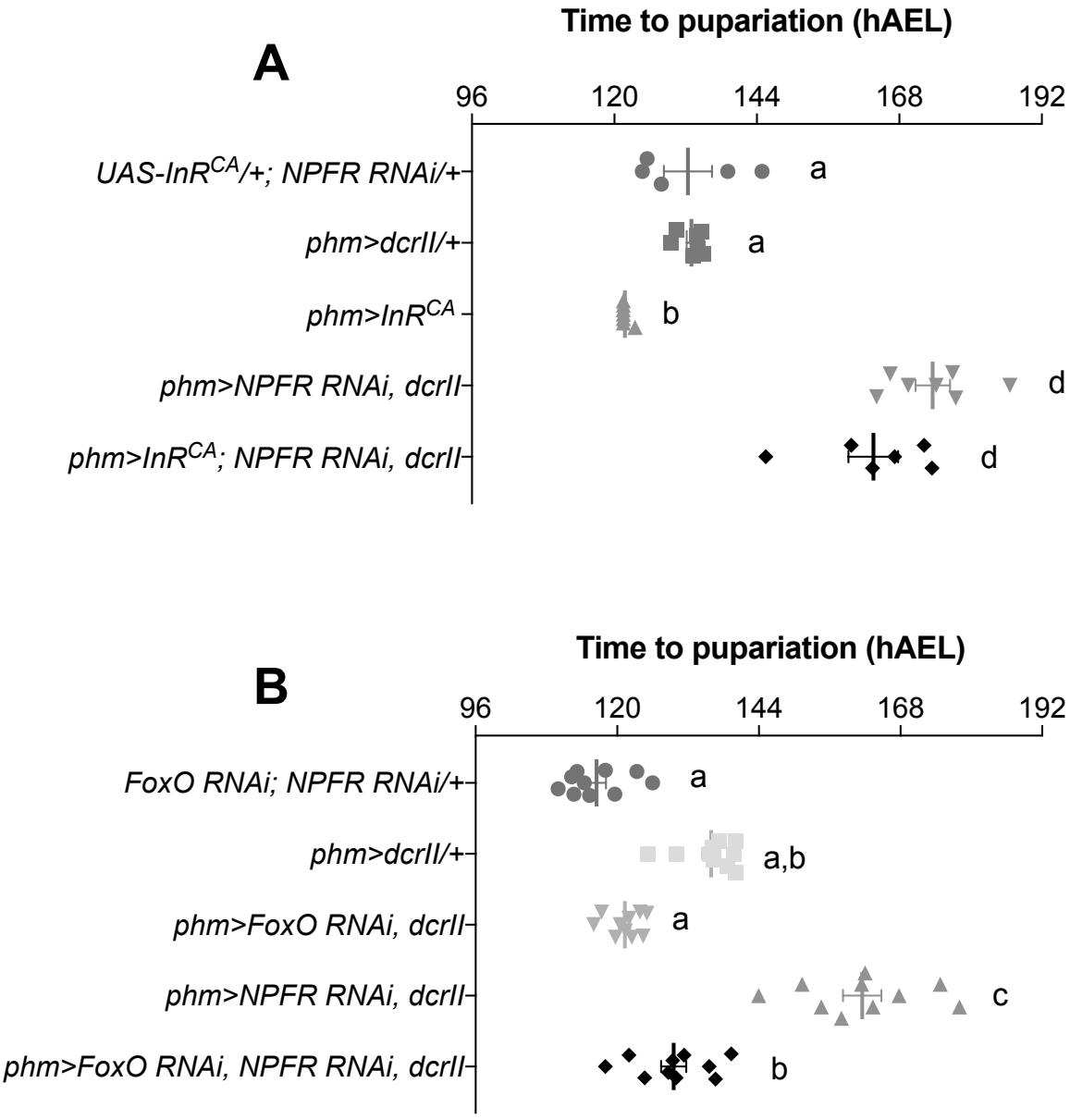

A
Time to pupariation (hAEL)
96
120
144
168
192
UAS-InR^CA/+; NPFR RNAi/+
phm>dcrII/+
phm>InR^CA
phm>NPFR RNAi, dcrII
phm>InR^CA; NPFR RNAi, dcrII
a
a
b
d
d
B
Time to pupariation (hAEL)
96
120
144
168
192
FoxO RNAi; NPFR RNAi/+
phm>dcrII/+
phm>FoxO RNAi, dcrII
phm>NPFR RNAi, dcrII
phm>FoxO RNAi, NPFR RNAi, dcrII
a
a,b
a
c
b

Figure 3.5: NPFR interacts with the insulin signalling pathway to control developmental timing

(A) Knockdown of *NFPR* while simultaneously expressing a constitutively active form of *InR* (InR^{CA}) specifically in the PG results in a significant developmental delay ($p<0.0001$), similar to PG-specific knockdown of *NPFR* alone. **(B)** Knockdown of both *NPFR* and *FoxO* specifically in the PG partially rescues the developmental delay seen with PG-specific knockdown of *NPFR* alone ($p<0.0001$). hAEL= hours after egg lay. Error bars represent ±1 SEM. In each experiment, genotypes sharing the same letter indicate that they are statistically indistinguishable from one another, while genotypes with contrasting letters indicate that they are statistically different (ANOVA and pairwise *t* tests).

In summary, here we have described a new role for the conserved feeding regulator, NPFR, in the regulation of developmental timing, animal growth rate and body size. In the PG, our data supports a role for NPFR in negatively regulating the insulin signalling pathway and for regulating ecdysone biosynthesis. Our genetic interaction data suggests NPFR acts downstream of the insulin receptor in the PG, and perhaps plays a role in keeping the insulin signalling pathway in check to ensure that the ecdysone pulses are produced at the correct time. By contrast to the results we obtained in the PG, we found that whole animal loss of NPFR generates phenotypes resembling reduced insulin signalling. The simplest explanation of these contrasting phenotypes is that NPFR has a second role in regulating developmental timing and body size elsewhere in the fly, perhaps due to a role in regulating insulin-like peptide production or secretion. These data thus not only highlight a previously undescribed mechanism by which insulin signalling and ecdysone production are regulated in the PG, but also demonstrate how a single neuropeptide signalling pathway can have functionally diverse roles within an organism in response to the same environmental cue.

Our findings raise the strong possibility that NPF may indeed coordinate feeding behaviour and growth. How might it do so? Other peptides known to function in the regulation of ecdysone production are produced either in neurons that directly innervate the PG, such as PTTH (McBrayer *et al.*, 2007), or in other tissues and secreted into circulation, such as the Dilps (Ikeya *et al.*, 2002, Brogiolo *et al.*, 2001). Either a local or systemic source of NPF is therefore possible for activating NPFR in the PG. While neuropeptides such as NPF are best described as having local modes of action, in the adult fly NPF has recently been shown to be secreted from the midgut into the hemolymph, where it can act systemically (Ameku *et al.*, 2018). In the larva, NPF is known to be expressed in dopaminergic neurons in the brain, as well as cells in the midgut (Brown *et al.*, 1999). To our knowledge it has not been shown to be expressed in neurons that innervate the larval PG. This suggests that it is more likely that systemic rather than local NPF activates NPFR in the larval PG cells. Systemic NPF produced in response to nutritional stress could thus act on both NPFR neurons in the brain to regulate feeding behaviour and on NPFR in the PG to regulate developmental timing, and in so doing NPF could coordinate feeding behaviour and development.

In conclusion, this study has provided evidence to show that NPFR signalling, best known for its regulation of feeding behaviour, also functions in the *Drosophila* PG to control developmental timing and body size via regulation of insulin signalling and ecdysone

production. To our knowledge, NPF represents the first neuropeptide described to play a role in regulating both feeding behaviour and development in response to nutritional conditions, and thus first candidate for coordinating these processes in response to environmental cues. Given that the mammalian homologue of NPF, NPY, also has a role in regulating feeding behaviour in response to nutritional stress, it would be of great interest to explore if it too is a candidate for coordinating behaviour and development.

3.4. Acknowledgements

We would like to thank Karyn Moore, Emily Kerton and the Australian *Drosophila* biomedical research facility (OzDros) for technical support; Michael O'Connor for providing fly stocks; and Pierre Leopold for the FoxO antibody. M.A.H is a National Health and Medical Research Council (NHMRC) Early Career Fellow. This work was supported by an Australian Research Council (ARC) grant to C.G.W and C.K.M.

3.5. Author contributions

C.G.W and C.K.M conceived the experiments, interpreted the data and co-led the work. J.R.K conceived the experiments, interpreted the data, and performed the experiments. M.A.H interpreted the data and assisted with experiments. L.M.P interpreted the data. S.K. generated the NPFR mutant *Drosophila* strain. J.R.K, C.G.W and C.K.M wrote the manuscript, with assistance from L.M.P.

3.6. Declaration of interests

The authors declare no competing interests.

3.7. Materials and Methods

3.7.1. Drosophila stocks

The following stocks were used: w^{1118} (BL5905), *NPF*-Gal4 (BL25682), InR^{CA} (BL8263; a constitutively active form of InR) and UAS-FoxO RNAi (BL32993) from the Bloomington *Drosophila* Stock Centre, UAS-*NPFR* RNAi (v9605), UAS-*NPFR* RNAi (v107663), UAS-*NPF* RNAi (108772) from the Vienna *Drosophila* Resource Centre, $NPFR^{SK8}$ mutant (Ameku et al., 2018, Kondo and Ueda, 2013), *phm*-Gal4-22, UAS-*mCD8::GFP* and UAS-*dicerII*; *phm*-Gal4-22, gifts from Michael O'Connor, University of Minnesota, Minneapolis (Ono et al., 2006). All flies were maintained at 25°C on fly media containing, per litre: 7.14 g potassium tartrate, 0.45 g calcium chloride, 4.76 g agar, 10.71g yeast, 47.62g dextrose, 23.81g raw sugar, 59.52g semolina, 7.14mL Nipagen (10% in ethanol) and 3.57mL propionic acid.

3.7.2. Developmental timing assays and body size analysis

Parental flies were allowed to lay eggs on 25mm apple juice agar plates for 3-4 hours. Twenty-four hours later, 15 L1 larvae were picked into standard food vials. Ten replicates were collected from each cross. Time to pupariation of the F1 offspring were scored every 8 hours. Larvae for all experiments were raised inside an insulated, moist chamber at 25 degrees in the dark. Each set of genetic crosses included a UAS-RNAi or Gal4 control crossed to w^{1118}; the genetic background for the RNAi library from the VDRC. As a proxy for body size, following their eclosion photos of the pupal cases from the developmental timing assays were taken using a light compound microscope at 2.5x magnification. Pupal case length was measured using Fiji.

3.7.3. Immunocytochemistry

For PG morphology studies, wandering larvae from each genotype were collected, and anterior halves of the larvae were dissected and fixed for 30 minutes in 4% formaldehyde in PBTx (0.01% Triton-X in Phosphate Buffered Saline (PBS)). Samples were washed 4 times over one hour in PBTx and then incubated in 50ul RNAase for 20 minutes. Samples were incubated in DAPI (1ul in 400ul PBTx) for 2 minutes, and washed in PBTx. Samples were

stored in Vectashield (Vector laboratories) and PGs were dissected under a light compound microscope in PBS. Dissected PGs were mounted onto a slide and were visualised using confocal microscopy (Olympus CV1000). Measurements of PG area were quantified using Fiji. For FoxO staining, larvae were staged at L3 and at the appropriate time points, dissected and fixed in 4% formaldehyde in PBS for 45mins at room temperature. Samples were then washed in PBTx and blocked for 30mins in 5% goat serum in PBTx and rabbit anti-FoxO (a gift from Dr. Pierre Leopold, 1:500) was added to 5% goat serum in PBTx. Samples were allowed to incubate at 4 degrees overnight. We then washed the samples in PBTx, and anti-rabbit Alexa 488 (Invitrogen, 1:500) in 5% goat serum in PBTx was added in the dark and allowed to incubate for 1.5 hours at room temperature. Samples were then washed and incubated in DAPI (1:400 PBTx) for 2 minutes. After washing in PBTx again, we added Phalloidin (1:1000) to the samples and allowed them to incubate at room temperature for 20 minutes. Samples were washed and stored in Vectashield (Vector laboratories) before further dissection onto poly-L-lysine coated coverslips and analysed using confocal microscopy.

3.7.4. Growth rate

Parental flies were allowed to lay on 25mm apple juice agar plates for 3-4 hours. Parent flies were removed and the eggs were allowed to develop for a further 24 hours. 15-20 L1 larvae were picked into standard food vials and were allowed to develop for a further 72 hours. Six - eight replicates were picked for each genotype. Individual larvae were then floated in 20% sucrose to retrieve them from the vials, and one replicate was weighed using a microbalance (Mettler Toledo) each morning and evening until the larvae started to pupariate. Weight over time was recorded and analysed using Prism 7.

3.7.5. Ecdysone feeding

To make 20E food, a stock solution of 10mg/ml of 20E (Cayman Chemical) was dissolved in 96% EtOH. To reach a final concentration of 0.15mg/ml, 15ul of the stock solution was added per 1g blended fly media. For the control food, 96% EtOH was used without 20E addition. Ten young L3 larvae were picked into the vials and allowed to feed *ad libitum*. Time to pupariation was measured every 8 hours. 10 replicates were used per genotype.

3.7.6. Quantitative PCR (qPCR)

Total RNA was extracted from the anterior halves of 10-15 larvae using TRIsure (Bioline). After DNase treatment, total RNA concentration was quantified and no more than 5ug of total RNA was converted to cDNA using a 1:1 mix of oligo DT and random hexamer primers, and reverse transcriptase (Bioline). qPCR was performed using SYBR Green PCR MasterMix (Bioline). Primer sequences for *phm*, *dib* and *rpl23* were borrowed from McBrayer *et al*., 2007. Sequences for *e74b* are as follows: *e74B* (F- 5' CGGAACATATGGAATCGCAGTG, R- 5' CATTGATTGTGGTTCCTCGCTG 3').

3.7.7. Ecdysone Titre Quantification

Larvae were synchronized by collecting newly ecdysed L3 larvae every 2 hrs. A sample of eight to ten larvae was weighed on a microbalance (Mettler Toledo) and then preserved in methanol. Prior to assaying, the samples were homogenized and centrifuged, and the resulting methanol supernatant was dried. Samples were resuspended in 50ul of enzyme immunoassay (EIA) buffer (0.4 M NaCl, 1 mM EDTA, 0.1% bovine serum albumin, and 0.01% sodium azide in 0.1M phosphate buffer). 20E EIA antiserum and 20E acetylcholinesterase tracer were purchased from Cayman Chemicals.

3.7.8. Immunoblotting

Five L3 larvae were homogenised in 80µl of lysis buffer (50mM Tris-HCl (pH 7.5), 150mM NaCl, 2.5mM EDTA, 0.2% Triton X, 5% glycerol, complete EDTA-free protease inhibitor cocktail (Roche)) and spun at 500g for 5 min at 4°C. Reducing buffer was added to all samples before boiling and separation by SDS-PAGE (any kDa TGX, Biorad) followed by transfer onto an Immobilon-P membrane (Millipore). Membranes were probed with either 1:1,000 anti-phosphorylated *Drosophila* Akt (Cell Signalling, 4054S), or 1:1,000,000 anti-α-tubulin (Sigma, B-5-1-2), washed and incubated with HRP-conjugated secondary antibody (1:10,000, Southern Biotech). Immunoblots were developed using ECL prime (GE healthcare) and imaged using a chemiluminescence detector (Vilber Lourmat). pAkt blot images were quantified using Fiji and differences between genotypes determined by unpaired *t*-tests from six biological replicates.

3.7.9. Nutritional plasticity

Food of varied caloric concentrations was made by diluting our standard food (SF) as described above with 0.5% agar (Gelita). The food concentrations used were 0.1x (10% SF, 90% agar), 0.25x (25% SF, 75% agar), 0.5x (50% SF, 50% agar) and 1x (100% SF). Eggs were picked onto these diluted foods and pupal weight was measured using a microbalance (Mettler Toldeo). For each genotype at least ten replicates of 15 larvae were raised on each food concentration. Differences in genotypes was determined by linear regression analysis using GraphPad Prism.

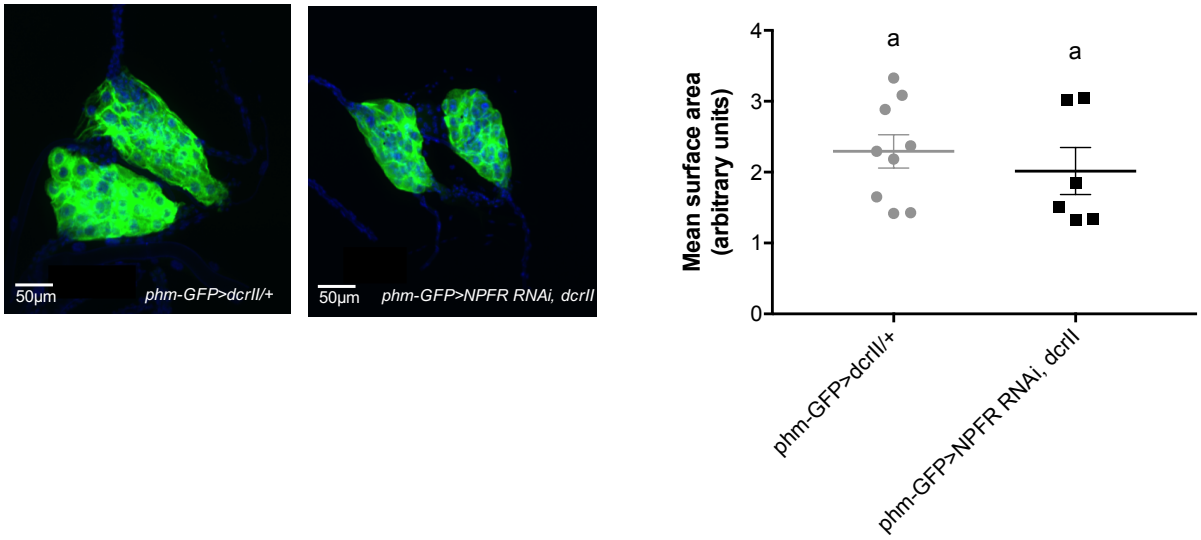
3.7.10. Quantification of food intake

Newly moulted third-instar larvae were transferred to freshly dyed food (4.5% blue food dye) and allowed to feed for 1 hr. After feeding, larvae were removed from food using 20% sucrose solution, washed in distilled water and dried. Replicates of 10 larvae were homogenised in 80µl of cold methanol and centrifuged for 10 min at 4°C. 60µl of supernatant from each sample was analysed in a spectrophotometer at 600nm. As standards, a two-fold dilution series of food dye, starting at a concentration of 4µl dye/ml methanol was used. Five-Six biological replicates were analysed per genotype.

3.8. Supplementary materials

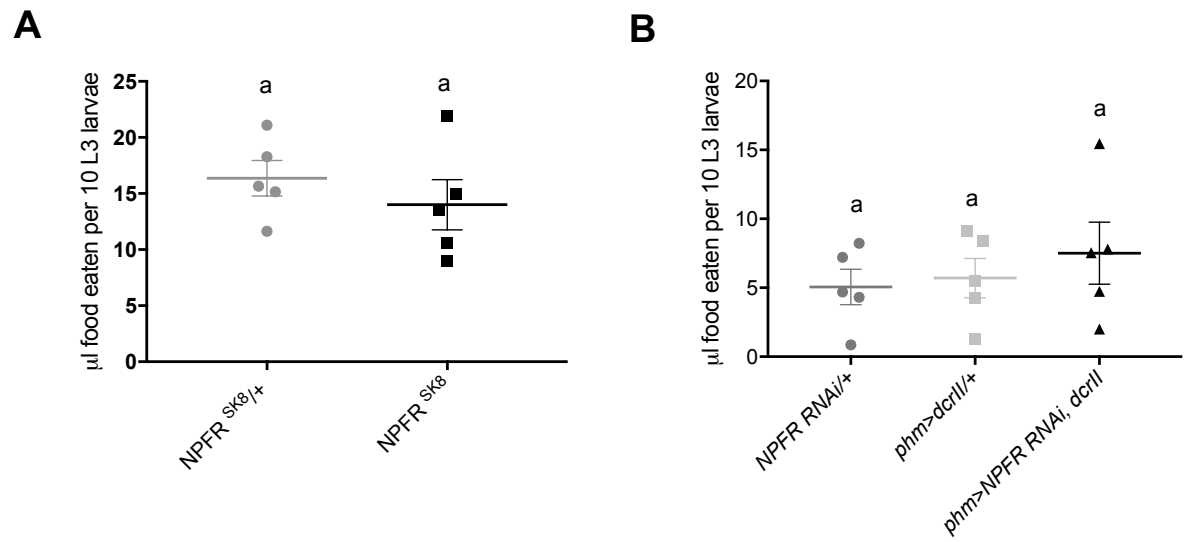
Supplementary Table 3.1: List of neuropeptide receptors known to bind to ligands that regulate feeding behaviour in *Drosophila* larvae

Neuropeptide	**Receptor(s)**	**Reference**
Allatostatin A (AstA)	Allatostatin Receptor 1 and 2 (AstA-R1 and AstA-R2)	(Wang *et al.*, 2012)
Drosulfakinin (Dsk)	Cholecystokinin-like receptor at 17D1 and Cholecystokinin-like receptor at 17D3 (CCKLR-17D1 and CCKLR-17D3)	(Söderberg *et al.*, 2012)
Neuropeptide F	Neuropeptide F Receptor (NPFR)	(Wu *et al.*, 2003, Wu *et al.*, 2005)
Hugin (Hug)	Pyrokinin 2 Receptor 1 and 2 (PK2-R1 and PK2-R2)	(Melcher and Pankratz, 2005, Schlegel *et al.*, 2016)
Short neuropeptide F (sNPF)	Short neuropeptide F Receptor (sNPFR)	(Lee *et al.*, 2004)



Supplementary Figure 3.1: Knocking down *NPFR* in the PG does not alter PG morphology

PGs from both control and *phm-GPF>NPFR RNAi; dcrII* animals are morphologically indistinguishable. Quantification of PG size from *phm>NPFR RNAi; dcrII* and *phm>dcrII/+* animals show that there are no significant size differences. *phm-GFP; UAS-dcrII* was used to both knock down *NPFR* and to visualise PGs. Error bars represent ±1 SEM. Scale bar = 50 µm. Genotypes sharing the same letter indicate that they are statistically indistinguishable from one another ($p > 0.05$, pairwise *t* tests). Eight-ten PGs were analysed per genotype.



Supplementary Figure 3.2: Reduction of NPFR both specifically in the PG and whole-animal wide does not affect amount of food consumed

(A) *NPFR*^{SK8} mutants do not consume significantly more or less food compared to controls**.** Similarly, **(B)** animals where *NPFR* has been knocked down specifically in the PG do not consume significantly more or less food compared to controls. Error bars represent ±1 SEM for all graphs. Genotypes sharing the same letter indicate that they are statistically indistinguishable from one another (p >0.05, ANOVA and pairwise t tests). Each point represents a biological replicate of 10-15 newly ecdysed L3 larvae.

Chapter 4:

Dh44-R1 plays a novel role in the *Drosophila* prothoracic gland to regulate developmental timing and body size

4.1. Introduction

As outlined in Chapter 1, ecdysone is the primary steroid hormone known to control the timing of developmental transitions in *Drosophila* (for review, see Yamanaka *et al.*, 2013). Ecdysone is produced in and secreted from the larval prothoracic gland (PG), and is under the control of neuropeptide signalling pathways that respond to environmental cues (for review, see Niwa and Niwa, 2014). Such pathways include the prothoracicotropic hormone (PTTH) pathway (McBrayer *et al.*, 2007), and the insulin signalling pathway (Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005). Disruptions in either of these pathways results in severe consequences such as developmental delays, changes in final body size, or even lethality (Gibbens *et al.*, 2011, McBrayer *et al.*, 2007, Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005). While these two pathways have proven to be very important in regulating these developmental transitions, there is increasing evidence to suggest that more peptide signalling pathways function in the *Drosophila* PG to carry out this same role (see Chapters 1 and 2).

To explore this, a PG-specific RNAi screen was carried out in an effort to identify novel peptide signalling pathways that function in the PG (Chapter 2). From this screen, it was found that knocking down *Diuretic Hormone 44-Receptor 1* (*Dh44-R1*) specifically in the PG results in developmental arrest at the first and second larval instars. A separate genome-wide screen targeting the PG also identified Dh44-R1 as a potential gene that may regulate ecdysone production, however its role in doing so was not further characterised (Danielsen *et al.*, 2016). In addition to this, antibodies raised against the ligand for Dh44-R1, Diuretic Hormone 44 (Dh44), in *Manduca sexta* were able to label axons that innervate the *Drosophila* ring gland (Zitnan *et al.*, 1993, Johnson *et al.*, 2004). This suggests that this ligand and its receptor may act in the *Drosophila* PG. However, whether this ring gland innervation included the PG was not shown. Despite this, together these data hint at a potential role for Dh44-R1 signalling in the PG to regulate ecdysone synthesis.

Dh44/Dh44-R1 signalling plays a variety of roles within the fly, including regulating nutrient sensing and feeding behaviour (Dus *et al.*, 2015, Schlegel *et al.*, 2016, Yang *et al.*, 2018), regulating fluid secretion and osmotic balance (Cabrero *et al.*, 2002, Zandawala *et al.*, 2018, Hector *et al.*, 2009), and regulating the circadian rhythm (Cavanaugh *et al.*, 2014, Cavey *et al.*, 2016, King *et al.*, 2017). This chapter aimed to determine whether Dh44-R1 signalling

also plays a role in regulating developmental timing by controlling the production of ecdysone in the PG.

4.2. Results

4.2.1. Dh44-R1 influences developmental timing

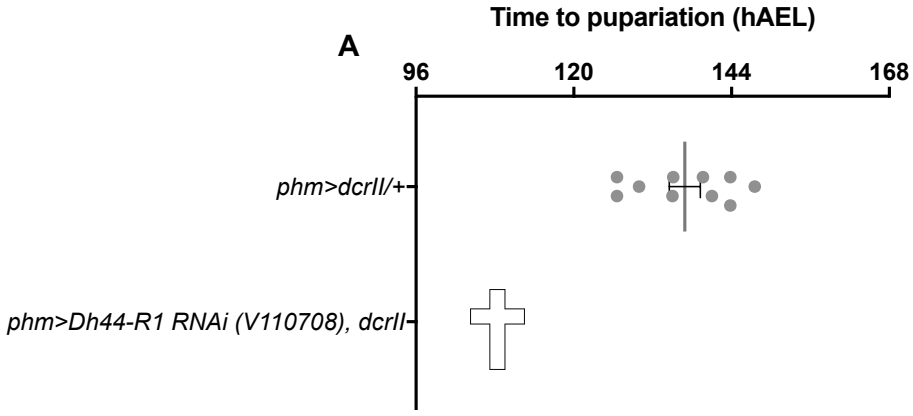
To identify novel regulators of ecdysone synthesis in the *Drosophila* PG, a small RNAi screen was conducted specifically in the PG, targeting receptors to neuropeptides and peptide hormones (see Chapter 2). From this screen, Dh44-R1 was identified as a preliminary hit. It was of subsequent interest to confirm this and thereby determine the role Dh44-R1 plays in regulating ecdysone synthesis in the *Drosophila* PG.

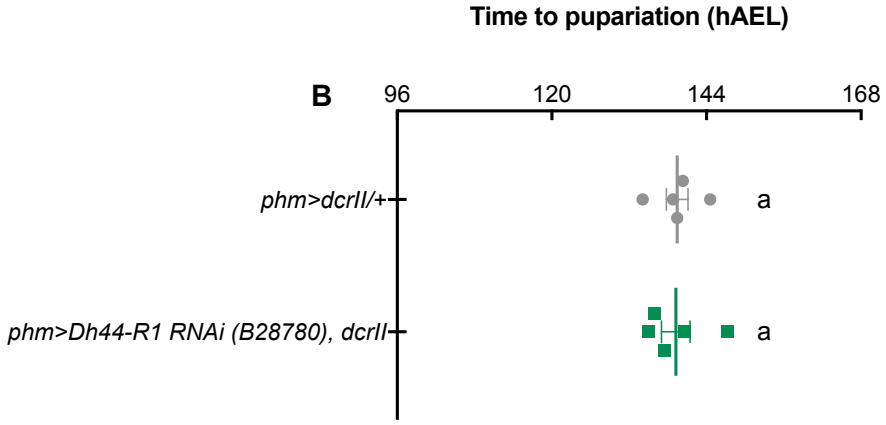
To validate the result seen in the screen (Chapter 2), and to begin to explore the role of Dh44-R1 in the PG, *Dh44-R1* was knocked down (using the UAS-*Dh44-R1* RNAi V110708 line) specifically in the PG using the PG-specific driver, *phantom*(*phm*)-Gal4. In addition to *Dh44-R1* knockdown, *phm*-Gal4 was also used to drive the expression of an enzyme responsible for cleaving double stranded RNA, *dicerII* (*dcrII*), which is known to enhance RNAi knockdown (Tomari and Zamore, 2005). As observed in the initial screen, developmental arrest at either the first or second larval stage occurred in these animals (Figure. 4.1A). These animals appeared to have the same morphology as control larvae at the first and second instar, however they failed to moult to the second/third instar and remained in their respective instar until they eventually die. The fact that these larvae died before reaching pupariation, the stage that marks the onset of metamorphosis, indicates a disruption in ecdysone synthesis or signalling (Gibbens *et al.*, 2011, Danielsen *et al.*, 2016, Di Cara and King-Jones, 2016). However, it cannot be ruled out that the lethality phenotypes could be caused by processes independent of developmental timing as it is difficult to elucidate the cause of developmental arrest.

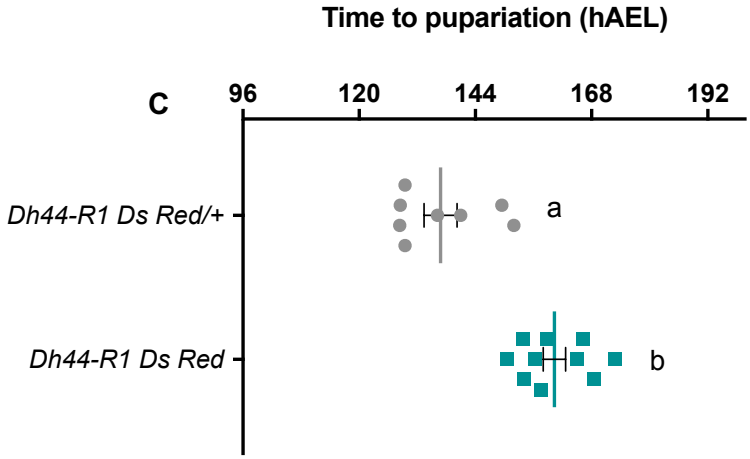
To confirm that the larval arrest phenotype seen in *phm>Dh44-R1, dcrII* animals is due to an endogenous role for Dh44-R1 in the PG, and not the result of an off-target effect, *Dh44-R1* was knocked down in the PG using a second, independent RNAi line (B28780; Figure 4.1B). However, these animals were not only able to survive until adulthood, but they also did not

exhibit any developmental delays – a sign that ecdysone synthesis is disrupted (McBrayer *et al.*, 2007, Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005). As it is predicted that up to 40% of publicly available RNAi lines are inefficient at gene knockdown (Heigwer *et al.*, 2018), it is therefore possible that this second RNAi line was unable to achieve sufficient knockdown of *Dh44-R1* to elicit an observable phenotype.
Therefore, an alternative approach was taken in order to validate the role of Dh44-R1 in regulating developmental timing, and time to pupariation was measured in *Dh44-R1* null mutants (*Dh44-R1 dsred*; King *et al.*, 2017). It was found that these animals exhibited a significant developmental delay of around 24hrs (Figure. 4.1C), supporting initial results suggesting that Dh44-R1 regulates developmental timing.

Next, the ligand to Dh44-R1*, Dh44*, was knocked down (using UAS-Dh44 v45054) specifically in *Dh44*-expressing neurons (using *Dh44*-Gal4), and time to pupariation was measured. No changes in development time were observed (Figure. 4.1D). As this may have been the result of an inefficient RNAi line, it was then explored whether *Dh44* is expressed in neurons that innervate the PG. If so, this would provide further evidence that Dh44-R1 plays a role in the PG via Dh44. To test this, *Dh44*-Gal4 was used to express GFP. This showed that *Dh44* is expressed in neurons that innervate the PG (Figure. 4.2), suggesting that it may be secreted there and play a role in regulating developmental timing via activation of Dh44-R1.


A
Time to pupariation (hAEL)
96
120
144
168
phm>dcrII/+
phm>Dh44-R1 RNAi (V110708), dcrII


B
Time to pupariation (hAEL)
96
120
144
168
phm>dcrII/+
a
phm>Dh44-R1 RNAi (B28780), dcrII
a


C
Time to pupariation (hAEL)
96
120
144
168
192
Dh44-R1 Ds Red/+
a
Dh44-R1 Ds Red
b

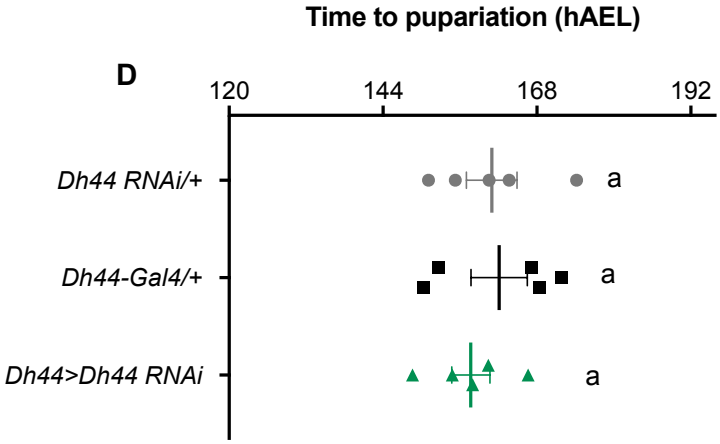

D
Time to pupariation (hAEL)
120
144
168
192
Dh44 RNAi/+
a
Dh44-Gal4/+
a
Dh44>Dh44 RNAi
a

Figure 4.1: Dh44-R1 regulates developmental timing

(A) Knockdown of *Dh44-R1* (using UAS-*Dh44-R1* RNAi, V110708) specifically in the PG using (*phm*-Gal4) results in failure to reach the pupal stage, with larvae arresting in the first and second larval instar. **(B)** Knockdown of *Dh44-R1* in the PG using a second, independent RNAi line (B28780) does not result in any observable developmental defect. However, **(C)** *Dh44-R1* null mutants (*Dh44-r1 DsRed*) exhibit a significant developmental delay compared to a heterozygous control. Finally, **(D)** knockdown of the Dh44-R1 ligand, *Dh44*, using *Dh44*-Gal, does not result in any developmental timing defects. hAEL= hours after egg lay. Error bars represent ±1 SEM for all graphs. Genotypes sharing the same letter indicate that they are statistically indistinguishable from one another, while genotypes marked with different letters are significantly different ($P < 0.05$ for panels **(A)** and **(C)**, while $P>0.05$ for panels **(B)** and **(D)**, ANOVA, pairwise *t* tests or two-tailed *t* tests). Each point represents a biological replicate of 15-20 animals.

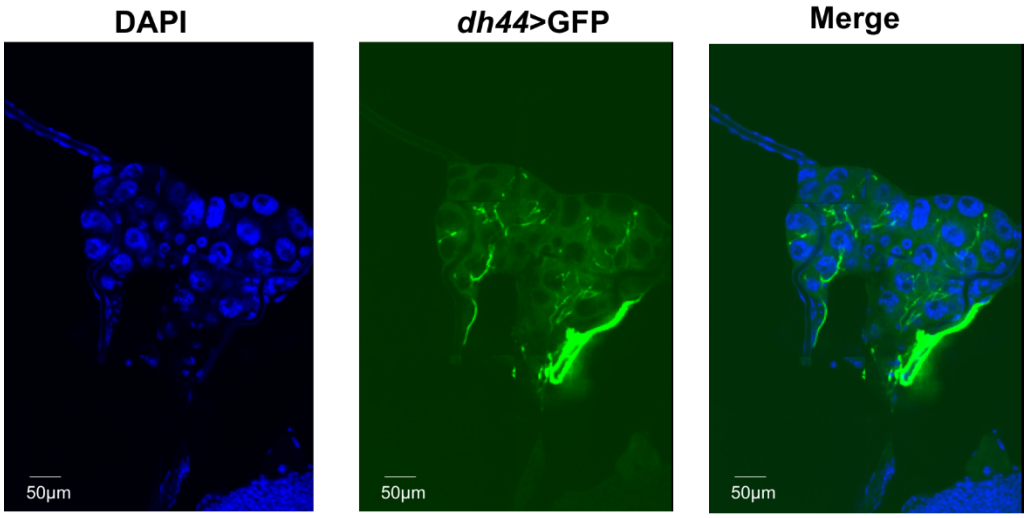


Figure 4.2: *Dh44* is expressed in neurons that innervate the PG

Dh44-Gal4 was used to drive the expression of UAS-GFP in late third instar larvae (green) and PGs were dissected and visualised using confocal microscopy. Nuclei were stained with DAPI (blue). From this, it was found that *Dh44* is expressed in neurons that innervate the PG. Scale bar represents 50µm. A total of ten PGs were dissected and visualised.

Dh44 is also known to bind to a second receptor, Diuretic Hormone 44 Receptor 2 (Dh44-R2; Hector *et al.*, 2009). Interestingly, a transcriptome analysis done on *Drosophila* ring glands identified that *Dh44-R2* is enriched in the ring gland compared to a whole larval sample (Ou *et al.*, 2016), however whether this enrichment is specific to the PG is unknown. Therefore, *Dh44-R2* was knocked down specifically in the PG and time to pupariation was measured in order to determine whether it also played a role in ecdysone synthesis. These animals pupariated at the same time as controls (see supplementary materials, Figure. 4.1). This same result was also observed in the genome-wide PG screen, which used a different RNAi line to knockdown *Dh44-R2* compared to this thesis (Danielsen et al., 2016). Taken together, these data suggest that it is exclusively Dh44-R1 signalling that plays a role in regulating developmental timing in the PG. However, it cannot be ruled out that the Dh44-R2 RNAi line was unable to efficiently knockdown expression in the PG, resulting in an absence of phenotype.

4.2.2. Knocking down Dh44-R1 in the PG with an inducible-Gal4 allows animals to reach pupariation

Because animals failed to reach the third instar when *Dh44-R1* was knocked down in the PG using the *phm*-Gal4 driver, an alternative approach was used to assess how PG-specific loss of Dh44-R1 affected time to pupariation and adult body size. This involved utilising the PG-specific inducible driver, *spookier*-GeneSwitch-Gal4 (*spok*-GS). In GeneSwitch Gal4 lines, Gal4 only becomes activated in the presence of the drug, Mifepristone (RU486; Osterwalder *et al.*, 2001). Furthermore, the amount of Gal4 activated is proportional to the amount of RU486 used (Koyama, unpublished). In this manner, it is possible to control the strength of UAS-RNAi knockdown.

In an effort to allow animals to survive to pupariation, *spok*-GS was used to knockdown *Dh44-R1* and animals were fed on diets either containing varying concentrations of RU486, or control food supplemented with ethanol. It was found that *spok-GS>Dh44-R1 RNAi* animals fed on food supplemented with 100nM RU486 were able survive to adulthood, and also exhibited a severe developmental delay of around 40hrs (Figure 4.3A). When only ethanol was added to the food, the *spok*-GS>*Dh44-R1 RNAi* larvae developed at the same rate as the control parental genotypes (*spok-GS /+, Dh44-R1 RNAi/+*). *spok-GS>Dh44-R1 RNAi*

animals were then fed on food supplemented with an increased concentration of RU486 (1000nM). These animals survived until adulthood and exhibited a significant developmental delay of around 50hrs compared to the control genotypes fed on the same diet, and all genotypes fed on the control diet (Figure. 4.3B). Finally, the concentration of RU486 was increased to 10000nM. *spok-GS>Dh44-R1 RNAi* animals fed on these high concentrations of RU486 exhibited a significant developmental delay of around 40hrs compared to the control genotypes fed on the same diet (Figure. 4.3C). The *spok-GS/+* parental control fed on 10000nM RU486 also exhibited a developmental delay, which may be attributed to the over-activation of *spok*-GS-Gal4. Additionally, only around 50% *spok-GS>Dh44-R1 RNAi* animals fed on 10000nM reached adulthood (data not shown). Given that *spok-GS>Dh44-R1 RNAi* animals fed on the diet containing 1000nM RU486 exhibited the greatest developmental delay while still permitting the majority of animals to survive to adulthood, this concentration of RU486 was used to activate the *spok*-GS driver for future experiments.

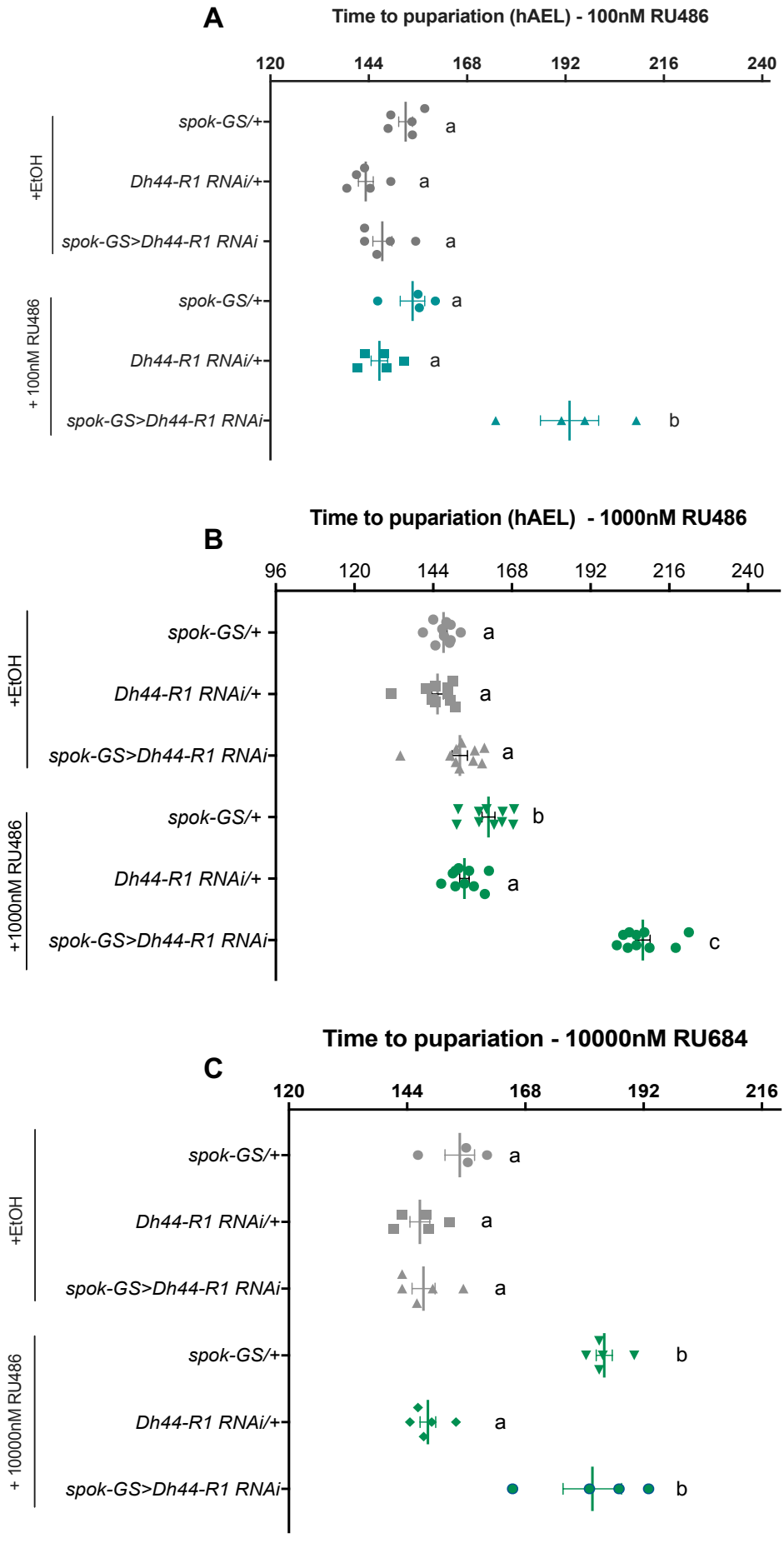

A
Time to pupariation (hAEL) - 100nM RU486
120
144
168
192
216
240
spok-GS/+
Dh44-R1 RNAi/+
spok-GS>Dh44-R1 RNAi
spok-GS/+
Dh44-R1 RNAi/+
spok-GS>Dh44-R1 RNAi
+EtOH
+ 100nM RU486
a
a
a
a
a
b
B
Time to pupariation (hAEL) - 1000nM RU486
96
120
144
168
192
216
240
spok-GS/+
Dh44-R1 RNAi/+
spok-GS>Dh44-R1 RNAi
spok-GS/+
Dh44-R1 RNAi/+
spok-GS>Dh44-R1 RNAi
+EtOH
+1000nM RU486
a
a
a
b
a
c
C
Time to pupariation - 10000nM RU684
120
144
168
192
216
spok-GS/+
Dh44-R1 RNAi/+
spok-GS>Dh44-R1 RNAi
spok-GS/+
Dh44-R1 RNAi/+
spok-GS>Dh44-R1 RNAi
+EtOH
+ 10000nM RU486
a
a
a
b
a
b

Figure 4.3: Inducibly knocking down *Dh44-R1* in the PG allows animals to survive to adulthood

Using **(A)** 100nM, **(B)** 1000nM or **(C)** 10000nM of RU486 to activate *spookier*-Geneswitch-Gal4 (*spok*-GS) to drive the knockdown of *Dh44-R1* specifically in the PG allows animals to reach adulthood and results in a significant developmental delay. hAEL= hours after egg lay. Error bars represent ±1 SEM for all graphs. Genotypes sharing the same letter are statistically indistinguishable from one another, while genotypes marked with different letters are significantly different ($P < 0.05$, ANOVA and pairwise *t* tests). Each point represents a biological replicate of 15-20 animals.

Another explanation for the developmental timing defects seen when knocking down *Dh44-R1* in the PG using *spok*-GS could be that Dh44-R1 regulates the development of the PG itself. To test this, *Dh44-R1* was knocked down in the PG using *spok*-GS and PG morphology and size was analysed (Figure 4.4 A-B). There was no difference in both morphology or size of the PG across all genotypes. This suggests that the developmental defects seen in *spok-GS>Dh44-R1 RNAi* animals are more likely due to a role for Dh44-R1 in ecdysone production in the PG, rather than in the development of the PG itself.

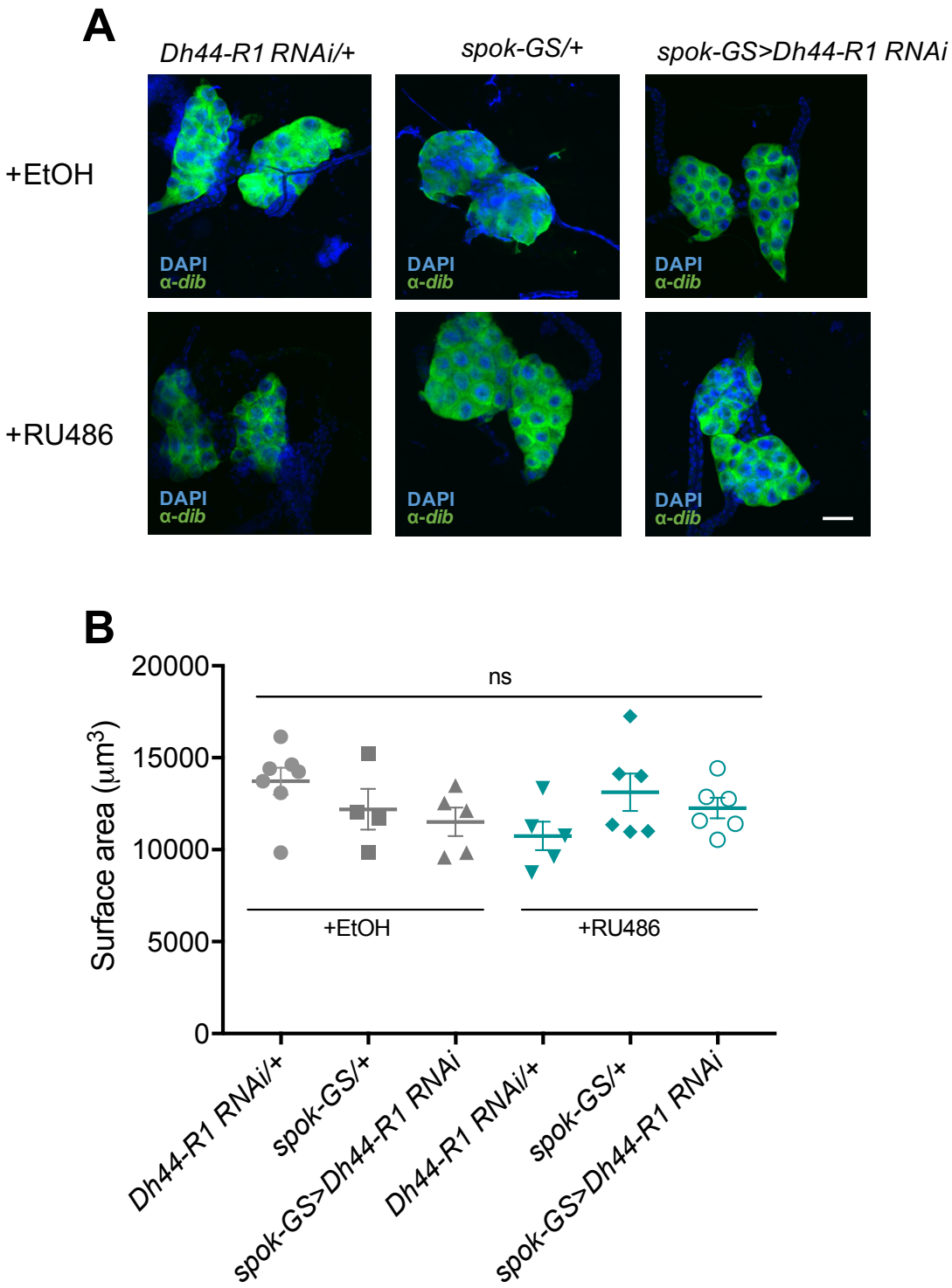

A
Dh44-R1 RNAi/+
spok-GS/+
spok-GS>Dh44-R1 RNAi
+EtOH
+RU486
DAPI
α-dib
B
Surface area (µm3)
20000
15000
10000
5000
0
ns
+EtOH
+RU486
Dh44-R1 RNAi/+
spok-GS/+
spok-GS>Dh44-R1 RNAi
Dh44-R1 RNAi/+
spok-GS/+
spok-GS>Dh44-R1 RNAi

Figure 4.4: PG-specific knockdown of *Dh44-R1* does not alter PG morphology or size

(A) Knocking down *Dh44-R1* specifically in the PG (using *spok*-GS-Gal4) does not alter the morphology of the PG compared to controls. For easy visualisation, PGs were stained with an antibody raised against the ecdysone synthesis gene, disembodied (α-dib), and nuclei were stained with DAPI. **(B)** PG size in *spok*-GS>*Dh44-R1* RNAi animals and controls, with or without RU486. Surface area of PGs were measured using Fiji. ns = not significant ($P < 0.05$, ANOVA). Error bars represent ±1 SEM for all graphs. n=5-7 for all genotypes. Scale bar = 50μm.

4.2.3. Dh44-R1 signalling regulates body size

As the PG is responsible for controlling developmental timing via the production of ecdysone, it regulates final body size by controlling the length of time larvae can grow. When animals delay development due to disrupted ecdysone production, they often have an increased final body size as a result of the prolonged larval period (Rewitz *et al.*, 2009, Mirth *et al.*, 2005, Caldwell *et al.*, 2005, Colombani *et al.*, 2005). Given that knocking down *Dh44-R1* in the PG using *spok*-GS resulted in a significant developmental delay, it was also of interest to examine the effect this had on final body size. *Dh44-R1* was therefore knocked down in the PG using *spok*-GS and pupal volume was determined as a measure of final body size. Upon activation of *spok*-GS by RU486, pupal size increased by approximately 15% when compared to the controls fed on diets containing either RU486 or EtOH (Figure. 4.5A).

Body size can change due to increased developmental time or due to changes in growth rate. While the data presented shows that Dh44-R1 activity in the PG alters developmental timing, in principle it could also change growth rate. To test this, *Dh44-R1* was knocked down in the PG using *spok*-GS and larval weight was measured over time during the third instar. Knocking down *Dh44-R1* in the PG did not significantly alter larval growth rate compared to the controls fed on diets containing either RU486 or EtOH. (Figure. 4.5B). This suggests that the increased body size phenotype seen when *Dh44-R1* is knocked down in the PG can be attributed to a prolonged larval period, rather than a change in growth rate.

Similarly, it was investigated whether *Dh44-R1* mutants also had larger body sizes. Interestingly, it was found that these mutants had an approximately 5% decrease in final body size compared to heterozygous controls (Figure. 4.5C). Animals can be developmentally delayed but have a smaller final body size if they also exhibit a reduced growth rate, as observed with insulin signalling mutants (Böhni *et al.*, 1999). Hence, it was of interest to determine if *Dh44-R1* mutants had a reduced growth rate. To test this, larval weight was measured over time in *Dh44-R1* mutants for the duration of the third instar. It was found that, as expected, these mutants had a significantly reduced growth rate compared to the heterozygous controls (Figure. 4.5D). This suggests that the smaller body size seen in *Dh44-R1* mutants is likely the result of a reduced growth rate. Together, these data show that Dh44-R1 influences final body size. However, given that PG-specific loss and whole animal loss of

Dh44-R1 have different effects on growth rate, this suggests Dh44-R1 plays an additional role in regulating growth rate/body size outside of the PG.

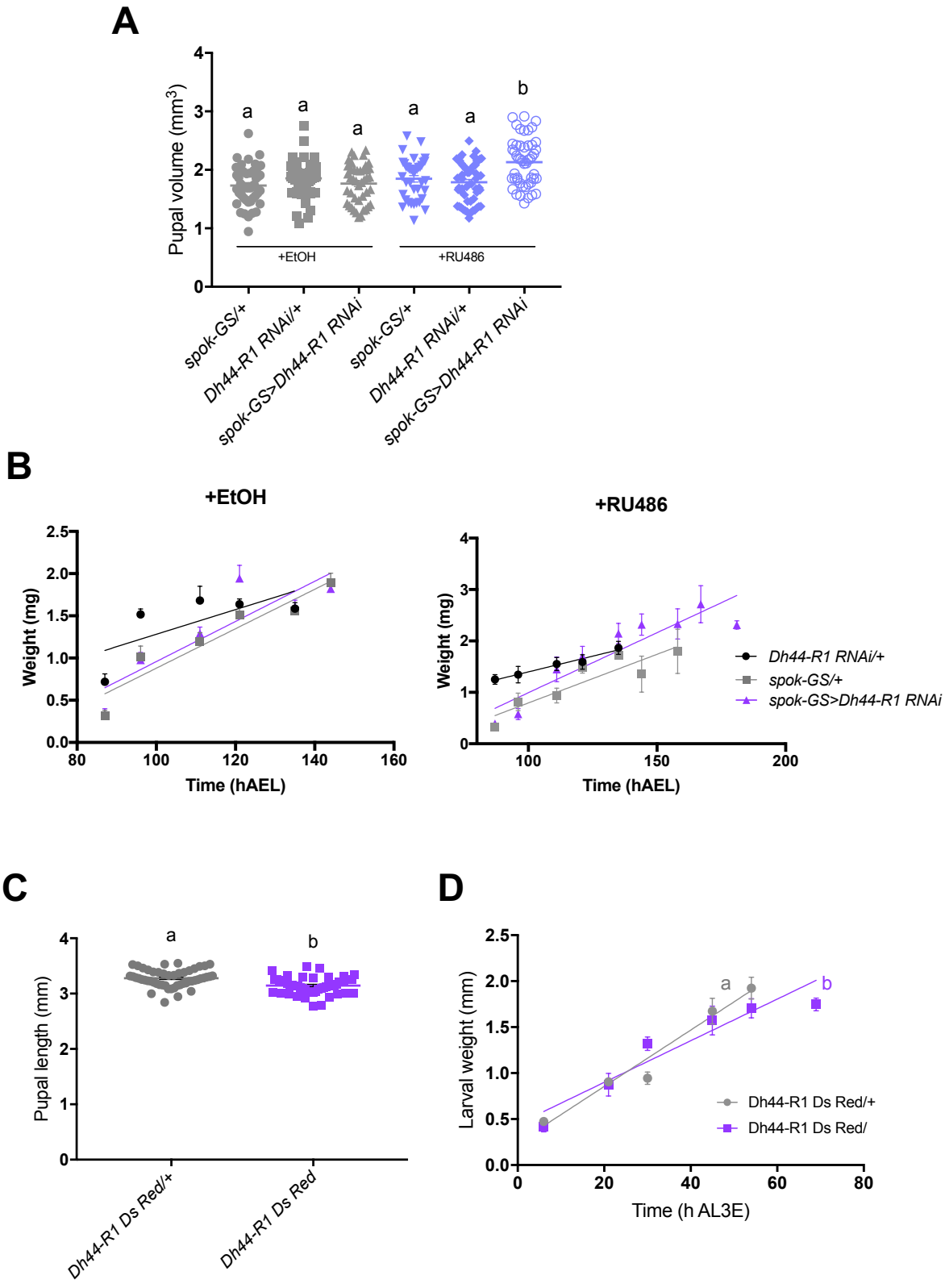

A
Pupal volume (mm3)
a
a
a
a
a
b
+EtOH
+RU486
spok-GS/+
Dh44-R1 RNAi/+
spok-GS>Dh44-R1 RNAi
spok-GS/+
Dh44-R1 RNAi/+
spok-GS>Dh44-R1 RNAi
B
+EtOH
+RU486
Weight (mg)
Time (hAEL)
Dh44-R1 RNAi/+
spok-GS/+
spok-GS>Dh44-R1 RNAi
C
Pupal length (mm)
a
b
Dh44-R1 Ds Red/+
Dh44-R1 Ds Red
D
Larval weight (mm)
Time (h AL3E)
a
b
Dh44-R1 Ds Red/+
Dh44-R1 Ds Red/

Figure 4.5: Dh44-R1 regulates final body size

(A) When *Dh44-R1* is knocked down specifically in the PG (using *spok*-GS-Gal4), an increase in body size is observed upon activation of *spok*-GS-Gal4 (in the presence of RU486). However, **(B)** PG-specific knockdown of *Dh44-R1* does not influence growth rate. **(C)** *Dh44-R1 Ds Red* mutants have a smaller final body size, which can be attributed to **(D)** a significant decrease in growth rate. hAL3E = hours after L3 ecdysis. Error bars represent ±1 SEM for all graphs. Genotypes sharing the same letter are statistically indistinguishable from one another, while genotypes marked with different letters are significantly different ($P < 0.05$, ANOVA and pairwise *t* tests). For growth rates, regression analysis was used to determine differences between slopes. For **(A)** and **(C),** each point represents an individual while for **(B)** and **(D)**, each point represents a biological replicate of 10-15 animals.

4.3.4. Feeding Dh44-R1-deficient animals ecdysone restores developmental timing

If the developmental delay observed by knocking down *Dh44-R1* in the PG results from reduced ecdysone synthesis, then animals should be able to pupariate normally if they are provided with another source of ecdysone. Two approaches were used to test if this was the case. Firstly, *Dh44-R1* was knocked down specifically in the PG using *spok*-GS and animals were fed food containing either ethanol or 20-hydroxyecdysone (20E), the active form of ecdysone. As expected, feeding *spok*-GS>*Dh44-R1* RNAi animals 20E resulted in these animals pupariating at the same time as controls (Figure. 4.6A). To provide further evidence that reducing *Dh44-R1* results in less ecdysone being produced, *Dh44-R1* mutants were fed food supplemented with 20E. The developmental delay was partially restored in these animals (Figure. 4.6B). Taken together, these data provide evidence to suggest that Dh44-R1 signalling regulates ecdysone synthesis in the PG.

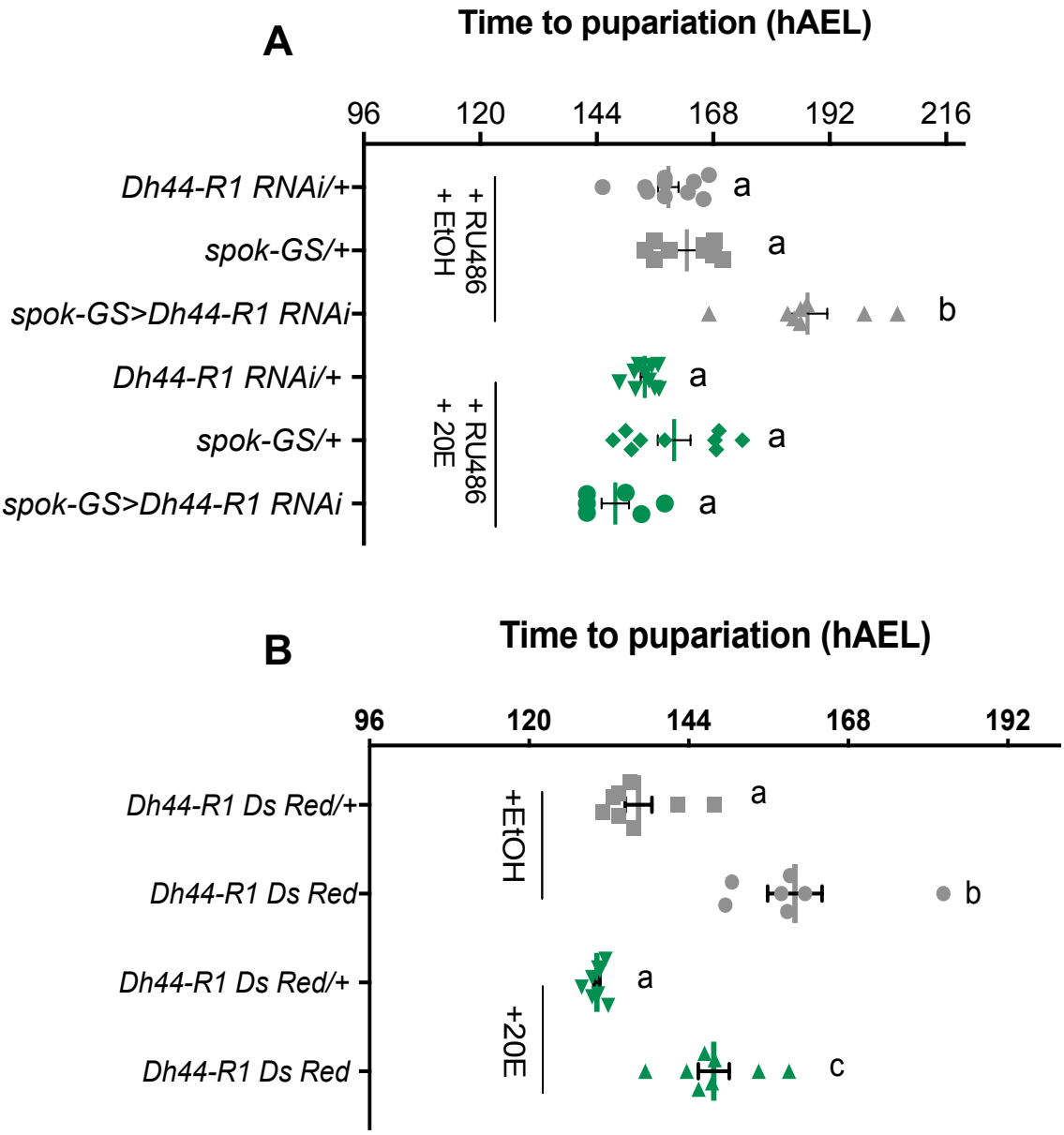

A
Time to pupariation (hAEL)
96
120
144
168
192
216
Dh44-R1 RNAi/+
spok-GS/+
spok-GS>Dh44-R1 RNAi
Dh44-R1 RNAi/+
spok-GS/+
spok-GS>Dh44-R1 RNAi
+ RU486 + EtOH
+ RU486 + 20E
a
a
b
a
a
a
B
Time to pupariation (hAEL)
96
120
144
168
192
Dh44-R1 Ds Red/+
Dh44-R1 Ds Red
Dh44-R1 Ds Red/+
Dh44-R1 Ds Red
+EtOH
+20E
a
b
a
c

Figure 4.6: Feeding ecdysone to animals with reduced *Dh44-R1* restores developmental timing

(A) Supplying *spok*-GS>*Dh44-R1* RNAi animals with 20E results in animals having a similar developmental timing to controls. **(B)** Similarly, supplying 20E to *Dh44-R1 dsred* mutants results in a partially reduced developmental delay. All genotypes were fed on diets containing 1000nM RU486 to activate *spok*-GS. hAEL= hours after egg lay. Error bars represent ±1 SEM for all graphs. Genotypes sharing the same letter indicate that they are statistically indistinguishable from one another, while genotypes marked with different letters are significantly different ($P < 0.05$, ANOVA and pairwise *t* tests). Each point represents a biological replicate of 15-20 animals.

4.3. Discussion

The regulation of ecdysone production must be tightly controlled to ensure that animals only transition between the various developmental stages at the correct time. While some of the peptide signalling pathways that control ecdysone production are well studied, there is increasing evidence to suggest that more function in the *Drosophila* PG. The data presented here provides preliminary evidence that the Dh44/Dh44-R1 signalling system plays a novel role in controlling developmental timing and body size in the PG, most likely through controlling the production of ecdysone. However, it must be noted that the evidence presented in this study is based on RNAi knockdown of Dh44-R1 using one RNAi line only, which has a predicted off target (See Chapter 2, Table 1). While the second RNAi line tested for Dh44-R1 did not replicate the phenotype seen with the first RNAi line used, studies of the Dh44-R1 null mutant show comparable results making it unlikely that the phenotypes observed are due to off target effects. However, moving forward, it is important that the results presented in this study are validated via determining the expression of Dh44-R1 transcripts upon RNAi knockdown, as well as knocking down Dh44-R1 using a second, independent RNAi line. Furthermore, validation of expression of the off-target, Toe, in animals where Dh44-R1 has been knocked down is needed.

Several pulses of ecdysone are produced during development, with a single pulse produced before each of the second and third larval moults (Riddiford, 1993). Given that knocking down *Dh44-R1* in the PG with the stronger *phm*-Gal4 driver resulted in arrest at either the first or second larval stage, it is highly possible that Dh44-R1 signalling plays a role in regulating these particular ecdysone pulses. As a result, the developmental delay seen with the weaker knockdown of *Dh44-R1* in the PG using the *spok*-GS driver may be due to delayed production of these ecdysone pulses. To confirm this hypothesis, it would be useful to determine if these animals have a prolonged first and second larval instar compared to controls, and to quantify ecdysone titres throughout the first and second instar. If this idea holds true, it provides an exciting new insight into the regulation of these earlier ecdysone pulses. While the Dilps and PTTH are known to control pulses of ecdysone in the third instar, very little is known about the regulation of ecdysone pulses in earlier instars.

While this study provided evidence that suggests that Dh44/Dh44-R1 signalling regulates ecdysone synthesis, either source of Dh44 or the environmental factor(s) to which it responds

are yet to be identified. Interestingly, this study found that *Dh44* was expressed in neurons that innervate the PG, suggesting that Dh44 may be secreted directly onto the PG from these neurons. Only one set of neurons, the PTTH-producing neurons, have been shown to innervate the entire PG (Siegmund and Korge, 2001). The PG-innervation pattern of the *Dh44*-expressing neurons therefore shares a striking resemblance to those that express *ptth* (Siegmund and Korge, 2001, McBrayer *et al.*, 2007). Therefore, this presents a possibility that *Dh44* may be expressed in the PTTH neurons. Supporting this argument, Dh44 has also been shown to play a role in regulating circadian rhythm (Cavanaugh *et al.*, 2014, Cavey *et al.*, 2016, King *et al.*, 2017), and PTTH is known to regulate ecdysone production in response to circadian rhythm (McBrayer *et al.*, 2007, Di Cara and King-Jones, 2016). Therefore, it is possible that Dh44 also signals to the PG in response to circadian rhythm, and may control ecdysone production by interacting with the PTTH pathway in the PG (Figure. 4.7). However further experiments such as co-localisation experiments with PTTH and genetic interaction studies are needed in order to confirm this. Additionally, analysing phospho-ERK, a downstream PTTH component, in PGs where *Dh44-R1* has been knocked down can also provide insight as to whether these two pathways interact.

Additionally, *Dh44* has been shown to be expressed in the neurosecretory cells which produce *Drosophila* insulin-like peptides (Dilps) 2, 3 and 5 (Schlegel *et al.*, 2016), and these cells innervate the a tissue adjacent to the PG, the corpora cardiaca (CC). Therefore, it is also possible that Dh44 is secreted from these cells and activate Dh44-R1 on the PG. Furthermore, Dh44 may also be secreted in response to nutrition, like the Dilps. In support of this, Dh44 has been shown to regulate nutrient sensing and feeding behaviour (Dus *et al.*, 2015, Schlegel *et al.*, 2016, Yang *et al.*, 2018). Perhaps Dh44 could signal to the PG in response to the consumption of a particular nutrient that is essential for development. If this is true, it is possible that the Dh44/Dh44-R1 signalling pathway interacts with the insulin signalling pathway in the PG (Figure. 4.7).

In addition to its role in circadian rhythm and nutrient sensing, Dh44 signalling is also known to be important for regulating fluid secretion and osmotic balance (Cabrero *et al.*, 2002, Zandawala *et al.*, 2018, Hector *et al.*, 2009). In the adult fly, *Dh44* is expressed in the CNS and in the malpighian tubules, where it controls fluid secretion (Cabrero et al., 2002). Dh44 is also important for maintaining osmotic balance, as loss of *Dh44* signalling results in reduced lifespan in flies exposed to osmotic stress (Hector et al., 2009). Furthermore, knockdown of

Dh44 in abdominal neurosecretory cells in the adult fly results in increased resistance to dessication, suggesting that it plays a role in water retention (Zandawala et al., 2018). It must be noted, however, that these roles have been shown to be primarily mediated through Dh44-R2 signalling. Additionally, these aforementioned functions of Dh44 signalling have only been explored in the adult fly, so it is unknown if it plays similar roles in larvae.

Nevertheless, given these roles in regulating fluid secretion, osmotic balance, and water retention, an intriguing hypothesis is that Dh44 signals to the PG in response to water availability. While it is known that water is essential for proper growth, as the animal will dehydrate and die without it, it is unknown if a certain amount of water must be ingested for the production of ecdysone to take place, as this could serve as an indication that the animal is in a healthy, moist environment. If this is true, it is possible that Dh44/Dh44-R1 signalling may co-ordinate water availability/water homeostasis with ecdysone production. This would be an interesting avenue to explore as regulation of ecdysone production by moisture/water is yet to be investigated.

Knocking down *Dh44-R1* in the PG induces either developmental arrest or developmental delay, depending on which Gal4 driver was used to drive the knockdown. Interestingly, the *Dh44-R1* null mutants displayed a much milder phenotype; they survive to adulthood but have a moderate developmental delay. This phenomenon, whereby PG-specific knockdown of a gene results in a more severe phenotype than a whole-animal mutant, has been observed in previous studies of other genes that function in the PG. For example, knocking down the clock component, *timeless*, in the PG results in lethality, whereas *timeless* mutants survive to adulthood (Di Cara and King-Jones, 2016). This is proposed to be due to genetic compensation, where in whole-animal mutants functionally related genes compensate for loss of a particular gene. This process does not occur with tissue-specific knockdown (Danielsen and Rewitz, 2016). Additionally, this study showed that *Dh44-R1* mutants are developmentally delayed and have a smaller body size compared to heterozygous controls. A caveat with this is that these heterozygous controls might display heterozygous advantage, suggesting that these phenotypes are not specific to loss of *Dh44-R1* but are rather the result of heterozygous controls displaying better fitness compared to their homozygous counterparts. Rescue experiments in which a UAS-*Dh44-R1* transgene is introduced into *Dh44-R1* mutants are needed to explore if these phenotypes can be rescued, as this would determine if these phenotypes are specific to Dh44-R1 signalling. However, the phenotypes

seen with PG-specific knockdown of *Dh44-R1* suggest that it does play a role in regulating developmental timing and body size, therefore it seems likely that the phenotypes observed in the *Dh44-R1* mutants are indeed due to loss of Dh44-R1.

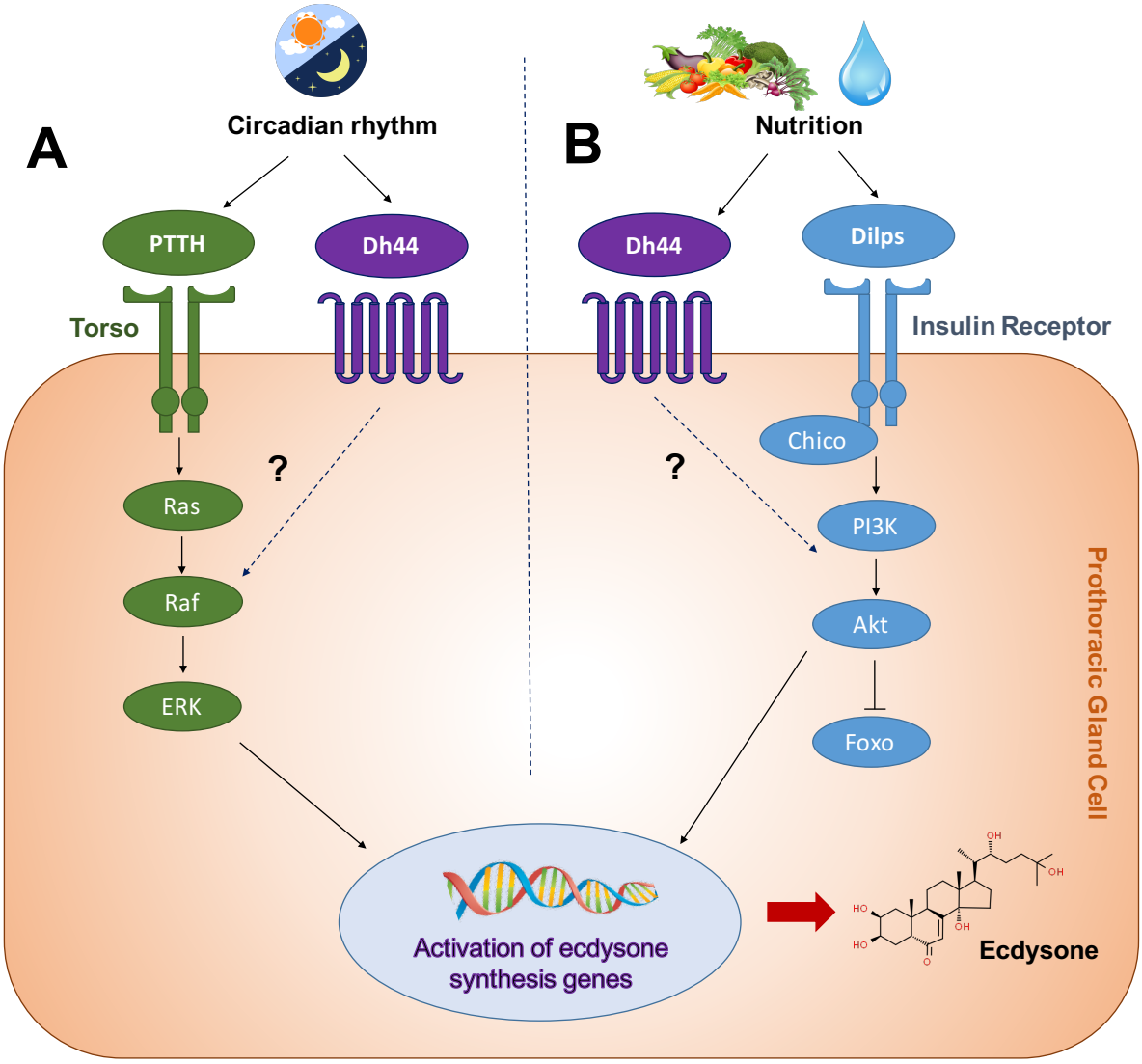


Figure 4.7: Possible ways in which Dh44-R1 signalling controls the production/secretion of ecdysone

(A) Dh44 signalling may potentially interact with the PTTH pathway in response to circadian rhythm to control the activation of ecdysone biosynthesis genes. Alternatively, **(B)** Dh44 signalling may interact with the insulin signalling pathway in response to nutrition and/or water availability to control the production of ecdysone. Dotted lines indicate potential interactions.

4.4. Conclusion

In conclusion, this study has provided evidence to suggest a novel role for Dh44/Dh44-R1 in regulating developmental timing in the *Drosophila* PG. While it was demonstrated that Dh44-R1 signalling is likely controlling the production of ecdysone in the PG, future experiments are needed to confirm this. Such experiments may include measuring ecdysone levels or conducting qPCR on ecdysone biosynthesis genes such as neverland, spookier, shroud, phantom, disembodied and shadow in animals lacking *Dh44-R1* in the PG. Similarly, antibody staining of the Halloween genes in the PG upon *Dh44-R1* knockdown would also complement qPCR analysis of these genes. Furthermore, determining what environmental factors Dh44/Dh44-R1 responds to would be of great interest in order to gain a more comprehensive understanding of not only all the environmental factors that control ecdysone production, but what molecular mechanisms underlie this. These findings may have implications for the regulation of steroid hormones in general, as Dh44 is homologous to the mammalian peptide hormone, corticotropin-releasing hormone (CRF) (Cabrero *et al.*, 2002), raising the possibility that CRF may play a role in the regulation of steroid hormones in mammals. Overall, this study has confirmed that there are indeed more peptide signalling pathways that function in the PG to regulate ecdysone production.

4.5. Materials and Methods

4.5.1. Drosophila stocks and maintenance

The following stocks were used: w^{1118} (BL5905) from the Bloomington *Drosophila* Stock Centre, *Dh44*-Gal4 from the Korean *Drosophila* Resource Centre (originally generated in Lee *et al.,* 2015), UAS-*Dh44-R1* RNAi (V110708), UAS-*Dh44-R2* RNAi (V43314) and UAS-*Dh44* RNAi (V45054) from the Vienna *Drosophila* Resource Centre, UAS-*Dh44-R1* RNAi (B28780) and UAS-IVS-GFP (B32197) from the Bloomington *Drosophila* Stock centre (UAS-IVS-GFP was originally donated from the Janelia Research Campus), UAS-*dicerII*; *phm*-Gal4-22, a gift from Michael O'Connor, University of Minnesota, Minneapolis (Ono et al., 2006), *spookier*-GeneSwitch, a gift from Dr. Takashi Koyama, University of Copenhagen, and *Dh44-R1 dsred*, a gift from Prof. Sehgal (King *et al*., 2017). All flies were maintained at 18°C on fly media containing, per litre: 7.14 g potassium tartrate, 0.45 g calcium chloride, 4.76 g agar, 10.71g yeast, 47.62g dextrose, 23.81g raw sugar, 59.52g semolina, 7.14mL Nipagen (10% in ethanol) and 3.57mL propionic acid.

4.5.2. Developmental timing assays and body size analysis

Flies from the parental generation were allowed to lay eggs on 25mm apple juice agar plates for 3-4 hours. Twenty-four hours later, 15 L1 larvae were picked into either standard food vials, food vials containing 1000nM RU486 (Cayman Chemical), or food vials containing an equivalent amount of 100% EtOH, depending on the experiment and genotype tested. Ten replicates were collected from each cross. Time to pupariation of the F1 offspring was scored every 8 hours. Larvae for all experiments were raised in incubators set to 25°C in the dark. Each set of genetic crosses included a UAS-RNAi or Gal4 control crossed to w^{1118}; the genetic background for the RNAi library from the VDRC. As a proxy for body size, following their eclosion photos of the pupal cases from the developmental timing assays were taken using a light compound microscope at 2.5x magnification. Either pupal case length or pupal volume was measured using Fiji.

4.5.3. Growth Rate

Flies from the parental generation were left to lay on 25mm apple juice agar plates for 3-4 hours. Parent flies were removed and the eggs were allowed to develop for a further 24 hours. 15-20 L1 larvae were picked into either standard food vials, vials containing food with 1000nM RU486 (Cayman chemical) or vials containing food with 100% EtOH, and were then allowed to develop for a further 72 hours to ensure they had reached the third larval instar. Six - eight replicates were picked for each genotype. Individual larvae were then floated in 20% sucrose to retrieve them from the vials, washed in water, dried, and one replicate was weighed using a microbalance (Mettler Toledo) each morning and evening until the larvae started to pupariate. Weight over time was recorded and analysed using Prism 7.

4.5.4. Ecdysone feeding

To make 20E food, a stock solution of 10mg/ml of 20E (Cayman Chemical) was dissolved in 96% EtOH. To reach a final concentration of 0.15mg/ml, 15ul of the stock solution was added per 1g blended standard laboratory food. For the control food, 96% EtOH was used without 20E addition. For experiments involving the *spok*-GS driver, food containing 1000nM RU486 (Cayman Chemical) was used in addition to either 20E or EtOH. Ten young L3 larvae were picked into the vials and allowed to feed *ad libitum*. Time to pupariation was measured every 8 hours. 10 replicates were used per genotype. For ecdysone-feeding experiments, parental flies were allowed to lay eggs on 25mm apple juice agar plates for 3-4 hours.

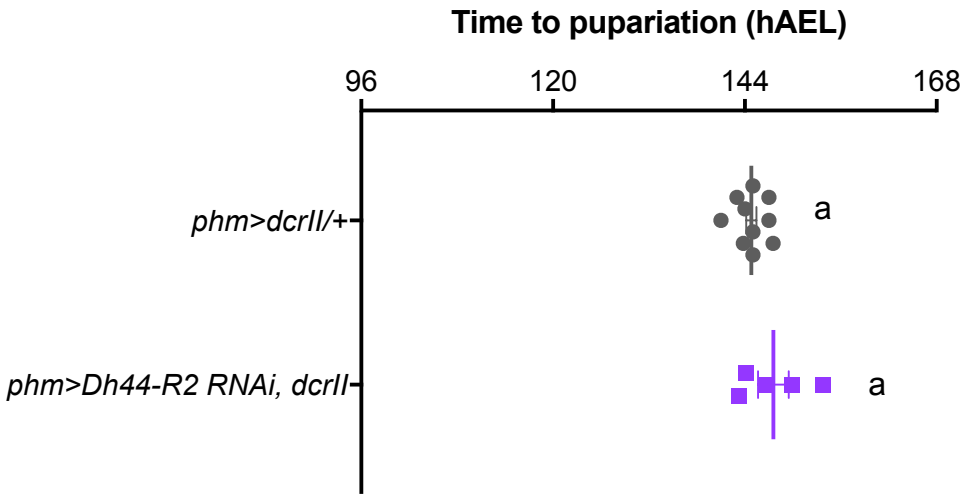
4.5.5. Immunocytochemistry

For PG morphology studies, wandering larvae from each genotype were collected and ring gland-brain complexes were dissected and fixed in 4% formaldehyde in PBS for 45mins at room temperature. Samples were then washed in PBTx (0.01% Triton-X in Phosphate Buffered Saline (PBS)) and blocked for 30mins in 5% goat serum in PBTx and rabbit anti-*dib* (a gift from Prof. Michael O-Connor, originally from Parvy et al., 2005, 1:500), was added to 5% goat serum in PBTx. Samples were allowed to incubate at 4 degrees overnight. We then washed the samples in PBTx, and anti-rabbit Alexa 488 (Invitrogen, 1:500) in 5% goat serum

in PBTx was added in the dark and allowed to incubate for 1.5 hours at room temperature. Samples were then washed and incubated in DAPI (1:400 PBTx) for 2 minutes. After washing in PBTx, samples were washed and stored in Vectashield (Vector laboratories) before further dissection onto poly-L-lysine coated coverslips and analysed using confocal microscopy. PG surface area was measured using Fiji.

For *dh44* expression studies, *dh44*-Gal4 was crossed to UAS-GFP, and progeny were allowed to develop until the wandering stage. Anterior halves of the larvae were dissected and fixed for 30 minutes in 4% formaldehyde in PBTx. Samples were washed 4 times over one hour in PBTx and then incubated in 50ul RNAase for 20 minutes. Samples were incubated in DAPI (1ul in 400µl PBTx) for 2 minutes, and washed in PBTx. Samples were stored in Vectashield (Vector laboratories) and PGs were dissected onto coverslips coated in poly-L-lysine under a light stereomicroscope in PBS. Dissected PGs were mounted onto a slide and were visualised using confocal microscopy at 40x and 60x magnification (Olympus CV1000).

4.6. Supplementary Materials



Supplementary Figure 4.1: Knockdown of *Dh44-R2* specifically in the PG results in normal developmental timing

When *Dh44-R2* is knocked down specifically in the PG (using *phm*-Gal4), animals are able to pupariate normally. hAEL= hours after egg lay. Error bars represent ±1 SEM for all graphs. Genotypes sharing the same letter indicate that they are statistically indistinguishable from one another (P>0.05, ANOVA and pairwise *t* tests). Each point represents a biological replicate of 15-20 animals and no fewer than 100 individuals were tested per genotype.

Chapter 5:

General discussion and Final conclusions

In *Drosophila*, developmental transitions are regulated by specifically-timed pulses of the steroid hormone, ecdysone. These pulses are produced in response to several environmental cues such as photoperiod and nutrition (Selcho *et al.*, 2017, Shimell *et al.*, 2018, McBrayer *et al.*, 2007, Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005), which are relayed to the ecdysone-producing PG via the action of neuropeptides and their receptors. While previous studies have shown that the PTTH signalling pathway (McBrayer *et al.*, 2007, Rewitz *et al.*, 2009) and the insulin signalling pathway (Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005) are important for regulating developmental timing via control of ecdysone production, the results presented in this thesis have demonstrated that more neuropeptide signalling pathways function in the *Drosophila* PG than previously thought. This highlights that there is still much to learn about how neuropeptides regulate developmental timing, and raises the question as to why there is a need for so many pathways to regulate the production of the same hormone.

5.1 Multiple environmental signals regulate ecdysone production

The PG produces and secretes ecdysone in response to various environmental cues. As previously mentioned, photoperiod, tissue damage, and nutrition are known to regulate the production of ecdysone in the PG (Selcho *et al.*, 2017, Jaszczak *et al.*, 2016, Shimell *et al.*, 2018, Colombani *et al.*, 2012, McBrayer *et al.*, 2007, Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Mirth *et al.*, 2005). However, there are also other environmental factors known to regulate developmental timing, such as oxygen, temperature and larval density – the number of larvae present in a defined area of space (Callier *et al.*, 2013, French *et al.*, 1998, Ghosh *et al.*, 2013, Henry *et al.*, 2018). Presumably, these additional environmental conditions also elicit their effects on developmental timing by altering the production of ecdysone, however the underlying molecular pathways which relay this type of environmental information to the PG are unknown. Given that many neuropeptide signalling pathways are known to respond to environmental signals, it seemed plausible that screening their function in the PG might reveal new pathways that communicate environmental information to the PG to regulate ecdysone production. While other screens and transcriptome studies of the ring gland have identified suites of genes that regulate ecdysone synthesis (Danielsen *et al.*, 2016, Christesen *et al.*, 2017, Ou *et al.*, 2016), this is the first study to focus specifically on uncovering new neuropeptide signalling pathways in the PG.

From the RNAi screen conducted in Chapter 2, it was found that individually knocking down eight neuropeptide receptors specifically in the PG resulted in developmental delays or lethality, suggesting a potential function for these receptors in ecdysone synthesis. While the roles of some of these receptors in the PG were further validated, the roles of NFPR and Dh44-R1 in the PG were extensively explored. NPFR appears to be regulating ecdysone production in response to nutrition (Chapter 3). Other genes from the screen have been implicated in detection of other environmental cues in different contexts. For example, Dh44/Dh44-R1 signalling has been implicated in regulating osmotic balance (Cabrero *et al.*, 2002, Hector *et al.*, 2009). Therefore, it is possible that Dh44/Dh44-R1 signalling controls the production of ecdysone in response to water levels within the fly. As water is presumed to be critical for animal growth, it certainly seems possible that an animal should only be able to progress to the next developmental stage if it has acquired the appropriate amount of water. In this manner, it is possible that the other receptors identified from this screen respond to specific environmental cues to regulate ecdysone production in the PG.

5.2. Regulation of ecdysone production by nutrition may require several peptide signalling pathways

Nutrition itself is known to regulate ecdysone synthesis by regulating insulin secretion. Insulin secretion itself responds to the levels of stored nutrients. While the Dilps have been shown to be important in regulating the production of ecdysone in response to nutrition (Caldwell *et al.*, 2005, Colombani *et al.*, 2005, Rewitz *et al.*, 2009, Mirth *et al.*, 2005), the data presented in Chapter 3 suggests that they may not be the only peptides that do so. The findings in Chapter 3 demonstrated that NPFR signalling is sensitive to nutrition, and that it negatively interacts with the insulin signalling pathway in the PG to regulate the production of ecdysone.

Like insulin signalling, NPFR signalling is known to regulate feeding behaviour across species (Wu *et al.*, 2003, Wu *et al.*, 2005, Mercer *et al.*, 2011). Interestingly, three other receptors identified from the RNAi screen (Chapter 2) bind to peptides that have also been implicated with roles in regulating feeding behaviour in larvae: AstA-R2, CCKLR-17D1, and sNPFR (Wang *et al.*, 2012, Söderberg *et al.*, 2012, Lee *et al.*, 2004). However, additional evidence is required to confirm roles for these genes in regulating ecdysone production, and

to demonstrate that the regulation of feeding behaviour and ecdysone synthesis might be well integrated by several peptide hormones.

Given that the aforementioned receptors regulate feeding behaviour in response to nutritional conditions, it is possible that they also regulate ecdysone production in response to different nutritional cues. To explore this hypothesis, nutrition itself can be broken down into several components such as water, salt, sugar, protein, and metals/vitamins. There are also other aspects of nutrition including food quality, food quantity, preferred foods etc. Therefore, it is possible that these other neuropeptide signalling pathways respond to these different aspects of nutrition. As the insulin signalling pathway responds to nutrition in the PG, these other peptide pathways may exert their effects on ecdysone production by interacting with the insulin signalling pathway, such as found in this study for NPFR signalling (Figure 5.1). Alternatively, it is also possible that they directly regulate ecdysone production. In any case, as it is vital that the animal receives all the required nutrients necessary for the morphological changes associated with developmental transitions, perhaps several nutrient-sensitive pathways are needed to fine-tune the insulin signalling pathway in the PG to tightly control the production of nutrient-sensitive ecdysone pulses.

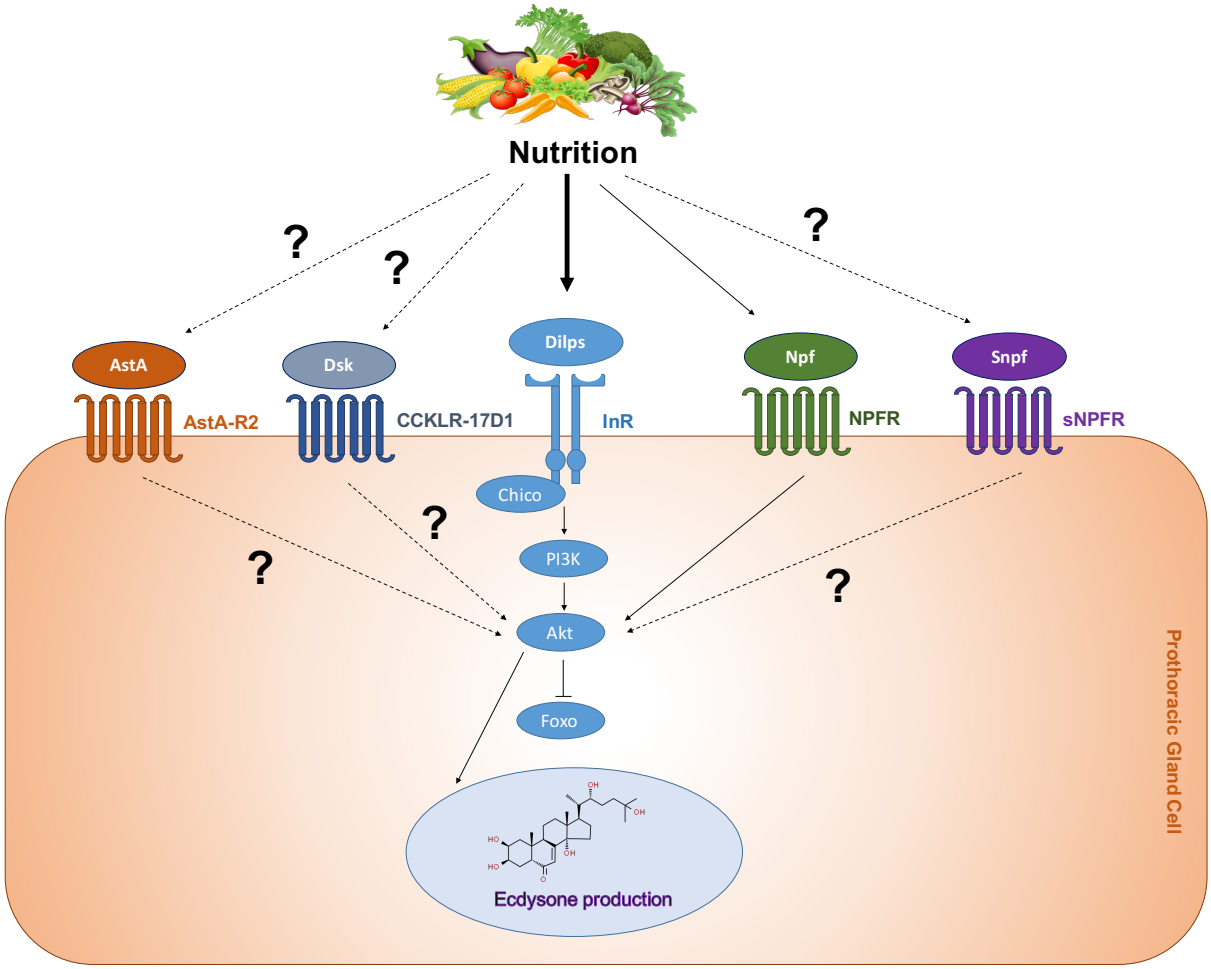

Nutrition
?
?
?
AstA
Dsk
Dilps
Npf
Snpf
AstA-R2
CCKLR-17D1
InR
NPFR
sNPFR
Chico
?
?
?
PI3K
Akt
Foxo
Ecdysone production
Prothoracic Gland Cell

Figure 5.1: Model for how neuropeptide pathways implicated in feeding behaviour might regulate ecdysone production via by responding to nutritional cues

Nutritional conditions dictate various feeding behaviours. Such feeding behaviours are under the control of four neuropeptide receptor signalling pathways (AstA-R2, CCKLR-17D1, NPFR and sNPFR) that were identified in the neuropeptide receptor screen (Chapter 2). Allatostatin A (AstA) binds to Allatostatin A Receptor 2 (AstA-R2) and is known to regulate foraging behaviour (Wang *et al.*, 2012), Drosulfakinin (Dsk) binds to Cholecystokinin-like receptor at 17D1 (CCKLR-17D1) and acts as a satiety signal in larvae (Söderberg *et al.*, 2012), Neuropeptide F (Npf) binds to Neuropeptide F Receptor (NPFR) and regulates food choice (Wu *et al.*, 2005) and short neuropeptide F (Snpf) binds to Short neuropeptide f receptor (sNPFR) and regulates food intake (Lee *et al.*, 2004). Given that nutrition regulates their roles in feeding behaviour, it is therefore possible that nutrition also regulates their function in the PG. If this is the case, these peptide signalling pathways may exert their effects on ecdysone production by interacting with the nutrition-sensitive insulin signalling pathway. Dotted arrows indicate potential interactions while solid arrows indicate known interactions.

5.3. Do multiple redundant or distributed signalling networks regulate ecdysone production?

This work has provided evidence that several neuropeptide signalling pathways that had not been previously shown to regulate ecdysone production, function in the *Drosophila* PG. At least two possible hypotheses could explain how these pathways may work together with the previously described PTTH and insulin signalling pathways to regulate ecdysone production. One is that they may work via a multiple redundant signalling network, where all signalling pathways perform the same function, sharing partial redundancy with each other (Figure 2; Wagner, 2005, Félix and Wagner, 2008). In this manner, is it possible that a range of peptide signalling pathways independently control the production of ecdysone. Secondly, it is possible that multiple neuropeptide signalling pathways function in a distributed signalling network, whereby alternative neuropeptide signalling pathways feed information into "central" signalling pathways to regulate ecdysone synthesis (Figure 2; Wagner, 2005, Félix and Wagner, 2008). In this way, it seems likely that PTTH and the insulin signalling pathway act as the "central" pathways that regulate ecdysone production, given their importance and conserved function in doing so across several insects (for review, see Rewitz *et al.*, 2013). Previous evidence and the findings from this thesis suggest that both multiple redundant and distributed signalling networks function in the PG, and that the dominant signalling networks change throughout development.

Evidence supporting the multiple redundant signalling network theory comes from observations during early larval development. For example, if both the PTTH and insulin signalling pathways are abolished in the PG, animals still survive up until the last instar (Gibbens *et al.*, 2011). This suggests that other signalling pathways can control the ecdysone pulses that occur earlier in development. Similarly, this thesis has shown that knocking down *Dh44-R1* in the PG using a stronger driver resulted in lethality at either the first or second larval instar (Chapter 4). This data suggests that Dh44-R1 signalling is required for the earlier pulses of ecdysone that direct larvae to moult to second and third instar, as the animal is unable to reach the final larval instar when Dh44-R1 is absent in the PG. Furthermore, it has been shown that reducing insulin signalling delays the first and second larval instars, but more strongly impacts the third instar (Shingleton *et al.*, 2005). This data demonstrates that insulin signalling has a much more minor role earlier in development. These examples suggest a multiple redundant signalling network, as it appears that PTTH/insulin signalling

are not essential for the earlier pulses of ecdysone, and therefore cannot act as central pathways with which other peptide signalling pathways simply interact. Therefore, it is more likely that other peptide signalling pathways, such as the Dh44-R1 pathway, directly control the production of the earlier pulses of ecdysone. This type of signalling network for earlier ecdysone pulses is plausible as it would seem certain that these earlier developmental stages may prioritise different environmental conditions and, to accommodate this, different neuropeptide signalling pathways are needed to regulate these ecdysone pulses. In this way, it would also appear that the dominant pathways that regulate ecdysone synthesis change over time.

Later in development, it seems more likely that control of ecdysone production is regulated by distributed signalling networks. In this hypothesis, it is likely that the PTTH and insulin signalling pathways act as the "central" pathways that regulate ecdysone synthesis, and other neuropeptide signalling pathways may feed information into these pathways. In support of PTTH/insulin signalling acting as the central pathways during the third instar, abolishing the activity of both of these pathways results in lethality in the third instar (Gibbens *et al.*, 2011), suggesting they are crucial during this developmental stage. Furthermore, other neuropeptide signalling pathways which function in the PG during this stage appear to exert their effects on ecdysone production by interacting with either the PTTH or insulin signalling pathway. For example, the Lgr3 signalling pathway, known for responding to imaginal disc damage, ultimately communicates with the PTTH-signalling pathway to control ecdysone production (Jaszczak *et al.*, 2016). In a similar manner, this study demonstrated how NPFR signalling is able to negatively regulate the insulin signalling pathway in the PG during the third instar to control the production of ecdysone (Chapter 3). In this way, these ecdysone pulses produced during the third instar are likely regulated by distributed signalling networks.

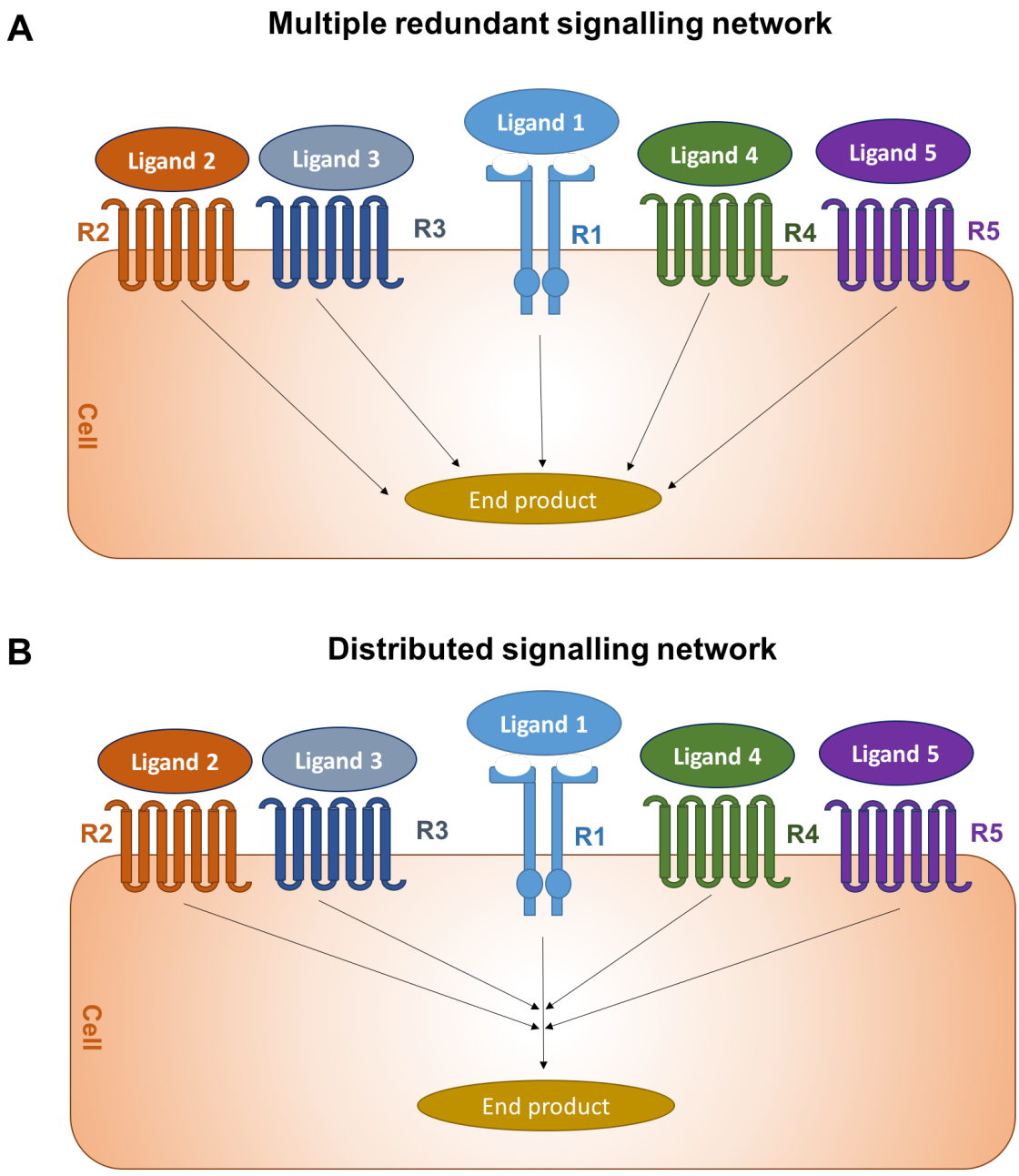


Figure 5.2: Multiple redundant signalling network vs. a distributed signalling network

There are two ways in which multiple signalling pathways could work together to achieve the same goal. **(A)** They may work in a multiple redundant signalling manner whereby all signalling pathways achieve the same function and share partial redundancy with each other. **(B)** Alternatively, in a distributed signalling network, multiple pathways feed information into one central pathway, which then goes onto achieve the end function (Wagner, 2005, Félix and Wagner, 2008). R = Receptor.

5.3.1. How do GPCR-activated signalling pathways regulate ecdysone production?

The neuropeptide receptors identified in the screen outlined in Chapter 2 all belong to the G-protein coupled receptor (GPCR) family. In order for these receptors to control ecdysone production, the signalling pathways they activate must ultimately regulate transcription factors responsible for activating ecdysone biosynthesis genes. While several transcription factors and complexes that regulate ecdysone biosynthesis genes have been identified, (Zeng *et al.*, 2018, Uryu *et al.*, 2018, Niwa and Niwa, 2016, Zhang *et al.*, 2018, Borsos *et al.*, 2015, Danielsen *et al.*, 2014), these are ones activated by Tor and InR, both receptor tyrosine kinases (RTKs). Whether the GPCRs identified here activate the same transcription factors remains to be elucidated. Understanding the molecular mechanisms by which GPCRs control the production of ecdysone is important as it can aid in understanding how ecdysone production is controlled, and will help determine if is via a multiple redundant signalling network or a distributed signalling network.

While it is unknown what specific transcription factors GPCR-activated signal transduction pathways regulate in the PG, what is known is that some protein kinases that are activated by GPCR-signalling pathways are also shared with RTK signalling pathways. GPCRs can activate different signal transduction cascades, depending on the individual GPCR, and more specifically, on the Gα subunit it activates (Bockaert and Pin, 1999, Gether, 2000). For example, $G\alpha_q$ signalling activates the ERK/MAP kinase phosphorylation cascade, just like the PTTH/Tor pathway (for review, see Luttrell *et al.*, 1999). In addition, the RTK insulin signalling pathway component, Phosphoinositide 3-kinase (PI3K), can also become activated via $G\alpha_s$ signalling (Nakano *et al.*, 2017). In this manner, it is possible that GPCR and RTK-activated signal transduction cascades may converge in the PG upstream of transcriptional activation, supporting a distributed signalling network (Figure 3).

Conversely, GPCR-activated signalling cascades can also activate factors that are not known to function in RTK-induced pathways. For example, as well as activating ERK/MAPK, the $G\alpha_q$ pathway can also result in an increase in cellular calcium levels (Figure 3). Calcium has shown to be important for ecdysone release (Yamanaka *et al.*, 2015), therefore in this manner, it is possible that GPCRs act in a multiple redundant fashion to control the secretion of ecdysone.

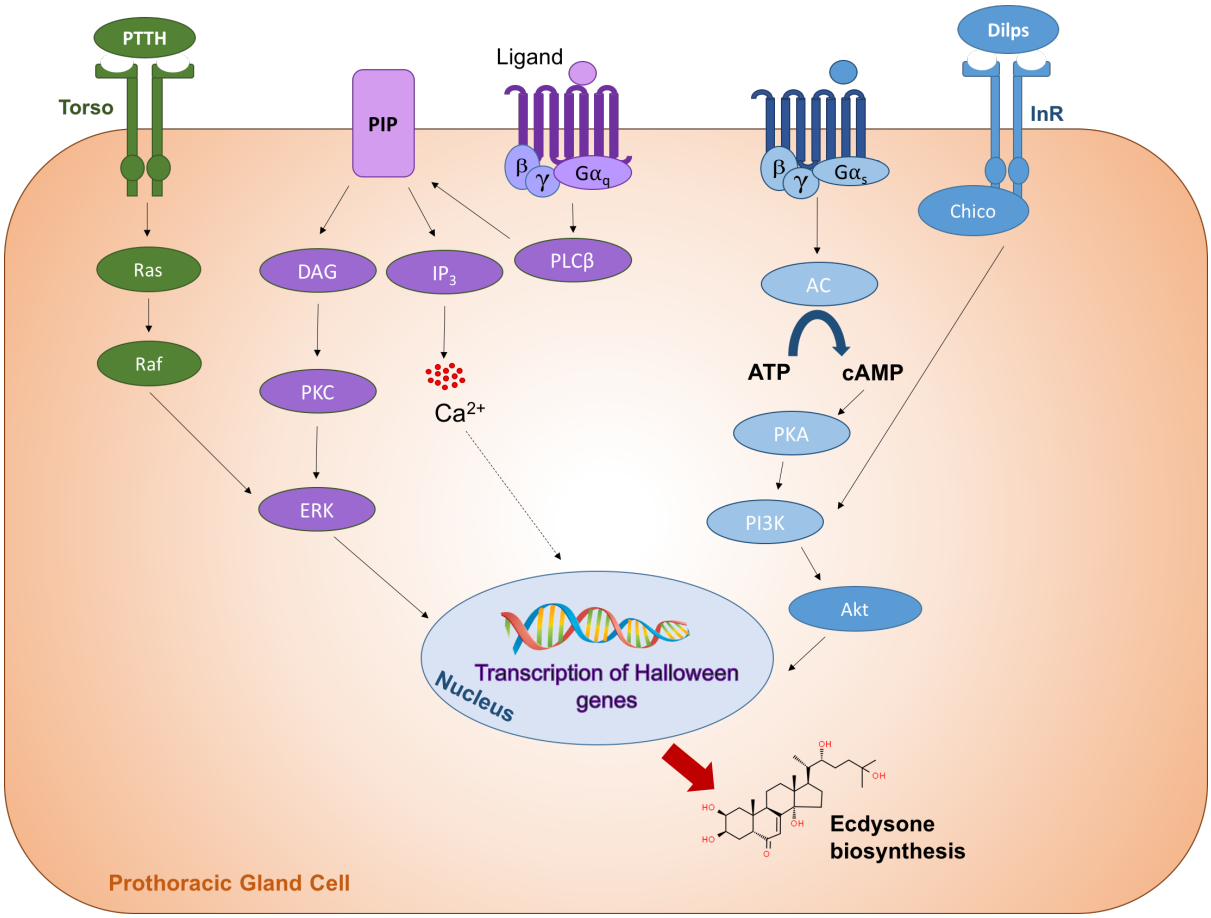

PTTH
Torso
PIP
Ligand
Dilps
InR
Chico
Ras
Raf
DAG
IP3
PLCβ
PKC
ERK
Ca2+
AC
ATP
cAMP
PKA
PI3K
Akt
Transcription of Halloween genes
Nucleus
Ecdysone biosynthesis
Prothoracic Gland Cell

Figure 5.3: Possible ways in which GPCR- activated signal transduction pathways may regulate the production of ecdysone in the PG

The $G\alpha_q$ second messenger pathway (purple) activates phospholipase C (PLCβ) which hydrolyses phosphatidylinositol 4,5-bisphosphate (PIP) into diacylglycerol (DAG) and Inositol trisphosphate (IP_3). DAG leads to the recruitments of Protein Kinase C (PKC) which in turn can activate ERK phosphorylation of transcriptional factors. Given that the PTTH pathway (green) can also trigger the ERK phosphorylation cascade, the PTTH and GPCR-induced pathways may converge to regulate the transcription of the same ecdysone biosynthesis genes (the Halloween genes), supporting a distributed network. Similarly, the $G\alpha_s$ second messenger pathway (blue) leads to an increase in cAMP via activation of adenyl cyclase (AC), which in turn can activate protein kinase A (PKA), which activates Phosphoinositide 3-kinase (PI3K), leading to the transcription of ecdysone biosynthesis genes. PI3K is also a component of the insulin signalling pathway (dark blue), so in this manner the $G\alpha_s$ and the insulin signalling pathways can converge to control the production of the Halloween genes, supporting a distributed model. Conversely, IP_3, induced by the $G\alpha_q$ pathway, also causes an increase in intracellular Ca^{2+} due to release from intracellular stores. This in turn can activate various kinases and phosphatases, leading to transcriptional control of ecdysone. In this manner, GPCR-induced pathways can directly activate the production of ecdysone via induction of their own second messenger pathway, supporting a multiple redundant pathway. For reviews, see Gether (2000), Luttrell *et al.* (1999).

5.4. Implications and Final Conclusions

In conclusion, this work has demonstrated that additional neuropeptide signalling pathways function in the *Drosophila* PG to regulate the production of ecdysone. The results presented in this thesis challenge the current model of how ecdysone is controlled by suggesting that several more peptide signalling pathways function in the PG than previously thought. Future work should focus on matching these neuropeptide signalling pathways to their environmental regulators to gain further insight into how many environmental factors regulate the production of ecdysone. Additionally, future experiments should aim to elucidate if different pulses of ecdysone are controlled by different peptide signalling pathways. This would help to distinguish whether these peptide signalling pathways function in multiple redundant or distributed networks, and when in development they do so.

Overall, the findings illustrated in this thesis have implications for research on steroid hormone synthesis in general. Steroid hormones are crucial regulators of growth across several species, and their dysregulation can lead to a variety of growth disorders. Therefore, understanding the molecular mechanisms by which steroid hormones are regulated aids in further characterisation of such disorders. Further to this, the added understanding this thesis has provided into insect development can be used to assist in developing novel pest-control strategies. In this manner, this thesis provides avenues for future research beyond the scope of *Drosophila* development.

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