



# AstA signaling functions as an evolutionary conserved mechanism timing metamorphosis and growth in *Drosophila Melanogaster*

Derya Deveci

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# THÈSE DE DOCTORAT

La voie de signalisation AstA, qui est conservée au cours de l'évolution, contrôle le déclenchement de la métamorphose et régule la croissance chez *Drosophila melanogaster*

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**Présentée en vue de l'obtention  
du grade de docteur en** Interactions  
moléculaires et cellulaires  
**d'Université Côte d'Azur**  
**Dirigée par :** Pierre Leopold  
**Soutenue le :** le 31 octobre 2018

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## Thèse

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## **AstA signaling functions as an evolutionary conserved mechanism timing metamorphosis and growth in *Drosophila Melanogaster***

Thesis advisor: Pierre Leopold

Defence on 31<sup>st</sup> of October 2018

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## Titre

La voie de signalisation de l'allatostatine A, conservée au cours de l'évolution, contrôle le déclenchement de la métamorphose et régule la croissance chez *Drosophila melanogaster*.

## Resume

La maturation sexuelle se fait en réponse à l'intégration de divers signaux internes homéostatiques et externes. Jusqu'à présent, on ignore en grande partie quels mécanismes sensoriels internes sont impliqués dans le couplage de ces signaux. Chez les mammifères, le début de la puberté est associé à des pulsations élevées de GnRH conduisant à un pic d'hormone stéroïdienne. Le système ligand/récepteur, KISS/KISSR, est un régulateur en amont des neurones producteurs de GnRH. Chez *Drosophila melanogaster*, un pic d'hormone prothoracicotrope (PTTH) produite par deux paires de neurones (PTTHn) conduit à la production de l'ecdysone, la principale hormone stéroïdienne chez les insectes. PTTH est l'un des premiers signaux à activer la cascade d'événements menant à la maturation. Si la production de PTTH est bloquée, un retard dans le début de la transition du stade juvénile au stade adulte est observé, tandis qu'une maturation précoce est observée lors de la surexpression de PTTH. Ceci indique donc un rôle important des PTTHn dans l'intégration des signaux. Afin de découvrir les signaux intégrés par les PTTHn, nous avons criblé une collection d'ARN interférents dans les PTTHn. Nous avons ainsi identifié le récepteur à l'allatostatine A (AstA-R1) comme un régulateur positif des PTTHn. La perte de fonction de AstA-R1 retarde la maturation avec une augmentation de la taille finale de l'organisme. Une réduction de la quantité du ligand allatostatine A (AstA) a également une incidence sur le déclenchement de la maturation. AstA est produite dans le cerveau par une paire de neurones bilatéraux qui étendent leurs axones vers les dendrites des PTTHn. De plus, les neurones AstA projettent également

leurs axones vers les cellules productrices d'insuline (IPCs), connues pour réguler le taux de croissance larvaire. L'inactivation d'AstA-R1 dans les IPCs donne des organismes plus petits. Nos résultats impliquent que les neurones AstA sont capables de réguler le rythme de croissance ainsi que le déclenchement de la maturation en jouant sur deux circuits différents ciblant les PTTHn et les IPCs. De façon intéressante, AstA/AstAR1 est homologue à KISS/KISSR (GPR54), un facteur d'entrée dans de la puberté humaine. Ceci suggère donc qu'un circuit neuronal est conservé au cours de l'évolution pour l'intégration des signaux qui contrôlent le déclenchement de la maturation sexuelle.

Mots clés: développement, maturation sexuelle, AstA, Kiss, Drosophila.

## Title

AstA signaling functions as an evolutionary conserved mechanism timing metamorphosis and growth in *Drosophila Melanogaster*

## Abstract

The onset of puberty occurs in response to the integration of various internal homeostatic and external signals. Up until now, it remains largely unknown which internal sensory mechanisms are involved in the coupling of those signals. In mammals, the onset of puberty is associated with elevated GnRH pulsations leading to a peak of steroid hormones. The KISS/KISSR system is a pivotal upstream regulator of GnRH producing neurons. In *Drosophila melanogaster* a peak of prothoracicotrophic hormone (PTTH) produced by two pairs of neurons (PTTHn) leads to the production of the insect steroid hormone ecdysone. PTTH is one of the first signals to activate the cascade of events leading to maturation. Once PTTH production is blocked, a delay is observed in the onset of the transition from juvenile to adult stage, whereas precocious maturation is observed upon PTTH over-expression, denoting an important role for PTTHn in the integration of cues. In order to uncover signals integrated by PTTHn we have conducted a biased RNAi screen in PTTHn. After two rounds of screening we identified the GPCR Allatostatin A receptor 1 (AstA-R1) as a positive regulator of PTTHn. AstA-R1 knock down delays maturation with a subsequent increase in final pupal size. Down regulation of its ligand, Allatostatin-A (AstA) on the brain is also affecting the timing of maturation. We found that AstA is produced in the central brain by a bilateral pair of neurons that extend their axons towards the PTTHn dendrites. In addition, AstA neurons also project their axons towards the *Drosophila* insulin producing cells (IPCs) that are known to regulate larval growth. Knockdown of AstA-R1 on the IPCs leads to smaller pupae. These findings imply that the AstA neurons are able to regulate growth and maturation timing by interacting with 2 different circuits: the

PTTHn and IPCs. Unexpectedly, AstA-R1 and AstA genes share a common evolutionary origin with KISSR and KISS, respectively, suggesting a common mechanism between insects and mammals for the integration of signals that control the onset of puberty.

Key words: development, sexual maturation, AstA, Kiss, Drosophila

*Sealed by a KISS..*



*'The KISS1 gene was discovered and named to the location of where it was discovered (Hershey, Pennsylvania, home of Hershey's Kisses)'*

September 2018

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## Abbreviations

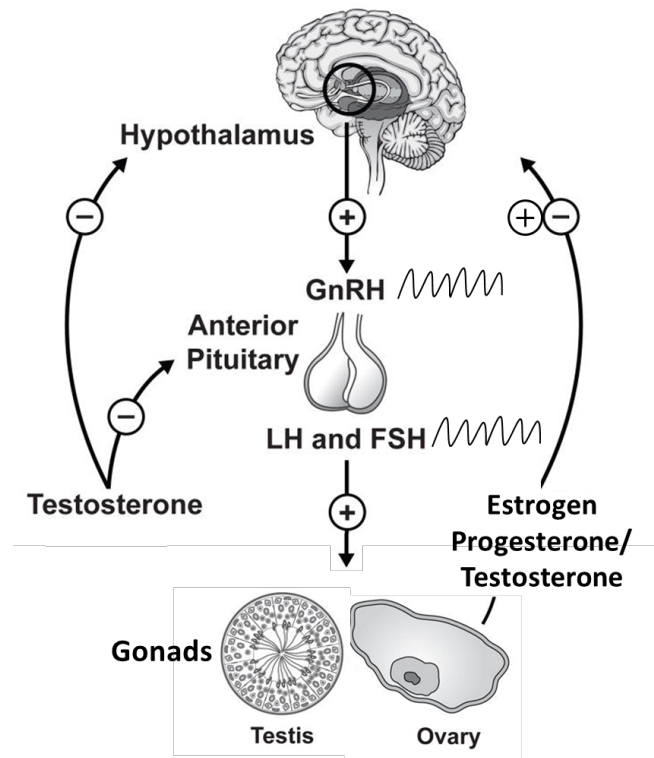
20-E	20-hydroxyecdysone
ACPR	AKH/CRZ-related peptide
AKH	adipokinetic hormone
AKHR	adipokinetic hormone receptor
ARC	hypothalamic arcuate nucleus
AstA	allatostatin- A
AstA-R1	allatostatin- A receptor 1
AVPV	anteroventral periventricular nucleus
CA	corpora allata
CC	corpora cardiaca
CPP	central precocious puberty
CRZR	corazonin receptor
DHR4	<i>Drosophila</i> hormone receptor 4
Dilp	<i>Drosophila</i> insulin-like peptides
EcR	Ecdysone receptor
EEDCs	estrogen-like endocrine disrupting chemicals
ER $\alpha$	estrogen receptor $\alpha$
ERK	extracellular signal-regulated kinase
FSH	follicle stimulating hormone
GCL	growth coordinating Lgr3 (GCL) neurons
GFP	green fluorescent protein
GH	growth hormone
GnRH	gonadotropin-releasing hormone
HH	idiopathic hypogonadotropic hypogonadism
IIS	insulin/insulin-like growth factor (IGF)-like signaling
InR	insulin receptor
IPCs	insulin- producing cells
JH	juvenile hormone
Kiss	kisspeptin
LH	luteinizing hormone
PG	prothoracic gland
PGS	pubertal growth spurt
PIP2	phosphatidylinositol bisphosphate
PLC	phospholipase C
PTTH	prothoracicotropic hormone
UAS	Upstream Activation Sequence

## 1. General introduction

An emerging trend in human physiology in the past decade is a decline in the average age of puberty onset. Whereas the average age in the USA used to be 14.2 in 1900, it dropped to 12.5 in the year 2000 (Bellis, Downing, and Ashton 2006). When children undergo puberty earlier, especially when compared to their peers, they could be more prone to psychopathologies such as depression that can persist into adulthood. Another possible health risk that is linked to precocious pubertal onset is the increased risk of breast cancer development in woman (Bodicoat et al. 2014). General improvements in health care and child nutrition could act as contributors to accelerate puberty. However, other factors such as obesity, increased animal protein, high dairy consumption and exposure to estrogen-like endocrine disrupting chemicals (EEDCs) have been reported to have a positive correlation with accelerated puberty onset (Berkey et al. 2000; Günther et al. 2010; W. Li et al. 2017; Roy, Chakraborty, and Chakraborty 2009). Due to the health risks associated with aberrant timing of puberty, it is important that puberty occurs at the right time. Therefore it is of fundamental interest to identify the underlying signals that interplay in pubertal onset to understand how puberty is triggered. Indeed, in the last decades, several permissive factors like leptin and estrogen have been identified that allow puberty to occur or to progress. Puberty onset is a complex process wherein multiple signals are integrated. Thus, rather than the existence of having one trigger signal, puberty seems to be the consequence of a large number of integrative factors. The identification of novel signals could therefore allow us to understand the emerging trend of early puberty.

## 1.1 Puberty is induced by steroid production

Puberty is the developmental transition of juveniles into sexually mature adults. Sexual maturation is indispensable for reproduction and is therefore tightly controlled by a sophisticated system that stems from an interplay of a complex network of signals integrated in the brain. The so called hypothalamic-pituitary-gonadal axis (HPG-axis) is the regulatory mechanism controlling the onset of sexual maturation relying on the intercommunication of these organs (**Fig. 1**). At the very top of this axis is gonadotropin-releasing hormone (GnRH) produced by GnRH producing neurons within the hypothalamus. GnRH is released in the hypophyseal portal bloodstream in the brain in a pulsatile manner and binds to its receptor GnRHR in the anterior pituitary. This subsequently stimulates the gonadotropes to release two gonadotropins: luteinizing hormone (LH) and follicle stimulating hormone (FSH). The pulsatile release of LH and FSH subsequently activates the gonads (testis and ovaries) to release the sex steroids estrogen, progesterone and testosterone to mature the gonads, inducing puberty. At the same time, estrogen and testosterone signal back to the hypothalamus to generate positive and negative regulatory feedback signals for the HPG- axis. Further details about these feedback regulatory mechanisms will be addressed in Chapter 1.7.



**Figure 1. Schematic of the hypothalamic-pituitary-gonadal axis.** During pubertal onset, gonadotropin-releasing hormone (GnRH) is produced from GnRH neurons in the hypothalamus and secreted in a pulsatile manner to stimulate the production and pulsatile release of LH and FSH from the pituitary gland. LH and FSH then activate the gonads (testis and ovaries) to produce sex steroid hormones, testosterone, estrogen and progesterone to mature the sexual organs and to provide feedback regulatory loops back to the hypothalamus. Adapted from (Bodicoat et al. 2014)

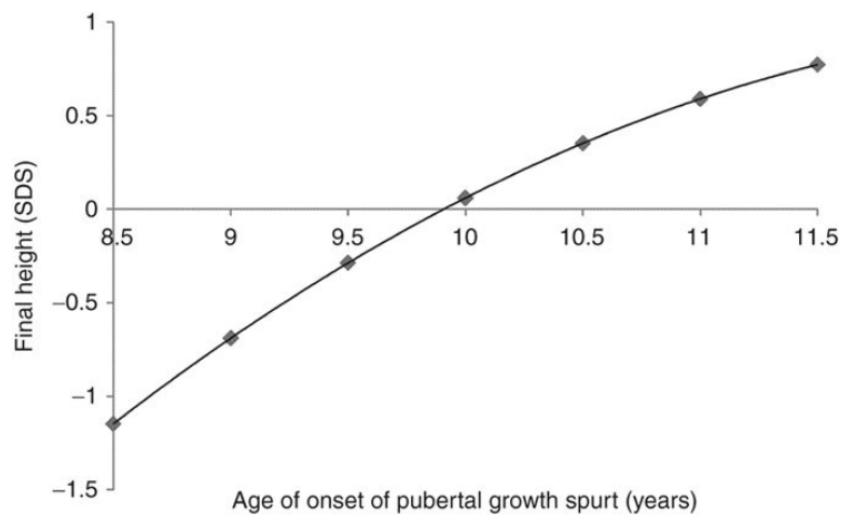
### 1.3 The effect of the time of pubertal onset on adult size

Humans are one of the few mammals that undergo a major pubertal growth spurt (PGS) during adolescence. After puberty, adolescents can still grow in height for up until an average of 2 years during this PGS phase. Statural growth, or growth in height is the result of elongation of long bones due to cartilage formation at the epiphyseal plates (growth plate). During puberty onset, growth velocity increased and decreases at late puberty when the growth plates fuse, halting bone growth (Shim 2015). The exact molecular mechanism underlying the fusion of

growth plates remains elusive since it is likely to involve interplay of multiple neuroendocrine factors. For instance, the sex steroid estrogen induces growth hormone (GH), that in turn stimulates the production of insulin-like growth factor-I (IGF-I). Both GH and IGF-1 function in bone growth. It is suggested from mice studies, however, that low doses of estrogen during early puberty is important for the growth spurt mediated by GH and IGF-1, whereas the high levels of estrogen signal to the growth plate cartilage to induce growth plate fusion at late puberty (Börjesson et al. 2013).

It has therefore been proposed that precocious puberty could have a negative correlation with total height gain due to early fusion of the growth plate. Conversely, adolescents that undergo delayed puberty would likely have increased height gain. Clinical reports, however, claimed the opposite effect, suggesting that adolescents undergoing puberty early undergo their PGS earlier, that in turn also lasts longer. This would result in an increase in total height gain. On the contrary, adolescents that undergo puberty at a late age would delay their PGS that would also last shorter, leading to lesser height gain. Nevertheless, these same studies have remarked that similar adult height is reached. Interestingly, a more recent mathematical approach revealed no correlation between final height and age of puberty onset from a linear regression analysis based on an observational retrospective study (Limony, Koziel, and Friger 2015). However, when the authors took into account the initial height of PGS onset, there was a significantly high correlation between final height and PGS onset (**Fig. 2**). These findings suggests that when adolescents with the same height at PGS, the absolute loss in the final height gain is greater in adolescents that undergo maturity earlier versus adolescents that undergo maturity later, both in girls and boys. These reports together highlight the controversy in the field as well as the complexity of the exact mechanism behind the time of pubertal onset, statural growth and final adult height.





**Fig. 2 Mathematic regression analysis of the relationship of final height and average age of pubertal growth spurt in girls.** The difference of final height corresponding to the average of pubertal onset is depicted as height- standard deviation score (SDS). It is assumed that the SDS value at pubertal onset is zero. When girls undergo pubertal growth spurt (PGS) at a young age, the final height obtained as an adult will be lower compared to girls that undergo PGS later. Adapted from (Limony, Koziel, and Friger 2015)

## 1.2 GnRH regulates pubertal onset

The discovery of gonadotropin- release hormone (GnRH) in neuroendocrinology as a gatekeeper of puberty was recognized by a Nobel Prize of Medicine in 1977. Mutations in either GnRH or in GnRHR have been implication in causing a large number of serious congenital defects, including hypogonadotropic hypogonadism (HH), as reviewed in (Maggi et al. 2016). This condition is defined by disrupted gonadal function due to an absence or impaired secretion of GnRH, that can result in absent, or delayed puberty.

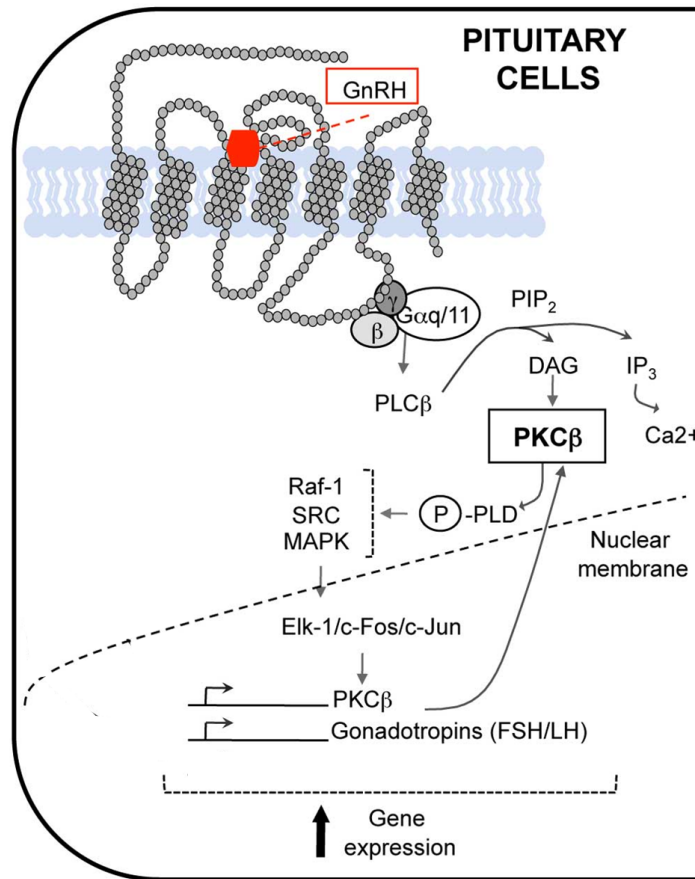
GnRH is synthesized from a large precursor consisting of 92 amino acids that is enzymatically cleaved into multiple transcript variants. GnRH is a small 10 amino acid long neuropeptide with a short half life of a few minutes due to peptidase cleavage (Wetsel and

Srinivasan 2002). GnRH is produced by GnRH neurons located in the arcuate nucleus of the medial basal hypothalamus. GnRH neuronal activity is already detectable at late gestation, or around the time of birth, when GnRH secretion is very high, before it drastically drops during juvenile stages (T M Plant 1982). It is only until the pre- pubertal stages when GnRH neurons become reactivated again, secreting GnRH in a pulsatile manner after which a large surge is observed during puberty onset, which is the hallmark of pubertal onset.

GnRH is secreted and binds to its receptor GnRHR, which is a rhodopsin-like G protein-coupled receptor located in the pituitary gonadotropin cells (Chi et al. 1993). Once activated, GnRHR undergoes a conformational change, activating phospholipase C (PLC) that in turn transmits its signal to diacylglycerol (DAG) and inositol 1,4,5-trisphosphate (IP3) (**Fig. 3**) (Kraus, Naor, and Seger 2001). Next, IP3 stimulates the release of  $\text{Ca}^{2+}$ , that in turn binds and activates Calmodulin (CaM) and then  $\text{Ca}^{2+}$ /calmodulin-dependent protein kinase (CaMK) to induce gonadotropin gene expression and secretion. In parallel, DAG activates the intracellular protein kinase C (PKC) pathway, including the mitogen-activated protein kinases (MAPKs), including extracellular-signal-regulated kinase (ERK) and p38 MAPK that ultimately translocate to the nucleus to induce transcription factors to activate gene expression of gonadotropins (LH/FSH) in the pituitary gonadotrope cells.

GnRH pulsatile activity is important for subsequent pulsatile release of FSH and LH. High frequency GnRH release is important for LH pulses, whereas low frequency GnRH pulsations stimulate FSH release. Prolonged high- dose secretion of GnRH results in loss of GnRHR response caused by rapid uncoupling of GnRHR from intracellular signaling molecules and subsequent reduction in GnRHR mRNA levels (Olefsky and Webster 2010). Therefore, GnRH pulsations are under tight regulation by multiple positive and negative cues . Two major neurotransmitters modulate GnRH neuronal excitation and synchronous GnRH release: Gamma-amino butyric acid neurons (GABA) and glutamate. Both inputs of GABA and

glutamate trigger  $\text{Ca}^{2+}$  oscillations by acting through their receptors,  $\text{GABA}_A\text{R}/\text{GABA}_B\text{R}$  and AMPA/kainate glutamatergic receptors, respectively (Moore, Shang, and Wray 2002). Other important regulators of GnRH are estrogen and progesterone originating from the gonads, to provide both negative and positive feedback signals. These signals, however, do not directly signal to GnRH since their receptors are not expressed in GnRH. Instead, these steroids signal to neighboring intermediary neurons to exert their modulatory effects in GnRH secretion (such as glutamate-, GABA-, or Kiss-neurons). GnRH itself is also able to regulate its own secretion through autocrine signaling via its receptor GnRHR (Krsmanovic et al. 1999). The main synchronizing regulator of GnRH pulsations, however, is agreeably the small neuropeptide kisspeptin that is produced by Kiss neurons. It is proposed that Kiss neurons are responsible not only for the activation of GnRH neurons to induce pulsatile GnRH release, but also to induce the large GnRH surge during puberty.



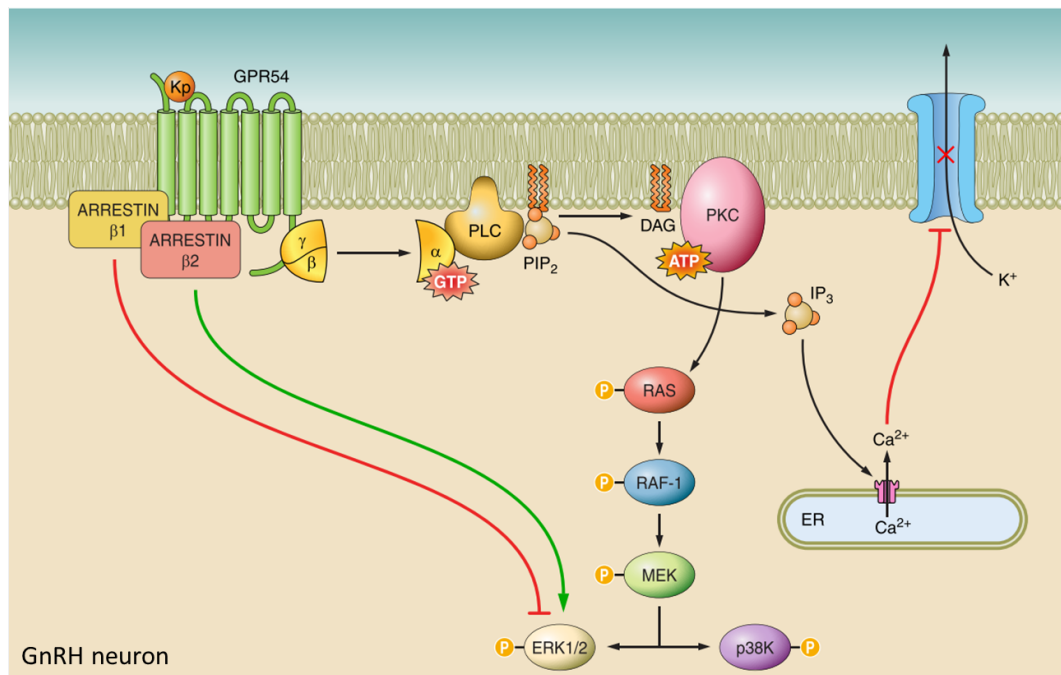
**Fig 3. GnRHR signal transduction pathway.** GnRH binds to GnRHR which is a seven transmembrane  $G_{\alpha q/11}$ -coupled receptor. Once activated, it activates phospholipase C $_{\beta}$  (PLC $_{\beta}$ ), converting converting phosphatidylinositol bisphosphate (PIP $_2$ ) into inositol 1,4,5-trisphosphate (IP $_3$ ), causing  $Ca^{2+}$  mobilization. PIP $_2$  also induces diacylglycerol (DAG) that activates the intracellular protein kinase C (PKC) pathway, leading to the phosphorylation of Raf-1, protein tyrosine kinase SRC and MAPK. This ultimately leads to the phosphorylation of Elk-1, c-Fos and c-Jun, inducing transcription of the gonadotropins follicle-stimulating hormone (FSH) and Luteinizing hormone (LH) as well as PLC $_{\beta}$ . Adapted from (Aguilar-Rojas, Pérez-Solis, and Maya-Núñez 2016)

### 1.3 Kiss/GPR54 signaling times puberty

Kisspeptins were originally discovered in 1996, owing its name to its discovery in Hershey, Pennsylvania, home to the famous Hershey's chocolate brand. The gene encoding for kisspeptins, *KISS1*, is an important regulator of GnRH secretion, both in the pulsatile behavior during pre- pubertal stages as well as the preovulatory gonadotropin surge during puberty onset. In mice models, inactivating mutations in *KISS1* or in its receptor *GPR54* results in failure of gonadal maturation that is associated with low FSH and infertility (Lapatto, Pallais, Zhang, Chan, Mahan, Cerrato, Wei, et al. 2007). In humans, inactivating mutations in *KISS1* or *GPR54* can cause idiopathic hypogonadotropic hypogonadism (HH), which is a disorder characterized by delayed puberty, or a complete absence of puberty (Seminara et al. 2003; Topaloglu et al. 2012). On the contrary, central precocious puberty (CPP) has been observed with a *GPR54* activating mutation (Arg386Pro), due to prolonged activation of intracellular signaling pathways revealed by *in vitro* studies (Trarbach et al. 2008).

The *KISS1* gene encodes for a precursor peptide of 145 amino acids long that is subsequently cleaved and processed to give rise to four kisspeptin: Kp-54, Kp-14, Kp-13 and Kp-10. Kisspeptins are produced by kisspeptin neurons (*Kiss1* neurons) located in the hypothalamic brain nuclei (Brock and Bakker 2013; Clarkson and Herbison 2006; Smith et al. 2005). All four kisspeptin peptides possess an Arg-Phe-NH<sub>2</sub> motif on the C-terminal, that allows them to bind to their receptor, GPR54/KISS1R that is expressed in the hypothalamus and in a number of other organs. GPR54 is a member of rhodopsin family and is a seven transmembrane G<sub>q/11</sub>-coupled receptor. Once bound to kisspeptin, phospholipase C (PLC) is activated, subsequently converting phosphatidylinositol bisphosphate (PIP<sub>2</sub>) into inositol 1,4,5-trisphosphate (IP<sub>3</sub>) (**Fig. 4**) (Castaño et al. 2009). This results in the immobilization of Ca<sup>2+</sup> from the endoplasmic reticulum (ER), causing changes in ion channel permeability, inducing depolarization. In parallel, the rise of PIP<sub>2</sub> also leads to diacylglycerol (DAG) formation,

inducing protein kinase C (PKC) which phosphorylates mitogen-activated protein kinases (MAPKs), Ras, Raf and MEK, to induce phosphorylation of ERK1/2 and p38. Since pharmacological blockade of ERK1/2 and p38 kinase reduces kisspeptin- induced GnRH secretion, it is proposed that not only  $\text{Ca}^{2+}$  mediated depolarization is required for GnRH secretion but also the recruitment of ERK1/2 and p38 through the MAPK pathway. (Castellano et al. 2006). In addition, kisspeptin and GPR54 modulate proliferation and migration in a tumoral setting since GPR54 signaling also plays role in metastasis suppression (Cho et al. 2012).



**Fig 4. Kiss/GPR54 signaling in GnRH neurons.** Kisspeptin (Kp) binds to the seven transmembrane G<sub>q/11</sub>-coupled receptor GPR54. Upon Kp binding, phospholipase C (PLC) becomes activated, converting phosphatidylinositol biphosphate (PIP<sub>2</sub>) into inositol 1,4,5-trisphosphate (IP<sub>3</sub>), causing  $\text{Ca}^{2+}$  mobilization from the endoplasmic reticulum (ER). PIP<sub>2</sub> also induces diacylglycerol (DAG), activating intracellular protein kinase C (PKC) leading to the phosphorylation of Ras, Raf-1 and MEK. This ultimately leads to the phosphorylation of ERK1/2 and p38 kinase (P38K). In parallel, GPR54 activation induces arrestin-β1 and -β2, which provide negative and positive feedback on receptor regulation, respectively. Adapted from (Pinilla et al. 2012)

Even though *KISS1* and *GPR54* are obvious candidates in stimulating sexual maturation as described in a large number of studies conducted in rodents (Colledge 2008), a small number of studies reported variable hypogonadism phenotypes or a complete absence of phenotype in *KISS1*- and *GPR54*- knockout mice (Lapatto, Pallais, Zhang, Chan, Mahan, Cerrato, Le, et al. 2007; Mayer and Boehm 2011). This, however, could possibly be explained by residual activity in the HPG- axis, or compensatory mechanisms since acute disruption of Kiss neurons does alter fertility as described in the same study. These pathologies uncover the pivotal role of kisspeptin/GPR54 signaling for pubertal onset as well as for timing.

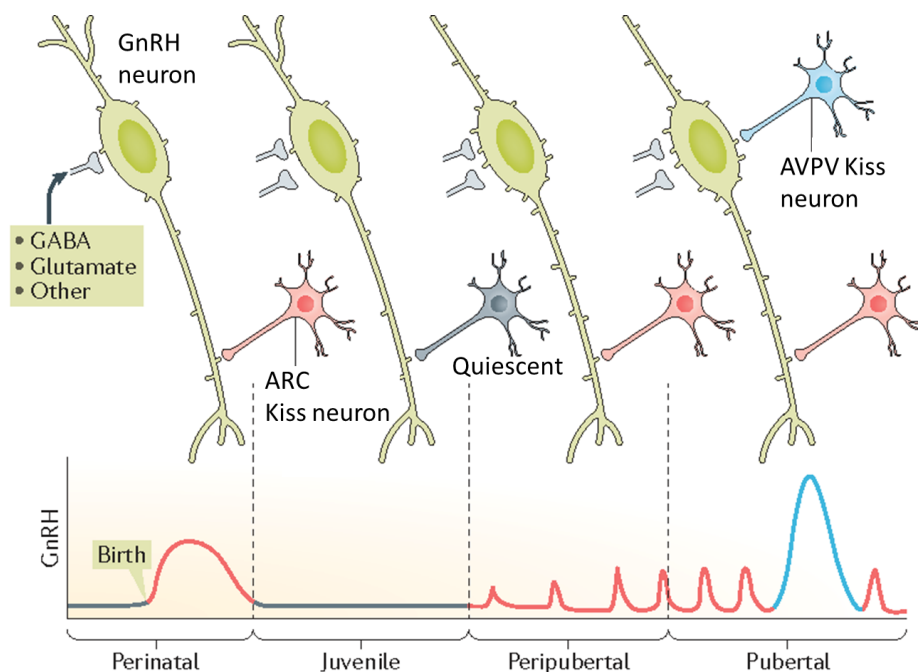
#### **1.4 Kiss/GPR54 signaling regulates GnRH pulsations during pubertal stages**

Kisspeptin plays a crucial role in controlling puberty through its regulatory function of GnRH pulsatile secretion (S.-K. Han 2005; K et al. 1999). Pulsatile secretion of kisspeptin induces the pulsatile behavior of GnRH secretion, that in turn regulates the pulsatile release of LH/FSH necessary for maturation of the sexual organs. It is known that disruptions in GnRH pulse frequencies are often associated with delayed, or precocious puberty (Balasubramanian et al. 2010). Similar to GnRH, kisspeptin secretion has a pulsatory behavior of interpulse interval of approximately 60 minutes that is correlated with subsequent LH pulses (Keen et al. 2008).

Kisspeptins are produced by kisspeptin neurons (Kiss neurons) that are subdivided into two populations, depending on the location in the rodent hypothalamic brain nuclei: hypothalamic arcuate nucleus (ARC) and anteroventral periventricular nucleus (AVPV) (Brock and Bakker 2013; Clarkson and Herbison 2006; Smith et al. 2005). It is proposed that the Kiss population in the ARC is more important in the robust pulsatile secretion of GnRH during pre-

pubertal stages, whereas the population in AVPV are more involved in the large preovulatory gonadotropin surge during puberty (**Fig. 5**).

The AVPV Kiss neuronal population increases towards early pubertal stages, which is proposed to increase kisspeptin- induced GnRH secretion. Rodent studies have revealed that the *KISS1* mRNA levels as well as the number of Kiss neurons in the AVPV increases towards late- juvenile stages (Bentsen et al. 2010; Clarkson and Herbison 2006). Moreover, a previous study observed significantly more Kiss neuronal fibers towards the GnRH neurons located in the preoptic area (POA), going in line with the previous findings that suggest Kiss neurons mature towards pre- pubertal stages in order to maximize kisspeptin- stimulated GnRH secretion (Clarkson and Herbison 2006).



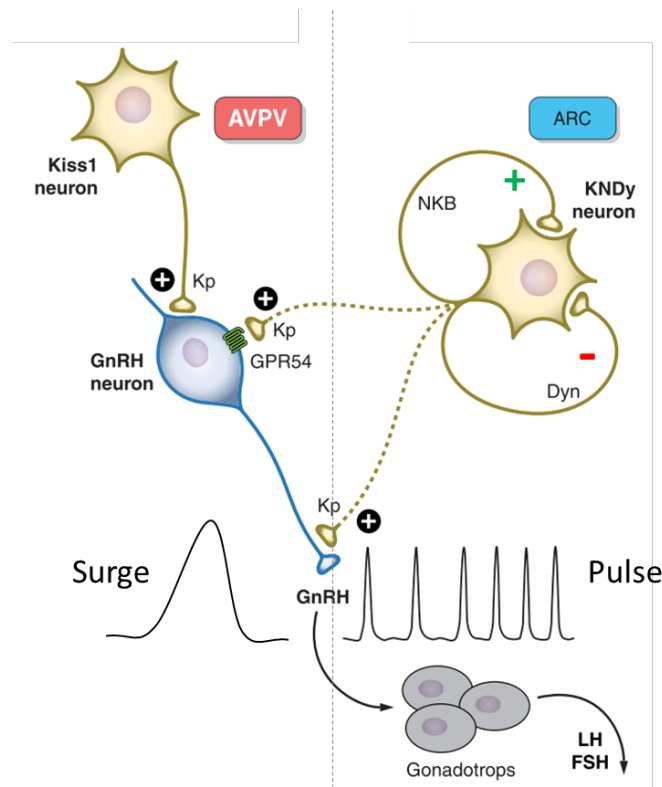
**Fig 5 Postnatal development of GnRH- and Kiss neuronal network.** GnRH secretion is high during birth, before drastically dropping during juvenile stages. GnRH neurons (green) receive inputs from the ARC Kiss neurons (red), as well as from other neurons (GABA, Glutamate). It is suggested that the ARC Kiss neurons become inactivated during early juvenile stages (in grey) before being re-activated again in pre- pubertal stages. During pre- pubertal stages, GnRH is released in a pulsatile manner. At the same time, the number of dendritic spines in the GnRH neurons increases. The GnRH surge occurs in the pubertal period, suggested to be enabled by the AVPV Kiss neuronal population (blue). Adapted from (Herbison 2016b)



The other Kiss neuronal population is located in the ARC and are also referred to as KNDy neurons, owing its name to the co-expression neurokinin-B/tachykinin-3 (NKB/TAC3) and dynorphin (Dyn) in these Kiss neurons (Goodman et al. 2007). NKB acts as a stimulator of kisspeptin release and subsequent GnRH secretion, acting through its receptor neurokinin-3 receptor/tachykinin receptor-3 (NK3R) that are also expressed on the ARC neurons (Amstalden et al. 2010). Similar to defects in *KISS1/GPR54*, inactivating mutations of human *NK3R* have been reported to cause delayed or absent puberty (Topaloglu et al. 2009). Dyn, on the contrary, acts as an inhibitory signal for kisspeptin secretion which might be coordinating the complex pulsatile release of kisspeptin, and subsequent pulsatile release of GnRH. Whether or not the Dyn receptor is expressed in the ARC Kiss neurons is yet to be confirmed. Since kisspeptin is released in a pulsatile manner from the ARC Kiss neural population due to the integration of positive and negative signals, it is proposed that these neurons have a more prominent function in synchronizing the GnRH pulsatile release from the GnRH neurons (**Fig. 6**). In vitro rodent coronal brain slices containing GnRH neurons reveal that once kisspeptin is administered in a pulsatile manner, *GnRH* pulsatile expression became robustly synchronized accompanied with pulsatile secretion of GnRH, revealing that pulsatile kisspeptin stimulation is important for synchronizing the pulsations of GnRH release (Choe et al. 2013).

Both ARC and AVPV Kiss neuronal populations express receptors for the sex steroids estrogen and testosterone, nevertheless, the response of *KISS1* acts in an opposite manner. Estrogen and testosterone signaling downregulates *KISS1* in the ARC, whereas they induce the upregulation of *KISS1* in the AVPV. The difference in response could be mediated by differential recruitment of transcriptional coactivators or corepressors during estrogen signaling in the different Kiss populations. The steroid- induced feedback mechanism in the HPG- axis will be further discussed in Chapter 1.6.

Even though it is suggested the two Kiss neuronal populations could have different functions in stimulating GnRH, the direct effects of these Kiss neuronal populations on GnRH pulsations remain unknown since most studies conducted so far are done in rodents and by administration of kisspeptin *in vivo* and have mainly used the pulsatile LH levels in the blood as an indirect measure to study GnRH. Further research is required to elucidate the regulatory effects of ARC- and AVPV- Kiss neurons on GnRH secretion.



**Fig. 6 A proposed mode of action of two Kiss neural populations in regulating GnRH secretion.** A hypothetical model of the differential regulation of the GnRH surge during puberty and the preceding pulsations in pre- pubertal stages. The AVPV Kiss neural population increases in size towards puberty onset, and mainly provides positive regulation to GnRH neurons. The ARC Kiss population mediates both inhibitory (Dyn) and positive (NKB) signals to generate a pulsatile release of kisspeptin and therefore a pulsatile secretion of GnRH from GnRH neurons. This in turn signals to the gonadotrophs to secrete LH and FSH. Based on data obtained from rodent studies. Adapted from (Pinilla et al. 2012)

Concomitant with drastic remodeling of the AVPV Kiss neuronal populations to enhance GnRH secretion, its receptor GPR54 is also subjected to changes to enhance kisspeptin/GPR54 signaling. Studies conducted in monkeys revealed that *GPR54* mRNA levels in the hypothalamus during juvenile stages increases by approximately three fold in mid-pubertal stages (Shahab et al. 2005). Further studies have revealed that kisspeptin- induced LH secretion is more persistent during pubertal stages, compared to adulthood. Chronic infusion of kisspeptin has a remarkable stimulatory effect on LH secretion, even after seven days of administration in pubertal mice (J. Roa et al. 2008). This effect might be mediated by reduced desensitization of the GPR54 receptor during pubertal stages, in order to achieve maximum kisspeptin signaling. Similarly, a study conducted in early postnatal stages of rats showed that kisspeptin administration already has a potent stimulatory function on GnRH and LH, whereas LH responsiveness to low doses of kisspeptin was significantly enhanced during pubertal stages (Castellano et al. 2006). These findings suggest that kisspeptin and GPR54 undergo dramatic changes during early- juvenile to pre-pubertal stages in order to achieve maximum signaling of GnRH.

## 1.5 Metabolic signals modulate the HPG- axis

The HPG- axis induces not only puberty, but also controls the reproductive ability since a surge in LH in adult females triggers ovulation. Especially in females, extreme underfeeding can decrease LH levels, postponing the reproductive axis until conditions improve. It is proposed that the HPG- axis that controls puberty can also be regulated by nutritional and internal metabolic cues that regulate the reproductive axis. It is proposed that metabolic signals originating from peripheral tissues can signal to GnRH, mostly indirectly through other neurons. Kiss neurons are the most widely studied neurons to integrate these hormones since Kiss expresses receptors for leptin and ghrelin (Elias 2011; Forbes et al. 2009a).

Leptin, or the ‘starvation hormone’, is an important hormone produced by the adipose tissues in response to fat stores to regulate energy balance by signaling to the brain to suppress appetite (**Fig. 7**). Leptin plays a crucial role in sexual maturation as it was demonstrated that leptin deficient mice do not sexually mature and show lower levels of *KISS1* expression (Chehab, Lim, and Lu 1996). Leptin administration in leptin deficient mice restored sexual maturation, but did not rescue *KISS1* expression. Furthermore, further research revealed that sexual maturation is not altered when leptin receptor is specifically removed from Kiss neurons (Donato Jr. et al. 2011). Re- introduction of leptin receptor specifically in Kiss neurons in leptin receptor knock- out mice also did not rescue the absent sexual maturation phenotype (Cravo et al. 2013). These findings together demonstrate that leptin signaling in Kiss neurons is not essential nor necessary for puberty, and likely acts through intermediate neurons.

Another important hormone that controls energy balance and food intake is ghrelin, that opposes the actions of leptin, acting as the ‘hunger hormone’ that is produced from the gut in response to negative energy balance to promote food intake. Ghrelin is suggested to be important in suppression of the HPG-axis in woman who over- exercise or that suffer from anorexia. *In vitro* experiments revealed that ghrelin suppresses the pulsatile release of GnRH

and LH (Cravo et al. 2013). This effect might be mediated through ghrelin- induced suppression of *KISS1* mRNA levels in the AVPV Kiss neurons but not in the ARC neurons that are suggested to be crucial for the GnRH surge during puberty onset (Forbes et al. 2009b). This suggests that ghrelin might be more important in suppressing starvation- induced ovulation, more than affecting puberty onset itself.

Insulin has also been suggested to be involved in regulating GnRH since *in vitro* studies on hypothalamic neurons revealed a stimulatory effect on GnRH secretion as well as expression (Forbes et al. 2009b). However, silencing insulin receptor specifically in the GnRH neurons, as well as in other neurons projecting to GnRH, did not alter puberty (Forbes et al. 2009b).

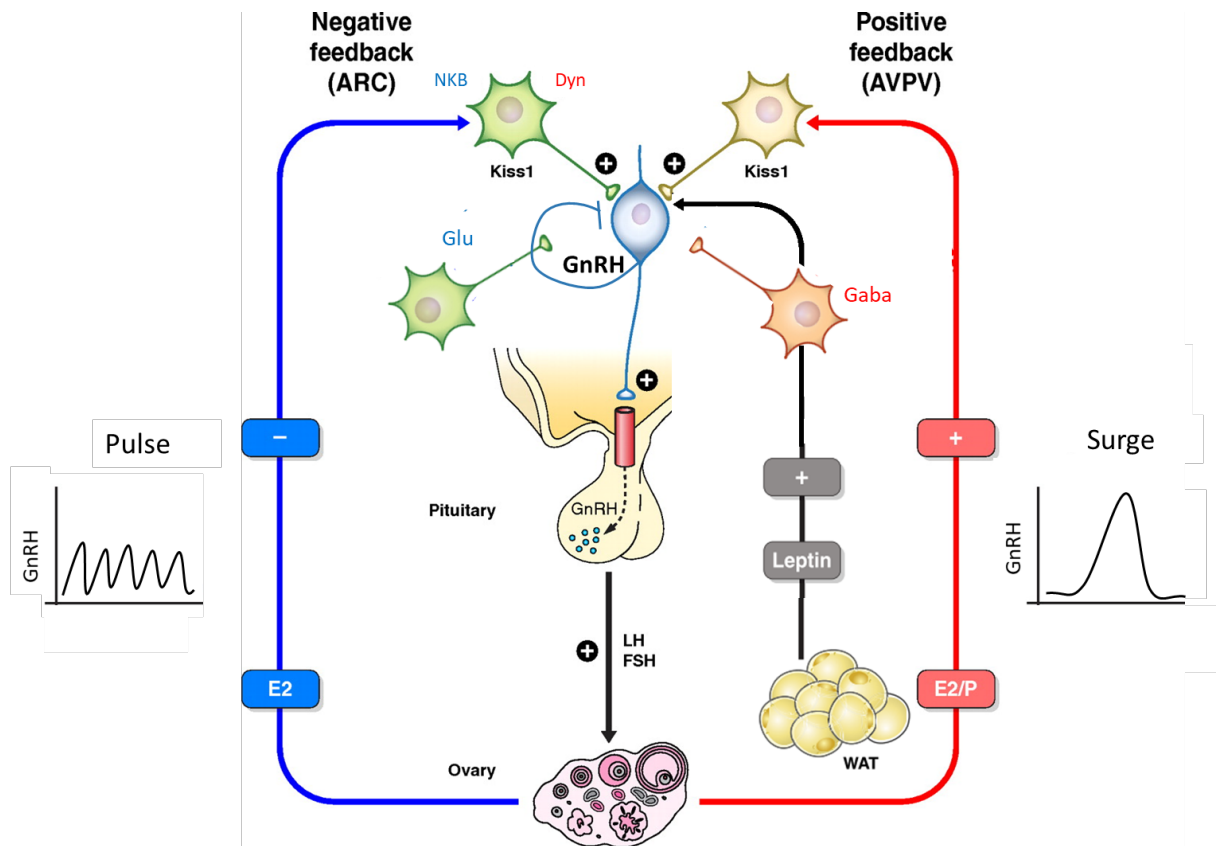
## 1.6 Steroidal feedback is important in HPG- axis

Sex steroids are produced by the gonads in response to GnRH- induced LH/FSH secretion. These steroids play a pivotal role in providing feedback regulatory loops onto the GnRH secretory behavior by signaling through the Kiss neurons (**Fig. 7**). Kiss neurons express several sex steroid hormone receptors, such as neuronal estrogen receptor  $\alpha$  (ER $\alpha$ ) and ER $\beta$ , androgen receptor, and progesterone receptor. Of these receptors, ER $\alpha$  is suggested to be important in timing puberty since specific ablation of ER $\alpha$  in Kiss neurons in female mice accelerated puberty onset due to a loss of GnRH inhibition from the ARC Kiss neurons (Mayer et al. 2010). In addition to this E2/Estrogen inhibitory signal in the ARC Kiss neurons, studies in rodents have demonstrated the existence of a positive feedback where E2 stimulates *KISS1* mRNA and kisspeptin levels in the AVPV (Forbes et al. 2009b). It is proposed that the Kiss neural network acts as an E2 dependent amplifier of the HPG- axis during puberty.

Disruption of the E2 feedback regulatory loops can have drastic changes in timing the onset of puberty as stated above. Estrogen-like endocrine disrupting chemicals (EEDCs) are substances that disrupt the endocrine system by interfering with the endogenous synthesis,

metabolism or receptor signaling of E2. These chemicals are present in pesticides, plastic products, flame retardants, pharmaceuticals and more products that are used on a daily basis. The EEDCs that act as E2 mimicker or blocker both have been proposed to cause precocious puberty onset, genital abnormalities as reviewed in Roy, Chakraborty, and Chakraborty 2009. The effect of human exposure to EEDCS present in the environment needs to be further elucidated. However, the EEDCs effect on pubertal onset currently lacks a good model since rodent *KISS1* mRNA levels are only affected at very high doses (Roy, Chakraborty, and Chakraborty 2009). However, whether E2 truly functions as a permissive or driving signal still remains to be elucidated, especially since specie dependent differences of E2 sensitivity exist.

To summarize, E2 from the gonads plays an important function in providing a regulatory feedback mechanism to the HPG- axis. It acts in two distinct manners, depending on the stage of pubertal onset. During pre- pubertal stages, E2 acts on the ARC Kiss neuronal population by providing negative feedback in order to generate kisspeptin pulsations to induce puberty. Once puberty is induced, E2 acts as an amplifier of the HPG- axis by providing positive feedback signals to the AVPV Kiss neurons.



**Fig. 7 A proposed mode of action of two Kiss neural populations in regulating GnRH secretion.** A hypothetical model of the differential regulation of the GnRH surge during puberty and the preceding pulsations in pre-pubertal stages. The AVPV Kiss neural population increases in size towards puberty onset, and mainly provides positive regulation to GnRH neurons. The ARC Kiss population mediates both inhibitory (Dyn) and positive (NKB) signals to generate a pulsatile release of kisspeptin and therefore a pulsatile secretion of GnRH from GnRH neurons. This in turn signals to the gonadotrope to secrete LH and FSH. Based on data obtained from rodent studies. Adapted from (Pinilla et al. 2012)

## 1.7 There is possibly not one ‘trigger’ for puberty

Since the reproductive maturity of an organism is a mechanism with no point of return, its temporal regulation is of fundamental importance. Given the hierarchical function of kisspeptin and GnRH act as potent activator of the HPG- axis, it is important to understand in response to which signals they may act to activate the cascade of events leading to the onset of puberty. All the regulatory signals of the HPG- axis summarized above highlight the multifaceted regulatory network involved in the pubertal onset. These signals seem to be acting more in a permissive manner rather than as a driving force to directly induce the onset of puberty. These signals act more in a permissive manner rather than as a driving force suggesting that puberty is under the control of a dynamic interplay between endogenous and environmental cues that are integrated in the HPG- axis to allow pubertal plasticity. This would ensure the complex energy demanding process of puberty to occur under optimal conditions. The nature of this precise and multifaceted mechanism timing puberty has only begun to be elucidated where several signals and feedbacks currently remain unknown.

Other than the known metabolic, nutritional and E2 signals as described before it could be plausible for the HPG-axis to also receive intrinsic signals originating from other organs. For instance, there could be a coupling between growing organs and the HPG- axis to ensure they have grown to an appropriate size before puberty starts and growth is fixed by closure of the bone plates.

In addition, in 1969 it was suggested that pheromones may signal into the HPG-axis since prepubertal female mice would undergo puberty earlier when they were in the presence of adult males (Vandenbergh 1969). Further studies in *C. elegans* and in mice showed similar results when females were kept in a place that was previously exposed to males (Aprison and Ruvinsky 2016; Flanagan, Webb, and Stowers 2011). However, the exact pheromone and pheromone receptor in both cases remains unknown and requires further research. Nevertheless,

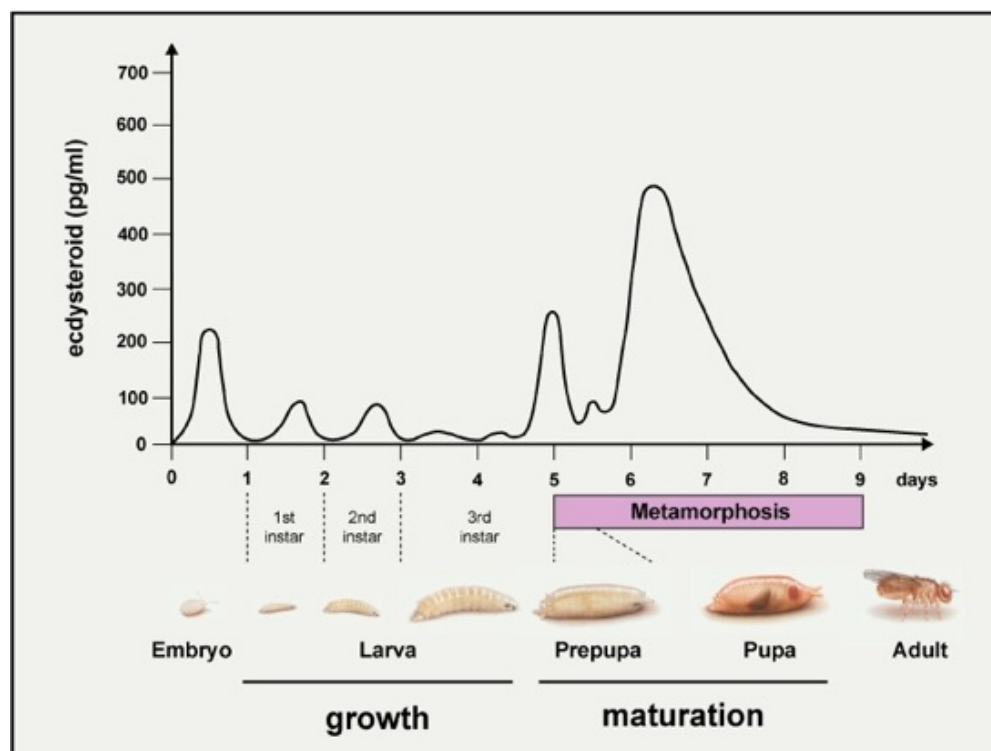


these findings suggest that vertebrates and invertebrates could potentially sense similar signals and integrate them to modulate sexual maturity. Due to the conservation of steroids timing the onset of sexual maturation, the large number of genetic tools and the ease of conducting genetic screens, we propose to use *Drosophila melanogaster* as a model organism to dissect the timing mechanism of puberty.

## 2. *Drosophila melanogaster* as a model to study the regulation of steroid production

### 2.1 *Drosophila* has a life cycle of two weeks

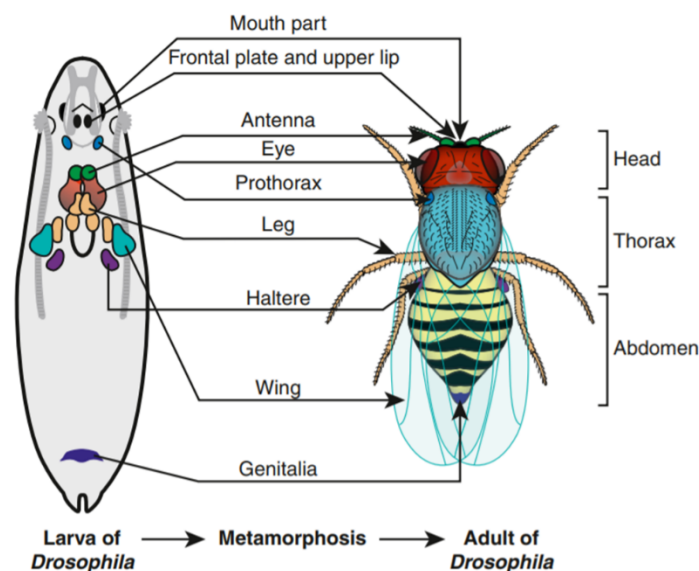
*Drosophila melanogaster* is extensively used as a model organism in biology due to its ease in use for genetic modifications that enabled the identification of novel genes in human pathologies. Its rapid development, short life cycle and easy upkeeping brings major advantages for fundamental research. A single fertile pair of flies can give rise to fertile offspring in only 10 to 12 days when kept at 25°C. The duration of the life cycle can be shortened or accelerated by temperature of nutrient availability. The life cycle of *Drosophila* can be classified in four distinct phases: the embryonic developmental phase, juvenile growth phase, sexual maturation (metamorphosis) and adult stage (**Fig. 8**).



**Fig. 8 Developmental stages of *Drosophila melanogaster*.** The developmental progression of *Drosophila* is under the control of the steroid hormone ecdysone. Ecdysone titers are high during embryonic stages and during larval molts. Growth occurs exclusively during larval stages and halts at the end of the 3<sup>rd</sup> instar larval stage that is induced by a large titer of ecdysone, causing the larvae to pupariate and undergo metamorphosis. Sexual maturation takes place during maturation stages, giving rise to mature adult flies. The entire life cycle of *Drosophila* takes approximately 9 days. Adapted from King-Jones: [http://www.biology.ualberta.ca/kingjones\\_lab/KKJ\\_lab/steroids.html](http://www.biology.ualberta.ca/kingjones_lab/KKJ_lab/steroids.html)

Upon egg deposition, *Drosophila* embryonic development takes place in a time window of around 22 hours. Once eclosed (hatched), larvae feed and grow extensively, undergoing approximately 200 fold increase in body mass (Robertson 1963). Larvae contain larval tissues and organs as well as small sac- like epithelial structures that are named imaginal discs that act as precursors of the adult fly organs (**Fig. 9**).

During the juvenile growth phase larvae undergo three instar developmental transitions that are marked by visible molts. These molts are induced by production of the insect steroid hormone ecdysone. Larvae spend around 24 hours in first larval instar stage, another day in second larval instar stage and the 3<sup>rd</sup> and final larval instar stage takes two to three days, before undergoing pupariation. Sexual maturation in *Drosophila* and other holometabolous insects occurs during pupariation stages through a process named metamorphosis. Upon pupariation, larval tissues are broken down or undergo autophagy whereas the imaginal discs undergo differentiation to give rise to adult organs. During this time sexual organs also undergo maturation after which adult flies emerge after approximately 4 days.



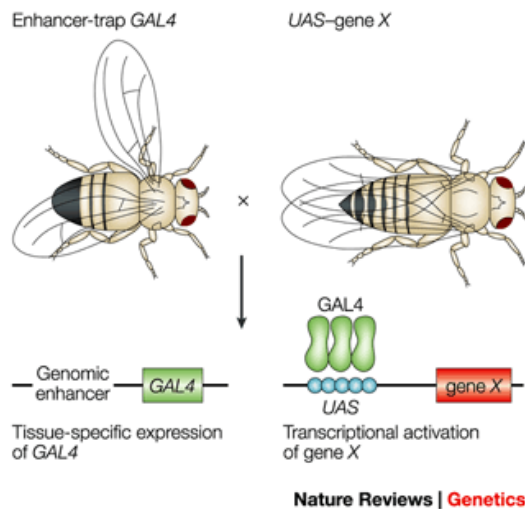
**Fig. 9 The larvae imaginal discs in the 3<sup>rd</sup> larvae instar stage and the corresponding adult structures.** The schematic representation and localization of the imaginal disc structures in the last instar larvae stage. During metamorphosis, a large number of larval tissues degenerate whereas the imaginal discs give rise to the corresponding adult structures. There are 19 discs in total, of which 9 bilateral pairs that give rise to the adult epidermis of the head, thorax and limbs, whereas the genitalia is derived from one medial imaginal disc. Adapted from (Aldaz and Escudero 2010)

## 2.2 *Drosophila* offers a wide variety of genetic tools

The whole genome of *Drosophila* was sequenced in 2000, and subsequently compared to the human sequence one year later (Adams et al. 2000). *Drosophila* only has four chromosomes: Chromosome I containing one pair sex- chromosomes (two X- chromosomes for females, one X and one Y for males), and three pairs of autosomes (II-IV), of which IV is the smallest, containing only 2% of the fly genome. The entire *Drosophila* genome encodes for roughly ~13.600 genes, versus ~22.000 genes in humans. It has been proposed that approximately 60-75% of all genes involved in human pathologies could have functional orthologous in *Drosophila* (Pandey and Nichols 2011).

*Drosophila* is a commonly used model to study gene expression and function due to the binary Gal4- UAS system that was developed by Brand and Perrimon in 1993. This system can allows tissue specific manipulation (**Fig. 10**). The system is based on the interplay of two parts: Gal4, encoded by the yeast transcriptional activator protein, and UAS (Upstream Activation Sequence). Gal4 containing flies have an activator protein but do not have the UAS- target gene to activate. The UAS- target gene fly lines, on the other hand, have a silent target gene since the activator gene is absent. Only when the UAS- and Gal-4 containing fly lines are crossed with each other, they will give rise to progeny containing the activated target gene. Gal4 is placed under a specific gene promoter or driver gene to be expressed in a specific subset of cells or tissues, referred to as the reporter or driver line. UAS is placed next to the desired gene, such as a gene RNA (for gene knock- down), a gene of interest (to induce overexpression) or GFP (to visualize tissues), for example.

Additionally, a second binary system, named the LexA- LexOP system can be used simultaneously with the Gal4-UAS system to allow multiplexing.



**Fig. 10 The *Drosophila* Gal4-UAS system.** UAS (Upstream Activation Sequence) acts as an enhancer for Gal4 (yeast transcriptional activation protein) to induce gene transcription. Gal4 is generally placed under a specific gene promoter or driver gene to be expressed in a specific subset of cells or tissues (enhancer-trap). UAS is placed next to the desired gene, such as a gene RNAi (for gene knock-down), GFP (to visualize cells/tissues), or different other tools. Once these lines are crossed with each other, it induces tissue specific manipulations in the progeny. Adapted from <https://media.nature.com/full/nature-assets/nrg/journal/v3/n3/images/nrg751-i1.gif>

### 2.3 Steroid production induces sexual maturation in *Drosophila*

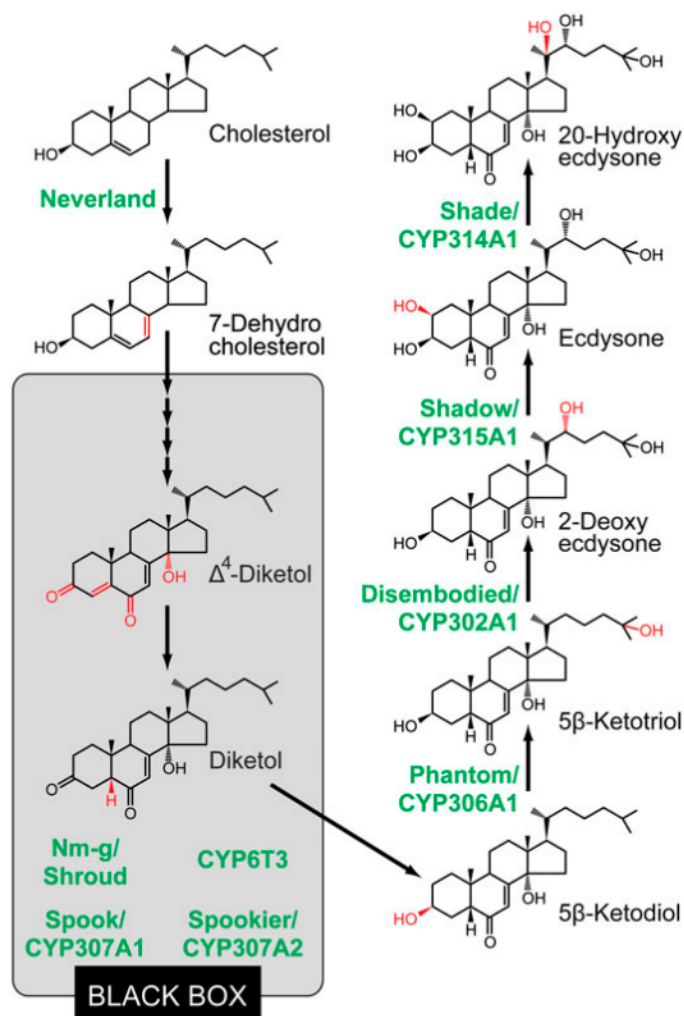
Similar to vertebrates, sexual maturation of *Drosophila* is initiated by a surge of steroid hormone. It takes place during metamorphosis, marking the transition of a growing juvenile larvae into a non-feeding pupae (**Fig. 11**). The production of the insect steroid hormone ecdysone takes place in the insect endocrine tissue prothoracic gland (PG), and controls the time of the two larval molts and metamorphosis (Warren et al. 2006).

Upon activation of the PG, a large number of enzymatic steps are required for the biosynthesis of 20E (active ecdysone). These steps are mediated by a group of ecdysone biosynthetic enzymes encoded by the Halloween genes that are members of the cytochrome P450 enzyme superfamily as reviewed extensively by Niwa and Niwa in 2014. First, cholesterol

is biosynthesized into 7- dehydrocholesterol by an enzyme named Neverland (*nvd*) and then becomes synthesized into ketodiol by a yet unknown mechanisms, but are known to involve the enzymes Shroud (*sro*), Spook (*spo*) and Spookier (*spok*). Next, Phantom (*phm*) synthesizes ketotriol and then is converted into 2- deoxyecdysone via Disembodied (*dib*). Finally, Shadow (*sad*) converts it into ecdysone (E) before being released from the PG into the hemolymph, the fly equivalent of blood. E becomes hydroxylated by peripheral tissues such as the fat body, via Shade (*shd*) into the bioactive form 20-hydroxyecdysone, 20E, or also referred to as ecdysone in the rest of the thesis.

Similar to vertebrates, steroid signaling is mediated through activation of the nuclear receptor superfamily that are ligand- regulated transcription factors. Bioactive ecdysone binds to its receptor EcR, that heterodimerizes with a second nuclear receptor, Ultraspiracle (USP)(L M Riddiford, Cherbas, and Truman 2000). Once activated, ecdysone signaling induces a larval stage- specific response by transcriptionally regulating a subset of nuclear receptors as well as regulating the expression of its receptor, *EcR*. Other downstream targets are *Drosophila hormone receptor 3* (DHR3), DHR4, E75B (primary response gene), E78, and ftz transcription factor 1 ( $\beta$ FTZ-F1), that in turn also regulate each other, as reviewed in King-Jones and Thummel 2005.

Other than its function in initiating metamorphosis due to a large increase at the late larval instar stages, ecdysone is also released at continuous low levels during larval development. These basal levels of ecdysone have been suggested to function in negatively finetuning larval growth rate and positively in promoting imaginal disc growth during larval development. Further studies need to be conducted to better understand the mechanisms underlying these functions. A more detailed description will be addressed in Chapter 2.7.



**Fig. 11 The biosynthesis of ecdysone.** The conversion of cholesterol into the bioactive form of ecdysone is the result of multiple steps involving biosynthetic enzymes that are encoded by a group of genes that are referred to as 'Halloween genes'. The first biosynthetic step is mediated by Neverland to convert cholesterol into 7-dehydrocholesterol. Next, 5 $\beta$ -Ketodiol is produced by a yet an unknown mechanism, that is referred to as the 'Black Box'. These steps are mediated by the biosynthetic enzymes Shroud, Spook, Spookier. 5 $\beta$ -Ketodiol is then converted into 5 $\beta$ -Ketotriol via the enzyme Phantom, before being converted into 2-Deoxyecdysone by Disembodied. The last step involves Shadow to produce ecdysone (E), that in turn is released in the hemolymph where it is hydroxylated into the bioactive form 20-Hydroxyecdysone (20E) via Shade. Adapted from (Niwa and Niwa 2014)

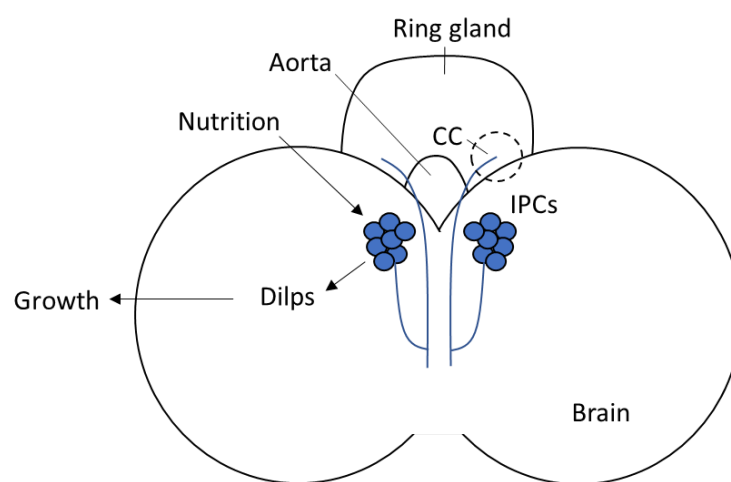
## 2.4 Insulin signaling controls systemic growth and determines final size

The onset of sexual maturation coincides with the end of the larval growth period. Therefore, it is important to have a coordination between metamorphosis and growth mechanisms to ensure properly sized adult flies. In almost all organisms insulin/insulin-like growth factor (IGF)-like signaling (IIS) relays nutritional information to organ and tissue growth and plays a fundamental role in physiology, governing growth, metabolism, longevity and development. As described above, insulin signaling in flies plays a pivotal role in controlling larval growth and subsequently final adult size. IIS signaling in *Drosophila* is under the control of *Drosophila* insulin-like peptides (Dilps). To date, eight Dilps (Dilp1-8) have been identified and characterized and with the exception of the newly identified Dilp8, all signal through the fly canonical IIS pathway to promote growth. Dilp1-7 bind to one common tyrosine kinase receptor, the *Drosophila* insulin receptor (InR), that is the homologue of the mammalian insulin-like growth factor type 1 receptor (IGF-1R). Overexpression of any of these Dilps leads to an increase in larval size, of which the most dramatic phenotype was seen in *Dilp2* overexpression, giving rise to adults that are 51% heavier (Ikeya et al. 2002)

Dilps exhibit specific temporal and spatial expression patterns and can be differentially regulated, either at transcriptional level or at the level of secretion. In fact, only four Dilps, *Dilp1*, *Dilp2*, *Dilp3* and *Dilp5* are expressed from the insulin-producing cells (IPCs) (**Fig. 12**). IPCs are two clusters of seven neurosecretory cells located in each side of the brain hemisphere with axonal projections towards the corpora cardiaca (CC), and aorta. The IPCs are the functional homologous of the mammalian islet  $\beta$ - cells, the insulin producing cells within the pancreas. Interestingly, it was described that not only the neuroblast progenitors of the IPCs are homologous to the islet  $\beta$ - cells, but also a second pair of neuroblast progenitors were found of the neurosecretory cells of the CC to share homology with the islet  $\alpha$ - cells that secrete glucagon which counterbalances the actions of insulin (Wang et al. 2007). The identification of these



parallels provides evidence that the endocrine axis of both flies and mammals might have evolved from a common ancestor. Genetic ablation of the IPCs results in growth retardation and extended life span, similar to the phenotype observed in mice when IGF-1R receptor is silenced (Holzenberger et al. 2002; Rulifson, Kim, and Nusse 2002). Out of all Dilps expressed from the IPCs, Dilp2 is the most prominently expressed and is considered to be the most potent growth promoter. Dilp2 overexpression alone is sufficient to rescue the growth perturbation phenotype and reduction in adult size caused by IPC ablation (Rulifson, Kim, and Nusse 2002).



**Fig. 12 Schematic representation of the IPCs in the *Drosophila* larval brain.** The insulin producing cells (IPCs) consist of two pairs of seven median neurosecretory cells in the brain. The IPCs project towards the corpora cardiaca (CC) in the ring gland, and aorta. In response to nutrition, *Drosophila* insulin-like peptides (Dilps) are secreted, inducing larval growth. IPCs express Dilp1,2,3 and 5.

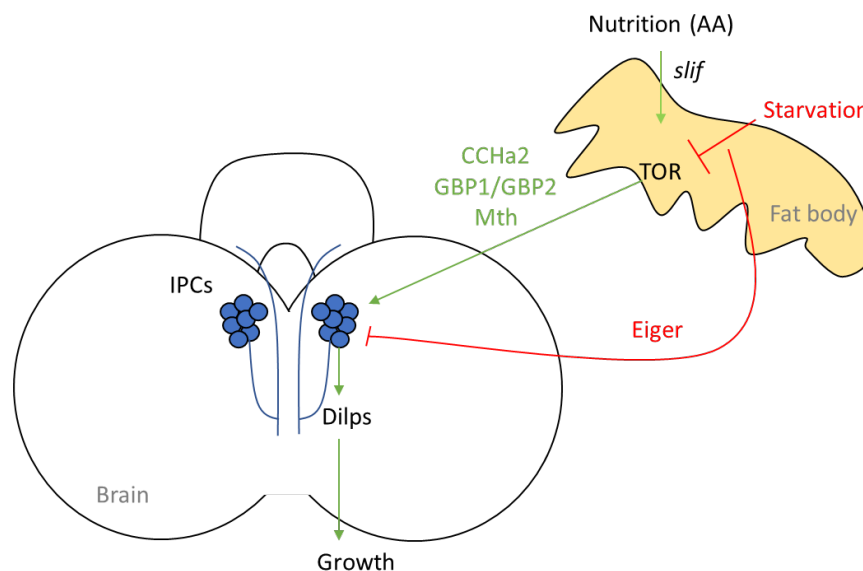
Out of the Dilps that are discovered, Dilp2, Dilp3, Dilp5 and Dilp6 are under the control of nutritional availability. Whereas *Dilp3* and *Dilp5* transcript levels decrease upon starvation, *Dilp2* expression is not affected (Ikeya et al. 2002). It was only until later when it was found that Dilp2 is regulated at the level of secretion since amino acid starvation caused Dilp2 to be retained in the IPCs (Rulifson, Le, and Ge 2009). *Dilp6* is expressed in the fat body at high levels during the non-feeding wandering stage of 3<sup>rd</sup> instar larvae as well as the pupal stage (Slaidina et al. 2009). Animals carrying a deletion of the *Dilp6* gene are smaller in size and are

even smaller upon starvation when compared to wild-type animals under the same conditions. Therefore, *Dilp6* expression is induced upon nutrient deprivation and functions to induce growth during the nonfeeding stages of *Drosophila*. This highlights the importance of the fat body in controlling growth in response to nutritional inputs.

## 2.5. Fat body is a nutritional relay for systemic growth control

The *Drosophila* fat body is the functional equivalent to the mammalian liver and adipocytes. Old studies showed that when the fat body was co- cultured with larval imaginal discs or quiescent imaginal neuroblasts, nonautonomous tissue growth and cellular proliferation were induced, respectively (Britton and Edgar 1998; Davis and Shearn 1977). This suggested at that time that the fat body possesses growth promoting signals. In the recent years, the fat body has become an extensively studied organ in *Drosophila* when it was revealed that the fat body acts as a relay of nutritional availability to modulate growth. It was discovered that when the amino acid transporter *slimfast* (*slif*) was downregulated in the fat body, it would cause a perturbation in systemic growth that is mediated by TOR1 signaling in the fat body (Colombani et al. 2003). The underlying molecular mechanism that couples growth with *slif* and TOR1 signaling was only revealed later when it was shown that when *slif* or *TOR1* were perturbed in the fat body, *Dilp2* was accumulated in the IPCs, inhibiting systemic growth (Rulifson, Le, and Ge 2009). This suggests that the fat body signals nutritional information to the IPCs to modulate systemic growth by acting on Dilp secretion, possibly through releasing an x- factor in the hemolymph that is able to signal to the IPCs (**Fig. 13**). Studies over the recent years focused on identifying these x- factors and this lead to the identification of several humoral signals controlling growth that will be summarized below.

In 2015, *Drosophila* CCHamide-2 (CCHa2) was identified as a fat body derived signal that relays protein availability to control insulin signaling in the IPCs. Through its receptor CCHa2-R, CCHa2 regulates Dilp2 and Dilp5 secretion in the IPCs and therefore controls systemic growth (Sano et al. 2015). A year later, another two fat body derived peptides were discovered to promote systemic growth, Growth-Blocking Peptide (GBP1) and CG11395 (GBP2). In response to amino acids and TOR signaling GBP1 and GBP2 stimulate Dilp2 and Dilp5 secretion from the IPCs to induce systemic growth (Koyama and Mirth 2016). That same year, a genetic screen in the IPCs led to the identification of a secretin-incretin receptor subfamily member Methuselah (Mth) to couple nutrition with growth (Renald Delanoue et al. 2016). Its ligand is identified as Stunted (Sun) that is a fat body secreted factor required for the IPCs to release Dilp2 and induce growth. Sun is released in the hemolymph under fed conditions and not in starved larvae, suggesting that Sun acts as a nutritional relay for the IPCs to induce growth.



**Fig. 13 The fat body acts as a nutritional relay to control growth through the IPCs in the *Drosophila* larval brain.** In response to nutrition, the amino acid transporter *slimfast* (*slif*) in the fat body activates TOR signaling that controls systemic growth by acting on the insulin producing cells (IPC's). The fat body does so by secreting *Drosophila* CCHamide-2 (CCHa2), Growth-Blocking Peptide 1 and 2 (GBP1/GBP2) and Methuselah (Mth) that positively regulate Dilp secretion from the IPCs under fed conditions. In response to starvation, the fat body secretes Eiger that negatively affects the Dilps from the IPCs. The green arrows represent positive regulation, whereas the red arrows represent negative regulation.

These signals described above all describe positive cues for the IPCs that are important to promote growth when nutritional availability is favorable. Under starvation, however, these positive regulators are lost, inhibiting growth. It is therefore possible to have additional signals that can signal from the fat body when starvation takes place to provide a stronger growth inhibitory response. In line with this, a genetic study was conducted to identify fat body derived growth inhibitory signals under poor nutrition. This led to the identification and characterization of the *Drosophila* TNF- $\alpha$  protein, Eiger (Agrawal et al. 2016). Under a normal diet, Eiger is inhibited by TOR in the fat body and is not released in the hemolymph. However, when larvae are deprived of proteins, Eiger is released and signals to the brain where it binds the TNFR Grindelwald to reduce Dilp expression in the IPCs, subsequently reducing larval growth. Together, these findings show that the fat body integrates nutritional cues to modulate systemic growth by controlling the Dilps in the IPCs.

Fat body mediated systemic growth effects can also occur in a nutrient independent manner, under the control of ecdysone signaling. Ecdysone signaling within the fat body causes inhibition of systemic growth induced by insulin signaling. Ecdysone induced EcR signaling blocks PI3K that subsequently allows FOXO translocation to the nucleus to activate the downstream target genes of dDOR, functioning as a feed, and Dilp6 to promote nutrient independent growth. Simultaneously, EcR signaling in the fat body also inhibits the transcription factor dMyc, that plays a pivotal role in promoting growth. It has been proposed that EcR induced FOXO and dDOR signaling is important to prepare larvae to undergo metamorphosis.

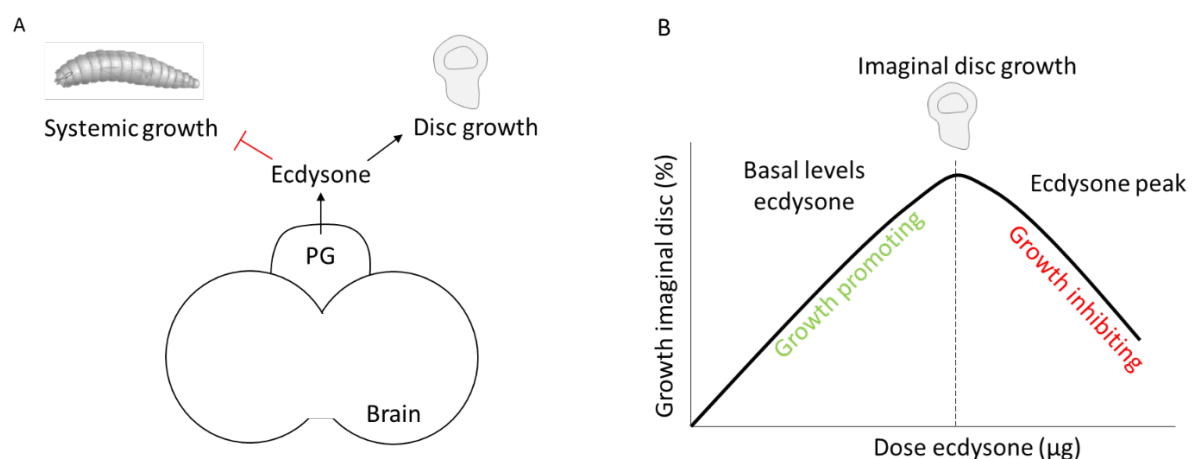
## 2.5 Steroid signaling controls growth

In *Drosophila*, and in other holometabolous insects, the onset of metamorphosis marks the end of the larval growth period so that growth is halted, subsequently fixing the final adult size of flies. Thus, the time of metamorphosis onset is crucial in determining the final size. A large production of ecdysone at the end of the 3<sup>rd</sup> instar larval stage induces the transition from feeding behavior (foraging) to the so called ‘wandering’ stage (non- feeding) preceding the metamorphosis state (Lynn M Riddiford and Truman 1993). An early onset of the ecdysone peak gives rise to smaller sized flies, whereas delayed ecdysone production causes larvae to grow longer and become larger as adults. The high ecdysone surge at the onset of metamorphosis ends the larval growth period and regulates expression of genes that are required for apoptosis of larval tissues and differentiation and development of the adult structures from the imaginal discs (Thummel 1995). The peak of ecdysone, however, inhibits growth of the imaginal discs at the end of the larval stages, before it resumes again during pupariation. As shown in the tobacco hornworm *Manduca sexta*, there is a negative correlation with the ecdysone peak and wing imaginal disc and cell number (H. Frederik Nijhout and Grunert 2010). Dose dependent injecting ecdysone during the wandering stages reveals that low titers of ecdysone induces growth of the wing imaginal disc, whereas at the highest dose inhibits growth (**Fig. 14**). This reveals a differential growth regulatory function of ecdysone that is dose dependent. The relevance of this study converges on that ecdysone is released continuously at low levels during larval development (basal levels), before peaking at the end of development.

The effect of ecdysone basal levels on systemic growth was addressed in a study in which ecdysone signaling was reduced by modulating the size of the ring gland, including the PG (Colombani et al. 2005). In this scenario, lower ecdysone levels corresponded to larger sized adults, that was reversed upon ecdysone feeding. In contrast, when larvae were fed with ecdysone, they were smaller as adult flies. This shows that ecdysone basal levels inhibit

systemic larval growth (**Fig. 14**). In an attempt to determine how ecdysone could regulate growth, the authors silenced the receptor for ecdysone (EcR) in the fat body. Surprisingly, these animals were significantly larger in size caused by an increase in larval growth rate, without affect the duration of growth. This effect is mediated by increased *dMyc* in the fat body, which is a regulator of systemic growth through ribosome biogenesis (Rénald Delanoue, Slaidina, and Léopold 2010). This suggests that the basal ecdysone levels during larval development regulates systemic growth mediated through the fat body.

Conversely, ecdysone has an additional function in promoting growth of the imaginal discs, particularly in the mid- late 3<sup>rd</sup> instar larvae (Herboso et al. 2015). Larvae that fail to synthesize ecdysone fail to undergo pupariation and continue to grow for an extended time, giving rise to big larvae. Interestingly, however, the imaginal discs in these larvae failed to grow and were not proportionate to their body size. Upon close examination of the wing discs it was revealed that cell number and cell size were greatly reduced. This suggests that ecdysone also has a growth promoting effect on imaginal discs (**Fig. 14**).



**Fig. 14 Differential growth regulatory function of ecdysone signaling .** (A) Ecdysone is produced from the prothoracic gland (PG) at basal levels during larval development that inhibits systemic growth. For the imaginal discs, however, ecdysone basal levels promotes growth of imaginal discs. (B) Conversely, high levels of ecdysone (at the end of larval development) negatively regulates growth of the imaginal discs. Schematics of (B) is extrapolated from data in published in H. Frederik Nijhout and Grunert 2010

## 2.6 Ecdysone production in the PG is tightly regulated

The insect endocrine gland PG plays a fundamental role in timing the commitment for larvae to undergo metamorphosis. This commitment results from ecdysone biosynthesis in the PG, and therefore many studies are focused on internal and external factors that can signal to the PG to modulate the timing of metamorphosis (**Fig. 15**). The first neurons that were identified to directly innervate the PG and to induce ecdysone production are the PTTH neurons (Mizoguchi et al. 1990). PTTH is a small neuropeptide that is produced by the PTTH neurons that once released, it acts on its receptor Torso to activate the MAPK pathway in the PG to ultimately activate ecdysone biosynthesis (Rewitz et al. 2009). Due to its central role in inducing ecdysone production, PTTH will be discussed in further detail in the next chapter.

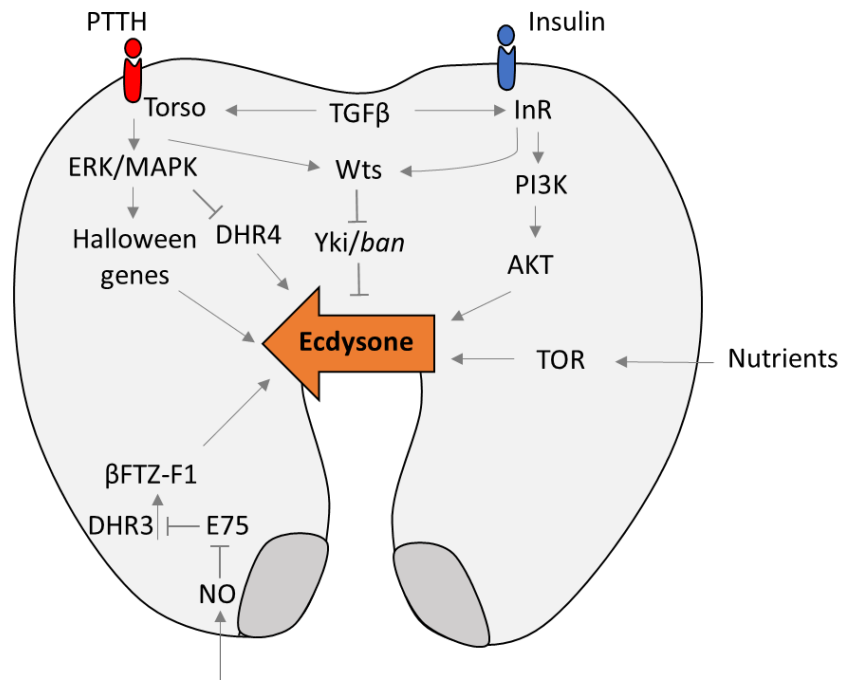
TOR signaling can also modulate ecdysone levels in the PG to couple nutritional state with maturation (Layalle, Arquier, and Léopold 2008). Inhibition of TORC1 specifically in the PG prolongs the larval growth period, without affecting growth rate, contrary to what is observed when insulin signaling is perturbed by silencing PI3K in the PG. This suggests that TORC1 acts exclusively to induce metamorphosis onset by altering the ecdysone peak at the end of larval development. Low nutrient conditions reduce TOR signaling in the PG, whereas the extended growth period caused by poor nutrition can be rescued by overexpressing TORC1 in the PG. This indicates that the PG possesses an intrinsic mechanism to directly sense nutrient availability to modulate the ecdysone peak and subsequent metamorphosis. Upon the integration of these signals, how does the PG commit to ecdysteroid biosynthesis? A recent study in 2017 revealed the underlying molecular mechanism of nutrient- dependent TOR signaling in the PG to induce ecdysteroid production (Ohhara, Kobayashi, and Yamanaka 2017). Here, it was observed that TOR inhibition in the PG during CW halted endocycling in the PG cells, preventing animals to undergo metamorphosis due to reduced levels of the ecdysteroid biosynthesizing enzymes. These effects could be rescued by overexpressing Cyclin

E, which is an important nuclear protein to trigger cells to progress into the S- phase of mitosis. This shows that TOR- mediated endocycling, in response to nutrients, acts as a checkpoint in the PG to couple systemic growth with maturation.

Nitric oxide (NO) is another signaling molecule in the PG that is required to induce metamorphosis. NO is a free radical gas is catalyzed by nitric oxide synthase (NOS) and acts in important cellular signaling pathways. In the PG, NO exerts its function through interacting with the *Drosophila* nuclear receptor E75, releasing the inhibitory effect on *Drosophila* hormone receptor 3 (DHR3). This subsequently induces the nuclear receptor  $\beta$ FTZ-F1, which is an important transcriptional inducer of two ecdysteroidogenic enzymes, Phantom (*phm*) and Disembodied (*dib*) (Parvy et al. 2005). Reducing the expression of NO synthase (NOS) specifically in the PG results in the inability of larvae to enter metamorphosis (Cáceres et al. 2011).

Other factors that can influence ecdysteroid production in the PG is TGF $\beta$ /Activin signaling (Gibbens et al. 2011). When TGF $\beta$ /Activin is blocked, larvae fail to undergo metamorphosis, giving rise to giant larvae . This is accompanied with failure to induce the ecdysone surge at the end of larval development due to low levels of ecdysteroid biosynthesizing enzymes. Moreover, close examination of possible target genes reveals reduction in the levels of *torso* and *inr* which are the receptors for PTTH and insulin signaling, respectively. This could suggest that the failure to biosynthesize ecdysone could be due to impaired PTTH and insulin signaling in the PG. TGF $\beta$ /Activin therefore functions to coordinate growth and maturation at the level of PTTH and insulin signaling in the PG. Interestingly, this reveals an evolutionary conserved mechanism of TGF $\beta$  to control maturation since inactivation of the TGF $\beta$  pathway in *C. elegans* induces a developmental arrest through dauer formation (Ren et al. 1996).





**Fig. 15 Signals regulating ecdysone production in the insect prothoracic gland.** The neuropeptide PTTH acts through its receptor Torso mediate MAPK signaling pathway through activation of Ras that subsequently leads to phosphorylation of ERK/MAPK. This is associated with an upregulation of the Halloween genes that encode for cytochrome P<sub>450</sub> enzymes that ultimately biosynthesize ecdysone. Another important player is insulin, that binds to insulin receptor (InR) that subsequently activates the PI3 kinase (PI3K) to convert PIP<sub>2</sub> into PIP<sub>3</sub>, activating AKT that correlates with ecdysone production. Nitric Oxide (NO) signals to the PG and inhibits E75, an inhibitor of *Drosophila* hormone receptor 3 (DHR3), activating the nuclear receptor βFTZ-F1, required for the expression two Halloween genes, *phm* and *dib* to biosynthesize ecdysone. TGFβ/activin signaling modulate expression levels of *tor* and *inr*. Warts (Wts) inhibits ecdysone production acting through Yorki (Yki) and the miRNA *bantam* (*bam*). Wts acts downstream of PTTH and insulin signaling.

## 2.6 Sexual maturation is coordinated with systemic growth

It is proposed that the time of metamorphosis onset is directly under the control of larval growth or a size assessment mechanism (C. Mirth, Truman, and Riddiford 2005). The attainment of the so called ‘critical weight’ is an important checkpoint at larval development, to ensure larvae have obtained enough mass in order to survive metamorphosis. When starved before reaching critical weight, most larvae die and those who survive undergo pupariation much later. Conversely, post- critical weight starvation does not affect the time of pupariation. However, further research is required to better understand how growth or the critical size assessment mechanism affects the time of maturation. It is proposed that the PG acts as a size assessment organ due to its crucial role of inducing maturation through ecdysone production.

In 2005, three studies have conducted experiments in the PG, specifically altering insulin signaling in order to determine a link between growth and maturation. Activation of the insulin receptor is required at cellular level for growth to occur, and promotes autonomous growth when activated at tissue specific level (Roy, Chakraborty, and Chakraborty 2009). Once insulin signaling is activated, PI3 kinase (PI3K) converts phosphatidylinositol-4,5-bisphosphate (PIP2) to PI-3,4,5-triphosphate (PIP3). When *PI3K* was overexpressed in the PG, using Phantom (phm) Gal4, a strong PG driver, PG size was increased causing larvae to undergo metamorphosis earlier which shortened the growth period, giving rise to smaller sized adults. (C. Mirth, Truman, and Riddiford 2005). Conversely, when PG size was suppressed using *PTEN*, a phosphatase converting PIP3 back to PIP2, caused larval lethality. These findings suggest that the PG size is likely the decisive factor to determine critical weight has reached, allowing larvae to undergo metamorphosis by inducing ecdysone signaling.

A similar study confirmed that constitutive *PI3K*, *Ras* and *Raf* overexpression in the PG using a different Gal4 driver (*amn<sup>c65l</sup>*) also decreased the larval growth period, leading to smaller sized animals (Caldwell, Walkiewicz, and Stern 2005). It is of note, however, that only

*Ras* and *PI3K*, and not *Raf* overexpression caused larger sized PG cells. *Ras*- mediated precocious metamorphosis onset was also accompanied with early ecdysone signaling, as determined by measuring expression levels of ecdysone target genes: *E74A* and *E74B*. Conversely, expressing dominant negative *PI3K*, *Ras* and *Raf* in the PG prolonged the larval stages, subsequently allowing larvae to grow bigger. Interestingly, only the dominant negative *PI3K* expression in the PG had a negative effect on PG cell size. This suggests that ecdysone signaling from the PG can be regulated in two different ways, one as a consequence of insulin-dependent PG cell growth in a *PI3K* growth dependent manner, and the other in a *Raf* dependent mechanism that likely acts to activate the transcription of ecdysone biosynthesis genes.

Nevertheless, there is compelling evidence showing that PG cell size alone is not the determining factor of initiating ecdysone signaling. For instance, artificially increasing PG size by silencing nitric oxide synthase (NOS) does not induce ecdysone production (Cáceres et al. 2011). Moreover, artificial induction of PG cell size in an insulin independent manner, using dMYC or cyclinD/cdk4 overexpression does not affect larval growth or body size (Colombani et al. 2005). This suggests that insulin signaling in the PG itself is a necessary factor for ecdysone signaling. It is of note that in this same study, unlike the other studies, a weaker driver was used to manipulate *PI3K* in the PG which resulted in changes only in larval growth rate, without affecting the timing of metamorphosis. Interestingly, this phenotype was accompanied with alterations in basal ecdysone levels only, without changing the peak level of ecdysone that occurs at the end of larval development. It is therefore likely that due to the differences in strength of the PG drivers, ecdysone levels are differentially altered, affecting either only growth rate or both growth and the timing of metamorphosis onset. These findings confirm the notion that ecdysone has two distinct functions in a dose- dependent manner, one by inhibiting larval growth rate through its basal activity during developmental stages, whereas the peak of ecdysone at the end of larval development promotes the developmental transition and thereby

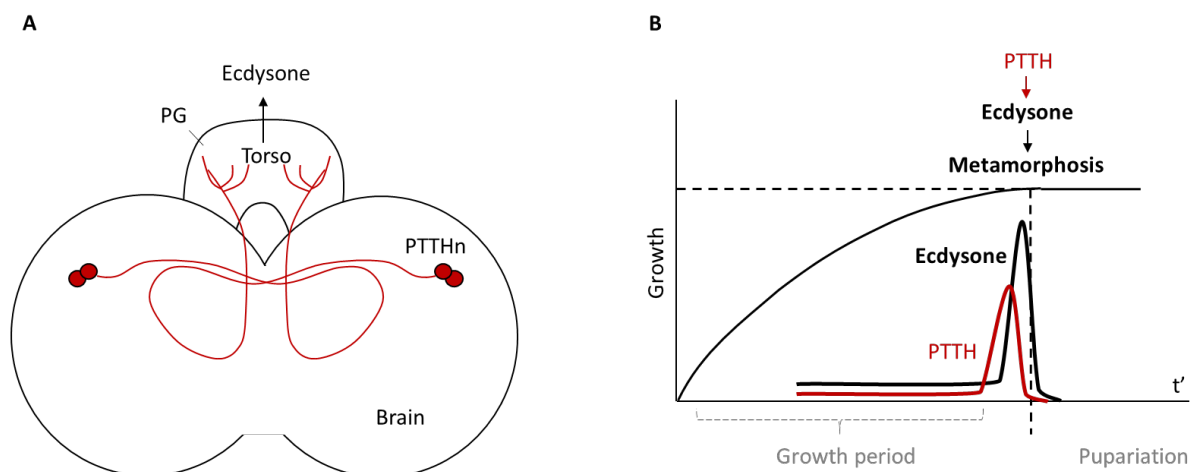
halting systemic growth entirely. In this context, insulin signaling in the PG is important in promoting ecdysone production, thereby reducing the adult size mediated by a reduction in larval growth rate, as well as by shortening the growth period.

Further research revealed that insulin induced ecdysone production is mediated through *bantam* miRNA that acts by repressing the basal levels of ecdysone in the PG (**Fig. 15**) (Sano et al. 2015). Recently, Warts (Wts) was identified to regulate ecdysone basal levels in the PG by coupling insulin signaling with ecdysone signaling, acting through Yorkie (Yki) and *bantam* (Moeller et al. 2017). Silencing of *Wts* in the PG results in larval overgrowth, that is caused by a reduction in the larval growth rate and not due to an extension of the growth period. This phenotype was accompanied by reduced ecdysone levels, suggesting that Wts in the PG inhibits growth by inducing ecdysone basal levels. Concomitantly, animals with silenced *Wts* in the PG also have increased insulin signaling. This suggests a growth regulatory function of Wts signaling by reducing systemic growth through inducing ecdysone basal levels, and negatively acting on insulin signaling, and therefore negatively acting on growth. It was further revealed that Wts function in the PG is mediated by Yki activity, that is a transcriptional co-activator of the Hippo pathway in which Wts is a downstream effector. Wts was also shown to negatively regulate *bantam* that represses ecdysone basal levels. Interestingly, *Wts* expression could be induced by activating insulin signaling in the PG, through overexpression of *InR*, or by activating PTTH signaling in the PG, by overexpressing the PTTH-Torso downstream signaling protein *Ras*. This suggests that Wts function is mediated by insulin and PTTH signaling in the PG to ultimately control ecdysone production. These findings together show that antagonistic interactions of ecdysone and insulin signaling coordinates systemic growth and maturation.

### 3. PTTH as a gatekeeper of sexual maturation in *Drosophila*

#### 3.1 PTTH times onset of metamorphosis

The first insect neuropeptide that was discovered to induce insect pupation (metamorphosis) is the small secreted peptide, prothoracicotropic hormone (PTTH) in 1922 (Kopeć 1922). PTTH was initially studied in the silkworm *Bombyx mori* in 1987 and later also characterized in all holometabolous insects. The tobacco hornworm *Manduca sexta* was extensively used as a model to study PTTH function (Kataoka et al. 1987, 1991). In *Drosophila*, PTTH was first purified in 1997 and then characterized in 2007 after which a large number of studies have addressed the function of PTTH in metamorphosis (Kim et al. 1997; Z McBrayer et al. 2007). PTTH is produced and secreted by two bilateral neurosecretory cells named PTTHn neurons (PTTHn), which are the first neurons discovered to directly innervate the PG (**Fig. 16**). Transcriptional profiling reveals a large titer of *ptth* at the end of larval development, preceding the ecdysone pulse before metamorphosis (Z McBrayer et al. 2007; Rewitz, Yamanaka, and O'Connor 2013). This suggests that PTTH could have function in timing metamorphosis by stimulating ecdysone signaling from the PG.



**Fig. 16 PTTH signaling controls ecdysone production and times metamorphosis.** (A) PTTH neuropeptide is produced from two pairs of neurons located in the larval central brain (PTTHn). These PTTHn project towards the prothoracic gland (PG) where PTTH is released. When PTTH binds to its receptor Torso, it activates the production of ecdysone. (B) PTTH titers anticipate ecdysone levels, inducing metamorphosis at the end of larval development.

The first functional experiment of PTTH was conducted by ablating PTTHn, that resulted in a drastic prolongation of the 3<sup>rd</sup> larval instar stage (Zofeyah McBrayer et al. 2007). These larvae entered metamorphosis five days later, compared to control larvae. Ablation of PTTHn also caused a delay in ecdysteroid titer at the end of larval development. This is likely caused by impaired transcriptional upregulation of several genes that are required for ecdysteroid biosynthesis. These genes encode for cytochrome P<sub>450</sub> biosynthesizing enzymes that are also referred to as ‘Halloween genes’. Expression levels in *spok*, *phm*, *nvd*, and *dib* were impaired, whereas expression of *sad* and *shd* were delayed but still surged right before metamorphosis. *shd*, however, is one of the biosynthesizing enzymes to not expressed in the PG and therefore is likely not under the control of PTTH. Accordingly, feeding larvae with ecdysone rescued the developmental delay, suggesting that the impaired timing of metamorphosis is caused by impaired ecdysone production due to PTTHn ablation (Zofeyah McBrayer et al. 2007).

The highest peak of *ptth* is observed during pupal stages, however, its function during pupal stages seems to be redundant since larvae without functional PTTHn still undergo metamorphosis (Graveley et al. 2011). These findings highlight the importance of PTTHn in timing developmental transition that is likely caused by loss of function of PTTH. Indeed, downregulation of *ptth* in PTTHn is sufficient to delay metamorphosis, showing that the developmental delay phenotype in PTTH ablated neurons is due to PTTH (Yamanaka et al. 2013). Surprisingly, overexpression of *ptth* causes animals to pupariate earlier. More recently, the first *ptth* null mutant flies were generated and characterized, again showing that the growth period was prolonged, delaying the onset of metamorphosis without affecting metamorphosis itself (Shimell et al. 2018). However, the total amount of delay that is caused by mutating the *ptth* gene is only one day, as opposed to a delay of five days when PTTHn are ablated, suggesting the presence of other factors in PTTHn affecting metamorphosis timing. This was

further addressed by genetic manipulation experiments conducted in *ptth* null mutants. Inhibiting secretion of PTTHn in a *ptth* mutant background resulted in an additional delay in metamorphosis by approximately one day. Accordingly, genetically activating PTTHn in *ptth* mutant larvae advanced metamorphosis, and largely rescued the delay that was caused by *ptth* loss of function. This suggests that PTTHn are able to produce another secreted factor that can also time metamorphosis independently of PTTH. These findings propose a key role for PTTH to time metamorphosis. PTTH neurons could therefore represent an analogy with the mammalian GnRH neurons, by acting as the key signal to induce steroid production and sexual maturation.

### 3.2 PTTH does not have a homology in vertebrates

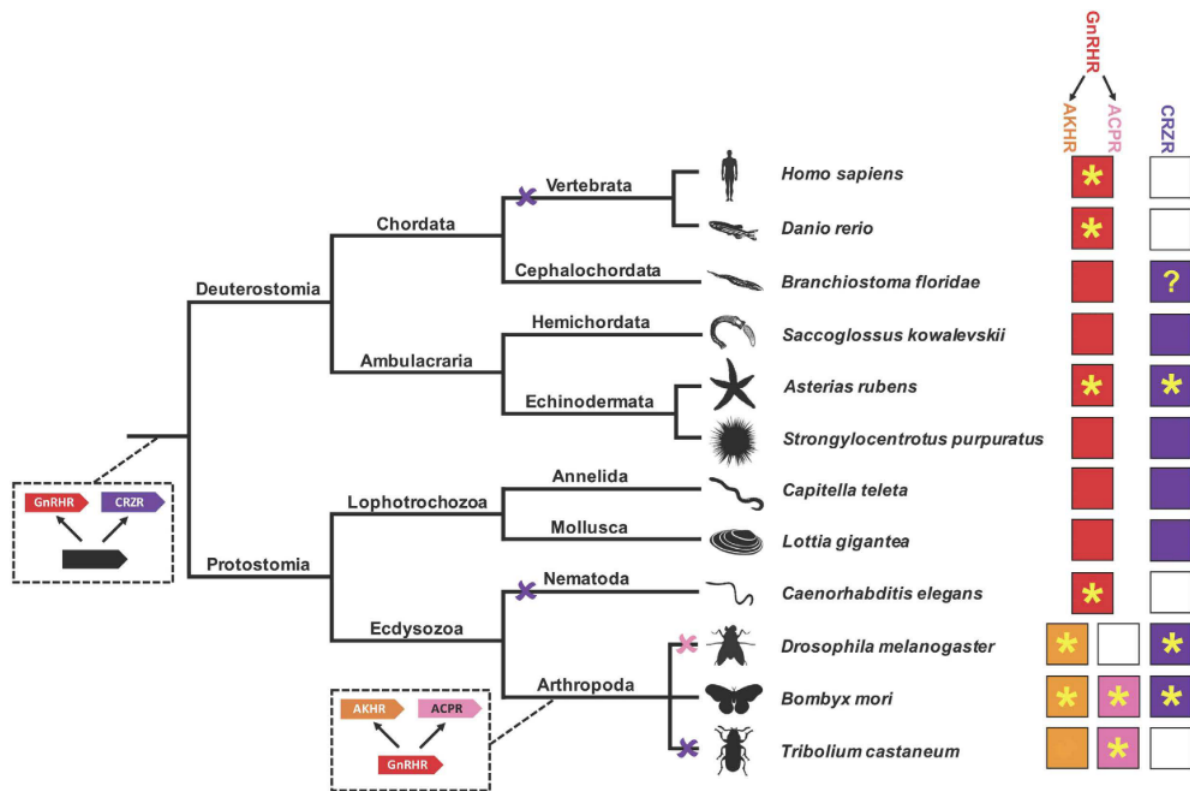
Metamorphosis and puberty are two major events in development of vertebrates and invertebrates where sexual maturation is attained. While they present common features regarding the central role of the brain controlling steroid production, they are controlled by different neuroendocrine axes. The gonadotrophic axis signals through intermediary cells to ultimately promote steroid production, whereas in insects there is a direct stimulus of neurosecretory cells onto the steroid producing gland. The initial signal the gonadotropic axis starts within the brain at the level of GnRH neurons in the hypothalamus that induces the release of sex hormones from the gonads. Similarly in insects, the initial signal controlling metamorphosis is also a brain derived signal (PTTH) that, in this case, directly signals to the gonads to induce steroid (ecdysone) production. Even though in both cases brain derived signals (GnRH and PTTH) are regulating the glands to produce steroids, there is no homology between these hormones, receptors and neuronal progenitors.

The receptor for GnRH, GnRHR, on the other hand did evolve in Arthropoda, including *Drosophila* (**Fig. 17**) Initially, a common ancestor gene in the Bilateria family gave rise to

GnRHR and corazonin receptor (CRZR) following a gene duplication. A second gene duplication had then occurred from GnRHR in Arthropoda, giving rise to Adipokinetic Hormone Receptor (AKHR) and AKH/CRZ-related peptide (ACPR), of which AKHR was kept in *Drosophila* whereas ACPR was lost. The precursors of the ligands for AKHR and CRZR, AKH and corazonin share similarities, suggesting an evolutionary conservation between these hormones. These ligands, however, only share short sequence similarities with GnRH residues. Moreover, functional experiments in *Drosophila* show an important role of AKH in energy/metabolism homeostasis, acting as the equivalent of the mammalian glucagon, providing evidence that AKH does not function as the ortholog of GnRH (Bharucha, Tarr, and Zipursky 2008). Similarly, the ligand of CRZR, corazonin, regulates metabolism and energy homeostasis by altering food intake during starvation- induced stress response (Kubrak et al. 2016). Even though there might be a common functional regulatory mechanism of corazonin/AKH/GnRH in response to metabolic stress, there is no evidence suggesting that corazonin and AKH induce steroid production like GnRH does in mammals.

Due to a lack of conservation at the level of neuronal progenitors of the HPG- axis as well as the level of neuropeptide ortholog, it is likely that steroid production and therefore sexual maturation is differentially regulated in *Drosophila*. Nevertheless, it is still plausible that flies could respond to the same internal or external signal to that of mammals to induce steroid production using different species- specific molecular sensing mechanisms.

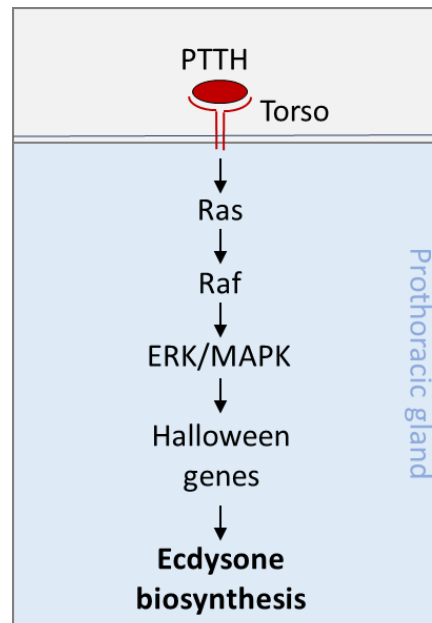




**Fig. 17 Conservation of GnRH receptor during evolution.** A common ancestral gene in the Bilateria family (black) gave rise to GnRHR and corazonin receptor (CRZR) through gene duplication. CRZR was lost in the vertebrates and Nematoda (purple cross), whereas GnRHR was lost in Arthropoda. In Arthropoda, another gene duplication took place from GnRHR, giving rise to AKHR (yellow) and AKH/CRZ-related peptide (ACPR, pink), former being lost in *Drosophila* (pink cross). Colored boxes depict the conservation of the subsequent receptor in given species. Empty boxes represent the loss of the given receptor. Asterisk within boxes represent the identification of neuropeptide ligands for given receptor type. Adapted from (Tian et al. 2016)

### 3.3 PTTH signaling induces metamorphosis by promoting ecdysteroid signaling

PTTH is secreted from the PTTH neurons upon which it binds and activates the tyrosine kinase receptor Torso that is expressed in the PG (Rewitz et al. 2009). Once Torso is activated, it stimulates the canonical MAPK cascade by activating the small GTPase Ras that subsequently triggers the phosphorylation of Raf/MAPK ultimately leading to phosphorylation of extracellular signal-regulated kinase (ERK). Loss of function of Torso in the PG, or in any of the core members of the MAPK pathway delays metamorphosis, mimicking the loss of function PTTH (**Fig. 18**). The downstream targets of this signaling pathway remain to be identified, though it involves the transcriptional upregulation of a group of selected Halloween genes that are required for ecdysteroid biosynthesis (Niwa and Niwa 2014). A newly identified target acting downstream of PTTH- Torso signaling is *Drosophila* Hormone Receptor 4 (DHR4). It was revealed that the nuclear receptor DHR4 oscillates within the PG nucleus and cytoplasm in response to PTTH signaling. Within the nucleus, DHR4 acts to suppress the uncharacterized cytochrome P<sub>450</sub> gene *Cyp6t3*, establishing the low ecdysone pulsations (Ou, Magico, and King-Jones 2011). Loss of function of *ptth* or *torso*, causes DHR4 to accumulate in the nucleus, abolishing the oscillatory behavior of ecdysone, providing evidence that DHR4 is responsible for fine tuning the ecdysone pulsations during the 3<sup>rd</sup> instar larval stage. Further research in the downstream targets of PTTH is required to establish the signaling pathway responsible for upregulation of the Halloween genes.

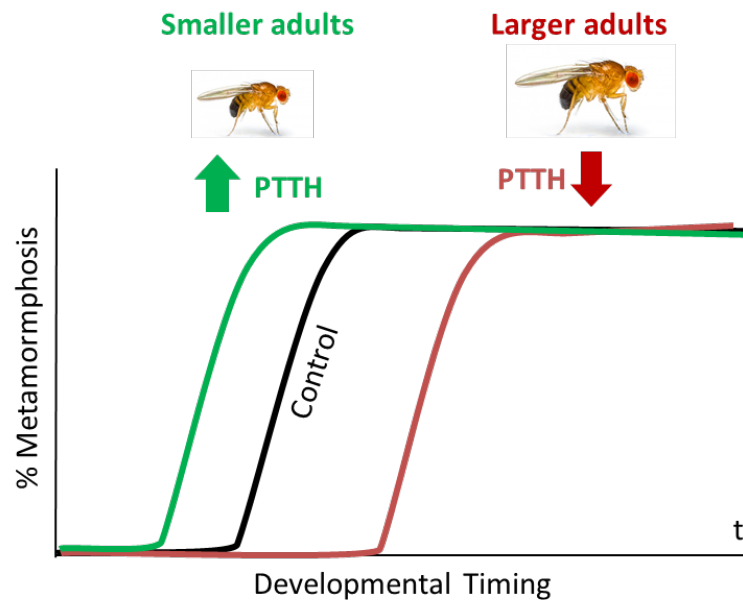


**Fig. 18 PTTH signaling in the PG.** Once PTTH is secreted in the prothoracic gland (PG), it binds its receptor Torso activating downstream signaling proteins. Once Ras is activated, it phosphorylates Raf, that in turn phosphorylates ERK/MAPK. The consequence of this signaling cascade is upregulation of the ecdysteroid biosynthesizing enzymes, encoded by the so called ‘Halloween genes’ (more on this in Chapter 2.3 in this thesis). This ultimately results in ecdysone biosynthesis.

### 3.4 PTTH regulates final body and organ size

In *Drosophila*, PTTH loss of function delays the onset of metamorphosis, giving rise to larger sized larvae in 3<sup>rd</sup> instar stage, and subsequently larger adult flies. This larval overgrowth phenotype is not the direct result of increased larval growth, since loss of PTTH does not change in larval growth rate. Instead, the larval growth period is extended, in which larvae are able to feed and grow during a longer time (McBrayer et al. 2007; Shimell et al. 2018). Additionally, PTTH loss of function also delays the time for larvae to reach critical weight, which is an important nutrition- dependent developmental checkpoint to ensure larvae have attained enough weight before undergoing pupariation. These mechanisms lead to larval overgrowth (**Fig. 19**). The delay in pupariation observed in PTTHn ablation and in the *ptth* null mutant is caused by a delay in ecdysteroid titers. Feeding these larvae with ecdysone rescues the overgrowth

phenotype and the delay in metamorphosis. As mentioned earlier, *ptth* overexpression accelerates the timing of metamorphosis. As expected, these larvae give rise to smaller sized adults since they spend less amount of time in the growth period



**Fig. 19 PTTH determines *Drosophila* adult size by timing the growth period.** Loss of PTTH (RNAi, PTTHn ablation, mutant) delays metamorphosis onset, giving rise to larger sized adults. Overexpressing *ptth* advances metamorphosis, giving rise to smaller sized adult flies.

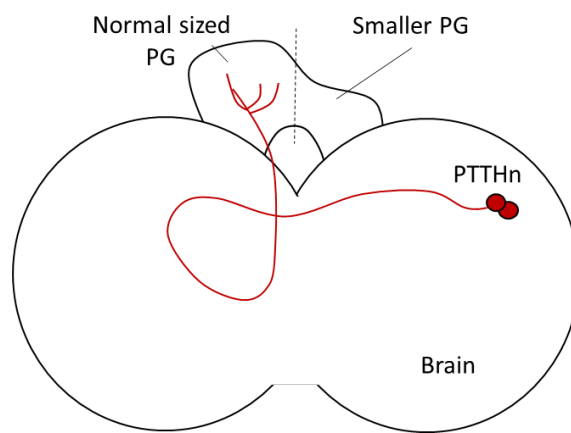
In addition to larval overgrowth, loss of PTTH also causes a reduction in the growth rate of the wing imaginal disc that, surprisingly, do not give rise to smaller wings. Instead, wing discs grow longer due to the extended larval growth period and are therefore larger in adults, compared to control wings. The reduction of the growth rate of imaginal wing discs is likely caused due to impaired ecdysone signaling in the absence of *ptth* since ecdysone has previously been proposed to promote growth of imaginal discs (Herboso et al. 2015). The increased duration of growth, however, likely overcomes the decreased growth rate in wing discs, still giving rise to larger sized wings. These findings suggest that PTTH is an important player in determining final adult size by affecting the growth period, as well as directing organ growth

by acting on imaginal disc growth rate through ecdysone signaling. In line with this, another question has opened up in the field concerning the function of ecdysone basal levels and larval growth rate. Previous studies have shown that the ecdysone basal levels during development inhibits larval growth rate (Boulant, Martín, and Milán 2013; Colombani et al. 2005; Rénald Delanoue, Slaidina, and Léopold 2010; Moeller et al. 2017; Slaidina et al. 2009). In the *ptth* null mutant, however, both ecdysone peak and ecdysone basal levels are lower but larval growth rate is unaffected. The underlying mechanism causing this effect remains to be further elucidated to understand the function of ecdysone and PTTH signaling in systemic growth and imaginal disc growth.

Other than its function in determining final size by adjusting the growth period, PTTH also exhibits trophic function on PG cell size. This function was uncovered by close examination of larvae with a hypomorphic mutation in the gap gene *giant* (*gt*). In *Drosophila*, *gt* mutants were previously described to have disrupted ecdysone titers which result in a developmental delay, giving rise to large (giant) flies. Later it was revealed that partial loss of *gt* caused a random loss of PTTH, either in one, or more PTTH neurons (Ghosh, McBrayer, and O'Connor 2010). Larvae showing a disruption in all four PTTH neurons would show the developmental delay phenotype, suggesting that PTTH is responsible for this effect. Interestingly, however, *gt* mutant larvae that randomly lose PTTH also lack the innervation to the PG, causing stochastic unilateral innervation which allows the close examination of the subsequent effect of not having PTTH on one side of the PG. This revealed that the PG lobe that is not innervated has PG cells with smaller nuclear size, whereas the PTTH innervated PG lobe has significantly larger sized cells resulting in an asymmetrically sized PG (**Fig. 20**).

To further elucidate whether these effects are due to PTTH coming from the PTTH producing neurons, double mutants were generated with the previously described *ptth* mutant, along with the *gt* mutation (Shimell et al. 2018). Accordingly, the same PG cell size difference

was observed in this double mutant background. The same effect was observed when PTTH was downregulated in the PTTH neuron from only one brain hemisphere, having the other brain hemisphere as a control. This provides more evidence supporting the notion that PTTH acts a PG growth factor. It is also interesting to note that the PG with innervated PTTH cannot rescue the loss of PTTH on the other side of the PG, showing that PTTH acts in a local manner. These findings together suggest an autonomous function of PTTH to induce PG cell size



**Fig. 20 PTTH acts as a trophic factor to promote PG growth.** Downregulating PTTHn in one brain hemisphere results in unilateral innervation of PTTHn in the prothoracic gland (PG). This causes asymmetric sized gland. The PTTH innervated PG is bigger due to larger sized PG cells compared to the PG side without PTTH innervations.

### 3.5 PTTH acts as a modulator of environmental adaptive plasticity

Environmental plasticity, or phenotypic plasticity, is the ability of an organism to adapt to their changing environments to ensure optimal fitness (Parsons 1987). Environmental factors that can induce plasticity generally occur in stressful environments such as food deprivation, crowding and temperature changes. In *Drosophila*, for instance, larvae that are grown in an environment with poor food, will minimize their developmental time at the expense of their body size, ovariole number and survival to optimize survival. Female *Drosophila* that are grown in poor food conditions as larvae are smaller in size, yet still lay bigger eggs with offspring that can grow faster when subjected to poor nutrients (Vijendravarma, Narasimha, and Kawecki 2010). When larvae are grown under crowded conditions, common to what happens in nature when *Drosophila* larvae grow on rotten food, they are exposed to larger amounts of larval waste products such as urea which is toxic for *Drosophila* (Joshi, Shiotsugu, and Mueller 1996). Under these conditions larvae minimize their developmental growth period, reducing their final size, since there is a negative correlation of fitness with developmental time and size, meaning that faster growing larvae have increased fitness (Horvath and Kalinka 2016).

Since developmental time is controlled by PTTH there could be a possibility that PTTH may have a function in allowing larvae to adapt to their changing environment by modulating the developmental time and final size. This was addressed in two scenarios, mimicking stress conditions by growing larvae in crowded environments, as well as by growing larvae under food deprivation (Shimell et al. 2018). The study shows that when PTTH mutant larvae are grown in an environment with insufficient amounts of food, they have a lower survival rate and take longer to undergo metamorphosis compared to animals that do have PTTH. This suggests that PTTH is required in nutrient deprived conditions to shorten the time to undergo metamorphosis in order to increase survival.

Similar effects were observed when larvae were grown in a crowded tube with a large amount of larvae. In this case, larvae lacking PTTH did not minimize the time of development nor adult size as they were bigger in size when grown in crowded tubes. This suggests that PTTH loss of function causes larvae to be unable to adapt to stressful environment caused by over crowdedness or by poor nutrition. Another interesting observation to note is that PTTH mutant adult flies have a higher ovariole number compared to wild-type flies, implying that PTTH plays an additional role in insect fecundity. Taken together, these findings suggest that PTTH is required in flies to be able to undergo a plastic response by modulating the growth period, organ growth and ovariole number in order to increase fitness and survival of the species. Whether or not this function has evolved only in *Drosophila*, or also among other insects, remains to be further elucidated.

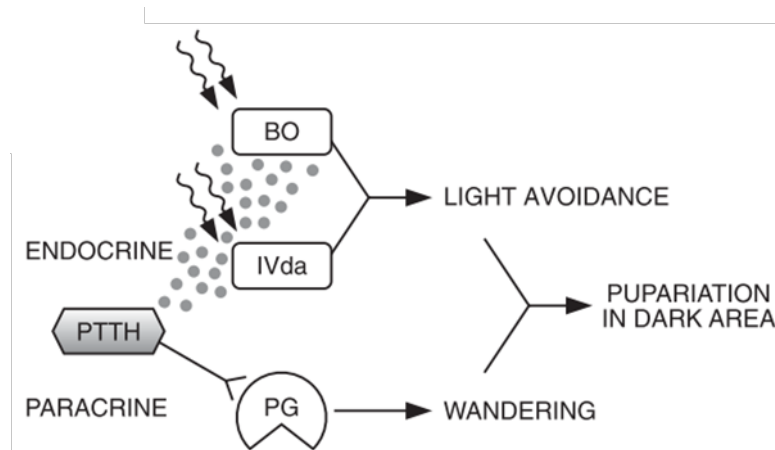
### **3.6 PTTH mediates light avoidance behavior**

The decision of *Drosophila* to undergo metamorphosis is accompanied with large number of behavioral changes in the larvae. During larval growth stages, larvae remain in the food, whereas late 3<sup>rd</sup> instar larvae crawl out of their food source during wandering stages, right before pupariation. Wandering larvae crawl extensively for a few hours, going in and out of the food in order to find the optimal spot to immobilize and pupariate. Larvae possibly will have a preference to pupariate in the dark in order to have protection from possible predators. The underlying molecular mechanisms causing these behavioral changes during wandering stages is subjected to a limited amount of studies. Previous work proposed that larvae exhibit a negative phototaxis, avoiding light, whereas larvae in wandering stages lose this light avoidance and attain a more photoneutral behavior. The authors suggest that this behavior could be important to facilitate larvae to exit from the food to enter wandering stages (Sawin-



McCormack, Sokolowski, and Campos 1995). The underlying mechanism, however, remained largely unknown.

In light of this, new research had identified a set of neurons located in the central larval brain that control larval light avoidance (Gong et al. 2010). Surprisingly, it was later revealed that these neurons are in fact the PTTH expressing neurons (Yamanaka et al. 2013). Indeed, downregulating PTTH in the PTTH neurons impaired light avoidance. Interestingly, PTTH mediated effect on light avoidance acts independently of the PG, since removal of the PTTH receptor *torso* in the PG does not affect light avoidance. This suggests that PTTH could, other than acting as an autonomous trophic factor for the PG, also have a non-autonomous function. In line with this, the authors discovered low levels of PTTH in the hemolymph and showed that inactivation of the PTTH neurons delayed the light avoidance behavior. These findings confirm that PTTH neurons likely do not exert their function in light avoidance by direct, physical interaction with target tissues (**Fig. 21**).



**Fig. 21 PTTH functions in two distinct pathway mediated by Torso.** PTTH functions as a paracrine factor for the prothoracic gland (PG) to produce ecdysone and induce developmental transition. In a separate manner, PTTH has an endocrine function signaling to the light sensors Bolwig's organ (BO) and class IV dendritic arborization neurons (IVda) to mediate larval light avoidance behavior. These PTTH mediated mechanisms ultimately cause larvae to pupariate in the dark. Adapted from (Yamanaka et al. 2013).

The authors then focused on identifying the target tissue responsible for this behavior and found that PTTH signals to previously described light sensors. These are the Bolwig's organ (BO), that are small photoreceptor bundles in the larval eye, and the class IV dendritic arborization (da) neurons that are tiling the larval body wall (Mazzoni, Desplan, and Blau 2005; Xiang et al. 2011). Specific down regulation of *torso* in the BO or da neurons abolished light avoidance behavior, showing that PTTH signals to these targets through its receptor Torso to promote light avoidance. Despite previously suggested, PTTH titers in the hemolymph are high during early wandering stages, whereas the light avoidance behavior mediated by PTTH persists during the entire wandering stage. As mentioned earlier, wander larvae will most likely search for a dark spot to become immobile and to pupariate to be protected from predators. In line with this hypothesis, the authors subjected wandering larvae to a light/dark preference assay and observed that when given a choice, larvae have a strong preference to pupariate in the dark. This behavior was abolished upon PTTH loss of function.

These findings suggest that PTTH functions in two independent mechanisms that coincide during larval wandering stages. PTTH acts in a paracrine manner to activate the PG to induce ecdysteroid production, that is followed by wandering behavior, whereas it also has an endocrine function in signaling to light- sensitive sensors to mediate light avoidance behavior that ultimately causes larvae to pupariate in the dark.

### 3.7 PTTH acts as a signal integrator of Dilp8 and critical weight

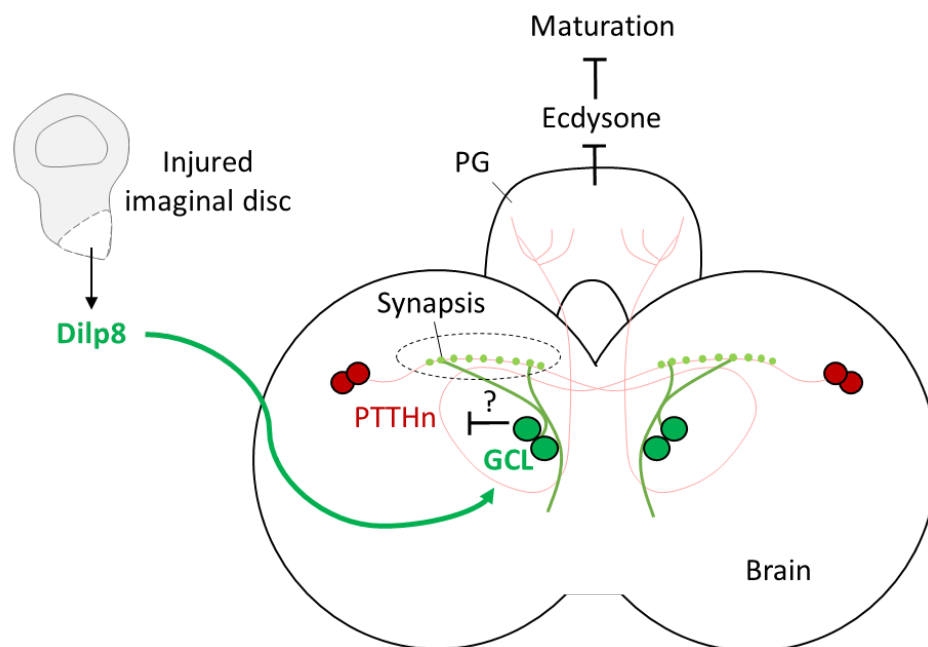
The maintenance of appropriate organ sizes in the event of genetic (internal) or physical perturbations (external) is fundamental to ensure optimal reproductive success and fitness. Maturation is not only coupled with nutritional cues, but also by intrinsic cues coming from damaged imaginal tissues. Experiments dating back to the 1970s have revealed that *Drosophila* larvae are delayed in metamorphosis upon cell- death induced damage to imaginal tissues (Simpson and A. Schneiderman 1975). This suggests a coupling of organ regeneration with timing of maturation to allow tissues to repair and attain appropriate size before entering adulthood. Intriguingly, removal of entire imaginal discs does not affect timing of metamorphosis, indicating that injured imaginal discs produce an inhibitory signal. This led to the search for a factor that can rescue the delayed developmental transition phenotype caused by abnormally growing tissues by conducting a genetic screen (Colombani, Andersen, and Leopold 2012). From this study, Dilp8 was identified as a secreted factor from imaginal discs upon tissue damage or growth perturbation and is sufficient by itself to delay metamorphosis from taking place. Co- culturing brains (with ring gland) and imaginal discs expressing wild type *Dilp8* or a non-secretable *Dilp8* showed that imaginal discs with wild type *Dilp8* significantly suppressed expression of an ecdysone downstream target, *E75B*. This suggests that Dilp8 that is produced by imaginal discs can remotely act on the PG or indirectly on the brain via a second relay system. One possibility could be that Dilp8 signals to PTTHn, inhibiting PTTH to subsequently reduce ecdysone biosynthesis.

In parallel, another study identified Dilp8 as a negative factor that links imaginal disc growth to developmental timing (Garelli et al. 2012). The authors artificially induced a tumor like phenotype in larval eye discs causing massive overgrowth and metastasis. By conducting a microarray study in these tumor discs, the authors identified *Dilp8* as a highly expressed factor from these discs. Examining Dilp8 induction in other conditions in which perturbed discs cause

delayed pupariation, the authors observed that in both fast- and slow growing tumors, as well as in artificially induced slower growing discs Dilp8 was upregulated. This shows that Dilp8 is a common factor that is induced by abnormal disc growth. *Dilp8* loss of function in the injured discs is able to shorten the delay phenotype. Interestingly, *Dilp8* is expressed during all larval development, whereas expression drops before metamorphosis, suggesting a physiological role in timing metamorphosis. However, *Dilp8* loss of function does not cause larvae to pupariate earlier, suggesting that Dilp8 absence does not act as a trigger for metamorphosis, or does not function in timing metamorphosis in normal conditions. Cell- death induced damage in the wing pouch causes delayed metamorphosis that is accompanied with lower *ptth* expression. Interestingly, silencing *Dilp8* in the damaged wing pouches partially rescues *ptth* expression, suggesting that Dilp8 negatively acts on *ptth*. The question remains, however, how Dilp8 could signal to PTTHn.

Thanks to follow up studies that were focused on identifying the relay for Dilp8 signaling, the brain relaxin GPCR Lgr3 was identified as the receptor for Dilp8. It was uncovered that secreted Dilp8 from injured imaginal discs acts on the brain by activating two pairs of neurons expressing Lgr3 receptor, named growth coordinating Lgr3 (GCL) neurons to mediate a delay in metamorphosis (Colombani et al 2015; Garelli et al. 2015; Vallejo et al. 2015). Close examination of the neuroanatomy of the GCL neurons revealed extensive axonal arborizations in very close proximity to PTTHn circuitry (**Fig. 22**). A commonly used tool in neurophysiology is GRASP, that stands for GFP Reconstitution Across Synaptic Partners (Feinberg et al. 2008). This allows the detection of synapsis between two neural population since GFP is split, expressing one GFP half in one neural population and the other half in the other neural population. By itself, split GFP is not detectable by fluorescence but when the two neural populations are in very close proximity of each other, both split GFP halves form a visibly detectable GFP. The detected GFP signal therefore correlates for physical contact of the

two neural populations. By using this method in the GCL neurons and PTTHn, the authors detected visible GFP signal on the PTTHn arborizations in the brain. These findings suggest that the GCL neurons, expressing the receptor for Dilp8 connect with the PTTHn circuitry. The current working model is that injured discs delay metamorphosis onset by secreting Dilp8 that signals to GCL neurons. These neurons act as a relay to signal PTTHn in order to modulate timing of metamorphosis to ensure (damaged) tissue grow to appropriate size to maintain a homeostasis of tissue size. However, more (functional) studies are required to determine how the GCL neurons signal to PTTHn



**Fig. 22 Neural circuitry of PTTHn and GCL neurons.** Injured imaginal discs secrete Dilp8 that signals to the growth coordinating Lgr3 (GCL) neurons. These neurons halt maturation, allowing injured discs to regenerate. This is accompanied with lower ecdysone titers which is likely mediated through inhibition of PTTHn by GCL neurons. GCL neurons physically interact with the PTTHn circuitry with the predicted synaptic sites shown as small green dots (GRASP).

Other possibilities of signals that could integrate in PTTHn could be critical weight. The attainment of critical weight is an important nutrition- dependent developmental checkpoint that ensures larvae have obtained enough mass to survive metamorphosis. Starvation before the critical weight results in larval lethality, or delayed metamorphosis. Therefore it is important to have a mechanism that senses when larvae have attained critical weight. PTTHn could be an interesting candidate in receiving this information since PTTH acts as a timer of metamorphosis. PTTHn ablation and *ptth* null mutants dramatically increase the time that is required for larvae to reach critical weight (McBrayer et al. 2007; Shimell et al. 2018). It is likely that PTTH could function as a critical size assessment system, and that when PTTH is lost, larvae lose their responsiveness to critical weight. This would go in line with PTTH regulation in *Manduca* where critical weight acts upstream of PTTH. In this model, once critical weight is attained in *Manduca* larvae, juvenile hormone (JH) declines and releases its inhibitory signal for PTTH secretion. In this case, PTTH secretion is prevented until critical weight is reached. A similar mechanism could be existing in *Drosophila*, in absence of JH, however, since *Drosophila* JH does not function similarly (further discussed in Chapter 3.8).

However, there could also be the possibility that critical weight is downstream of PTTH in which PTTH sets the time when larvae undergo critical weight. Further research is needed to test these possibilities.

### 3.8 PTTH is differentially regulated in other insects

Given the biological relevance of PTTH in timing maturation, its function in *Drosophila* still remains largely unknown. PTTH mRNA levels show temporal differences during larval development, thus it is likely that transcription of PTTH is controlled by a yet unknown factor. It remains unknown if and how PTTH is regulated at the level of splicing/processing and secretion. Studies of PTTH in other insects could therefore give more insight in the regulatory signals of PTTH. Juvenile hormone (JH) is considered to be the main regulator of PTTH since its function in inhibiting PTTH function has been well established in *Manduca*. JH drops upon attaining critical weight, which is an important nutrient dependent developmental checkpoint, allowing PTTH titers to rise to induce metamorphosis (H F Nijhout and Williams 1974). In *Drosophila*, however, removal of the JH producing gland (corpora allata, CA) delays metamorphosis rather than advancing it by releasing the potential JH inhibitory function on PTTH (C. K. Mirth et al. 2014). In line with this, feeding *Drosophila* larvae JH decreased final adult size, rather than having a growth promoting effect (Thomas, Meng-Ping, and Marc 2005). However, further research is required to elucidate the regulatory function of JH on PTTH in *Drosophila*.

In *Manduca*, PTTH secretion is suggested to be under the influence of the circadian clock, since PTTH release occurs when the photoperiodic gate is open, otherwise PTTH is released in the next photoperiodic gate (Bollenbacher et al. 1987). In *Drosophila*, however, the circadian regulation of the periodic transcriptional profile of PTTH has not been fully established (Z McBrayer et al. 2007). The pigment dispersion factor (PDF), is a neuropeptide that controls circadian rhythm and is produced by PDF- neurons that are in close proximity to the PTTH neurons in *Drosophila*. PDF null mutant flies exhibited higher levels of *ptth* transcript and a change in the periodic transcriptional profile of *ptth* (Z McBrayer et al. 2007). *Drosophila* metamorphosis, however, is not gated by photoperiod and artificially inducing the PTTH pulse

induces metamorphosis even when the photoperiodic gate is closed (unpublished data from the lab). In pupae, however, time information from the central clock is transmitted to PTTHn upon which these neurons forward time information to the PG clock to induce circadian rhythmicity of eclosion (Palacios-mun et al. 2017).

Based on experimental evidence from other insect models, PTTH was proposed to act in response to the nutritional state of the organism, as shown in two hemipterans, *Oncopeltus* and *Dipetalogaster*. In these insects, abdominal swelling stimulates ‘stretch’ receptors to signal to PTTH production in the brain (H.Frederik Nijhout 1979; H F Nijhout 1984). In *Drosophila*, however, artificial inflation of larvae does not induce metamorphosis, as reviewed in (H. F. Nijhout 2003).



## Aim of the thesis

The question that will be addressed in this thesis is how puberty, or sexual maturation is timed. The average age of pubertal onset is dropping over the past decades without a clear explanation. Sexual maturation and insect metamorphosis is the transition of juvenile into adulthood and is the result of a hormonal cascade peaking with the production of steroid hormones. Despite the differential regulation of the hormonal cascade in flies and mammals, steroid- induced maturation in both models could respond to the same evolutionary conserved signals. In light of this, I used *Drosophila melanogaster* as a model organism to identify novel signals that are integrated in the brain to induce steroid production.

During my thesis I focused on PTTH, that acts as a timer for metamorphosis by inducing steroid production. Since PTTH does not have a known regulation in *Drosophila*, I conducted a genetic screen by using RNAi- knock down to specifically silence genes in PTTHn. This lead to the identification of the GPCR Allatostatin A Receptor- 1 (AstA-R1) that delays metamorphosis once silenced in PTTHn. In addition, due to the extended growth period, larvae grow larger and become larger as adult. The ligand that is responsible for AstA-R1 signaling is AstA that is produced by two pairs of AstA neurosecretory cells in the *Drosophila* larval brain. AstA neurons form physical contact with PTTHn circuitry and regulate PTTH secretion into the prothoracic gland (PG), responsible for steroid production. Another interesting finding in my PhD is that AstA-R1/AstA has an additional function in the insulin producing cells (IPCs). Once AstA-R1 is specifically silenced in these neurons, larval growth rate is reduced, giving rise to smaller sized adult flies. This is due to impaired secretion of Dilp2 from the IPCs.

To summarize, this PhD lead to the identification of AstA/AstA-R1 regulating larval growth and timing metamorphosis. Surprisingly, AstA/AstAR1 is homologous to KISS/GPR54, an initiating factor of human puberty, suggesting that an evolutionary conserved neural circuitry controls the onset of maturation.

## **AstA signaling functions as an evolutionary conserved mechanism timing juvenile to adult transition**

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Key words: development, sexual maturation, AstA, Kiss, *Drosophila*,

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## **Abstract**

Sexual maturation is the result of a hormonal cascade peaking with the production of steroid hormones. However, the exact signals that time the onset of maturation remain elusive. Here, we present the identification of the *Drosophila* neuropeptide AstA and its receptor AstAR1, as a developmental signal that triggers maturation by promoting PTTH secretion and, in turn, the biosynthesis of ecdysone, the main insect steroid. AstA/AstAR1 also controls larval growth by directly promoting insulin-like peptide secretion from the *Drosophila* brain, therefore coordinating juvenile growth and the onset of maturation. Interestingly, AstA/AstAR1 is homologous to KISS/GPR54, an initiating factor of human puberty, suggesting that an evolutionary conserved neural circuitry controls the onset of maturation.

## Introduction

In vertebrates, the onset of puberty is marked by an increased production of steroid hormones as a consequence of the activation of the hypothalamic–pituitary–gonadal (HPG) axis (Ellison et al. 2012; Tony M Plant 2015). In the hypothalamus, this transition coincides with the pulsatile secretion of gonadotropin-releasing hormone (GnRH) from GnRH neurons (GnRHn). GnRHn project their axons onto the pituitary gland where FSH and LH intermediate hormones are produced, which in turn stimulate the production of steroids at the gonads. GnRH pulsatile secretion is the result of several excitatory and inhibitory inputs from afferent neural circuitries (Ojeda and Skinner 2006). Among them, Kisspeptin (KISS) produced by hypothalamus neurons plays a leading role (Victor M. Navarro and Tena-Sempere 2012; Pinilla et al. 2012; Juan Roa et al. 2008; Tena-Sempere 2008). Indeed, knock-down of the KISS receptor GPR54 in GnRHn induces hypogonadotropic hypogonadism and impaired onset of puberty (Herbison 2016a). Therefore, Kiss neuron activity and the regulation of GnRH secretion are crucial for steroid hormone production and puberty initiation. Despite its importance, the timed regulation of KISS and GnRH secretion relies on multifaceted mechanisms whose nature is still poorly understood (Lomniczi et al. 2013; Sangiao-Alvarellos et al. 2013). In many invertebrates, the control of sexual maturation also requires the production of steroid hormones. In *Drosophila*, the transition from juvenile to adult (i.e. metamorphosis) is marked by an increase in the production of ecdysone by the prothoracic gland (PG). Pioneering work in *Bombyx* and *Manduca* identified the Prothoracicotropic Hormone (PTTH) as a brain neuropeptide controlling ecdysone production. In *Drosophila* larvae, PTTH is produced by a pair of bilateral neurons (PTTHn), which project their axons on the PG (Kopeć 1922; Zofeyah McBrayer et al. 2007; Wigglesworth 1934). *ptth* gene transcription significantly increases just before the juvenile/maturation transition (JMT) and ablation of PTTHn delays

the time to metamorphosis by 4-5 days, highlighting the importance of this neural circuit in controlling JMT.

The onset of sexual maturation also coincides with the end of the growth period. Therefore, a coordination of growth and sexual maturation is needed to ensure proper adult size and fitness. Larval growth relies on the production and release of insulin-like peptides (Dilps) by specialized neurosecretory cells called insulin-producing cells (IPCs). As in other models, insulin-IGF signaling (IIS) relays nutritional information to organ and tissue growth, and the release of Dilps by the IPCs, relies on the presence of amino acids in the food (Renald Delanoue et al. 2016; Géminard et al. 2006; Koyama and Mirth 2016). Under limiting food, animals grow slowly and present delayed JMT. However, the coupling mechanism between growth and maturation is not fully understood.

Recent work has highlighted the role of a hormone called Dilp8 in another coupling between organ growth and maturation. In response to tissue damage, Dilp8 activates a neural circuit that interferes with PTTHn, ensuring extra time for tissue repair before the JMT takes place (Colombani, Andersen, Boulan, Boone, Romero, Virolle, Texada, and Leopold 2015; Vallejo et al. 2015). However, removal of Dilp8 during development does not induce premature pupariation. This suggests that in the absence of this checkpoint signal, inducer signals are still required for the timed activation of PTTHn.

In this study we performed a genetic screen aimed at identifying regulators of PTTHn. We found that Allatostatin-A (AstA) and its receptor Allatostatin A Receptor 1 (AstAR1) control PTTH secretion, thereby determining the time of metamorphosis. Interestingly, AstA/AstAR1 also controls animal's growth rate by acting on Dilp2 secretion from the IPCs. We find that AstA/AstAR1 is developmentally regulated with a peak of activation at the onset of metamorphosis. AstAR1 is homologous to the mammalian Kisspeptin receptor, GPR54. Therefore we propose that AstA neurons (AstAn) provide an evolutionary conserved

developmental signal that controls juvenile growth and maturation, ensuring proper adult size and fitness.

## Results

### AstAR1 induces growth and maturation

To explore the mechanism timing the onset of JMT, we conducted an RNAi-based screen for the modulation of PTTHn activity. For this, we used the *NP423-Gal4* (*423>*) and the *ptth-Gal4* (*ptth>*) drivers, both targeting the PTTHn. When combined with *UAS-dicer2* and *UAS-ptth-RNAi*, these driver lines induce a delay at the larva-to-pupa transition by 24 and 12 hours, respectively (Fig. 1A and Supplementary Fig 1B). We screened a collection of 1300 RNAi constructs targeting genes encoding membrane-associated proteins, nuclear receptors, and synaptic components, for their ability to delay the onset of metamorphosis when targeted to PTTHn (Supplementary Fig 1A). One of our best hit identified Allatostatin-A receptor 1 (*AstAR1*), a rhodopsin- like 7 transmembrane G-coupled protein receptor, as a regulator of PTTHn function. As shown in Figure. 1A and Supplementary Figure 1B, reduced *AstAR1* expression in PTTHn (*TshG80, 423>AstAR1-RNAi dcr2* or *ptth>AstAR-1-RNAi dcr2*) induces a pupariation delay similar to that observed upon *ptth* silencing (*TshG80, 423>ptth-RNAi dcr2* or *ptth>ptth-RNAi dcr2*). As a consequence, larvae grow longer, resulting in pupae that are approximately 12% larger than controls (Fig. 1B and Supplementary Fig. 1C).

To determine *AstAR1* expression, we used an *AstAR1-Gal4* knock-in line to drive GFP expression (Yamagata et al. 2016). No signal was observed in larval tissues including the gut, fat body, PG and mitotic tissues, suggesting that *AstAR1* is mainly expressed in the brain, as

previously reported by high-throughput anatomy RNA-Seq data (Brown et al. 2014). Indeed, we observed a signal within the brain lobes in PTTH cell bodies and PTTH axons that extend to the PG (Fig. 1C). We validated *AstAR1* expression in PTTHn with two other driver lines expressing *Gal4* under the control of fragments of the *AstAR1* regulatory region (Tirian and Dickson 2017) (Supplementary Fig. 1D and data not shown). Interestingly, we also detected a specific GFP signal in the IPCs, raising the possibility that *AstAR1* carries some function in these cells (Fig. 1C and Supplementary Fig. 1D). Indeed, silencing *AstAR1* in the IPCs using *Dilp2-Gal4* (*Dilp2>*) induces an important reduction in pupal size (-14%) due to a reduced larval growth rate (Fig. 1E and F), but does not affect the timing of the developmental transition (Fig. 1D).

Altogether, these data provide evidence that *AstAR1* acts both in the IPCs and the PTTHn, exerting a dual function on the animal growth rate and the time of maturation.

### ***AstAR1* controls *Dilp2* and PTTH secretion**

To understand the function of *AstAR1* signaling in the IPCs, we first analyzed *Dilp2* accumulation profile as an indicator of the ability of these cells to promote systemic growth (Géminard et al. 2006). Upon *AstAR1* silencing (*Dilp2>AstAR1-RNAi*), we observed an increase in *Dilp2* staining in the IPCs (Fig. 2A) despite normal *Dilp2* transcript levels (Supplementary Fig. 2A), as previously described in starved conditions and corresponding to impaired *Dilp2* secretion (Fig. 2A). Indeed, *Dilp2>AstAR1-RNAi* larvae show significantly reduced levels of *Dilp2* in the hemolymph, as observed in starved animals (Fig. 2B).

A similar approach was used to evaluate *AstAR1* function in PTTHn. Since the *ptth* gene is transcriptionally controlled during development (Zofeyah McBrayer et al. 2007), we first sought to analyze *ptth* transcription but did not observe any change in *TshG80, 423>AstAR1-RNAi dcr2* animals compared to controls (Supplementary Fig. 2B). However,

PTTHn present extensive remodeling of their axonal termini, with enlarged PTTH-containing boutons at the surface of PG cells (Fig. 2C). PTTH immunostaining in the cell bodies of PTTHn remains unaffected (data not shown). This suggests that AstAR1 controls PTTH secretion from PTTHn boutons located on the PG. In support of this hypothesis, the change in morphology of PTTHn synapses after *AstAR1* silencing was similar to the one observed after blocking the activity of PTTHn using the inward rectifier channel Kir2.1 (Fig. 2C) (Baines et al. 2001; Paradis, Sweeney, and Davis 2001). Finally we performed ELISA tests for circulating PTTH and detected significantly lower levels of circulating PTTH in the hemolymph in *TshG80 423>AstAR1-RNAi* larvae when compared to their controls (Fig. 2D).

We therefore conclude from these experiments that AstAR1 directly regulates neuropeptide secretion in both IPCs and PTTHn.

### **AstAR1 functions are mediated by Allatostatin A-expressing neurons (AstAn)**

The small neuropeptide Allatostatin A (AstA) is the only known ligand for AstAR1 receptor (Birgül et al. 1999; J. Chen et al. 2016). Antibody stainings against AstA reveal two pairs of bilateral AstA-positive neurons (AstAn) in the larval brain (Yoon and Stay 1995; Zoephel et al. 2012). In order to characterize these neurons, we used an *AstA-Gal4* line from the GAL4 Fly line collection (Janelia Farm), which presents an expression pattern similar but stronger than the previously described *AstA-Gal4* (Hergarden, Tayler, and Anderson 2012). Driving RFP expression with this *AstA-Gal4*, reveals two neural cell bodies in the basolateral protocerebrum, with dense arborizations in both dorsolateral and dorsomedial protocerebrum (Fig. 3A and Supplementary Movie 1). Interestingly, PTTHn and IPCs are located in these arborization areas, suggesting that AstAn could contact both populations of neurons (Fig. 3B). In order to test direct connexions between AstAn and PTTHn or IPCs, we used the GFP Reconstitution Across Synaptic Partners (GRASP), which generates a GFP signal when two



neuronal circuits are in molecular proximity (Gordon and Scott 2009). When pairing *AstA-LexA>GFP11* with *423-Gal4>GFP1-10*, a cloud of GFP signal appears in the area of the PTTHn (Fig. 3C). Additionally, we observed a robust GFP signal surrounding the IPCs when *AstA-Gal4>GFP1-10* is paired with *Dilp2-LexA>GFP11* (Figure 3D). These data suggest that AstAn make direct contact with both the PTTHn and the IPCs. By using DenMark (which marks dendritic arborisations) and anti-AstA immunostaining, we observed some AstA neuropeptide in the vicinity of both PTTHn and IPCs dendrites, suggesting that AstA could physically interact with its receptor in these neurons (Fig. 3E and F). Silencing *AstA* in the whole larval brain with the *synaptobrevin-Gal4* driver (*syb>AstA-RNAi dcr2*) causes a delay in metamorphosis (Fig. 3G), similar to the one observed after silencing *AstAR1* in the PTTHn (see Fig. 1A). Despite having an extended growth period, *syb>AstA-RNAi dcr2* animals are slightly reduced in size (-6%) (Fig. 3H), similar to what is observed in *Dilp2>AstAR1-RNAi* animals (see Fig. 1E). These results strongly suggest that AstA, by activating AstAR1 concomitant in the PTTHn and IPCs, controls larval growth and maturation. To further elucidate this hypothesis, we simultaneously silenced AstAR1 function in PTTHn and IPCs by using *423>Dilp2>AstAR1-RNAi*. Consistently, these larvae were delayed in the onset of metamorphosis and with a slight reduction in pupae size of about 4% (Fig. 3I and J). Thus, here we demonstrate that the AstA neuropeptide regulates both growth and maturation through the AstAR1 in IPCs and PTTHn, respectively.

### **AstA/AstAR1 differentially controls growth and maturation through AstA-N1 and AstA-N2 neurons**

Our previous results suggest that AstAn form a neural circuit with the IPCs and the PTTHn to control growth and sexual maturation, but it is not clear whether this is achieved commonly by both AstA neurons or if each neuron has a distinct role. The Coin-Flp technique, which allows mosaic visualization of patterns (Bosch, Tran, and Hariharan 2015), revealed the

morphological diversity of AstA neurons. The main posterior AstA neuron, referred to as AstA-N1, projects anteriorly towards the PTTH projections, and to some IPC projections (Supplementary Fig. 3B). The second AstA neuron, referred to as AstA-N2, projects towards the dorsomedium protocerebrum where IPCs cell bodies and PTTH projections are located (Fig. 3A and Supplementary Fig. 3A-C). These morphological structures suggest that AstA-N1 and AstA-N2 could signal to both PTTHn and IPCs.

In order to dissect the differential functions of AstA-N1 and AstA-N2, we use an intersection of *Gal4* and *Gal80* expression to silence individual AstA neurons. When both AstA neurons are inactivated using *Kir2.1* (*AstA>Kir2.1*), pupariation is delayed (Fig. 4A) and pupal size is reduced by 7% (Fig. 4B). This phenotype is similar to the one observed after silencing *AstA* in all the brain using *syb-Gal4*. Combining *AstA-Gal4* with *Tsh-G80*, which suppresses Gal4 activity mostly in the ventral chord, we could selectively silence AstA-N1 neurons, while maintaining AstA-N2 neurons active (as revealed by *GFP*, see Supplementary Fig. 3D). In these conditions, we observed a similar developmental delay as in *AstA>Kir2.1* animals, and no effect on pupal size (Fig. 4A and B). This indicates that only AstA-N1 neurons control the timing of pupal transition. In addition, since pupae are of normal size despite a prolonged larval period, our data suggest that AstA-N1 also affect animal's growth rate. Therefore, these experiments suggest that AstA-N2 control larval growth only, while AstA-N1 control both growth and the timing of maturation.

### **AstA/AstAR1 achieve maximal signaling activity in PTTHn prior to maturation**

Growth takes place during larval development and the cessation of growth occurs concomitant with the onset of maturation when PTTH levels raise (Zofeyah McBrayer et al. 2007). Thus, we test the temporal relevance of AstA/AstAR1 signaling by evaluating *AstAR1* expression both in IPCs and PTTHn during early and late larval development. When driven

with *AstAR1-Gal4*, GFP accumulates in the IPCs at constant levels during larval development, suggesting that *AstAR1* expression is not controlled by developmental cues in these cells (Fig. 4D). By contrast, we detected progressively increasing GFP signal in the PTTHn during larval development (Fig. 4C). Indeed, an up-regulation of *AstAR1* transcript levels is observed by RT-qPCR on whole larvae towards the end of larval development (Supplementary Fig. 4A). We did not observe any obvious remodeling of AstAn, as shown using an *AstA>GFP* reporter (Supplementary Fig. 4B). However, AstA immunoreactivity within AstA-N1 dramatically increases during larval development (Fig. 4E). This is concomitant with the observed increase in PTTH as previously reported (Yamanaka et al. 2013). We could not quantify AstA peptide levels specifically in AstA-N2 as they are below detection threshold (Supplementary Fig. 4C). Since neuropeptides are often actively secreted by an increase in intracellular calcium (Sudhof 2012), we used the *CaLexA* calcium reporter (Masuyama et al. 2012) as a marker of neurosecretory activity in these neurons. Indeed, we found increasing levels of *CaLexA*-induced GFP in AstA-N1, suggestive of an increasing neurosecretory activity towards the end of larval development (Fig. 4F).

In summary, our data indicates that AstA signals in the IPCs promotes growth throughout larval development and in a temporally-regulated fashion in the PTTHn to induce maturation at the end of larval period (Fig. 4G).

## Discussion

### Coordination of growth and maturation by AstA signaling

During juvenile development, both the rate and the duration of growth affect final adult size. It is therefore important to clarify the mechanisms that coordinate growth rate and growth duration. Studies in *Drosophila* have revealed that PTTH plays a role in such coordination. After larvae reach a so-called critical weight, *ptth* transcripts raise, subsequently inducing a rise

in ecdysone biosynthesis. In addition to this transcriptional control, we now identify AstA/AstAR1 as a developmental signal controlling PTTH secretion. One question remains: how is the control of PTTH production coordinated with the growing status? A possible mechanism could use Dilp8/LGR3 signaling, which delays the onset of metamorphosis in response to tissue damage. Whether this signal interferes with PTTH function during normal development still needs to be clarified (Colombani, Andersen, Boulan, Boone, Romero, Virolle, Texada, and Leopold 2015; Garelli et al. 2015). In this study, we demonstrate that AstA signalling not only times the onset of maturation by regulating PTTH secretion, but also induces larval growth by promoting Dilp secretion. Interestingly, blocking AstA signaling in the IPCs reduces larval growth rate, but this is compensated for by extending the growth period through PTTHn, allowing animals to reach normal body weight. Interestingly, another receptor for AstA, AstAR2 has been recently reported to link AstA signaling with the metabolic status in the adult IPCs (Hentze et al. 2015). Thus, AstAn, PTTHn and IPCs likely define a homeostatic neural circuit that coordinates growth/metabolism and maturation timing.

### **AstA and KISS: a conserved neural circuitry controlling maturation**

AstA was initially described as an arthropod-specific hormone inhibiting food intake and juvenile hormone (JH) secretion (Lechner, Lein, and Callaway 2002). However, a comprehensive evolutionary study of the AstA receptor 1 gene revealed that it shares an evolutionary ancestor gene with the mammalian GPR54 receptor gene (Felix et al. 2015). Moreover, the *Drosophila AstA* presents a conserved gene synteny with the human *KISS* gene, suggesting that *AstA* and *KISS* could originate from a common ancestor. As previously mentioned, the KISS/GPR54 pathway promotes pulsatile secretion of GnRH, a necessary event for steroid production and sexual maturation in vertebrates (V. M. Navarro, Fernández-Fernández, et al. 2004). In this study, we identify the AstA/AstAR1 pathway as part of the timer

for PTTH secretion, ecdysone production and the onset of sexual maturation. Our findings present unexpected functional parallels with the role of the KISS/GPR54 pathway at the onset of puberty: (i) *KISS* expression rises during pre-pubertal stages to induce maximum secretion of GnRH (V. M. Navarro, Castellano, et al. 2004); (ii) hypothalamic levels of *GPR54* mRNA increases dramatically at pre-puberty stage (Seong-Kyu Han et al. 2005); (iii) an increase in KISS-GPR54 signaling occurs during puberty, due to an absence of desensitization to KISS stimulus (J. Roa et al. 2008). In parallel with this, we find that *Drosophila* AstA peptide levels rise at the end of development, anticipating the rise in PTTH levels and the onset of metamorphosis. Concomitantly, expression of *AstAR1* in PTTHn increases suggesting that these neurons become more sensitive to the AstA signal just before metamorphosis. Another feature observed in mammals is the distribution of two KISS neuron subpopulations in the hypothalamus: one in the arcuate nucleus (ARC), and one in the rostral periventricular area of the 3rd ventricle (RP3V). Different roles for the ARC and RP3V KISS neurons have been allocated in either initiation or progression of puberty (Mayer et al. 2010), but the regulation of these differential actions is poorly understood (Pinilla et al. 2012). Similar to this, we describe here two separate AstA neurons with different functions. The sole inactivation of AstA-N1 is sufficient to induce a delay, indicating that it has clear timing function. The role of AstA-N2 in timing maturation is not yet established. Future research will be needed to better understand the differential function of AstAn and incoming regulatory signals for AstA-N1 and AstA-N2. AstA/AstAR1 signaling increases with larval volume and could respond to a size threshold. Our experiments suggest a concomitant action of AstA/AstAR1 signaling on growth and maturation. Interestingly, several studies point to the role of Kisspeptin on growth hormone (GH) secretion from the anterior pituitary gland (Luque RM, 2011, Kadokawa H, 2008., Chang JP, 2012., Gutiérrez-Pascual E, 2007).

Given the remarkable molecular and functional conservation between KISS and AstA signaling for the control of maturation and growth, the regulation of *Drosophila* steroid production by AstA signaling should provide further mechanical insights on this major developmental transition.

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### **Author contributions**

D.D., N.M.R. and F.A.M. performed the experiments and N.M.R., D.D., P.L. designed and interpreted experiments. D.D., N.M.R., wrote the original draft. F.A.M, P.L., D.D. and N.M.R. wrote, edited and reviewed the manuscript.

### **Competing interests**

The authors declare no competing financial interests.

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## Figures

### Figure 1. AstAR1 as a positive regulator of PTTHn and IPCs

(A-B) *AstAR1* silencing in the PTTH neurons (*TshG80-NP423>AstA-RNAi*) causes a delay in metamorphosis and an increase in pupal size. (C) AstAR1 expression reporter in *Drosophila* larval brain. AstAR1 is expressed in PTTH neurons and IPCs. PTTH, IPCs and GFP are visualized by immunostaining against PTTH (yellow), Dilp2 (red) and GFP (green). (D-E) *AstAR1* silencing in IPCs (*Dilp2>AstA-RNAi*) decreases pupal size without affecting the timing of metamorphosis. (F) This decrease of pupal size is due to a reduction in larval growth rate. \* $P \leq 0.05$ , \*\* $P \leq 0.01$ , \*\*\* $P \leq 0.001$ , \*\*\*\* $P \leq 0.0001$ , unpaired t-test. Data are represented as mean  $\pm$  SEM.

### Figure 2. AstA-R1 signaling controls the secretion of Dilp2 and PTTH

(A) AstAR1 silencing in the IPCs (*Dilp2>AstA-RNAi*) causes accumulation of Dilp2 in the IPC cell bodies, as observed in acutely starved larvae (positive control). (B) Corresponding Dilp2 hemolymph levels measured by ELISA (see materials and methods). (C) *AstAR1* silencing in PTTHn (*TshG80, NP423>AstA-RNAi*) leads to an accumulation of PTTH in the PTTHn boutons projecting on the prothoracic gland, as observed upon PTTHn hyperpolarisation (*ptth>Kir2.1*). (D) *AstAR1* silencing in PTTHn reduced levels of circulating PTTH as measured using an HA-tagged PTTH (see materials and methods). Dilp2 and PTTH retention are measured by immunohistochemistry using rat anti-Dilp2 and guinea pig anti-PTTH.

### Figure 3. AstAR1-mediated growth/maturation effects rely on AstA

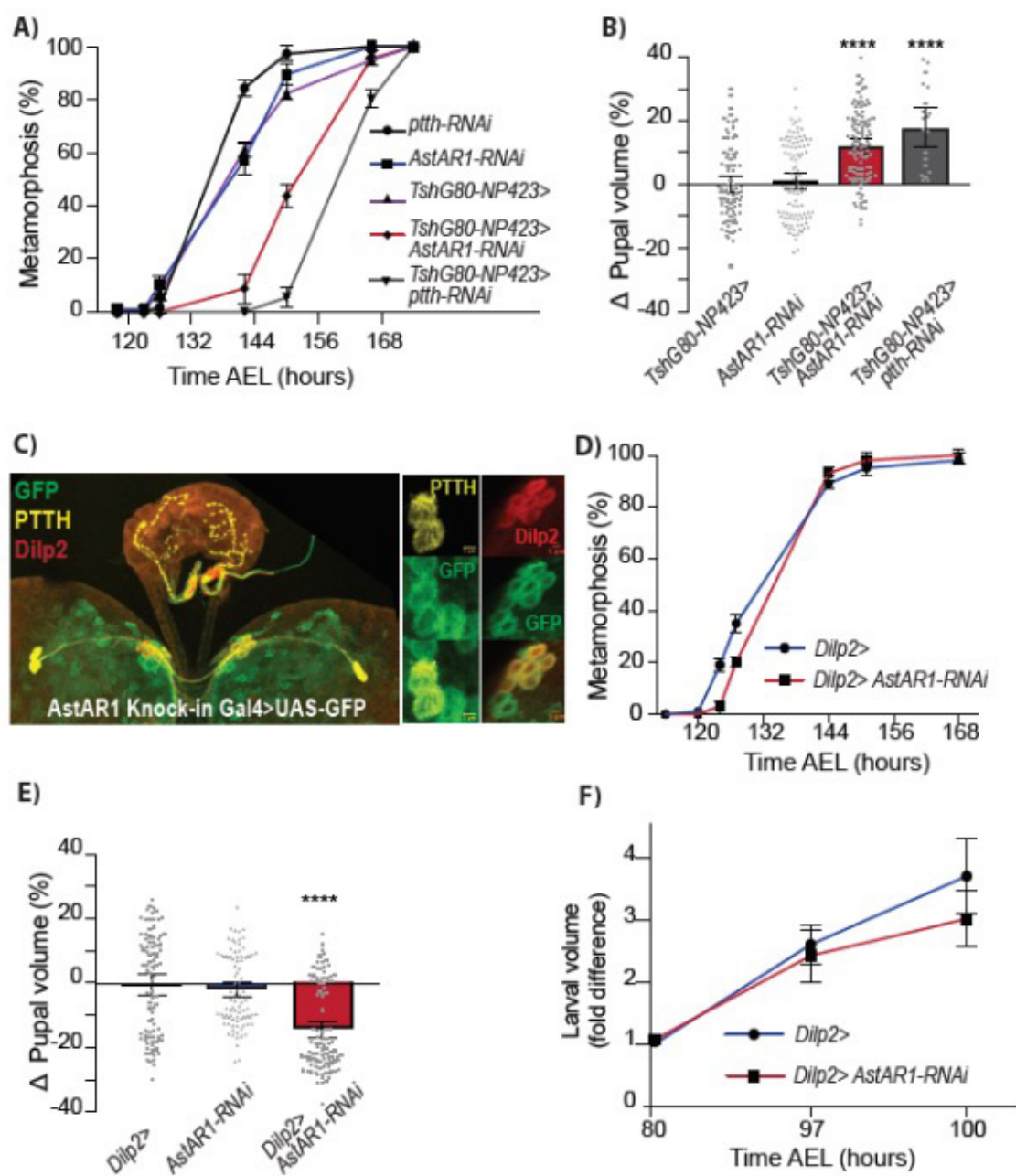
(A) The expression patterns of AstA and Dilp2 in larval brains are visualized using RFP and GFP, respectively (*AstA-Gal4>RFP; Dilp2-lexA>GFP*). PTTH is visualized by immunostaining (in cyan). Only one pair of bilateral neurons stain positive for GFP in the

central brain (N1 and N2 neurons). (B) XZ section of dissected larval brains labelled with RFP and GFP under the control of *AstA-Gal4* (red) and *Dilp2-LexA* (Green), and stained for PTTH (cyan). Axons of the AstA-positive cells intersect with those of PTTHn and IPCs. (C) GFP Reconstitution Across Synaptic Partners (GRASP) between AstAn and PTTHn. The GRASP signal (GFP) is in green after immunostaining against GRASP-specific GFP antibody. (D) GRASP signal is also detected between AstAn and IPCs. (E-F) AstA peptide localizes near the dendrites of PTTHn and IPCs, marked by *NP423>UAS-DenMark* (red). Dendrites were marked using *NP423-Gal4>UAS-DenMark* or *Dilp2-Gal4>UAS-DenMark* (red). AstA is visualized by immunostaining (green) (G-H) AstA silencing in the brain (*syb>AstA-RNAi*, *dcr2*) delays metamorphosis and reduces pupal size compared to controls. (I-J) Simultaneous *AstAR1* knock-down in PTTHn and IPCs delays metamorphosis and reduces pupal size (*Dilp2>NP423>AstAR1-RNAi dcr2*).

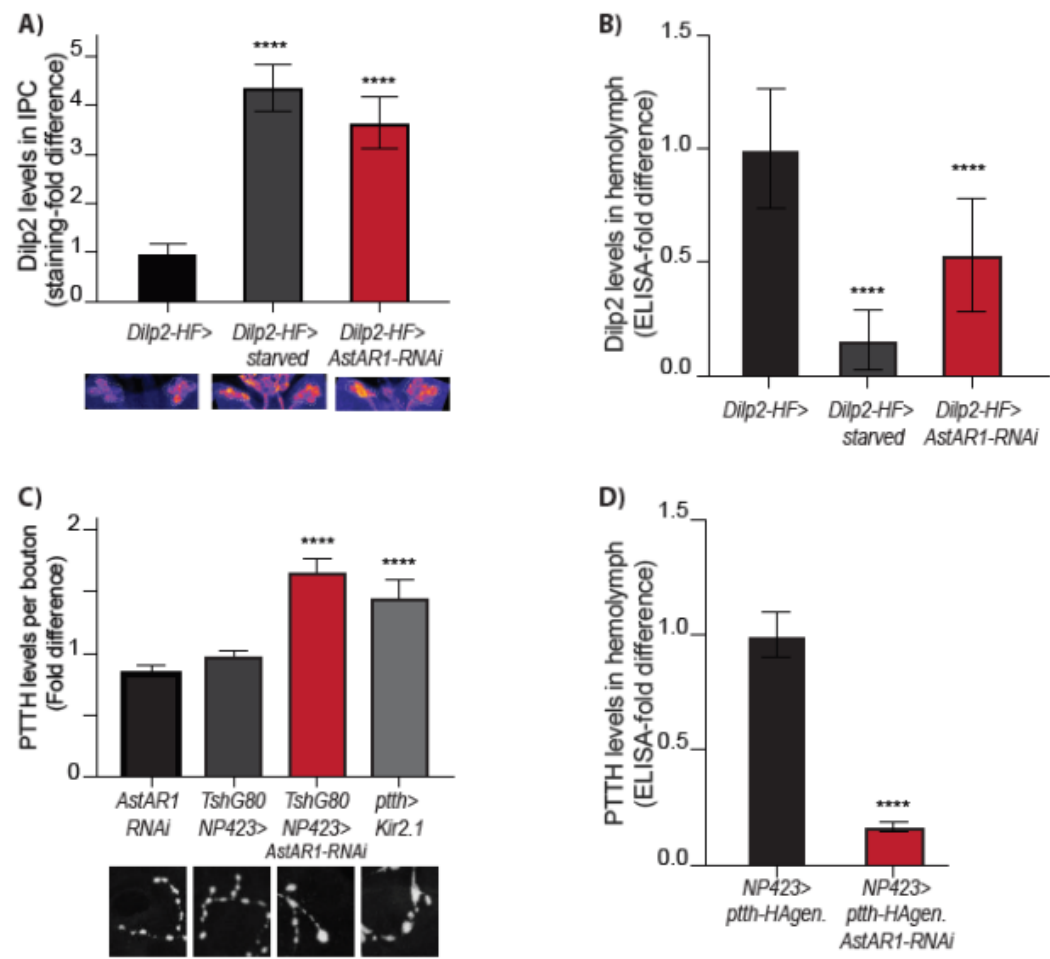
#### **Figure 4. AstA/AstAR1 signaling is developmentally regulated**

(A) Silencing both AstAn (*AstA>Kir2.1*) or AstA-N1 alone (*TshG80*, *AstA>Kir2.1*) delays metamorphosis. (B) Pupal size is decreased when both AstAn are silenced by *AstA>Kir2.1*. (C) GFP signal in *AstAR1*-knock-in *Gal4>UAS-GFP* in PTTHn shows gradual increase throughout larval development, whereas (D) it remains constant in IPCs. (E) AstA peptide levels increase in the cell bodies of AstA-N1 during larval development, as detected by immunostaining against AstA. (F) Neural activity of AstAn is also increased during larval development as labeled by the CaLexA system (*AstA-Gal4>UAS-mLexA-VP16-NFAT*, *LexAop-Cd8-GFP*). (G) Our current working model for AstA/AstAR1 signaling during larval development and schematics for AstAn/PTTHn/IPC neural network in the *Drosophila* larval brain.

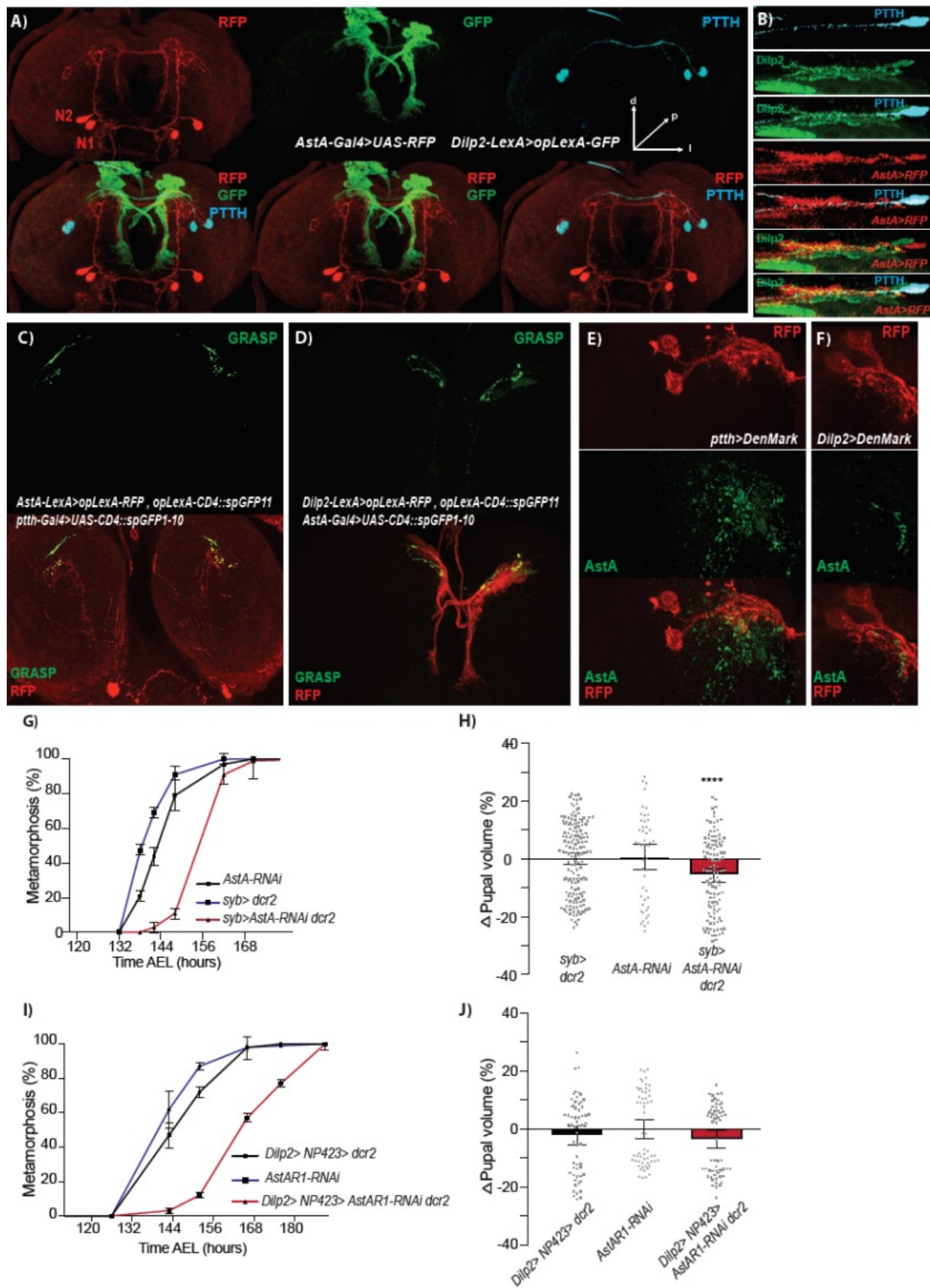
**FIGURE 1**



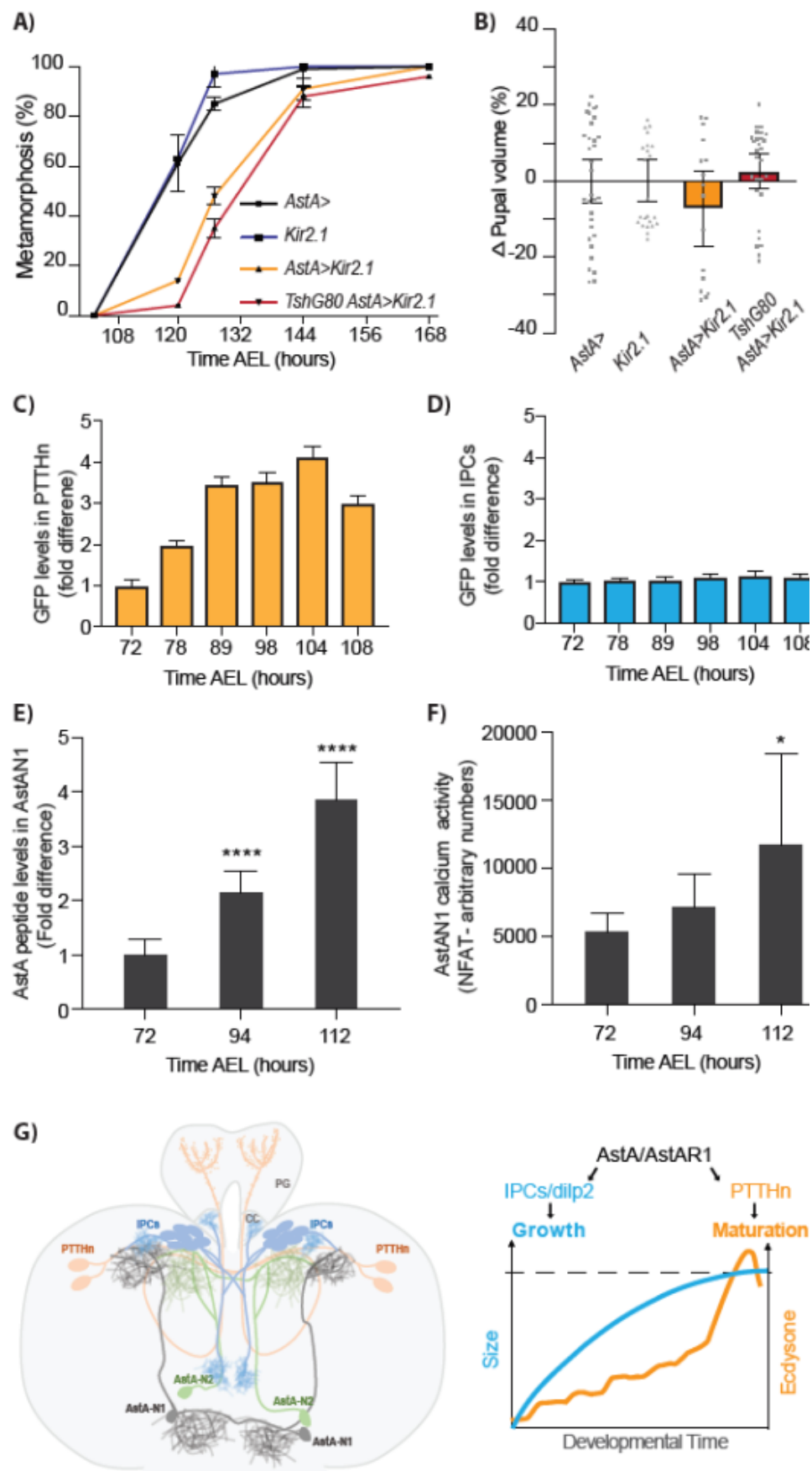
**FIGURE 2**



**FIGURE 3**



**FIGURE 4**





## Materials and Methods

### *Drosophila strains and food*

*Drosophila* larvae were raised at 25°C on standard cornmeal food containing, per liter, 17 g inactivated yeast powder, 83 g corn flour, 10 g agar, 60 g white sugar, and 4.6 g Nipagin. The following transgenic and mutant flies were used: *ptth-Gal4* (Zofeyah McBrayer et al. 2007), *Dilp2-Gal4* (Brogiolo et al. 2001; Rulifson, Kim, and Nusse 2002), *Tsh-Gal80* (Berni et al. 2012), *ptth-HA genomic* (Shimell et al. 2018), *Ilp2HF* (Park et al. 2014), *Dilp2-LexA* (Q. Li and Gong 2015), *AstARI-Gal4* (Yamagata et al. 2016) From Vienna Drosophila RNAi Center we obtained the following lines: *AstA-RNAi* (#14398), *AstARI-RNAi* (#39222) or (#48495), *ptth-RNAi* (#102043), *UAS-dicer2* (#60008) or (#60009). *NP423-Gal4* (#103614, Drosophila Genetic Resource Center, Kyoto). The following lines were obtained from Bloomington Drosophila Stock Center: *AstA-Gal4* (#39351), *op-LexA-GFP* (#58754), *UAS-RFP* (#58754), *CoinFLP-LexA::GAD* (#58754), *UAS-Kir2.1* (#6595), *UAS-DenMark* (#33061), *nSyb-Gal4* (#51635), *opLexA-RFP*, *opLexA-CD4::spGFP11*, *UAS-CD4::spGFP1-10*.

### *Genetic screen*

We performed a biased genetic screen to identify positive regulators of PTTHn. We used the *NP423-Gal4* line to screen a set of RNAi lines from the Vienna Drosophila RNAi Center (VDRC) for selected GO terms present in the brain: membrane targeted proteins, nuclear receptors, vesicular mediated neurotransmitter components. 1300 lines were tested on their ability to delay metamorphosis. We tested 300 lines per week by crossing 16 virgins containing the *NP423-Gal4* driver with 10 males of each RNAi lines. Three replicates were collected from each cross with collections of 4 h. 270 candidates significantly affected developmental timing. In a secondary screen, positive RNAi lines were combined with *ptth > ptth > dcr2* tester line. Only one hit presented a strong and robust developmental delay of metamorphosis, *AstARI*

### *Developmental timing*

All developmental timing experiments were conducted at 25°C on standard cornmeal food without added sugar. Embryos were collected after 4 hours of egg laying carried out on agar dishes containing 3% sugar and were incubated at 25°C for 21 hours after which the L1 larvae were transferred into tubes containing standard cornmeal without added sugar. Pupae number from 4 tubes per genotype were scored 3 times a day and ordered by progressive pupariation time and plotted in GraphPad or Microsoft Excel using non-linear regression curve fit.

### *Pupal volume measurements*

Pupae were aligned in a petridish and photographed. Pupal length and width were obtained with ImageJ by measuring the medial line between anterior-posterior, and by measuring along the axial line, respectively. Pupal volume was calculated by using the formula for prolate spheroid:  $(\pi/6)W^2*L$ . Data obtained was plotted in GraphPad or Microsoft Excel to obtain bar graphs.

### *Measuring growth rate*

Larvae were sorted at the transition of L2 into L3 and transferred to standard cornmeal food. At 80, 97 and 100 hours AEL, images of the larvae were captured and larval size was measured by ImageJ as described above.

### *Immunohistochemistry of larval brains*

Dissected brains were fixed in 4% formaldehyde (Sigma) for 25 min at RT, washed in PBT (0,1% Triton X-100 in PBS (137 mM NaCl, 2.7 mM KCl, 4.3 mM Na<sub>2</sub>HPO<sub>4</sub>, 1.47 mM KH<sub>2</sub>PO<sub>4</sub>, [pH 8])) and blocked in PBT containing 10% FBS for 40 min. Brains were incubated

in PBT containing the primary antibody overnight at 4°C, washed three times in PBT, incubated with the secondary antibody for 2 hours at 4°C, washed and mounted in Vectashield mounting media with DAPI (Vector laboratories). Fluorescent images were acquired with Leica TSC SP5 using the 20x or 40x objective and analyzed using ImageJ.

### *Antibodies*

The following primary antibodies were used: anti-PTTH at (1:400, guinea pig), anti-dilp2 (1:400, rat), anti- AstA (1:20, mouse anti Ast7 cockroach, DSHB #5F10), anti- AstA (1:100, rabbit against cockroach A-AST, Jena Bioscience #ABD-062) anti- GFP (1:10.000, chicken), anti- GFP specifically recognizing reconstituted GRASP (1:100, mouse, Sigma). Secondary antibodies that were used: Alexa Fluor 488 anti- chicken (1:400, Thermo Fisher Scientific), Alexa Fluor 488 anti- mouse (1:400), Alexa Fluor 546 anti- rat (1:400), Alexa Fluor 648 anti- guinea pig (1:400), Alexa Fluor 648 anti rabbit (1:400).

### *Quantitative RT-PCR*

Larvae were washed in PBS and frozen in liquid nitrogen. Total RNA was extracted by using RNeasy lipid tissue Minikit (QIAGEN) according to the manufacturer's protocol. RNA samples were treated with DNase and then reverse transcribed to cDNA using the SuperScript II reverse transcriptase (Invitrogen). cDNA samples were then used for RT-qPCR (StepOne Plus, Applied Biosystems) upon using PowerSYBRGreen PCR mastermix (Applied Biosystems). Rp49 was used to normalize the samples. Primers were designed by using the NCBI/Primer-BLAST. Primer sequences are available on request.

### *Dilp2 retention in IPCs*

Dilp2 levels were quantified upon staining larval brains for Dilp2 as described above. Confocal Z stack images were acquired at 12 bit using 0,5 µm step size with identical laser

power and scanning settings. Using ImageJ, sum- intensity of Dilp2 in the IPCs was measured to obtain the area and mean intensity value.

#### *Circulating PTTH and Dilp2 in the hemolymph*

Total circulating levels of PTTH and Dilp2 in the hemolymph were quantified by sandwich ELISA. F8 Maxisorp loose Nunc-Immuno modules (Thermo Scientific #469949) were coated and incubated overnight at 4°C with anti-FLAG (2.5 µg/ml, Sigma-Aldrich F1804) (for Dilp2) or anti-PTTH (1:10) in coating buffer (0,2M sodium carbonate/bicarbonate buffer, pH 9,4; BuPH Carbonate-Bicarbonate buffer packs, ThermoFisher #29892). Plates were washed with PBTw0,2% (0,2% Tween-20 in PBS) and blocked with filter sterilized PBS containing 2% BSA overnight at 4°C. The next day, plates were washed with PBTw0,2% before adding hemolymph.

Hemolymph was obtained by bleeding washed larvae on ice, and collected in 50 µL ice cold PBS. Tubes containing the hemolymph were centrifuged at 1,000 x g for one minute and supernatant was collected. The samples were then mixed with anti-HA-Peroxidase, High Affinity (3F10) (Roche #12013819001) at a dilution of 1:500 in PBTw0,2% and incubated in the blocked Nunc-Immuno modules overnight at 4°C. The next day, samples were aspirated and the plates were extensively washed with PBTw0,2%. Next, 1-Step Ultra TMB- ELISA Substrate (Thermo Scientific #34029) was added into the wells and incubated for 30 min at RT. The reaction was stopped by adding 2M sulfuric acid and absorbance was measured immediately at 450nm.

#### *Quantification PTTH bouton in PG*

PTTH content per bouton was quantified upon staining wandering larvae with anti-PTTH as described above. Confocal Z stack images were acquired at 12 bit using a 0,5 µm step

size with identical laser power and scanning settings. Using ImageJ, sum- intensity of the PTTH boutons was measured to obtain pixel intensity and area. PTTH per bouton was obtained by pixel intensity \* area.

### *Statistical Analysis*

P values are the result of ANOVA or Student's test provided by Graphpad Prism (\*p<0,05; \*\*p<0,01; \*\*\*p<0,001, \*\*\*\*p<0,0001).

## Supplementary figures

### Supplementary Figure 1. Identification of *AstAR1* from a genetic screen as a positive regulator of PTTHn

(A) 1300 RNAi lines were screened on their ability to delay metamorphosis when silenced in PTTHn using two different drivers (*NP423>* and *ptth>dcr2*). (B) When candidates from the primary screen were subjected to a second round of screening using *ptth>dcr2* we obtained one candidate that delayed metamorphosis (C) and gave rise to bigger animals, *AstAR1*. (D) Expression pattern of *AstAR1* in the central brain (3<sup>rd</sup> instar larvae) visualized by *VT-204065-GAL4>UAS-GFP*. PTTHn, IPCs and GFP are labeled by immunostaining.

### Supplementary Figure 2. *PTTH* and *Dilp2* expression levels after silencing *AstAR1* in PTTHn or IPCs

(A) *Dilp2* mRNA levels in control larvae (*Dilp2-Gal4*) and after silencing *AstAR1* in IPCs (*Dilp2>AstAR1-RNAi*) as measured by RT-qPCR (B) *ptth* mRNA levels in control larvae (*TshG80-NP423-Gal4*) and after silencing *AstAR1* in PTTHn (*TshG80-NP423>AstAR1 RNAi*).

### Supplementary Figure 3. Morphology of the AstAn circuit in the larval brain

(A-C) By expressing *AstA-Gal4* with the CoinFLP system we obtained mosaic expression of the AstA neurons, specifically visualizing the distinct projections of AstA-N1 and AstA-N2 towards IPCs and PTTHn. PTTHn, IPCs and GFP are visualized by immunostaining against PTTH (cyan), Dilp2 (magenta) and GFP (yellow). (A) All AstA neurons and their projections express GFP in both brain hemispheres. (B) Only AstA-N1 expresses GFP in each hemisphere. Note AstA-N1 projects anteriorly towards the PTTHn- and IPCs-projections (C) Only AstA-N2 in the right hemisphere expresses GFP, visualizing its projections towards the dorsomedium protocerebrum where IPC cell bodies and PTTHn projections are located. (D) Expression

pattern of *AstA-Gal4* and *AstA-Gal4>TshG80* by driving *UAS-GFP*. AstA-N2 GFP expression is silenced by *TshG80*. GFP is visualized after immunostaining.

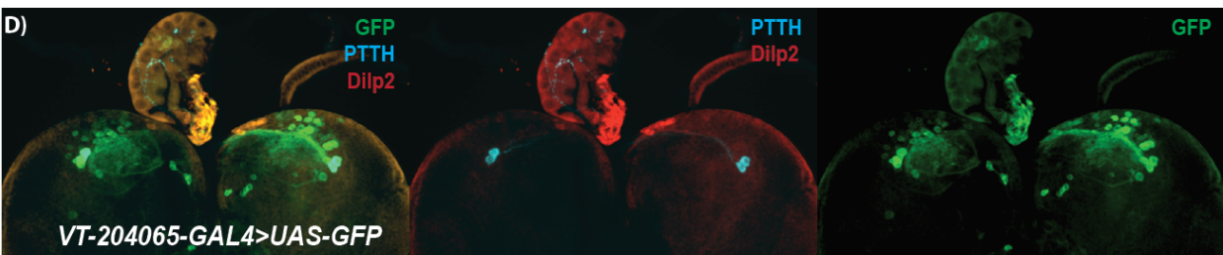
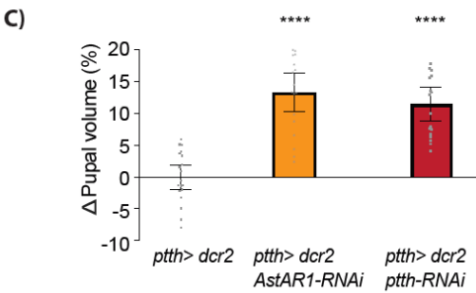
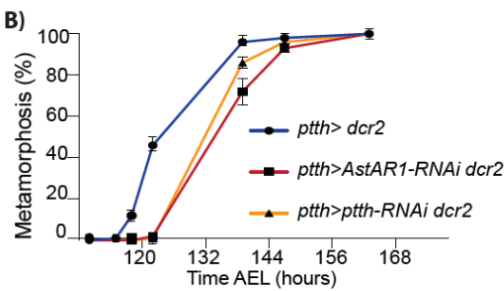
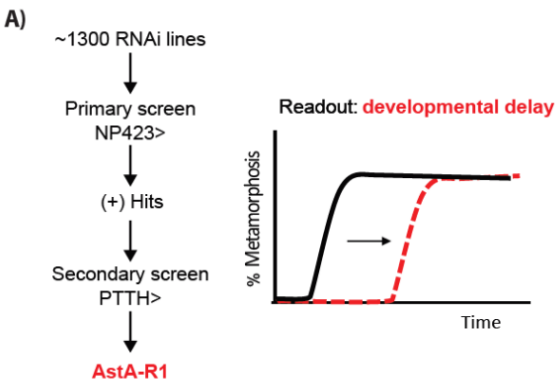
#### **Supplementary Figure 4. AstA/AstAR1 regulation during larval development**

(A) *AstAR1* mRNA expression levels of total larvae during development as measured by RT-qPCR. (B) AstA circuit morphology visualized by imaging larval brains at different developmental time points (*AstA-Gal4>UAS-GFP*). GFP is visualized by immunostaining. (C) Representative immunostaining of AstA in AstA-N1 and AstA-N2 in the larval brain.

#### **Supplementary Movie 1. AstAn arborization in the larval brain**

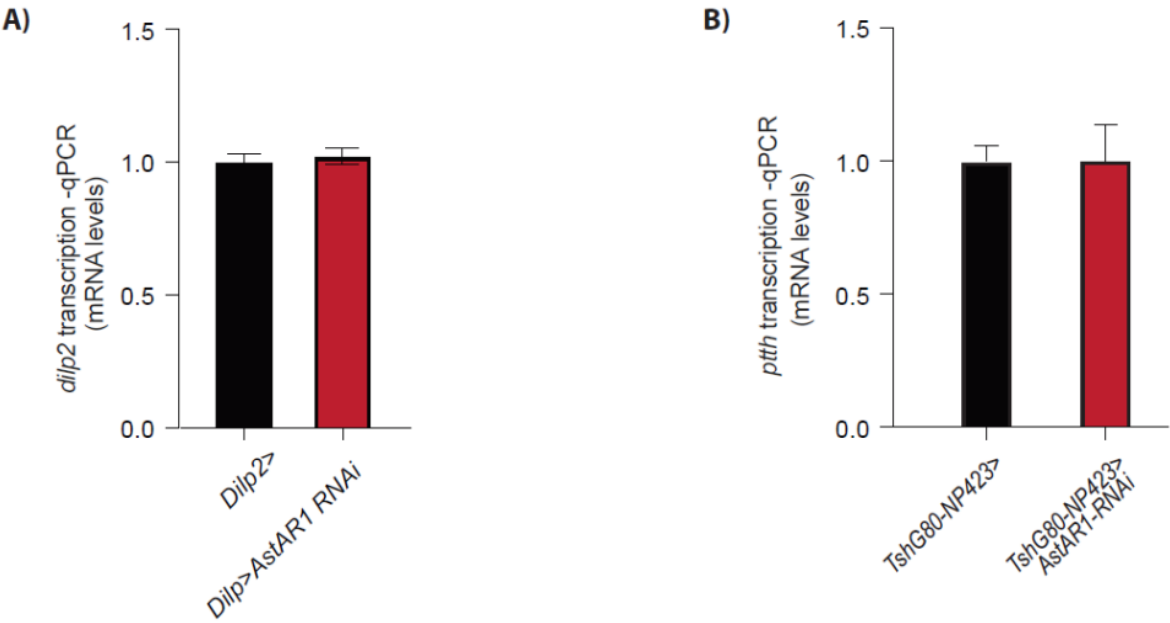
3D reconstruction of Z sections (0,5  $\mu$ m) of a larval brain (3<sup>rd</sup> instar larvae) expressing *AstA-Gal4>GFP*. AstA neurons and arborizations are visualized by GFP immunostaining in the basolateral protocerebrum

SUPPLEMENTARY FIGURE 1

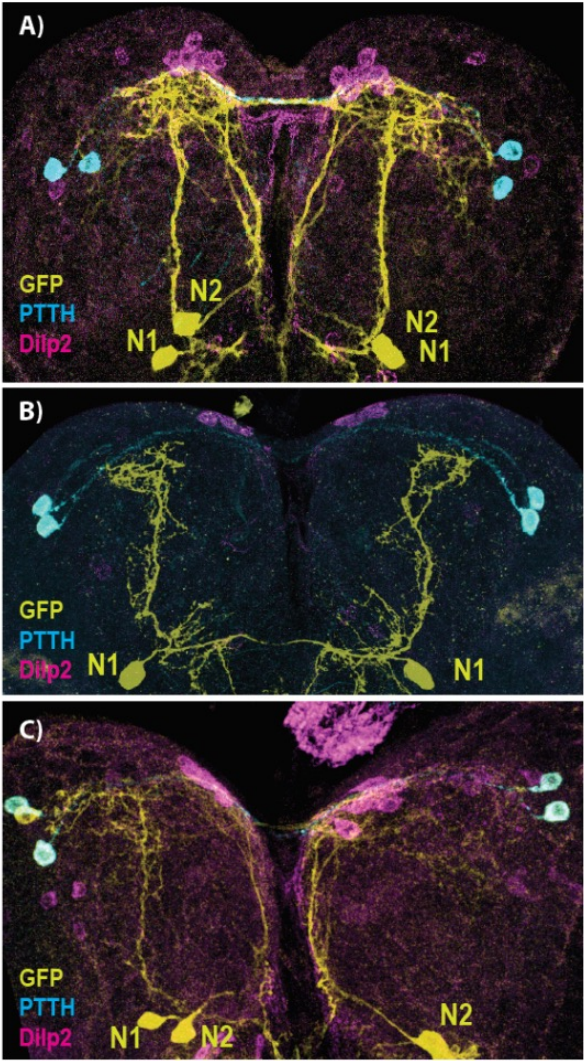




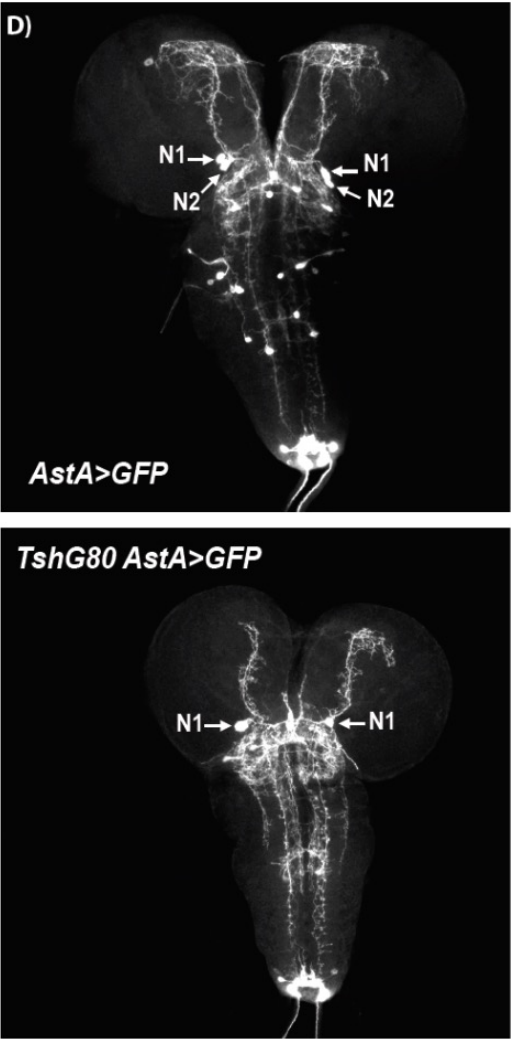
SUPPLEMENTARY FIGURE 2



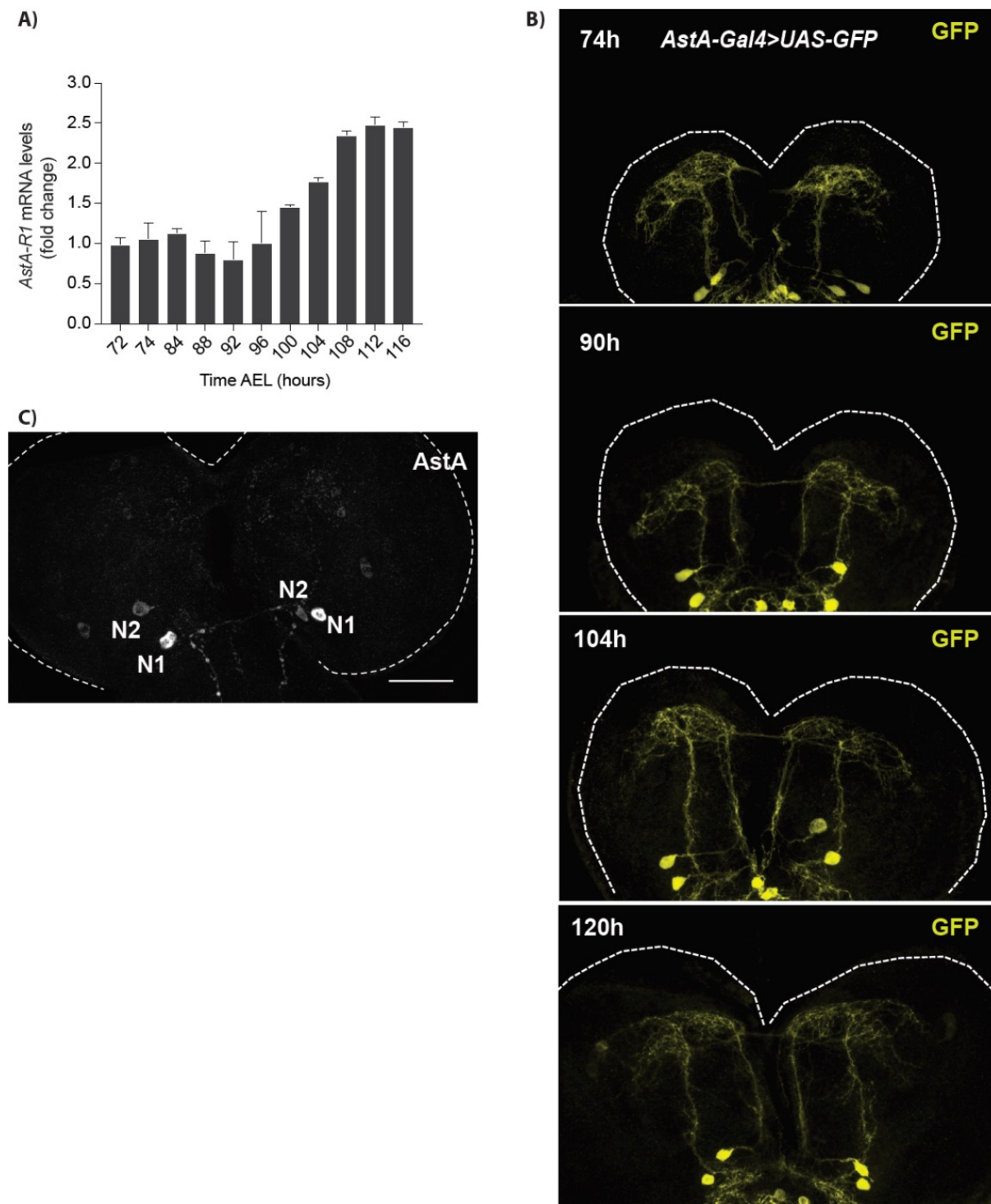
**SUPPLEMENTARY FIGURE 3**



*AstA-GAL4>UAS-FLP*  
*CoinFLP-LexA::GAD.GAL4>LexAop2-CD8+GFP*



# SUPPLEMENTARY FIGURE 4



## Complementary unpublished experiments

### 1. Regulation of AstA-R2 in controlling larval growth

Other than acting as a ligand for AstA-R1, AstA is also the ligand for AstA-R2. AstA-R2, or Dar-2, is expressed in the *Drosophila* larvae IPCs, as previously shown by Hentze et al. 2015 (**Fig. 23**). The authors have shown that silencing AstA-R2 in the IPCs leads to changes in metabolic target genes, including a decrease in *Dilp2* expression levels. I therefore reasoned that AstA-R2 could also have a function in regulating larval growth in the IPCs. To determine the function of AstA-R2 in the IPCs, *Dilp2>AstA-R2-RNAi* was used to silence AstA-R2 in the IPCs (**Fig. 24**). AstA-R2 downregulation caused a slight reduction in pupal size of ~5%, smaller than the reduction observed when we downregulate AstA-R1 (~14%). Simultaneously silencing both AstA-R1 and AstA-R2 did not change pupal size (*Dilp2>AstA-R1-RNAi; AstA-R2-RNAi*). No change was observed in metamorphosis timing when AstA-R2 is silenced in the IPCs, whereas double down regulation of both receptors had a tendency of being slightly delayed

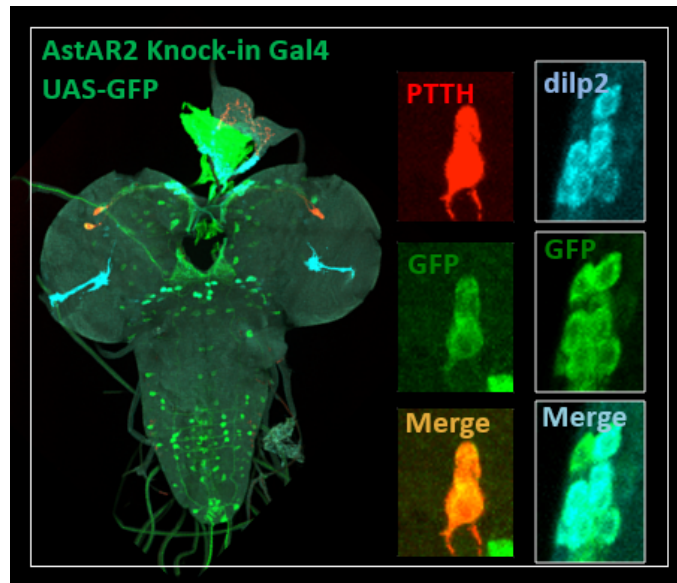


Fig. 23 Predicted expression profile of AstA-R2 in the larval brain.

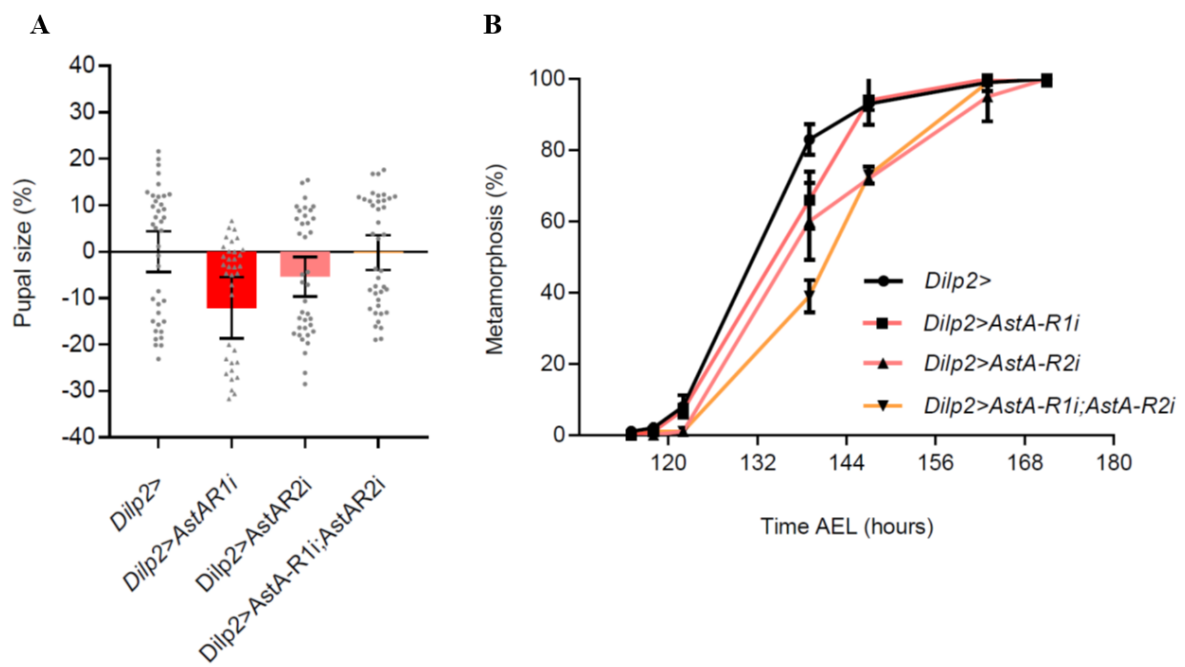
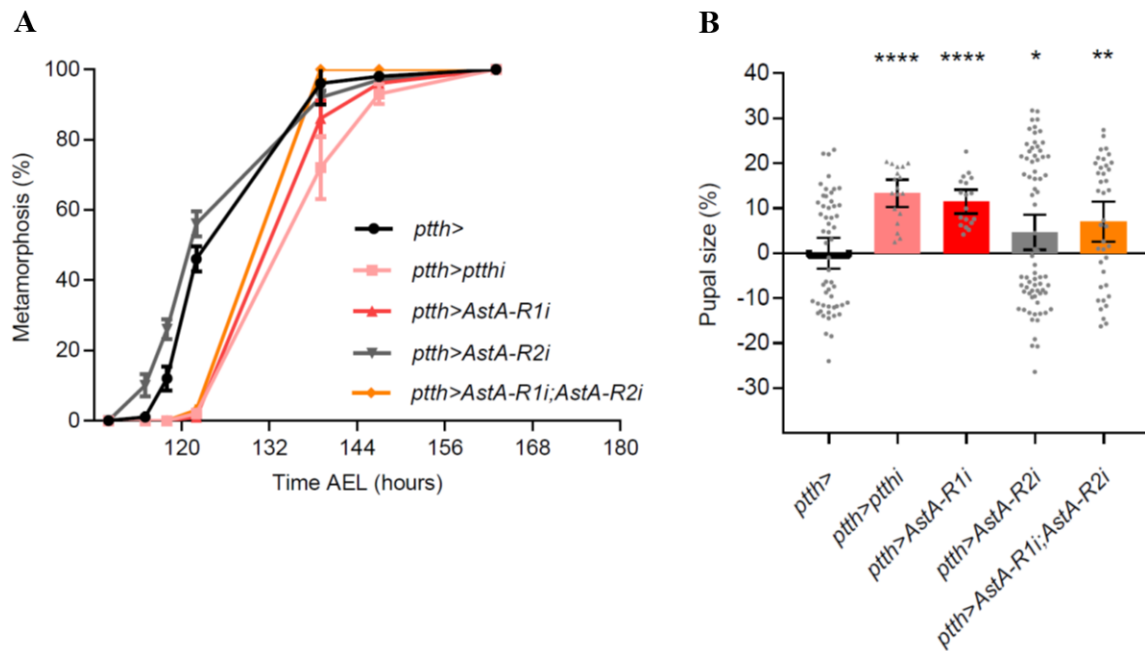


Fig. 24 AstA-R2 regulation in growth

## 2. Regulation of AstA-R2 in timing metamorphosis

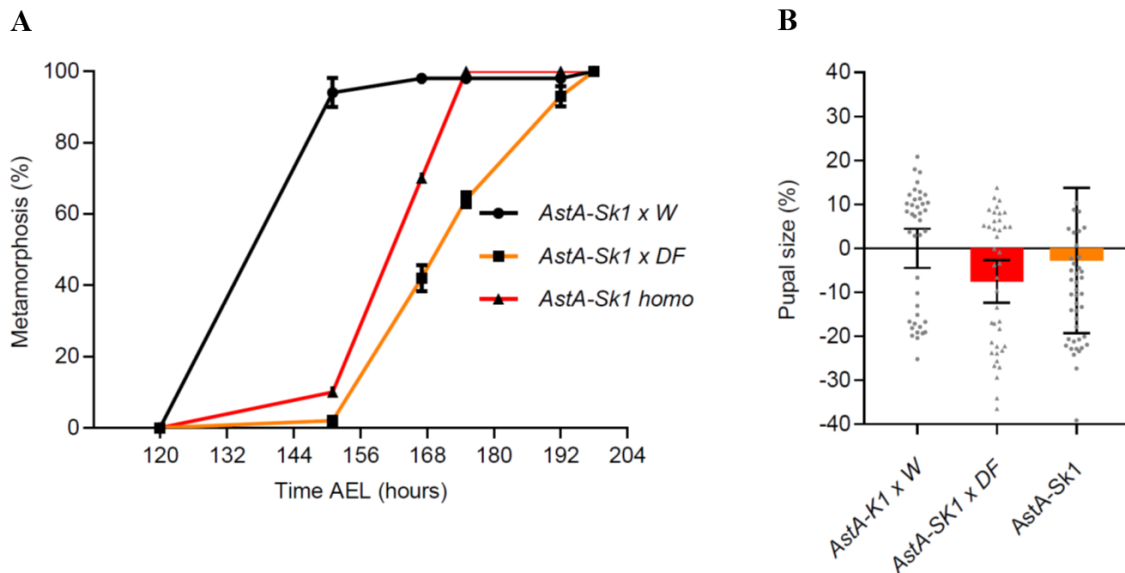
Visualization of the expression pattern of AstA-R2- knock in Gal4 (kindly provided by Kim Rewitz) in the larval central brain revealed expression in PTTHn (**Fig 24**). I therefore hypothesized that in addition to AstA-R1, AstA-R2 could act as an enhancer of AstA signaling in regulating PTTH. When AstA-R2 was silenced in PTTHn (*ptth>AstA-R2-RNAi*) larvae had were delayed in metamorphosis (**Fig 25**). Silencing AstA-R1 and AstA-R2 at the same time in PTTHn delayed metamorphosis but did give an additive phenotype. *ptth>AstA-R2-RNAi* pupal size, however, was not as much increased (~4%) as *ptth>AstA-R1 RNAi* (~11%), whereas double downregulation was only slightly elevated (~6%).



**Fig. 25 AstA-R2 regulation on timing metamorphosis**

### 3. AstA mutant larvae exhibit both timing and growth defects

AstA signaling times metamorphosis onset through PTTH, whereas it also affects larval growth by regulating Dilp2 secretion from IPCs. The question therefore remains what happens in larvae that do not express AstA in the entire body. To test this, I used the AstA null mutant that was generated by Hentze et al. 2015. As expected, AstA null mutant larvae were developmentally delayed (**Fig. 26**). Interestingly, however, these larvae were significantly smaller in size (~ -7,5%), possibly due to a reduction in growth rate since the growth period is extended.

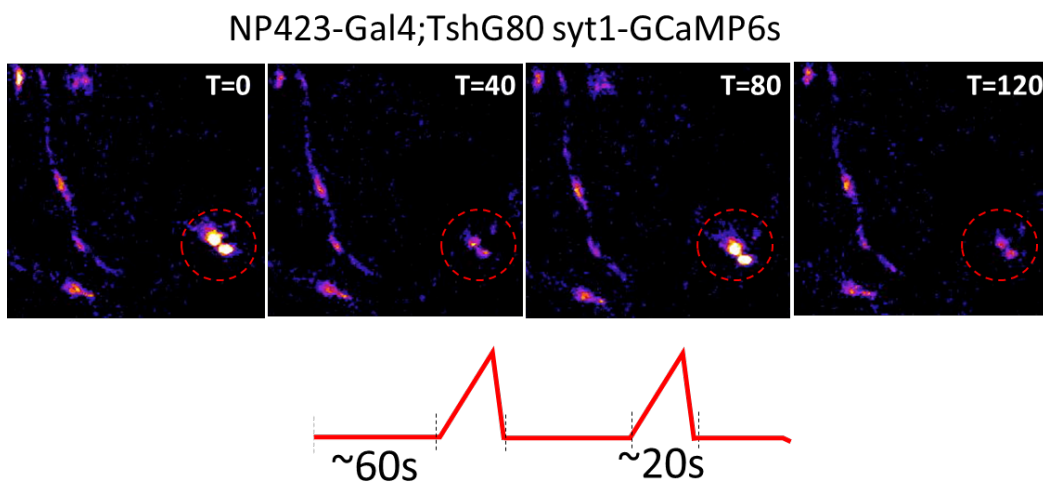


**Fig. 26** AstA regulation in timing metamorphosis and growth



#### 4. Pulsatile activity of PTTHn in PG

GnRH neurons in the HPG- axis release GnRH in a pulsatile manner, accompanied with oscillations in intracellular calcium levels (Constantin, Klenke, and Wray 2010). PTTH does not have mammalian homology, nevertheless it is analogous to the GnRH neurons since both neurons regulate steroid production. To determine whether PTTHn also has pulsatile oscillatory behavior, I did time lapse measurements of calcium imaging using the calcium sensor GCaMP6s, combined with syt1 (T. W. Chen et al. 2013). Syt1, or synaptotagmin-1, is a component of synaptic vesicles and is involved in triggering neurotransmitter release, once bound to  $\text{Ca}^{2+}$  (T. W. Chen et al. 2013). By examining *NP423-Gal4;TshG80>syt1-GCaMP6s* larval brains, I observed oscillations of calcium at the synaptic sites of PTTHn in the PG. These observations, however, were observed in only third of all larval brains (10 out of 30 brains).



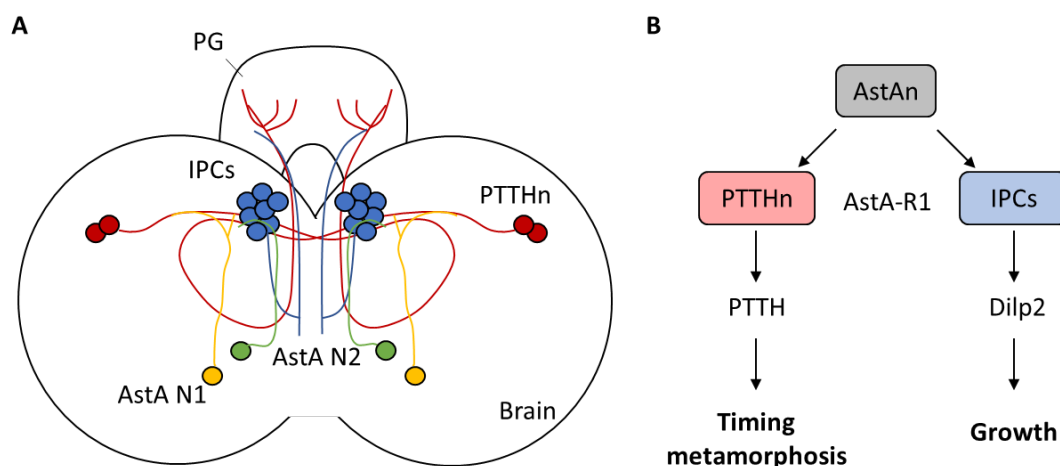
**Fig. 27** Live calcium imaging of PTTHn synapsis in the PG



## General conclusion

During the course of my PhD, I focused on the identification of novel signals which are able to time pubertal onset. In order to answer to this question, I conducted a genetic screen using the model organism of *Drosophila melanogaster*. By doing so, I identified Allatostatin A Receptor-1 (AstA-R1) as a regulator of PTTH, an important neuropeptide that times metamorphosis (**Fig. 28**). AstA-R1 promotes PTTH secretion and thereby times metamorphosis onset. AstA-R1 is activated by the neuropeptide AstA that is produced by two pairs of neurons that physically contact the PTTH neural circuitry, suggesting that AstA neurons mediate metamorphosis by regulating PTTH signaling. Concomitantly, AstA-R1/AstA signalling plays a role in insulin signalling by regulating Dilp2 secretion from IPCs, affecting larval growth.

Surprisingly, AstA-R1/AstA has a mammalian homology with GPR54/KISS1, that play a pivotal role in puberty onset since its mutations are linked with delayed/absent puberty in humans. These findings together suggest an evolutionary conserved mechanism governing sexual maturation.



**Fig. 28 Working model of AstA/AstA-R1 signaling.** (A) Schematic representation of the AstA neural circuitry in the central brain. AstA-N2 neurons mainly project towards IPCs, whereas AstA-N1 neurons project to both IPCs and PTTHn. (B) AstA/AstA-R1 signaling regulates both timing of metamorphosis and larval growth by acting on PTTHn and IPCs, respectively. AstA/AstA-R1 signaling controls secretion of PTTH and Dilp2.

## Discussion

Here, I provide evidence that AstA/AstA-R1 signalling regulates both larval growth and metamorphosis onset. AstA/AstA-R1 signalling positively regulates metamorphosis by acting on PTTHn to induce secretion of PTTH in the PG. At the same time, the same signalling pathway promotes larval growth rate by acting on IPCs to promote Dilp2 secretion.

### There is an evolutionary conserved mechanism in the brain controlling puberty

In an attempt to identify incoming signals controlling steroid production by conducting an RNAi screen in the PTTH neurons in *Drosophila melanogaster*, we identified the AstA-R1/AstA system as a positive regulatory signal for PTTH secretion. We did not predict to discover conservation at this point since *Drosophila* PTTH and its receptor Torso share no homology with any of the HPG-axis components. Remarkably, however, *AstA-R1* diverged from the same ancestral gene that gave rise to the mammalian *GPR54*, following a gene duplication, as shown in a recent comprehensive study analyzing the origin of *AstA/AstA-R1* throughout evolution (**Fig. 29**) (RC Felix 2015). *GPR54* is important in regulating pubertal onset due to its regulatory role in GnRH secretion to activate the HPG- axis. Moreover, it is described that *AstA* has a conserved gene synteny with the human *KISS1* gene that encodes the ligand for GPR54, kisspeptin. Both AstA and kisspeptin peptides also share the highly conserved FGL- amide motif at the C- terminus that is required for peptide binding and activation of the human GPR54. This suggests that in addition to *AstA-R1*, *AstA* could originate from the same evolutionary ancestor as *KISS1*.

Previous phylogenic studies, however, did reveal the existence of a homologue of GnRH and GnRH in insects (Hauser and Grimmelikhuijzen 2014). Adipokinetic hormone (AKH) and corazonin are homologues of GnRH, that have arisen from a gene duplication from

a shared common ancestor, as reviewed in Zandawala, Tian, and Elphick 2017. *Drosophila* AKH is the equivalent of the mammalian glucan, playing a crucial role in energy homeostasis, but also in mediating stress responses, whereas corazonin functions mainly in stress responses but also in metabolism (Bharucha, Tarr, and Zipursky 2008; Kubrak et al. 2016; Zemanová, Stašková, and Kodrík 2016). Interestingly, the mammalian GnRH/GnRHR system in reproduction also seems to be responsive to stress and metabolism to regulate the HPG- axis in reproduction, after pubertal onset (Ciechanowska et al. 2016). Nevertheless, AKH and corazonin do not function in mediating metamorphosis onset, suggesting that these peptides are not the functional homologues of GnRH in controlling pubertal onset. Therefore, for the first time, we provide evidence of the existence of a functional homology at the level of Kiss/GPR54 in the neuroendocrine system of *Drosophila* that controls sexual maturation.

Our findings in *Drosophila* metamorphosis unexpectedly share common ground with a few major characteristics of mammalian kisspeptin regulation in puberty.

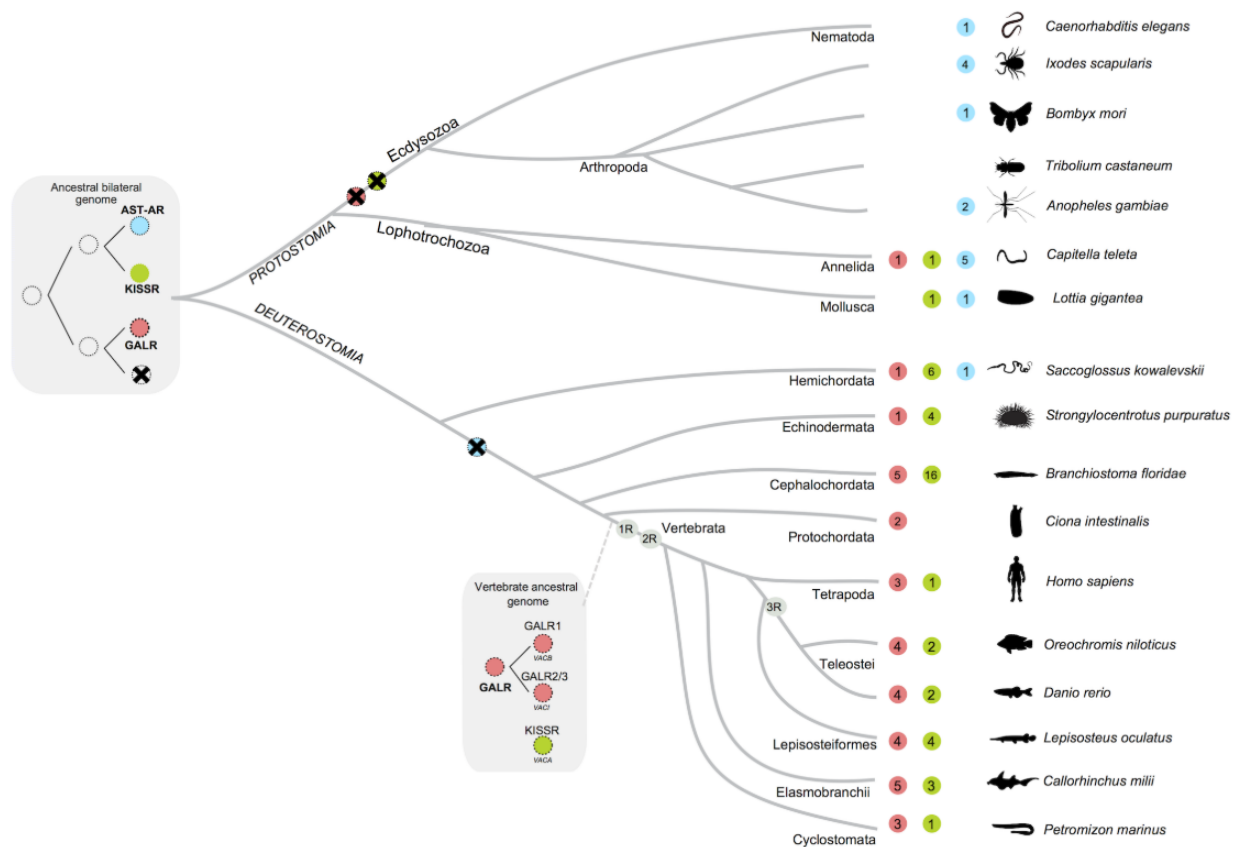
1. The first one being that kisspeptin expression rises during prepubertal stages that is found to be sufficient to induce the maximum secretory release of GnRH (Bentsen et al. 2010; Clarkson and Herbison 2006). In line with this, we find that in both AstA neurons intracellular calcium levels as well as AstA peptide gradually increases towards the end of metamorphosis, likely becoming more active to exert its stimulatory effect on PTTH secretion to induce metamorphosis.
2. At the same time, expression of AstA-R1 on the PTTH neurons increases throughout development, with the highest level being right before metamorphosis, suggesting that the neurons seemingly become more sensitive to the AstA- induced secretory effect on PTTH. This goes in line with the second characteristic of kisspeptin, being that the

kisspeptin stimulated GnRH secretory effect becomes more prominent during prepubertal stages compared to early juvenile stages (Castellano et al. 2006).

3. Another characteristic of pubertal onset is the increase in Kiss-Gpr54 signaling efficacy during pubertal timing, possibly due to absence of desensitization to ubiquitous stimulation of kisspeptin (J. Roa et al. 2008). Also in *Drosophila* we could predict the existence of a similar mechanism based on the observation that AstA-R1 and AstA neuropeptide levels increase at the time when PTTH is secreted onto the PG. More research, however, is needed to determine whether AstA-R1 loses desensitization towards the end of larval development.
4. The final important characteristic of kisspeptin in mammals is the distribution of two Kiss neuronal subpopulations in the hypothalamus: one being in the arcuate nucleus (ARC), whereas the other subpopulation is located in the rostral periventricular area of the 3rd ventricle (AVPV). It is proposed that the ARC Kiss neurons are important for continues kisspeptin pulsations, whereas the neurons in the AVPV are mainly required for the big surge during pubertal onset. In our study, we describe the existence of two AstA neuronal populations in the larval central brain that both project their axons onto the IPCs and PTTHn. More research, however, is needed to determine whether these neuronal groups act in a similar way, or whether they respond to different inputs. These projections, as well as our experiments show a coordination of both growth and maturation at the level of AstA/AstA-R1 signaling that might or not be *Drosophila* specific

The findings presented in this thesis provide evidence that AstA/AstA-R1 signaling are the functional homologues of kisspeptin/GPR54 in controlling metamorphosis. Interesting follow ups studies would be to answer whether the mammalian kisspeptins are also able to bind and

activate *Drosophila* AstA-R1 to activate PTTHn using GCaMP experiments. Another important finding in this thesis is that similar to the pulsatile Kiss neural activity, PTTHn might also be activated in a pulsatile manner, as shown in **Fig. 27**. Silencing AstA-R1 in the PTTHn did not abolish pulsatile calcium activity (data not shown), however, it is highly likely that AstA-R1 might act in synchronizing the duration or intervals of the pulsations, rather than inducing the pulses itself. Additional research is needed to elucidate whether the function of pulsatile oscillations in steroid production plays a significant role in metamorphosis.



**Fig. 29 Origin and evolution of GPR51 and AstA-R1.** GPR54 (KISSR in this scheme) and AstA-R1 (AST-AR) is proposed to have arisen from the same common ancestor gene during evolution. GPR51 has been lost and AstA-R1 was kept in Ecdysozoa, including Arthropoda. AstA-R1 on the other hand, was lost in most Deuterostomia. The receptor for galanin (GALR) is also proposed to have arisen from the same ancestral gene, that gave rise to the the common ancestor gene of AstA-R/GPR54. The function of galanin in sexual maturity remains unknown. Adapted from (RC Felix 2015)

## Interplay of steroid production on maturation and growth

In *Drosophila*, adult size is determined during the larval growth period that is under de control of steroid production. The cessation of larval growth upon ecdysone production at the end of larval development is well established in the field. Findings presented in this thesis show that the kisspeptin homologue AstA acts both in the neuroendocrine pathway controlling metamorphosis as well as growth in parallel. Therefore, AstA signaling acts as a developmental signal to coordinate both events at the same time, in response to yet an unknown signal.

Growth in humans, on the contrary, occurs during juvenile stages after which a growth spurt is observed during pubertal stages that is accompanied by elevated sex steroid levels stemming from the activated HPG- axis. It could therefore be possible that the kisspeptin dependent control of the gonadotrophic axis could also function to promote growth. In this context, some pioneers in the field had looked more deeply into kisspeptin/GPR54 signaling in the somatotrophs located in the anterior part of the pituitary, the part that produces growth hormone (GH). The anterior pituitary also includes the gonadotrophs, the part that produces the steroids LH and FSH in response to GnRH. Interestingly, they found that kisspeptin was able to stimulate  $\text{Ca}^{2+}$  response in both gonadotrophs and somatotrophs that was accompanied with dose- dependent LH and GH secretion, though lower compared to stimulation with GnRH and GH promoting factors (Gutiérrez-pascual et al. 2007). Nevertheless, this raises the possibility that kisspeptin can directly activate both steroidogenesis and growth without going through the intermediate step of promoting GnRH release. Following this, more studies have followed up on the potential function of the direct pituitary effects by kisspeptin. Several in vitro experiments conducted in monkey, cattle, goldfish and rat pituitary cells have shown that kisspeptin administration stimulates GH release (Chang et al. 2012; Gutiérrez-pascual et al. 2007; Kadokawa, Suzuki, and Hashizume 2008; Luque et al. 2011). However, these effects were not seen when kisspeptin was administered in swine, goats and cattle, highlighting that

kisspeptin- mediated GH stimulation still needs additional research but could be species-specific (Ezzat et al. 2009; Hashizume et al. 2010; Lents et al. 2008). The biological relevance of having a system that regulates both growth and times metamorphosis might be a way to coordinate growth with sexual maturation. Further research is required to elucidate whether these molecular pathways in humans can interact to coordinate both events, like in *Drosophila*.

### What is upstream of AstA?

Since AstA signaling seems to act as a developmental signal to control both PTTHn and IPCs, it is important to discover the regulatory factors controlling AstA signaling. One possibility would be that AstA signaling could be responsive to metabolic signals or nutritional status. This would allow that whenever nutrition is not available, AstA signaling would halt growth and metamorphosis simultaneously. In 2015, Hentze *et al.*, had described the effect of different nutritional states on *AstA* and *AstA-R2* transcript levels in adult flies. They showed that upon starvation (1% sucrose) for 48 hours, there was a slight reduction in *AstA/AstA-R2* expression. When these starved flies were re-fed with a carbohydrate rich cornmeal diet, *AstA* and *AstA-R2* expression levels were significantly higher. It is unknown whether these nutrition dependent transcriptional regulations of *AstA* and *AstA-R* also exist in the larvae. Nevertheless, it remains unknown which signals are required for AstA signaling during normal developmental progression. Another possibility would be that AstA signaling is developmentally regulated and might be halted in response to stress responses. A genetic screen in the AstA neurons might be required to identify novel regulatory signals of AstA signaling.

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## Abstract

There is an emerging trend in the declining age of pubertal onset over the past decades. The underlying mechanism that times puberty, or sexual maturation, however, remains elusive. Sexual maturation is the result of a hormonal cascade peaking with the production of steroid hormones. Here, we propose to use the fruitfly *Drosophila melanogaster* as a model organism to identify novel signals that induce steroid production. By conducting a genetic screen, we identified the neuropeptide Allatostatin A (AstA) and its receptor Allatostatin A Receptor- 1 (AstAR1), as a developmental signal that triggers maturation by promoting PTTH secretion and, in turn, the biosynthesis of ecdysone, the main insect steroid. AstA/AstAR1 also controls larval growth by directly promoting insulin-like peptide secretion from the *Drosophila* brain, therefore coordinating juvenile growth and the onset of maturation. Surprisingly, AstA/AstAR1 is homologous to KISS/GPR54, an initiating factor of human puberty, suggesting that an evolutionary conserved neural circuitry controls the onset of maturation.

Key words: development, sexual maturation, AstA, Kiss, *Drosophila*