

THE DROSOPHILA PROPROTEIN PROCESSING PROTEASE AMONTILLADO IS
REQUIRED FOR GLUCOSE HOMEOSTASIS AND DEVELOPMENTAL PROGRESSION

by

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(Under the Direction of Michael Bender)

ABSTRACT

Neuropeptides are an important class of chemical messengers that allow an organism to alter behavior, development, and other physiological processes in response to a constant barrage of stimuli from both internal and external environments. Most peptide hormones are synthesized as larger, inactive precursor molecules that require proteolytic processing by one or more members of a family of subtilisin-like proprotein convertases (PCs) to generate a biologically active molecule. Differential processing due to cell specific expression of different PCs can result in production of distinct sets of active peptide hormones from the same precursor peptide. Thus, processing by PCs represents an important regulatory mechanism of peptide hormone signaling. This thesis describes a genetic dissection of prohormone processing in the model system

Drosophila by *amontillado* (*amon*), the homolog of mammalian PC2. PC2 is a PC expressed in the regulated secretory pathway, and is one of the two main convertases responsible for the proteolytic activation of endocrine and neuroendocrine hormones. Here, we provide evidence that *amon* regulates glucose homeostasis and development in *Drosophila* by proteolytically activating peptide hormones involved in these processes, including the *Drosophila* insulin-like peptides (*dilps*) and adipokinetic hormone (*akh*) – the vertebrate glucagon analog. We have demonstrated that *amon* function is both necessary and sufficient in the AKH producing cells to maintain normal glucose homeostasis. *amon* function is also important in the insulin producing cells (IPCs) as a cell-type specific loss of *amon* results in hyperglycemic larvae. In addition to regulating physiological processes, we demonstrated a molting requirement for *amon* in a subset of cells in the CNS. Together, these studies support a regulatory role for *amon* in *Drosophila* metabolism and development. We also establish *Drosophila* as a useful system in which to study the cell-type specificity of prohormone processing, to identify the function of known neuropeptides, and to potentially identify novel hormone signals. Given the highly conserved nature of developmental and metabolic pathways, these studies in *Drosophila* have the potential to provide invaluable insight into the development of human diseases and metabolic disorders.

INDEX WORDS: Proprotein convertase, *Drosophila melanogaster*, adipokinetic hormone, insulin like peptides, glucose homeostasis, molting, development

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DEDICATION

To God for His love and care in fulfilling this goal.

To Ron who has been my most adamant supporter. For that I am forever yours.

To my parents for their love, endless support, patience, and encouragement.

To my siblings for their constant motivation by reminding me how long I've been in
school.

And to my puppies for their unconditional love and unspoken support.

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CHAPTER 1

INTRODUCTION

Complex multicellular organisms can be composed of billions of cells—some that are within micrometers and others that may be meters apart. These cells are often organized into tissues and organs that are specialized to perform specific functions. Consequently, the very survival of organisms depends on their ability to regulate and coordinate the activities of these tissues, leaving cells with the daunting task of communicating a tremendous amount of information in an efficient and rapid manner. Surprisingly, there are only two basic types of physiological signals that have evolved – electrical and chemical (Silverthorn, 2001). The nervous system elicits short, rapid responses through direct innervations of its target tissues and release of neurotransmitters into the synaptic cleft following an electrical impulse. Binding of the neurotransmitter to receptors on the neighboring cell may result in a change in ion permeability (if the receptor is an ion channel) or activation of intracellular second messenger cascades (Silverthorn, 2001).

While the nervous system coordinates rapid and precise responses to stimuli via action potentials, the endocrine system maintains homeostasis and long-term control using chemical signals. This signaling system utilizes hormones as chemical

messengers that are delivered to target tissues typically by the circulation.

Unlike neurotransmitters, a single hormone may have different effects on various tissues depending on the cell's receptor and signal transduction pathways for the hormone (Silverthorn, 2001). Oxytocin, for example, is a nine amino acid hormone released from the posterior pituitary gland that controls the ejection of milk during breastfeeding. When this same hormone binds its receptor on the uterus, however, it stimulates contractions during labor and delivery.

Hormones can be classified into three main classes: amine, steroid, and peptide hormones (Berne and Levy, 1999). Amines are derived from a single amino acid and include melatonin, catecholamines, and the thyroid hormones. All steroid hormones are derived from cholesterol and are small lipophilic molecules that can diffuse easily across membranes (Sanderson, 2006). Given this property, steroid hormones cannot be stored in secretory vesicles so they are synthesized as they are needed. Once a stimulus is received by the endocrine cell, its synthetic pathway is activated and precursors in the cytoplasm are rapidly converted to active steroid hormones. As the concentration of active hormone in the cytoplasm increases, it moves out of the cell by simple diffusion (Berne and Levy, 1999).

Finally, peptide hormones are synthesized from amino acids and are secreted into the bloodstream (Berne and Levy, 1999). Most peptide hormones are synthesized as prepropeptide molecules that are targeted to the secretory pathway by a signal

sequence (Martoglio and Dobberstein, 1998). Once in the lumen of the rough endoplasmic reticulum (rER), the signal sequence is removed by a signal peptidase and the propeptide is folded into its proper conformation. Next, it undergoes the first posttranslational modifications, including glycosylation and the formation of disulfide bridges. Following these modifications, the propeptide is transferred to the Golgi apparatus where endoproteolytic cleavage of the precursor molecule typically occurs at paired basic residues (most commonly Lys-Arg and Arg-Arg) by the action of a family of endoproteases known as the proprotein convertases (PCs) (Seidah and Chretien, 1997). Additional processing events include removal of exposed basic residues by the carboxypeptidases and amidation of the C-terminus (Czyzyk et al., 2003; Steiner, 1998). Because endoproteolytic cleavage is required to activate the precursor molecules, the PCs responsible for these events play an important role in intracellular processing, both as part of the biosynthetic process, as well as a regulatory step. Finally after being proteolytically processed, the neuropeptide and peptide hormones are sorted either to the constitutive or regulated secretory pathways. Proteins targeted to the constitutive pathway are packaged within constitutive secretory vesicles and transferred directly to the cell membrane. On the other hand, proteins of the regulated secretory pathway are packaged in large dense core vesicles and stored until they are secreted following either external or internal stimuli (von Eggelkraut-Gottanka and Beck-Sickinger, 2004).

Although the biological activation of peptide hormones has been extensively characterized, much remains to be learned about the family of enzymes responsible for the proteolysis of inactive precursors. Since the identification of the first member of the PC family almost twenty years ago (see below), hundreds of secretory precursors have been added to the list of proven or suspected substrates (Khatib et al., 2002; Seidah and Chretien, 1999), thus reinforcing the biological importance of these proteolytic enzymes. Among this list are endocrine hormones, growth factors, receptors, adhesion molecules, bacterial toxins, and viral envelope proteins, indicating that the PCs are at a center of a variety of normal physiological processes, and more likely than not, responsible for the pathophysiology of multiple illnesses. Given the wide variety of substrates and tissue expression patterns of the different PCs, it is important to determine how the control of PC expression and activity contributes to developmental defects and the pathology of diseases such as diabetes and cancer. Understanding these aspects of PC activity can then in turn facilitate the development of new mechanism-based therapeutic agents.

Identification of the proprotein convertases

The first bioactivating enzyme responsible for cleavage of peptide hormones was identified in 1984 as the yeast lysine-arginine cleaving endopeptidase kexin (Julius et al., 1984), which cleaves at dibasic residues to activate the yeast alpha-mating factor. Database searches using the *KEX2* sequence led to the discovery of furin as the mammalian ortholog of kexin (Fuller et al., 1989; Roebroek et al., 1986). Both Kex2p and

furin are classified as subtilisin-like proteases because of their similarity to the bacterial serine protease subtilisin (Siezen et al., 1994). The catalytic domains of subtilisin, kex2, and furin are highly similar, suggesting that they all employ a similar catalytic mechanism. Several labs performed PCR using primers based on the highly conserved nucleotide sequences encoding the active site of Kex2p to clone PC1/3 and PC2 from mammalian endocrine cells. Eventually, other proprotein convertases were identified, including PC4, PACE 4, PC 5/6, and PC7. Three of these seven convertases act within regulated secretory pathway, including PC1/3, PC2, and PC4. PC4 has only a few known substrates, and its expression is limited to the testes. Thus, PC1/3 and PC2 are the two main convertases involved in the cleavage of peptide hormones and neuropeptides (Seidah et al., 1998; Steiner, 1998; von Eggelkraut-Gottanka and Beck-Sickinger, 2004).

All members of the subtilisin-like serine protease family share a common overall structure, including the N-terminal signal sequence (Pre domain), the Pro domain, the catalytic domain, the P domain, and the C-terminal extension (Figure 1.1). These proteins are targeted to the secretory pathway by the presence of the Pre domain (Muller and Lindberg, 2000), which is removed by a signal peptidase once the enzyme has successfully reached the endoplasmic reticulum (ER). The Pro domain is thought to function as an intramolecular chaperone, and its removal during enzyme maturation is necessary for protein activation (Seidah and Chretien, 1997). The most conserved

domain among the family of PCs is the catalytic domain, as vertebrate members display a 40-50% homology with subtilisin. Also included in this domain is the highly conserved catalytic triad, comprised of aspartate, histidine, and serine residues (Rouille et al., 1995), and an oxyanion hole that is essential for catalysis (Seidah and Chretien, 1997; Steiner, 1998). Finally, the P domain contributes to the pH requirement, calcium dependence, proper folding, and stability of the convertase (Zhou et al., 1998). The C-terminal extensions are not well conserved among the PC family, but for the four members of the family localized to the constitutive secretory pathway (Furin, PC6, PC7, and PACE4), it includes a transmembrane domain (Steiner, 1998).

Prohormone Convertase 2 is unique among the PCs

Several characteristics of PC2 make it a unique member among the family of PCs. Unlike the other convertases, proPC2 is retained in the ER resulting in delayed activation and secretion (Benjannet et al., 1998; Benjannet et al., 1992; Zhou et al., 1994). This delay may be due in part to differences in the maturation of PC2 as it traverses the secretory pathway. For most PCs, autocatalytic cleavage of the proregion occurs in the ER. The propeptide then remains attached to the convertase and is thought to be required for the proper folding of the enzyme and its exit to the Golgi apparatus (Anderson et al., 1997). proPC2, however, exits the ER with its proregion intact and does not undergo autocatalytic activation until either late in the *trans* Golgi Network (TGN) or in the immature secretory granules (Benjannet et al., 1993; Guest et al., 1992; Shen et al., 1993;

Zhou et al., 1994). Because cleavage of PC2 occurs following its exit from the ER, achieving the proper conformational shape may be complicated by the presence of the intact propeptide, resulting in longer retention in the ER (Muller et al., 2000).

Another key difference between PC2 and the other proprotein convertases is the change of a highly conserved and catalytically important asparagine (Asn) residue to an aspartate (Asp) in the oxyanion hole. The Asn residue is believed to stabilize the substrate during enzymatic processing by donating hydrogen bonds to the negatively charged intermediate (Seidah et al., 1998). When Asp is substituted for Asn in the subtilisins, catalytic efficiency is reduced at their alkaline pH optimum. Thus, the substitution of Asn for Asp may represent a mechanism for preventing activation of PC2 until it reaches the acidic post-Golgi compartments (Smeekens et al., 1991). An additional function of Asp may also facilitate the binding of the PC2-specific neuroendocrine protein known as 7B2 – the final distinguishing characteristic of PC2.

Transport of proPC2 out of the ER and subsequent activation in the post-Golgi compartments requires the presence of 7B2 (Muller et al., 1997). Once 7B2 is bound to proPC2, cleavage of the neuroendocrine helper protein into an inhibitory C-terminal (CT) fragment and a 21 kDa amino-terminal fragment occurs in the *trans* Golgi Network (TGN) (Benjannet et al., 1995; Lindberg et al., 1995; van Horssen et al., 1995). While it is known that the latter N-terminal fragment is essential for PC2 activation, it is not clear whether it is required as a cofactor for activation, or merely for protection of the proPC2

enzyme from premature propeptide removal (Muller and Lindberg, 1999; Steiner, 1998). Interestingly, if PC2's unique oxyanion hole residue is changed to Asn, proPC2 is unable to bind pro7B2 and only inactive enzyme is produced (Benjannet et al., 1998; Zhu and Lindberg, 1995).

Posttranslational cleavage of peptide hormones is an important regulatory mechanism

Endoproteolytic processing of peptide hormone precursors by the PCs is key to regulating hormone signaling for three main reasons. First, it gives organisms the ability to create diverse sets of peptides that have distinct functions from a single precursor. For example proopiomelanocortin (POMC) is a proprotein that is differentially processed (Figure 1.2), allowing the release of adrenocorticotrophic hormone (ACTH) from the anterior pituitary and alpha-melanocyte stimulating hormone (α -MSH) from the intermediate lobe of the pituitary (Pritchard and White, 2007b). Secondly, differential expression patterns of members of the PC family ensure that active molecules are only generated from tissues in which they are required. Cleavage of the POMC precursor into either ACTH or α -MSH is dependent on the specific proprotein convertases present in each tissue. PC1 is expressed in the anterior pituitary, and is responsible for the release of ACTH in addition to beta-lipotropin (β -LPH) (Benjannet et al., 1991; Pritchard and White, 2007a), whereas expression of both PC1 and PC2 in the intermediate lobe is required for the production of α -MSH from the

POMC precursor (Benjannet et al., 1991; Pritchard and White, 2007a). Impaired PC2 activity in the intermediate lobe of the pituitary results in overproduction of ACTH and the development of Cushing's syndrome (Shomali and Hussain, 2000).

Finally, recent data from studies of the thyrotropin-releasing (TRH) receptors suggest that endoproteolytic processing confers an additional layer of endocrine pathway control by coordinating the activation of TRH receptors with the rate and extent of TRH processing according to metabolic demands (Sanchez et al., 2004). Tissue specific regulation of PC1 and PC2 have been shown to be critical for this synchronization (Pritchard and White, 2007b). These three reasons taken together demonstrate the importance of how understanding the mechanism by which expression and activity of the PCs are regulated may provide insight into their influence of signaling by peptide hormones. In addition, recent data suggest that PC activity is regulated by serpin protease inhibitors, demonstrating an additional control point for peptide hormone production (Hook et al., 1994; Osterwalder et al., 2004).

PC2 is important for glucose homeostasis

Many characteristics of the mammalian brain make it a unique organ. Among these is its inability to utilize fatty acids, and consequently, its dependence on glucose as a sole source of energy. Because the brain also lacks the ability to synthesize glucose *de novo*, it relies on a constant supply of energy provided by the circulation. Under normal physiological conditions, the rate that glucose crosses the blood-brain barrier is

higher than the rate of brain glucose metabolism (Cryer, 2008). During periods of fasting, however, plasma glucose concentration decreases, thus reducing glucose availability to the brain and ultimately threatening survival. Given this requirement for a continuous supply of glucose, it is therefore not surprising that mechanisms to quickly correct and maintain plasma glucose concentrations have evolved.

Glucose is a six carbon sugar that can be derived from three main sources: absorption by the intestine following a meal including dietary carbohydrates, formation from glucose precursors such as lactate and amino acids through a process known as gluconeogenesis, and through the breakdown of glycogen (Cryer, 2008). Depending on the organ, glucose has several potential metabolic fates (Figure 1.3). When circulating levels of glucose are high, the liver can convert glucose to glycogen, oxidize it for energy, or convert it to fat to be stored in the liver or transported to other tissues as low density lipoprotein. Muscle tissue also stores glucose as glycogen or metabolizes it through glycolysis to pyruvate. During periods of fasting, however, muscles have the ability to reduce glucose uptake to zero and oxidize fatty acids for energy needs. In addition to muscle, adipose tissue uses glucose to synthesize fatty acids and can also use glucose in the formation of glycerol-3-phosphate which esterifies fatty acids to form triglycerides. Similar to muscle tissue, adipose cells also reduce their net uptake of glucose during periods of fasting, and oxidize fatty acids to satisfy energy needs. Of all

the organs in the body, the liver and kidneys have the sole capability to release glucose into the circulation as only they express glucose-6-phosphatase.

In mammals, the uptake and release of glucose is controlled by two key peptide hormones – insulin and glucagon. When circulating glucose levels are high, insulin is released into the bloodstream by the β -cells of the pancreas and acts on the liver and other peripheral tissues to decrease plasma glucose concentration. More specifically, insulin acts on the liver to inhibit gluconeogenesis and glycogenolysis, thereby converting the liver into an organ of net glucose uptake and energy storage. Tissues such as muscle and fat also respond by increasing glucose uptake, storage, and utilization. Defects in insulin production or secretion result in obesity and in serious diseases such as diabetes mellitus. As plasma glucose falls, it has an immediate inhibitory effect on insulin secretion and stimulates glucagon release from the α -cells of the pancreas. Glucagon acts antagonistically to stimulate the release of glycogen, resulting in an increase in blood sugar levels.

As with the majority of peptide hormones, the active forms of insulin and glucagon are generated from larger, inactive precursor molecules that are enzymatically processed and modified (Furuta et al., 2001; Rouille et al., 1997a; Rouille et al., 1994; Steiner et al., 1967). The mature insulin hormone consists of two polypeptide chains (A and B) linked by two disulfide bridges. Initially, however, insulin is synthesized as a 110 amino acid precursor molecule having a N-terminal signal sequence followed three

defined domains: the B, C, and A chains. As with other peptide hormones, the signal sequence directs the molecule into the lumen of the ER, thereby assuring its entry into the secretory pathway. Here, the signal peptide is cleaved co-translationally at a canonical signal peptidase cleavage site and the remaining peptide chain continues to the Golgi (Dev and Ray, 1990). Dibasic cleavage sites are found at the B/C junction (Arg-Arg) as well as the C/A junction (Lys-Arg), which are cleaved to release the 35 amino acid C peptide.

The involvement of PC1 in proinsulin maturation was demonstrated through the creation of a mouse lacking functional PC1. Elevated levels of a proinsulin intermediate *des* (lys₆₄arg₆₅) were observed in these mice (Zhu et al., 2002). The involvement of a second endoprotease, PC2, in proinsulin maturation was also demonstrated through gene knockout in the mouse. When function of PC2 is removed, proinsulin processing is impaired and the *des* (arg₃₁arg₃₂) insulin intermediate accumulates (Furuta et al., 1998). Some insulin is produced in both knockout models, suggesting that PC1 and PC2 are capable of cleaving at either processing site, but with reduced efficiencies (Lipkind and Steiner, 1999). These studies, however, cannot rule out the possibility that another protease may be sufficient to provide compensatory activity to produce active insulin.

Given the requirements for low pH and high calcium ion concentration for PC function, final activation of proinsulin occurs in the secretory granules of the pancreatic β cells (Orci et al., 1973; Sorenson et al., 1970) through the concerted activities of the

PC1, PC2, and carboxypeptidase E (CPE) (Davidson and Hutton, 1987). PC1 mediated cleavage at the B/C junction is thought to occur first, as binding of PC2 to the A/C junction may require a conformational change to the precursor. Supporting this is the observation that *des* (arg₃₁arg₃₂) insulin is the preferred substrate of PC2 *in vivo* (Rhodes et al., 1992). Following PC1 cleavage, CPE removes the exposed basic residues and PC2 then acts at the A/C junction to release the mature hormone.

The other key regulator of glucose metabolism is glucagon, which is also produced by the proteolytic processing of a precursor molecule. Unlike insulin, however, proglucagon is expressed in multiple tissue types and can be processed into a variety of peptide hormones. For instance, in the intestinal L endocrine cells proglucagon is proteolytically processed by PC1 to release glicentin, oxyntomodulin, intervening peptide (IP2), and glucagon like peptides-1 and -2 (Rouille et al., 1997b), whereas PC2 acts in the α -cells of the pancreas to release glucagon and major proglucagon fragment (Furuta et al., 2001; Rouille et al., 1997a). Between each of these peptides are pairs of basic amino acid residues (Lys-Arg or Arg-Arg) that are used by the PCs as cleavage sites during processing.

The mature 29 amino acid glucagon hormone acts on the liver through a seven transmembrane G protein coupled receptor to promote gluconeogenesis and glycogenolysis and to inhibit glycogen synthesis (Jelinek et al., 1993). Glucagon was identified as a key regulator of glucose metabolism when administration of exogenous

glucagon was shown to increase plasma glucose concentration in both fasted and fed animals (Myers et al., 1991; Young et al., 1993). This hyperglycemic response of exogenous glucagon could be abolished through administration of polyclonal glucagon-neutralizing antibodies (Tan et al., 1985). The same effect was also observed using a high affinity monoclonal anti-glucagon antibody (Brand et al., 1994). In both cases, circulating glucagon was reduced to undetectable levels by the glucagon antibodies (Brand et al., 1995; Brand et al., 1996; Brand et al., 1994).

Knockout of the PC2 gene in mice also revealed a function for glucagon in glycemic control. In the absence of PC2, glucagon was not detected in circulation and the mice suffered from chronic hypoglycemia and severe hypertrophy of the α -cells of the pancreas – a documented compensatory mechanism. Complicating the correlation between a loss of PC2 and hypoglycemia was the observation that these same animals also showed defects in proinsulin processing (Furuta et al., 1997; Furuta et al., 2001). Replacement of glucagon via an osmotic pump, however, restored normal plasma glucose concentration and reduced α -cell hypertrophy, thereby solidifying the role of glucagon in glucose homeostasis (Webb et al., 2002).

***Drosophila* as a model system to study peptide hormone maturation**

Drosophila melanogaster is an ideal system in which to study how prohormone processing contributes to normal development. Many neuropeptides and their functions have been identified, including eclosion hormone (EH), ecdysis triggering

hormone (ETH), crustacean cardioactive peptide (CCAP), adipokinetic hormone (AKH), and the *Drosophila* insulin like peptides (DILPs) (Ewer, 2005). In addition to the twenty-two known peptide hormones in *Drosophila*, fourteen others are predicted based on their presence in other insects (Hewes and Taghert, 2001). Previous studies have also demonstrated the importance of peptide hormones in development as mutations in the peptidylglycine α -hydroxylating monooxygenase (PHM) result in embryonic lethality. This enzyme is responsible for the C-terminal amidation of secretory peptides, which occurs in more than 90% of known and predicted peptide hormones (Jiang et al., 2000).

In *Drosophila*, excellent genetic tools such as the UAS/GAL4 system (Brand and Perrimon, 1993) exist for examining temporal and spatial gene function *in vivo*. Targeted ablation of endocrine cells using tissue specific expression of toxins such as reaper has contributed to the knowledge about the functions of many *Drosophila* peptide hormones and their role in development. Thus, using this knowledge and similar tools, we can build upon the foundation of insect endocrine signaling by studying the enzymes responsible for the proteolytic activation of *Drosophila* neuropeptides. The *Drosophila* homolog of PC2, *amontillado*, has been identified (Siekhaus and Fuller, 1999), and mutants of this gene suggest that *amon* is involved in hatching behavior (Siekhaus and Fuller, 1999), larval molting (Rayburn et al., 2003) and pupal development (Rayburn et al., 2008 in review), as well as in insulin signaling (Rhea and Bender, unpublished).

The short life cycle of *Drosophila*, which is generally completed in ten days, is characterized by three larval stages followed by pupariation and subsequent eclosion of the adult fly. Like most insects, each developmental stage is separated by a molt. Molting, the process whereby the old cuticle is replaced with a new cuticle, begins approximately one hour before the cuticle is actually shed during ecdysis (Park et al., 2002).

Embryogenesis is completed in 24 h, and concludes with the hatching of the egg and emergence of the first instar larvae. The first and second instar larval stages also last approximately 24 h, and are separated by a molt which must occur to accommodate the increasing body size of the animal. The final third instar larval stage is completed in 2 days and is followed by a molt that leads to pupariation. At this point, the third instar larval cuticle shortens, tans, and hardens to form the puparium, which will protect the animal for the duration of metamorphosis. During this stage, the animal undergoes metamorphosis to become an adult. Eclosion marks the completion of metamorphosis, and is characterized by the shedding of both the puparium and the pupal cuticle.

Progression through each molt of the *Drosophila* animal until eclosion of the adult fly requires a carefully orchestrated series of events that are regulated by the release and degradation of several steroid and peptide hormones from the endocrine axis (Mesce and Fahrbach, 2002; Truman, 1992). This axis includes the prothoracic glands (PG), corpus allatum (CA), corpus cardiaca (CC), and the brain. While steroid and

peptide hormone control of developmental progression is not completely understood, many key players and their targets have been extensively characterized.

The events leading to a successful molt are first initiated when neurosecretory cells of the brain respond to external and internal stimuli, including the photoperiod, temperature, and body size. In response to these cues, the neurosecretory cells release the peptide hormone prothoracicotropic hormone (PTTH) into the hemolymph. The PG glands respond to the PTTH signal by synthesizing and releasing ecdysteroid precursors that are ultimately converted into the molt and metamorphosis stimulating steroid hormone 20-hydroxyecdysone (20E). The levels of circulating 20E cycle throughout development; a rise in the steroid hormone 20E initiates molting, while a decline in these titers signal the completion of the molt. The CA also plays a role in regulating metamorphosis, as it synthesizes and releases the sesquiterpenoid juvenile hormone (JH), which prevents larval tissues and organs from prematurely undergoing metamorphosis in response to 20E titers. Additionally, activity of the CA is regulated by small neuropeptides that either stimulate (allatropins) or inhibit (allatostatins) production of JH.

Changes in 20E titers also serve to regulate the expression of genes important for molting and ecdysis. The product of one such gene, eclosion hormone was originally thought to be solely responsible for triggering ecdysis (Truman, 1992). EH was first identified in *Manduca sexta* in the 1980s as a 62 amino acid neurosecretory peptide, and

the *Drosophila* homolog was characterized in 1993 as a 73 amino acid prohormone.

Within the 73 amino acid *Drosophila* EH prohormone is a dibasic cleavage site Lys-Arg (K-R) characteristic of the recognition sequence of a subtilisin-like prohormone convertase. If cleaved at this site, products of 9 and 62 amino acids (the size of *Manduca* EH) would be produced. To date, however, no studies have determined whether or not the EH prohormone is cleaved, and if so, what enzyme may be responsible for this cleavage.

In 1996, Zitnan *et al.* identified a second peptide, the ecdysis triggering hormone (ETH), and showed it to be essential for ecdysis as animals lacking the *eth* gene fail to complete ecdysis at the end of the 1st instar larval stage and subsequently die. ETH is a 203 amino acid peptide that contains two possible endoproteolytic processing sites, a Lys-Arg (K-R) and Arg-Arg (R-R) site, each of which are potential prohormone convertase recognition sequences. While there is still some controversy as to how EH and ETH interact, there is a general consensus that these two peptides are responsible for the regulation of timing of ecdysis. Initially, there are thought to be low levels of circulating ETH as 20E titers fall. This ETH is thought to stimulate the release of EH, which in turn promotes further release of ETH from the Inka cells of the trachea. Thus, at the onset of ecdysis, stores of EH and ETH are entirely depleted (McNabb *et al.*, 1997).

The pathway downstream of the EH/ETH loop is not completely understood, but a third peptide, the Crustacean Cardioactive Peptide (CCAP), is thought to serve as the most proximate trigger of ecdysis behavior. Similar to EH and ETH, CCAP contains a putative processing site, suggesting that it too may require endoproteolytic activation. Increased circulating levels of EH in *Manduca* have been shown to trigger a second messenger cascade, resulting in elevated levels of cGMP within CCAP-expressing cells (Ewer and Truman, 1997), but such an effect is not observed in *Drosophila* (Ewer and Truman, 1996). CCAP, in turn, suppresses the pre-ecdysis events triggered by EH and activates the ecdysis program (Gammie and Truman, 1997). Initially, models placed CCAP downstream of both EH and ETH. Recent studies, however, using EH + CCAP knockout larvae have provided evidence that EH and CCAP may actually have partially redundant roles, as the double knockout displays more severe defects than either of the single knockouts (Park et al., 2003). The mechanism by which CCAP activates ecdysis, however, remains elusive to date.

Glucose Homeostasis and *Drosophila melanogaster*

Recently, seven genes encoding insulin-like peptides (*dilps*) have been identified in *Drosophila melanogaster* (Brogiolo et al., 2001). Of the products of these genes, the DILP2 protein is the most similar to vertebrate insulin (35% amino acid identity), and has been shown to function as a ligand of the *Drosophila* homolog of the insulin receptor (Brogiolo et al., 2001). Evidence that the insulin signaling pathway in flies controls cell

size and number comes from studies with animals homozygous for a mutation in *inr* (Brogiolo et al., 2001), larvae lacking the insulin producing cells (Rulifson et al., 2002), and investigations involving *Drosophila* homologues of insulin receptor substrates and other downstream components of the vertebrate pathway (Garofalo, 2002). Recent data also suggests that the *dilps* and *inr* participate in metabolism, as flies lacking the insulin producing cells (IPCs) have significantly higher levels of circulating sugar levels than do wild-type animals (Rayburn et al., 2003; Rulifson et al., 2002). Thus, these investigations indicate the presence of a functionally conserved insulin signaling pathway in *Drosophila* – a finding that is of particular interest, given the potential application to human diabetes. Given that PC2 plays a major role in insulin production in vertebrates, and that the *Drosophila* homolog of PC2 has been identified, we were interested in determining whether *amontillado* (*amon*) has an analogous role in insulin production in the fly.

Insects also have peptide hormones that act as functional homologs of vertebrate glucagon. Initially, the glucagon-like peptide in insects was referred to as hypertrehalosemic hormone (HTH) after injection of the peptide into the cockroach resulted in an increase of hemolymph trehalose levels (Bedford, 1977; Steele, 1961). Injection of this peptide in locusts, however, resulted in systemic increase of both carbohydrate and lipid levels (Beenakke, 1969). Thus, the alternative name adipokinetic

hormone refers to this family of neuropeptides that includes more than forty known isoforms spanning almost all major insect orders (Gade and Auerswald, 2003).

The gene structure of the *akh* genes is similar and includes two exons with a small intervening intron. In *Drosophila*, the first exon encodes the signal sequence and the first amino acid of the mature AKH peptide. The second exon encodes the remaining seven amino acids of the mature hormone, followed by a dibasic processing site and a C-terminal AKH Associated Peptide (AAP) (Noyes et al., 1995). In *Drosophila*, the *akh* gene is expressed in the corpora cardiaca portion of the ring gland at all stages of development (Lee and Park, 2004) and is directed to the regulated secretory pathway by the signal sequence where it is translated and packaged into secretory vesicles (Lee and Park, 2004). Once in these vesicles, cleavage occurs at the dibasic processing sites, releasing the glucagon-like peptide AKH and AAP, which currently has no known biological function. In addition, all known AKHs are blocked at the N-terminus by a pyroglutamate residue, and all but one are C-terminally amidated (Noyes et al., 1995).

AKH acts through a single G-protein coupled receptor to control lipid and sugar mobilization (Gade et al., 1997). This receptor was initially identified as an orphan receptor and was categorized as a gonadotropin-releasing hormone receptor-related (GnRHR) based on structural similarities to the vertebrate gonadotropin-releasing hormone (GnRH) receptors. Thus, it was originally hypothesized that the ligand of the GnRHR receptor would be related to one of the vertebrate GnRHs and would play a

role in reproduction. Subsequent biochemical studies, however, identified AKH as the endogenous ligand of the GnRHR receptor, and it was renamed to Drm-AKH receptor (Staubli et al., 2002).

Identification and characterization of *amon* mutants

In 1999, Siekhaus and Fuller identified the *Drosophila* homolog of mammalian PC2, *amontillado* (*amon*). Subsequent studies demonstrated that, similar to its vertebrate counterpart, *amon* requires association of a neuroendocrine specific 7B2 protein (*d7B2*) for maturation and activity (Hwang et al., 2000). Northern analysis of RNA from each developmental stage showed that *amon* expression is first detectable in the embryo, and expression continues throughout the lifecycle of the fly. Initial observations of *amon* mutants showed that these animals were defective in behaviors necessary for hatching (Siekhaus and Fuller, 1999). The *amon* mutation was initially characterized using animals carrying a deletion of the 97D1-2 region. Because this deletion contains at least one previously characterized gene, *Toll*, in addition to *amon*, an ethyl methane sulfonate (EMS) screen was performed to identify point mutations affecting only the *amon* gene (Rayburn et al., 2003). Ultimately, 9 mutants falling within the *amon* interval were identified. Of these 9 *amon* mutants, three of the mutations induced by EMS result in truncated proteins, while the other six mutations are missense mutations predicted to impair the protein's activity.

Depending on the point mutation, *amon* mutants display varying degrees of lethality, ranging from 37% (*amon*^{E601sa}) and 84% (*amon*^{C254Y}) of expected larvae surviving at 36 hrs after egg laying (AEL). All mutants that successfully complete embryogenesis and hatch die during larval development, as none are able to pupariate or eclose. Arrested mutant larvae show a phenotype indicating they fail to complete ecdysis. This phenotype consists of the presence of two sets of mouth hooks in the *amon* mutants. These structures, used by the animal for feeding, are shed during ecdysis and replaced with a new set characteristic of the new larval stage. Based on morphology, the double mouth hooks seen in *amon* mutants represent first and second instar larval stages, suggesting that mutants are unable to complete ecdysis which is necessary for developmental progression (Rayburn et al., 2003).

The mutations described above can be partially rescued using a heat-shock-inducible AMON transgene (*yw; hs-amon; amon/Df(3R)Tl-X*). *amon* mutants expressing this transgene are able to survive in greater numbers not only past the embryonic stage (71% with transgene vs. 43% with no transgene), but also to the 60 and 96 hr AEL time points. Many of the *amon* mutants carrying the *hs-amon* transgene die between the 60 and 96 hr time points with the double mouth-hook phenotype. Analysis of these mouth-parts shows that they are representative of the second and third larval instar stages, again suggesting that *amon* is required to complete ecdysis between the second and third instar larval stages (Rayburn et al., 2003).

Additional experiments demonstrate that the *hs-amon* transgene can be used to rescue *amon* mutants to adulthood and that these mutants are fertile. If heat-shock is removed, however, during the late third instar stage at 108 hrs AEL, these mutants fail to eclose. Detailed analysis of the *amon* mutants that arrest during pupal development show that these animals have several developmental defects, including failure to evert the head, failure to extend the legs and wings, and a failure of the abdomen to differentiate. In spite of these defects, development of the pupa continues as events that normally occur after head eversion and leg and wing extension were observed (Rayburn *et al.*, 2008, in review). These data, therefore, suggest that *amon* may process peptide hormones that are required to coordinate events of pupal development.

Western blot analysis of the *amon* protein throughout development indicates that the *amon* protein is present during all developmental stages, consistent with *amon* RNA expression reported previously (Siekhaus and Fuller, 1999), and consistent the requirements of this protein during embryonic (Siekhaus and Fuller, 1999), larval, and pupal stages (Rayburn *et al.*, 2003). Immunocytochemistry using a polyclonal antibody against the *amon* protein shows that *amon* localizes to cells of the central nervous system, as well as to endocrine cells of the larval mid-gut (Rayburn *et al.*, 2003). This expression data, therefore, suggests that *amon* has multiple potential targets in *Drosophila*.

Hypotheses

In the following chapters, the role of the proprotein convertase *amon* in regulating sugar homeostasis and developmental progression is examined. In Chapter 2, I address the hypothesis that AKH is a potential substrate of AMON. I describe the generation of three new transgenic fly strains used genetically establish this link. Chapter 3 addresses the hypothesis that AMON regulates DILP activation, and utilizes the tools described in Chapter 2 to identify *amon* as a key player in the *Drosophila* insulin signaling pathway. Together, these two chapters define cell-specific regulatory roles for *amon* in maintaining glucose homeostasis during *Drosophila* development and demonstrate a powerful approach to dissecting how proprotein convertases contribute to the regulation of important physiological processes. In Appendix A, we demonstrate the use of our new genetic tools to identify a subset of CNS cell in which *amon* is sufficient to promote developmental progression. A portion of this data appears in a co-author manuscript currently in prep for *Developmental Biology*. Appendix B reports on a potential regulatory role for *amon* on the synthesis and/or release of the steroid hormone ecdysone – the key initiator of pupariation and metamorphosis. Together, this dissertation contributes to our understanding of the importance of peptide hormone signaling in physiological and developmental processes, and how proteolytic activation of such signals may provide a key regulatory mechanism in the generation of hormone messengers. More importantly, it establishes *Drosophila* as a model system in which to

elucidate cell specific requirements for the proteolytic activation of peptide hormones and provides tools that could potentially be useful in identifying previously unknown functions of these signals.

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Figure 1.1. Typical structure of subtilisin-like proprotein convertases. All PCs are active within the secretory pathway and require the presence of an N-terminal signal sequence to be transported into the endoplasmic reticulum. Initially, PCs are synthesized as precursor proteins, and cleavage at the sites indicated above within the Pro domain results in an active enzyme. The conserved catalytic triad consisting of an Asp, His, and Ser residue are indicated above, as well as a conserved Asn required to stabilize the oxyanion intermediate. In PC2, this residue is an Asp. Figure adapted from (Thacker and Rose, 2000).

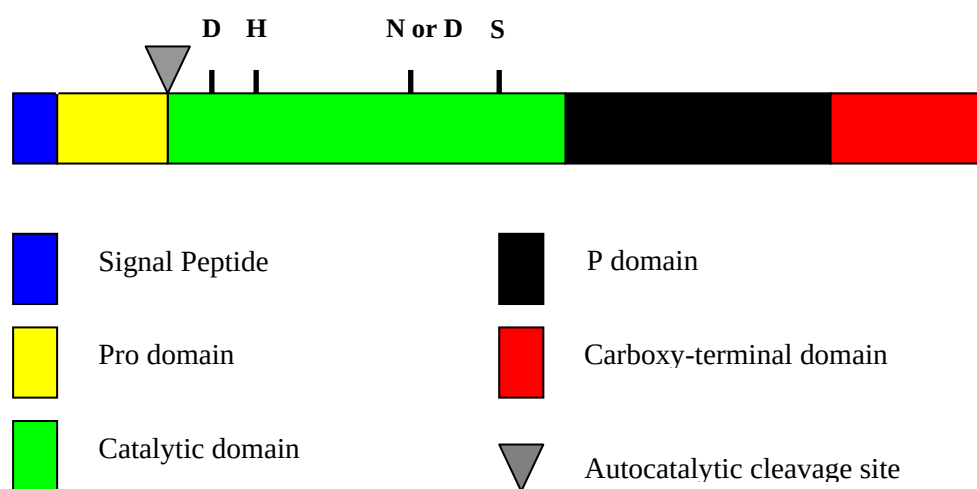


Figure 1.2. Differential proteolytic cleavage of the POMC precursor. The POMC precursor is produced in both the anterior and intermediate lobes of the pituitary. In the anterior lobe, PC1 proteolysis releases ACTH which acts on the adrenal gland to regulate steroidogenesis. Both PC1 and PC2 are expressed in the intermediate lobe resulting in the release of α -MSH which controls melanogenesis in the skin. Figure adapted from (Shomali and Hussain, 2000).

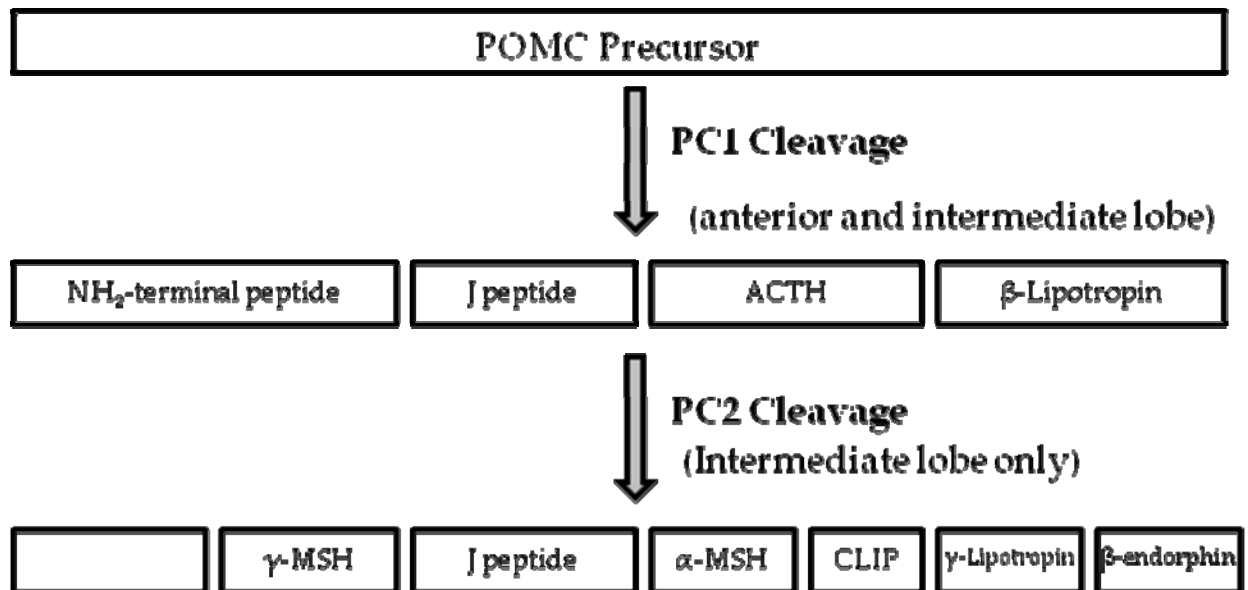
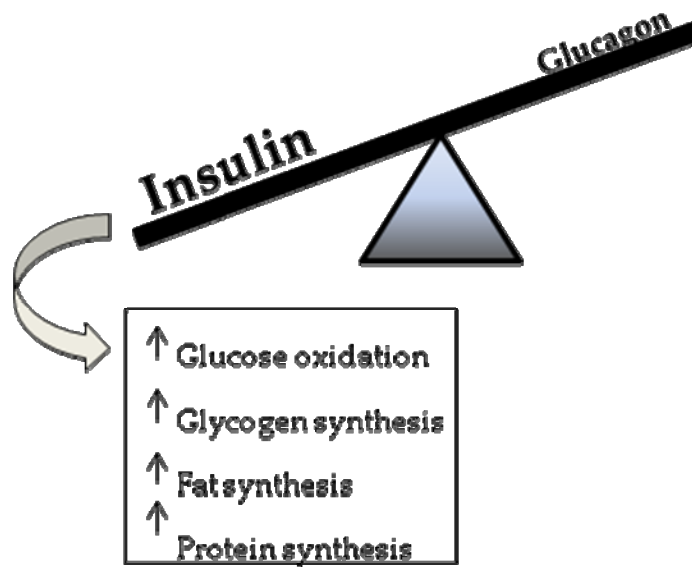


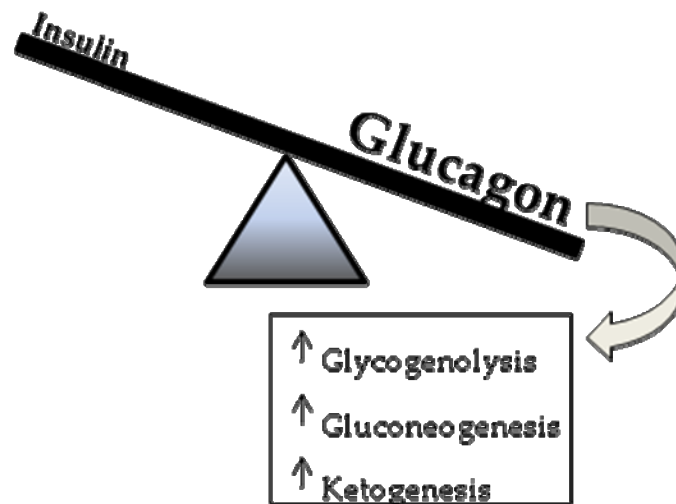
Figure 1.3. Insulin and glucagon act antagonistically to maintain plasma glucose

levels. (A) In the fed state, insulin is the dominant hormone that acts to promote net anabolism. Ingested glucose is used for immediate energy needs while insulin stimulates the storage of excess glucose. (B) In between meals and during periods of starvation, glucagon is the dominant hormone stimulating the synthesis of glucose from glycogen and non-glucose intermediates. Figure adapted from (Silverthorn, 2001).

A. Fed state: insulin dominates



B. Fasted state: glucagon dominates



CHAPTER 2

THE PROPROTEIN CONVERTASE AMONTILLADO (AMON) IS REQUIRED FOR GLUCOSE HOMEOSTASIS IN DROSOPHILA¹

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ABSTRACT

Peptide and neuropeptide hormones are potent signaling molecules that coordinate animal physiology, behavior, and development. A key step in activation of many peptide hormones is the proteolytic cleavage of prohormone precursors by a family of proteases, the subtilisin-like proprotein convertases (SPCs). Here, we report the functional dissection of the *Drosophila* SPC *amontillado* (*amon*) using cell-type specific inactivation and rescue experiments and show that *amon* is required in a subset of endocrine cells for normal glucose homeostasis. Like vertebrates, insects employ peptide hormone signals to regulate circulating glucose levels. In *Drosophila*, adipokinetic hormone (AKH) acts to increase circulating glucose levels from energy stores, while insulin-like peptides (DILPs) act antagonistically to decrease glucose levels. *amon* mutant larvae have significantly reduced hemolymph sugar levels, and thus phenocopy larvae where the AKH producing cells have been ablated. Reduction of *amon* expression in these cells via cell-specific RNA inactivation also results in larvae with reduced sugar levels. Expression of *amon* in AKH cells in an *amon* mutant background rescues *amon* hypoglycemia. Hypoglycemia in larvae resulting from *amon* RNA inactivation in the AKH cells can be rescued by global expression of the *akh* gene. Finally, mature AKH is not detectable in *amon* mutants by direct mass spectrometric peptide profiling. Our data indicate that *amon* function in the AKH producing cells is

necessary and sufficient for normal glucose homeostasis and that *amon* functions upstream of *akh*. These observations support the model that the AKH propeptide is a direct proteolytic target of the *amon* proprotein convertase.

Introduction

Many peptide hormone signals are synthesized as larger inactive precursor molecules that must be enzymatically processed and modified by the subtilisin-like proprotein convertases (PCs). This endoproteolytic cleavage is a key step in the regulation of hormone signaling for several important reasons. First, it represents a mechanism by which multiple sets of peptides, each with distinct functions, can be generated from a single precursor (Pritchard and White, 2007). In addition, the suite of peptides produced from a precursor peptide can be altered by differential expression patterns of members of the PC family (Pritchard and White, 2007). Finally, recent studies have demonstrated that receptor activation and nutritional input can influence the expression of proprotein convertase 1 and 2 (PC1 and PC2), thereby regulating the production of biologically active Thyrotropin Releasing Hormone (TRH) (Sanchez et al., 2004). These observations suggest that understanding the mechanism by which expression and activity of the PCs are regulated may provide insight into their influence on signaling by peptide hormones.

Of the nine mammalian proprotein convertases that cleave after basic amino acid residues, PC1/3 and PC2 are the main convertases involved in proteolytically activating

peptide hormones released in the regulated secretory pathway, and are most highly expressed in endocrine tissues (Hook et al., 1994; Scamuffa et al., 2006; Seidah and Prat, 2007; Zhou et al., 1999). Mutations in either PC1/3 and PC2 result in obesity (Jackson et al., 1997), defects in glucose homeostasis (Furuta et al., 1997; Furuta et al., 2001; Webb et al., 2004), and are linked to tumor progression and malignancy in an extensive list of cancers, including small cell lung carcinomas (Creemers et al., 1992; Schalken et al., 1987) and breast cancer (Cheng et al., 1997). Biochemical studies *in vitro* have examined the mechanism of PC cleavage and demonstrated the ability of this class of enzymes to cleave a variety of protein precursors at dibasic motifs (Scamuffa et al., 2006). In addition, physiological roles of the PCs have been identified using mice carrying null mutations in the various PC genes (Scamuffa et al., 2006). While these studies have provided valuable information regarding the biological role of PCs, it is important to remember that these proteolytic enzymes are expressed in a variety of tissues and are responsible for the endoproteolytic cleavage of an array of precursor molecules. Studying cell-specific functions of the PCs using animals lacking these enzymes only in specific tissues, therefore, may provide important additional insight into the regulatory role of the PC family in development, growth, and disease. The recent identification of the *Drosophila* homolog of mammalian PC2, *amontillado* (*amon*) (Siekhaus and Fuller, 1999), coupled with the genetic tools available in this model system, presents the opportunity for facile cell-specific dissection of PC activity *in vivo*.

Glucose serves as a major fuel source for organisms ranging from bacteria to humans, and as such, mechanisms to maintain a stable concentration of this sugar available to cells has been evolutionarily conserved from *C. elegans* to humans. In insects, trehalose is the major form of sugar found in the hemolymph along with monomeric glucose, and consists of two glucose molecules (Rulifson et al., 2002). Similar to humans, *Drosophila* employs two hormones, adipokinetic hormone (AKH) and the insulin like peptides (ILPs), which act antagonistically to maintain sugar homeostasis. AKH is the *Drosophila* analog of vertebrate glucagon and is known to regulate both lipid and sugar mobilization from the fat body during activities such as flight and locomotion (Kodrik et al., 2000; Kollisch et al., 2000; Lorenz et al., 2001; Schaffer et al., 1990; Schoofs et al., 1997; Siegert et al., 2000; Stone et al., 1976) or under conditions of starvation (Isabel et al., 2005; Kim and Rulifson, 2004; Lee and Park, 2004). It is synthesized in the corpora cardiaca (CC) portion of the ring gland as a preprohormone containing a signal peptide, a single AKH of 8-10 amino acids, and a carboxyterminal peptide (Noyes et al., 1995; Predel et al., 2004; Wegener et al., 2006). Before AKH is released, the mature AKH peptide is enzymatically cleaved from the carboxyterminal peptide at a dibasic processing site (Noyes et al., 1995), the preferred cleavage sites of the PCs. In contrast to AKH, the DILPs act to lower glucose levels in the hemolymph (Rulifson et al., 2002). The DILPs also possess dibasic cleavage sites similar to mammalian insulin (Brogiolo et al., 2001).

Here we demonstrate that *amon* mutant larvae show a hypoglycemic phenotype that is similar to larvae in which the AKH producing cells have been ablated (Isabel et al., 2005; Lee and Park, 2004; Rulifson et al., 2002) and that *amon* is expressed in the AKH cells. Reducing *amon* expression in the AKH producing cells shows that *amon* function is required in these cells to maintain sugar homeostasis. Restoring *amon* activity in the AKH cells in an *amon* mutant background is sufficient to rescue the hypoglycemia. Normal sugar levels can be restored to hypoglycemic larvae through broad expression of a *hs-akh* transgene, supporting a regulatory role for *amon* upstream of AKH. Together, these data indicate that *amon* function in the ring gland is necessary to maintain normal sugar homeostasis. Finally, mature AKH is not detectable in *amon* mutants as shown by mass spectrometric peptide profiling techniques, suggesting that AKH may be a proteolytic target of *amon*.

Materials and Methods

Generation of transgenic fly strains. To create the *amon-gal4* construct, the GAL4 cassette was removed from the pGATB vector (Brand and Perrimon, 1993) as a *Bam*HI/*Not*I fragment and cloned into the pCaSpeR4 transformation vector (Thummel and Pirrotta, 1992). Forward (5'-ATCCAACGCAGCTGAGCAGC-3') and reverse (5'-CGGAAGGAAAGCACAACAAG-3') PCR primers were used to amplify the 5' upstream region extending from the center of the 5' UTR (-331) of *amon* to +133 of the transcription start site. The PCR fragment was cloned into the *Bam*HI site of a pCaSpeR4

already containing the *gal4* gene. The recombinant vector was injected into *w*¹¹¹⁸ embryos by the Duke University Model Systems Genomics. A homozygous viable *amon-gal4* line, which has an insertion on the second chromosome, was used in this study.

To create the *UAS-amon* RNAi construct, forward (5'-TGGCGTTGCTTATGACAG-3') and reverse (5'-ATGTCCCGCCAAGTCAGC3') primers for the sense insert, and forward (5'-TGGCGTTGCTTATGACAG-3') and reverse (5'-ATGTCCCGCCAAGTCAGC-3') primers for the antisense insert were used to amplify exons 7 and 8 of the *amon* transcript. *amon* antisense products were cloned into the pUASTi vector (Amin Ghabrial, Krasnow Lab, Stanford University, Stanford, Ca) as *KpnI/XbaI* fragments. *amon* sense products were cloned into the vector as *BglIII/XhoI* fragments. The recombinant vector was injected into *w*¹¹¹⁸ embryos by the Duke University Model Systems Genomics. A homozygous viable *UAS-amon-RNAi*^{28b} line, which has an insertion on the second chromosome, was used in this study.

Finally, the *UAS-amon* construct was created by digesting the *amon* cDNA sequence out of the *amon* #5-8 vector (Siekhaus and Fuller, 1999) as a *EcoRI/EcoRV* fragment and subcloning it into pBluescript. The pBluescript vector containing the *amon* cDNA was then digested with *EcoRI* and *XhoI*, and the resulting fragment was cloned into pUAST (Brand and Perrimon, 1993). The recombinant vector was injected into

w¹¹¹⁸ embryos by the Duke University Model Systems Genomics. The homozygous viable *UAS-amon^{40L}* line, which has an insertion on the second chromosome, was used in this study.

Expression of *amon-GAL4*. *amon-GAL4* was used to drive expression of GFP in cell membranes using the *UAS-CD8-GFP* reporter. Ring glands were dissected from third instar larvae at approximately 108 hrs AEL, and mounted in glycerol. Tissues were visualized using a Hamamatsu Orca-ER digital camera (model #C4742-80).

***In situ* hybridization.** The generation of DIG-labeled probes and *in situ* hybridization to third instar larval ring glands was performed as described in (Cai et al., 1996).

Immunohistochemistry. Approximately 20 virgin *yw; uas-CD8-gfp* females were crossed to 15 *yw; amon-gal4^{91D}* males. For co-localization experiments, ring glands from wandering third instar larvae were dissected in TBS (25 mM Tris, 137 mM NaCl, 0.27 mM KCl) and fixed in 4% para formaldehyde for twenty minutes at 4°C. Tissues were then washed in TBS containing 0.5% Triton X-100 (TBST) two times for five minutes each and then blocked in TBST+5% bovine serum albumin (BSA) for one hour at room temperature. After blocking, tissues were incubated with AKH antiserum (1:1000, gift from M. Brown, University of Georgia) and anti-Green Fluorescent Protein antibody (1:1000, Molecular Probes, Invitrogen) overnight at 4°C. The following day, tissues were washed for one to two hours, and then incubated with Alexa Flour 568 goat anti-Rabbit

(1:1000) and Alexa Fluor 488 goat anti-mouse (1:1000) for one hour in the dark at room temperature. Tissues were washed for two hours in TBST, mounted on slides, and visualized.

Heat-shock rescue and trehalose assay of *amon* mutants. To obtain sufficient volumes of hemolymph, larvae were rescued to third instar larval stages by periodic heatshock driven *amon* expression. Approximately 150 *yw; +; amon*^{Q178ST/TM3 Sb Ser y+ e} virgin females carrying an *amon* and 150 *yw; hs-amon; Df(3R)Tl-X e/TM3 Sb Ser y+ e* males were placed in an egg collection chamber containing a grape juice agar plate with fresh yeast paste, and incubated at 25°C. After 24 hrs, a 4 hr egg collection was taken by replacing the old grape juice agar plate with a fresh plate containing yeast paste. This egg collection plate remained in the incubator for 36 hrs AEL, at which point the plate was heat-shocked at 37°C for 30 min, and then returned to the incubator. Subsequent heat shocks were performed at 60 hrs AEL, 84 hrs AEL, and in one assay at 108 hrs AEL. After the final heat shock, *yellow* larvae were selected from the egg collection plate, moved to a fresh grape juice agar plate with yeast paste, and returned to the incubator for either 3 hrs or 24 hrs. At either 87 hrs AEL, 108 hrs AEL, or 111 hrs AEL, trehalose and sugar measurements were done as previously described (Rulifson et al., 2002). Briefly, larvae were moved to a siliconized glass slide in groups of three where their cuticles were torn in order to allow the hemolymph to bleed onto the slide.

Approximately 0.2 µL of hemolymph were collected using a micro-pipette (Hamilton

model #701) with a teflon tip (Hamilton model #84255). Hemolymph samples were combined with 100 μ L of Infinity Sugar Hexokinase Liquid Stable Reagent with porcine kidney trehalase at 5 μ L per 5 ml. Samples were incubated overnight at 37°C, during which time trehalose is converted to sugar, and NADH is produced in equal molar proportion to sugar through the enzymatic endpoint reaction involving the coupled hexokinase and glucose-6-phosphate dehydrogenase. Following overnight incubation, total hemolymph concentration was determined by measuring NADH fluorescence at 340 nm (Synergy HT-I). For each experimental trial, standard curves were generated over a 0-2500 mg/dL range. Average concentration values for each hemolymph sample were determined using a linear regression of the standard curve. *Canton S* larvae were heat-shocked and assayed in the same manner as the *amon* mutants described above.

To assay combined glucose and trehalose levels of larvae in which *amon* function was removed in the AKH producing cells, approximately 50 *yw*; +; *akh-gal4* virgin females were crossed to either 25 control males (*w*¹¹¹⁸) or 25 experimental males (*w*; *UAS-amon-RNAi*^{28b}; +). As an additional control, approximately 50 *yw*; +; *akh-gal4* virgin females were crossed to 25 *w*; *UAS-reaper*; + males. Flies were raised on standard fly food and incubated at 25°C. At 108 hrs AEL, glucose and trehalose measurements were performed as described above using feeding third instar larvae.

To determine if *amon* functions upstream of AKH in controlling sugar homeostasis, approximately 50 *yw*; *hs-akh*; *akh-gal4* virgin females were crossed to either

25 control male flies (*w*¹¹¹⁸) or 25 experimental male flies (*yw*; *UAS-amon-RNAi*^{28b}; +). As an additional control, 50 *yw*; *hs-akh*; *akh-gal4* virgin females were crossed to 25 *w*; *UAS-reaper*; + males. Crosses were performed in egg collection chambers containing a grape juice agar plate with fresh yeast paste, and incubated at 25°C. Six hour egg collections were taken on fresh grape juice agar plates with yeast paste. At 36 hrs AEL, approximately 100 first instar larvae from control and experimental crosses were transferred to a vial containing standard fly food. Beginning at 36 hrs AEL, larvae were heatshocked at 37°C for 45 min every 8 hours. At 108 hrs AEL, glucose and trehalose measurements were performed as described.

To determine if *amon* function in the AKH producing cells is sufficient to maintain sugar homeostasis, approximately 150 *yw*; *UAS-amon*; *Df(3R) Tl-X e/TM3 Sb Ser y+ e* virgin females were crossed to 50 *yw*; *hs-amon*; *akh-gal4*, *amon*^{C241Y}/*TM3 Sb Ser y+ e* males. As a control cross, 150 *yw*; *UAS-amon*; *Df(3R)Tl-X e/TM3 Sb Ser y+ e* virgin females were crossed to approximately 50 *yw*; *hs-amon*; *amon*^{C241Y}/*TM3 Sb Ser y+ e* males. These crosses were performed in egg collection chambers containing a grape juice plate with fresh yeast paste, and maintained at 25°C. After 24 hrs, a 4 hr egg collection was taken by replacing the old grape juice agar plate with a fresh plate containing yeast paste. This egg collection plate remained in the incubator for 36 hrs AEL, at which point the plate was heat-shocked at 37°C for 30 min, and then returned to the incubator. Subsequent heat shocks were performed at 60 hrs AEL and 84 hrs AEL.

After the final heat shock, *yellow* larvae were selected from the egg collection plate, moved to a fresh grape juice agar plate with yeast paste, and returned to the incubator for 24 hrs. At 108 hrs AEL trehalose and glucose measurements were done as described previously.

Quantitative Real Time PCR. Approximately 20 *yw; UAS-amon-RNAi^{28b}* virgin females were crossed to 10 *hs-gal4* males in an egg collection chamber containing an apple juice plate with fresh yeast paste, and maintained at 27°C. As a control, 20 *w¹¹¹⁸* virgin females were crossed to 10 *hs-gal4* males in the same manner. After 48 hours, a four hour egg collection was taken. Beginning at 36 hrs AEL, egg collection plates were heatshocked every 12 hours for 1 hr at 37°C. Total RNA was isolated from 10 whole third instar larvae at 108 hrs AEL with TRIzol reagent (Invitrogen) and treated with DNaseI, and 5 µg were used for reverse transcription using the Transcriptor First Strand cDNA Synthesis Kit (Roche). The *amon* transcript was quantified using an Applied Biosystems 7500 Real-Time PCR system according to the manufacturer's instructions. PCR reactions consisted of 2 µl of each primer at 6.5 µM, 3.5 µL dH₂O, 12.5 µL of SYBR green mix, and 5 µL of cDNA. Two sets of primers were used, targeting either exon 10 or exon 11: exon 10 forward (5'-GCCGGCGCCATGGT-3') and reverse (5'-ATAGCGCGGTGGCACTGA-3'), and exon 11 forward (5'-TTCAACTCGCCCCAAACAC-3') and reverse (5'-ATGCAGGACCAAGGACCATTC-3'). Ribosomal protein 49 (rp49) was used as an endogenous control.

Lethal phase analysis of *amon*-RNAi animals. Crosses and egg collections were performed as described in Quantitative Real Time PCR. Beginning at 36 hrs AEL, both control and experimental animals were heatshocked at 37°C for 1 hr every 12 hrs until approximately 12 days AEL. Death was scored once every 24 hrs. Pictures were taken with a digital camera (Hamamatsu 3CCD) mounted to a Leitz dissecting scope.

Quantitation of growth defects. Approximately 150 *yw; UAS-amon; Df(3R)Tl-X e/TM3 Sb y+ e* virgin females were mated to either 75 control males (*yw; +; amon^{C241Y} e/TM3 Sb Ser y+ e*) or 75 experimental males (*yw; +; akh-gal4, amon^{C241Y} e /TM3 Sb y+ e*) and were placed in egg collection chambers at 25°C. After 24-48 hrs, 4 hr egg collections were made on grape juice agar plates and larvae were sorted at 36 hrs AEL into mutant or control classes using the *yellow* marker. *amon* mutant larvae (*yw; UAS-amon/ +; amon^{C241Y} e /Df(3R)Tl-X e*), rescued larvae (*yw; UAS-amon/ +; akh-gal4, amon^{C241Y} e /Df(3R)Tl-X e*) and control larvae (*yw; uas-amon; amon^{C241Y} or Df(3R)Tl-X e/Tm3 Sb Ser y+ e*) were washed and killed by microwave as described in (Rulifson et al., 2002). Length measurements were made from enlarged print-outs of photographs taken with a digital camera (Hamamatsu 3CCD) mounted to a Leitz dissecting scope.

Mass spectrometric profiling. Virgins of *yw; +/+; amon^{e241y}/TM3 Sb Ser y+ e* were crossed with *yw; hs-amon; Df(3R)Tl-X e/TM3 Sb Ser y+ e* males. Eggs were collected every morning, and heatshocked every 24h until the larvae had reached the 3rd instar. Three

days later, ring glands were dissected and profiled by MALDI-TOF mass spectrometry. For adult flies, heatshock was continued until eclosion and then stopped. Corpora cardiaca (CC)/hypocerebral ganglion were dissected out from 5 days old adult males. Dissection was performed as described (Wegener et al., in press). Tissues were transferred in pulled glass capillaries and spotted directly onto the MALDI target and left to dry. For the ring gland, matrix (saturated solution of recrystallized α -cyano-4-hydroxycinnamic acid in MeOH/EtOH/Aq.bidest 30/30/40% v/v/v) was added in small nanoliter volumes using a manual oocyte injector (Drummond Digital, Broomall, PA). For adult CC, 500 nl of matrix was added to each sample with a micropipette. For peptide quantifications, 400 nM heavy isotope-labeled AKH* (pGlu-Leu[$^{13}\text{C}_6$, ^{15}N]-Thr-Phe-Ser-Pro-Asp-Trp-amide, Mw = 982.5 Da, Iris Biotech, Marktredwitz, Germany) were added to the matrix. Only low protein-binding plastic ware was used during peptide handling.

MALDI-TOF mass spectra were acquired in positive ion mode on an Applied Biosystems Voyager DE RP MALDI-TOF (ring gland samples) or an Applied Biosystems 4800 *Plus* MALDI TOF/TOF mass spectrometer (adult CC). Samples were analyzed in reflectron mode with delayed extraction time. To suppress matrix signals, the low mass gate was set to 850 Da, focus mass was 1100 Da. For quantification, laser power was first adjusted with one sample to provide optimal signal-to-noise ratios, and

then kept constant for all samples on the MALDI target. For each spectrum, five subspectra with 50 shots each were acquired.

For the standard curve and quantification, only adult male flies were used; larvae were not sexed. For each *amon* fly, we measured a control fly taken from the same bottle that had either eclosed on the same day (adults) or originated from the same day of egg laying (larvae) to minimize possible age, food or population density effects on AKH levels. Raw data were analyzed using Data Explorer 4.3 software (Applied Biosystems). For the quantification, mass spectra were base-line corrected and de-isotoped. Then, the ratio of the resulting relative intensity of the monoisotopic peaks of AKH/AKH* and AKH intermediate/AKH* was calculated. Statistics were performed using GraphStat Prism 4.0 (GraphStat Software, San Diego, CA).

Results

***amon* mutants have reduced hemolymph sugar levels.** *amon* mutants die early in development, with most arresting as first instar larvae exhibiting molting defects (Rayburn et al., 2003). In order to obtain sufficient volumes of hemolymph for sugar level determination, we provided *amon* expression via a *hs-amon* transgene to rescue mutants past the early requirements for *amon* to the third instar larval stage. While a single heatshock during the first instar larval stage is sufficient to rescue larvae to the second instar larval state, it is not sufficient to rescue them to the third instar larval stage (data not shown), suggesting that *amon* protein turnover occurs in less than 24 hrs.

Thus, sugar levels of *amon* mutants were determined 24 hrs after the last heatshock treatment. Total hemolymph sugar levels were determined as described in the Materials and Methods section. When hemolymph is collected from *amon* mutants three hours after the final heatshock, combined sugar levels (1230 mg/dL, SEM=51.73) are similar to those seen in wild-type sibling controls (1163 mg/dL, SEM=76.64, Figure 2.1A, left bars). Twenty-four hours after the final heatshock, however, *amon* mutants (1281 mg/dL, SEM=44.71) are hypoglycemic compared to control larvae (1711 mg/dL, SEM=51.20, Figure 2.1A, center bars), indicating that *amon* mutants fail to properly regulate hemolymph sugar concentrations.

An additional heatshock at 108 hrs AEL and assay three hours later is sufficient to restore *amon* mutant sugar levels (1575 mg/dL, SEM=65.92) to control levels (1591 mg/dL, SEM=65.71, Figure 2.1A, right bars). This observation suggests that maintenance of normal sugar levels is dependent on expression of the *amon* gene. The hypoglycemia seen in *amon* mutants is similar to that seen in larvae in which the AKH producing cells have been ablated (Isabel et al., 2005; Lee and Park, 2004; Rulifson et al., 2002).

To determine if *amon* is expressed in the AKH producing cells, we created an *amon-gal4* transgenic construct in which 424 bp of *amon* promoter sequence drives expression of the yeast GAL4 protein. Combination of this construct with a *uas-cd8-gfp* construct promotes expression of GFP in the CNS in a pattern similar to that seen using the *amon* antibody (Rayburn et al., in revision and data not shown). Using this driver,

we also see expression of the GFP reporter in the CC portion of the ring gland (Figure 2.1B) where AKH is produced. Expression of the *amon* gene in these cells was verified by *in situ* hybridization using an RNA probe directed against *amon* (Figure 2.1C). Finally, we examined the expression patterns of *amon* and AKH using the *amon-gal4* construct and an AKH antibody (Kaufmann and Brown, 2006). Figure 2.1D-F show that *amon* and AKH co-localize to the CC cells of the ring gland.

***amon* expression in the AKH producing cells is necessary and sufficient for normal sugar regulation.** To ask whether *amon* expression in the AKH cells is required to maintain normal hemolymph sugar levels, we reduced *amon* expression in these cells by combining an *uas-amon*-RNAi transgene with the *akh-gal4* driver. Figure 2.2A-B shows that ubiquitous expression of the *uas-amon*-RNAi construct via a heatshock GAL4 driver reduces *amon* transcript levels by 90% (Figure 2.2A), and that ubiquitous expression of this transgene phenocopies *amon* mutants. Ninety-one percent of *amon* knockdown animals (n=182) die when the *uas-amon*-RNAi construct is expressed using *hs-gal4*; expression of the *uas-amon*-RNAi construct using an *actin-gal4* driver results in complete lethality (n=60). In addition, the phenotypes observed in knockdown animals that arrest during pupal development when the *hs-gal4* driver is used (Figure 2.2B, bottom) resemble *amon* mutants (Figure 2.2B, middle), including a failure to evert the head and a failure of the abdomen to differentiate.

Reduction of *amon* activity in the AKH cells results in a significant decrease in combined glucose and trehalose levels (Figure 2.2C, gray bar) relative to control larvae (Figure 2.2C, black bar). In these experiments, this difference was similar to AKH cell ablated larvae (Figure 2.2C, blue bar). Thus, *amon* activity is necessary in the AKH producing cells to maintain normal sugar homeostasis.

To ask whether *amon* activity in the AKH producing cells is sufficient to maintain hemolymph sugar concentrations, we expressed *amon* in the AKH cells in an *amon* mutant background. Expression of *amon* in the AKH cells was achieved by combining a *uas-amon* construct and the *akh-gal4* driver in an *amon* mutant background. *amon* function in these cells alone, however, is not sufficient to rescue the early developmental requirements for *amon* (data not shown). In order to obtain larvae large enough for sugar determination, we combined the *hs-amon* construct with the *uas-amon* and *akh-gal4* constructs in an *amon* mutant background. *amon* mutants were rescued by expressing *amon* via the heatshock promoter once every 24 hrs until the third instar larval stage. In this background, *amon* is expressed in the AKH cells by virtue of the *uas-amon* and *akh-gal4* constructs, allowing us to examine requirements for *amon* in sugar homeostasis. Sugar levels were assayed approximately twenty-four hours after the final heatshock as described above. Expression of *amon* via a *uas-amon* transgene in the AKH cells of an *amon* mutant results in larvae with wild-type sugar levels (Figure 2.3, black vs. gray bar). This observation indicates that restoring *amon* activity in the

AKH producing cells in an *amon* mutant background is sufficient to rescue the hypoglycemic defect.

Ubiquitous expression of AKH rescues hypoglycemia induced by *amon* knockdown in AKH producing cells. To ask whether the defect in sugar homeostasis observed in larvae in which *amon* expression has been reduced in the AKH cells *via* RNAi can be attributed to a lack of mature AKH, we expressed AKH in these larvae using the *hs-akh* transgene. It has been previously shown that the hypoglycemia induced by ablation of the AKH cells can be rescued through the expression of the *akh* gene throughout the larva using a *hs-akh* transgene (Kim and Rulifson, 2004). Since these animals lack detectable AKH cells, this observation suggests that other cell types possess the proteolytic machinery to produce functional AKH. We find that ubiquitous expression of *hs-akh* in control larvae (*yw; hs-akh/+; akh-gal4/+*) has no effect on sugar levels, and that heatshock treatment also does not affect hemolymph sugar levels (Figure 2.4, left black bars). In addition, we have recapitulated the rescue of hemolymph sugar levels in AKH cell ablated larvae (*yw; hs-akh/uas-reaper; akh-gal4/+*, Figure 2.4, center blue bars) demonstrated in earlier studies (Kim and Rulifson, 2004). Finally, we show that *hs-akh* expression in larvae with reduced *amon* function in the AKH cells (*yw; hs-akh/uas-amon-RNAi^{28b}; akh-gal4/+*) is sufficient to restore glucose and trehalose to control levels (Figure 2.4, right gray bar), indicating that *amon* functions upstream of AKH to prevent hypoglycemia.

Direct peptide profiling shows that *amon* mutants lack mature AKH peptide.

In mammals, PC2 processes a variety of peptide hormones into their active forms reviewed in (Scamuffa et al., 2006). Since *amon* is the homolog of PC2 (Siekhaus and Fuller, 1999), it is likely that *amon* is also responsible for the proteolytic activation of *Drosophila* peptide hormones. To determine whether or not *amon* mutants are defective in AKH processing we used direct mass spectrometric profiling to compare mature AKH levels in wild-type versus *amon* mutants. We detected mature AKH and a processing intermediate with the C-terminal extension GKR in approximately 89% of control (*ebony*) third instar larval ring glands compared to 0% of *amon* mutants (*yellow*) (Figure 2.5A, left graph). When *amon* mutants are rescued by continuous heatshock expression of *amon*, mature AKH production is rescued to control levels (Figure 2.5A left), indicating that *amon* is required for the proteolytic cleavage of AKH. The same was found when heatshocks were given on day 1-3 plus one day before dissection (100% detections, n=5). We next profiled the CC/hypocerebral ganglion complex of 5 days old adult males that were heatshocked until eclosion. In both control and *amon* flies, AKH and its intermediate could be detected (94% and 88% of the preparations, respectively, Figure 2. 5A right), although the spectra from *amon* flies showed decreased signal-to-noise ratios indicating a lower amount of AKH. To (semi)quantify the amount of AKH, we added a constant amount of heavy isotope-labeled AKH* with the matrix, and calculated the ratio of the relative signal intensity of native AKH or AKH intermediate

vs. AKH*. This is possible since the ratio of the relative intensities of native AKH and AKH* is linear when AKH* is present in a concentration of 50-500 nM (Figure 2.5C). Control flies showed a significantly higher ratio of AKH/AKH* than *amon* flies (Figure 2.5d). They also showed a significantly higher ratio of AKH intermediate/AKH*. Based on the obtained ratios and the amount of AKH* added, we estimate that the CC of control flies contain 500 ± 120 fmol AKH, whereas the CC of *amon* flies contain 50 ± 20 fmol.

Discussion

In mammals, maintenance of blood sugar levels is a fundamental physiological process accomplished by the antagonistic action of two endocrine hormones: insulin and glucagon. Failure to properly regulate blood sugar levels may result in serious health consequences, including diabetes and obesity. As with most peptide hormones, both insulin and glucagon are liberated from larger precursor molecules by the action of the subtilisin-like proprotein convertases, PC1/3 and PC2 (Furuta et al., 1998; Furuta et al., 2001; Rouille et al., 1997; Rouille et al., 1994; Westphal et al., 1999; Zhu et al., 2002). It has long been established that metabolic pathways change little over evolutionary time, thus it is not surprising that functional homologs of the two mammalian endocrine hormones are present in insects. This conservation, then, raises the possibility that similar mechanisms exist for the regulation of synthesis and release of peptide hormones between vertebrates and invertebrates.

In *Drosophila*, seven insulin-like peptides showing structural similarity to mammalian preproinsulin have been identified, each consisting of a B and A chain connected by an intermediate C peptide. Predicted cleavage occurs at the ends of the C-peptide between the B and A chains at the preferred processing site of the proprotein convertases to give rise to an active peptide (Brogiolo et al., 2001). In turn, *akh* is the functional analog of glucagon and is also synthesized as a prepropeptide (Noyes et al., 1995). Cleavage at a dibasic processing site yields the mature AKH peptide and a C-terminal peptide of unknown function (Noyes et al., 1995). Although the major convertases involved in the maturation of the vertebrate hormones have been identified, the enzyme responsible for the activation of the *dilps* and *akh* has yet to be established.

In *Drosophila*, there are three genes structurally resembling vertebrate PCs: *dfurin1*, *dfurin2*, and *amontillado* (Roebroek et al., 1992; Roebroek et al., 1991; Siekhaus and Fuller, 1999). Two of three, *dfurin1* and *dfurin2*, have enzymatic activity similar to that of mammalian *furin* as demonstrated through *in vitro* assays (Debie et al., 1995; Roebroek et al., 1992). *amon* is most similar to mammalian PC2 and is expressed in numerous peptidergic neurons (Siekhaus and Fuller, 1999). Phenotypic analysis of animals lacking functional *amon* have revealed a requirement for this gene during embryonic hatching (Siekhaus and Fuller, 1999), molting (Rayburn et al., 2003), and

metamorphosis (Rayburn et al., 2003), suggesting that *amon* may be required for the proteolytic activation of one or more secretory peptides.

We have shown here that *amon* mutants have reduced hemolymph sugar levels compared to wild-type control larvae (Figure 2.1A). This abnormal reduction in circulating carbohydrate levels of mutant larvae is reminiscent of larvae lacking the AKH producing cells (Isabel et al., 2005; Kim and Rulifson, 2004; Rulifson et al., 2002), indicating that *amon* function plays an important role in maintaining sugar homeostasis. Given that *amon* is expressed in the corpora cardiaca portion of the ring gland as seen by both *in situ* hybridization and expression of a GFP reporter using an *amon-gal4* driver (Figure 2.1B and C), we hypothesized that *akh* is a proteolytic target of *amon*.

We also show that *amon* function is necessary in the AKH producing cells of the CC to maintain circulating carbohydrate levels as cell-type specific reduction of *amon* in these cells results in larvae with approximately 30% less glucose and trehalose in circulation (Figure 2.2C). Previously, labs have correlated defects in sugar homeostasis with particular hormone signals using a heatshock inducible transgene containing the coding sequence of peptide hormones (Kim and Rulifson, 2004; Rulifson et al., 2002). For example, Kim and Rulifson (2004) demonstrate that expression of a *hs-akh* transgenes can completely rescue the hypoglycemic defect seen in larvae in which the AKH cells have been ablated (Kim and Rulifson, 2004). A similar rescue of hyperglycemia was also demonstrated in IPC ablated larvae using a *hs-dilp2* transgene

(Rulifson et al., 2002). Together, these studies indicate that the prohormones can be properly cleaved and released into circulation from other tissues, presumably due to the expression of important proteolytic enzymes in multiple cell types (Kim and Rulifson, 2004; Rulifson et al., 2002). Using a similar approach, we show that sugar homeostasis can be restored to hypoglycemic larvae produced by *amon*-RNAi expression in the CC portion of the ring gland using the *hs-akh* transgene (Figure 2.4), thereby attributing the hypoglycemic defect seen in *amon*-RNAi larvae to a lack of active AKH and supporting the idea that AKH is a substrate of *amon*.

In addition to being necessary in the AKH cells for sugar homeostasis, *amon* is sufficient in these same cells to rescue the mutant hypoglycemic phenotype as expressing *amon* in the AKH cells of an *amon* mutant rescues the hypoglycemic defect (Figure 2.3). Interestingly, rescued larvae also show a partial rescue of the growth defect seen in *amon* mutants (data not shown), suggesting that *amon* activity in the AKH cells may be sufficient to rescue growth, possibly through AKH or a novel peptide hormone. Finally, we have provided biochemical evidence through mass spectrometric techniques indicating a requirement for *amon* in the production of mature bioactive AKH (Figure 2.55).

The genetic tools described here that allow reduction of *amon* activity in a cell-specific fashion and the restoration of *amon* expression to specific cell types in an *amon* mutant background are potentially very powerful tools to identify cell-type specific

requirements of the *Drosophila* proprotein convertase *amon*. Here, using both genetic and biochemical approaches, we provide evidence to support the role of *amon* in sugar homeostasis through the production of mature AKH. This apparent evolutionarily conserved mechanism of peptide hormone production by the proprotein convertase family presents the opportunity to use a simple model system to discover the regulatory mechanisms behind peptide hormone synthesis, processing, and release and apply the knowledge to mammals. In the future, these tools may provide beneficial insight into the cellular requirements of proprotein convertases and their regulatory role in normal physiological processes as well as in the pathology of a variety of diseases.

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Figure 2.1. Larvae lacking functional *amon* have reduced hemolymph sugar levels.

(A) Bars indicate combined glucose and trehalose hemolymph levels in control siblings (black) and *amon* mutants (white). Hemolymph carbohydrate levels were measured in control and *amon* mutant larvae collected three hours (left bars) and twenty-four hours (center bars) after the last in a series of three heat shocks (at 36, 60, and 84 hours AEL) and in control and *amon* mutant larvae collected three hours (right bars) after the last of a series of four heat shocks (at 36, 60, 84, and 108 hours AEL). n=number of larvae assayed. **p<0.0001, Students T-Test. (B) *amon-gal4* drives expression of *UAS-CD8-GFP* in the corpora cardiac (CC) cells of the ring gland (white arrows). (C) *In situ* hybridization confirms *amon* expression in the CC cells of the ring gland. (D-E) Co-localization showing that *amon* is expressed in the same cells as AKH.

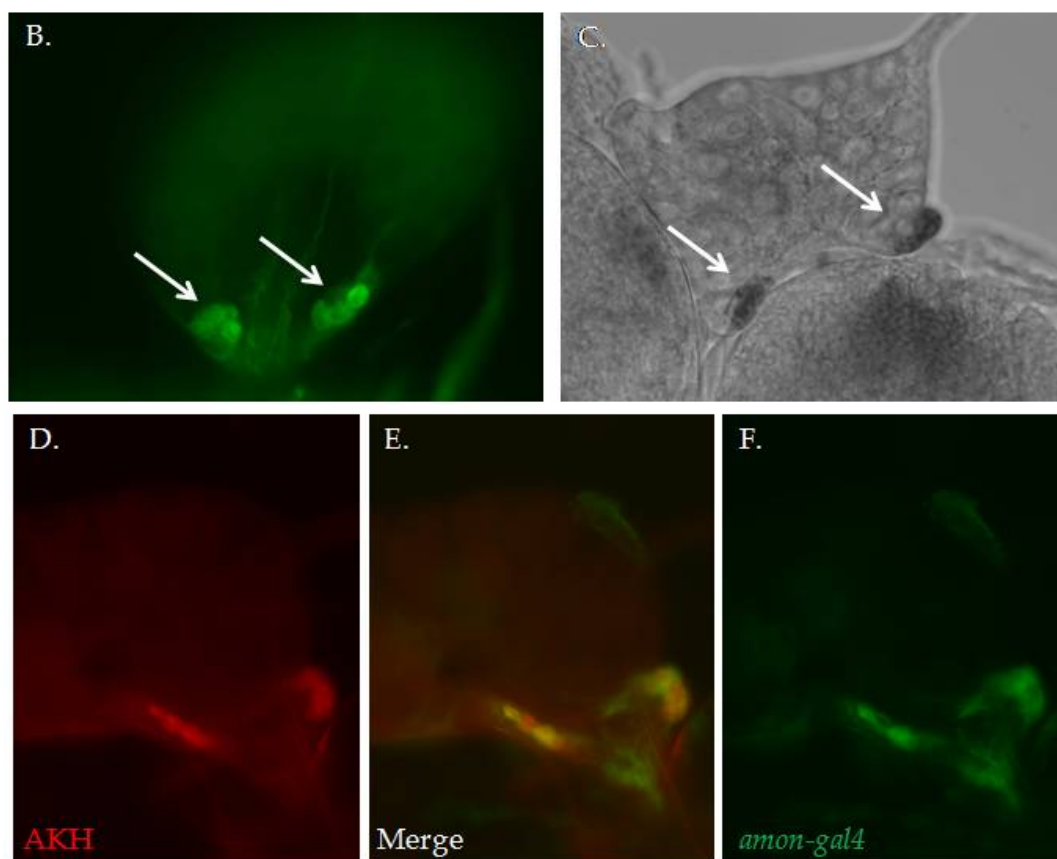
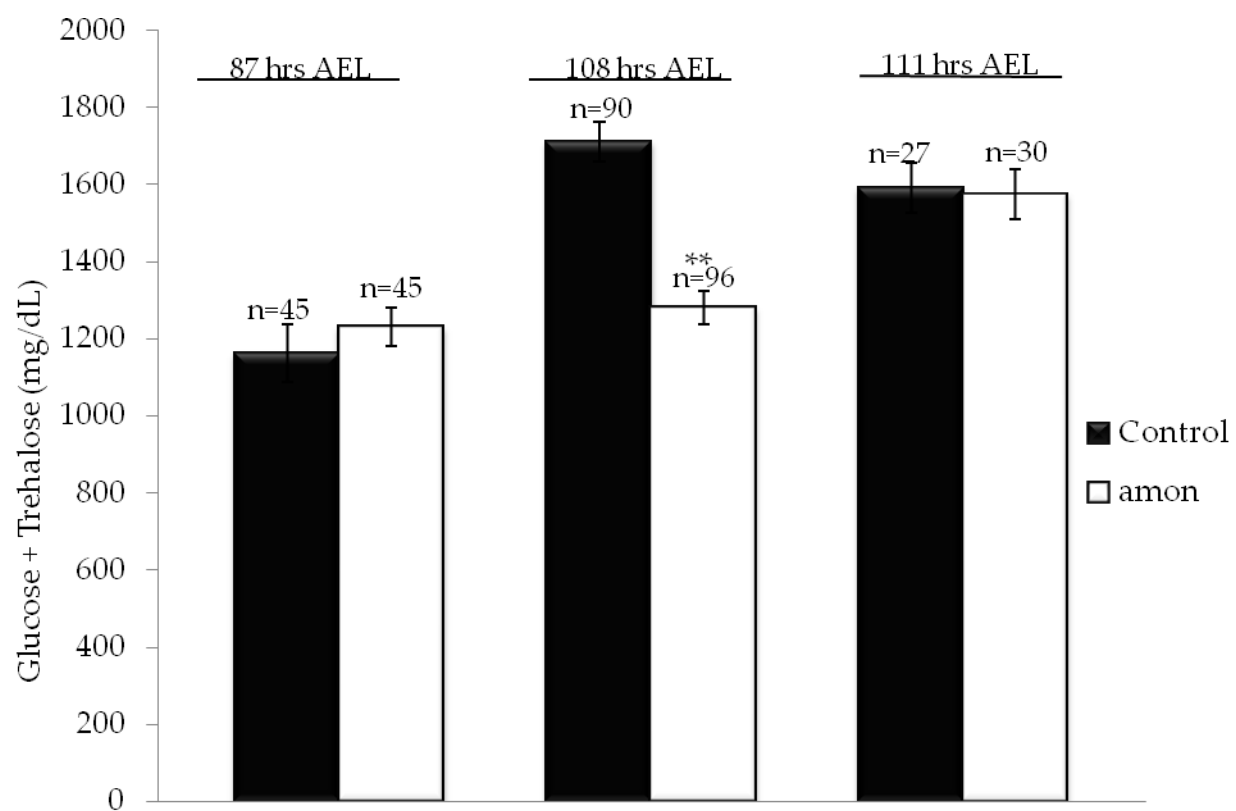


Figure 2.2. *amon* is required in the AKH producing cells for normal glucose regulation. (A) The Black bar indicates *amon* transcript levels in wild-type larvae, while the white bar indicates *amon* transcript levels when *amon*-RNAi is ubiquitously expressed. Primers specific to *amon* exon 10 were used to assess *amon* transcript levels. (B) Dorsal and ventral views of a wildtype pupa (top). Middle panels represent *amon* mutants that are unable to complete metamorphosis, and die with defects in head eversion and abdominal differentiation. *amon* RNAi knockdown animals also die with phenotypes similar to *amon* mutants (bottom). (C) Combined glucose and trehalose levels of control larvae are shown in the black bar. The center blue bar shows hemolymph sugar levels in AKH ablated larvae, while the gray bar represents animals in which *amon* expression has been reduced in the AKH producing cells by RNAi.

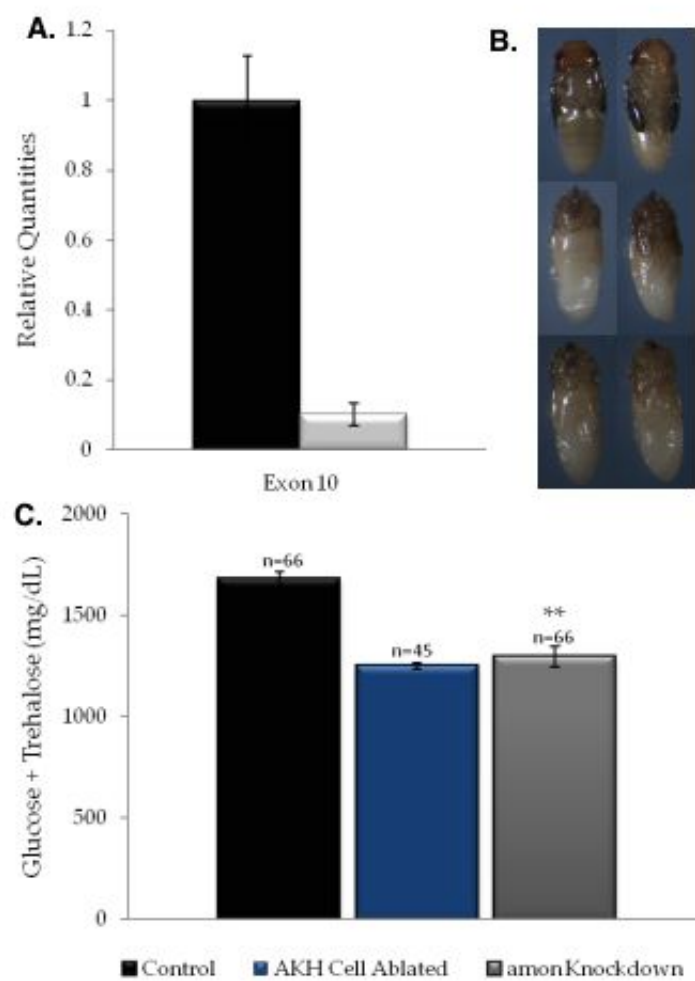


Figure 2.3. Expression of *amon* in the AKH cells of an *amon* mutant is sufficient to rescue hypoglycemia. (A) Schematic of the *UAS-amon* construct. (B) Gray bars represent larvae in which *amon* expression has been restored in the AKH producing cells as compared to *amon* mutants (white bar) and control siblings (black bar). n=number of larvae assayed.

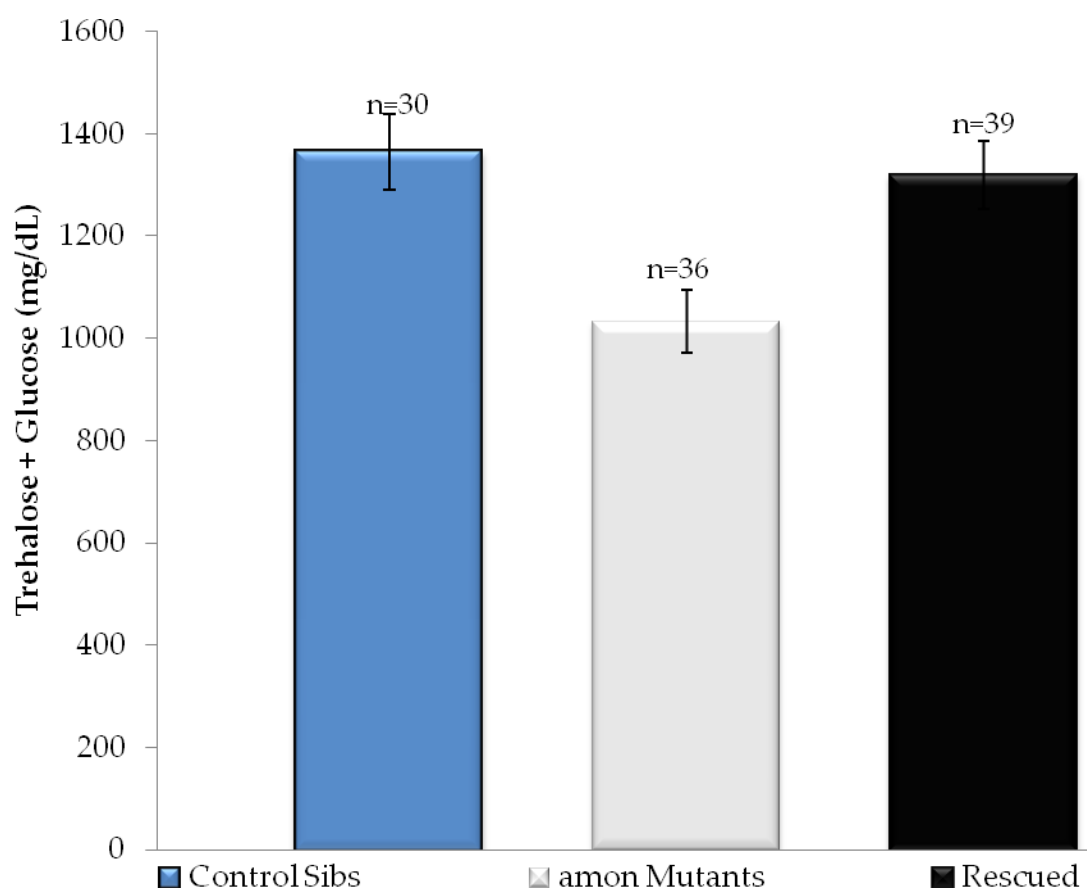


Figure 2.4. Ubiquitous expression of AKH rescues the hypoglycemic defect seen in *amon* knockdown larvae. The left black bars represent wild-type levels of combined glucose and trehalose. The center blue bars represent sugar levels of AKH ablated larvae compared to larvae in which *amon* has been reduced in the AKH cells by RNAi (right gray bars). Combined glucose and trehalose levels following ubiquitous expression of *akh* are found in bars in which the heatshock regime has been administered, indicated by a '+' below the graph. n=number of larvae assayed.

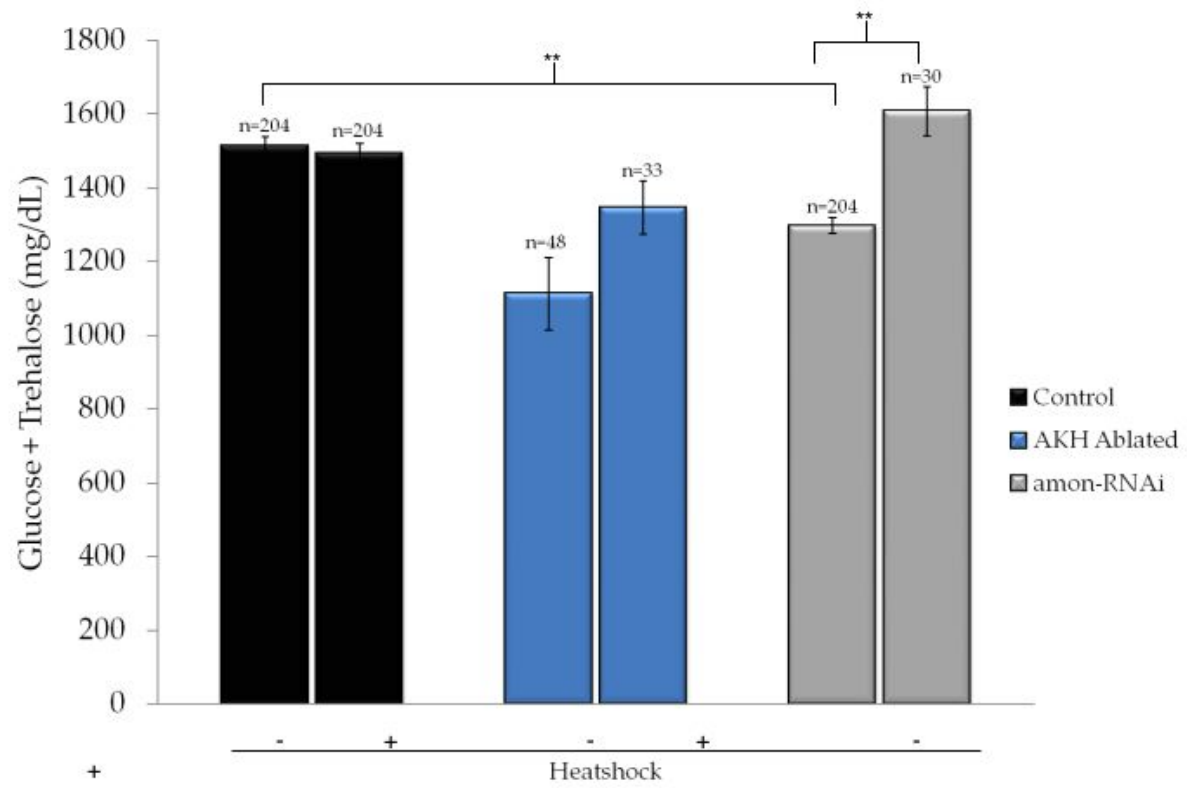
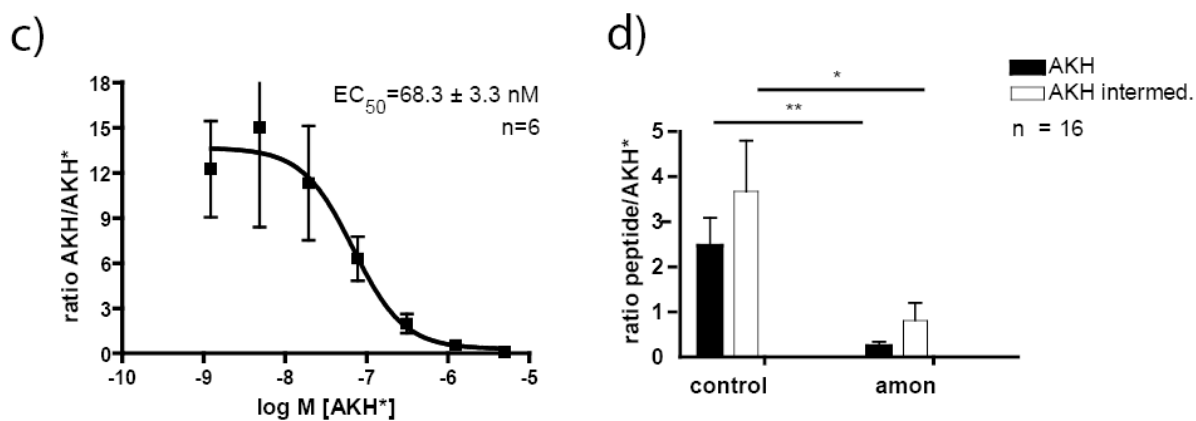
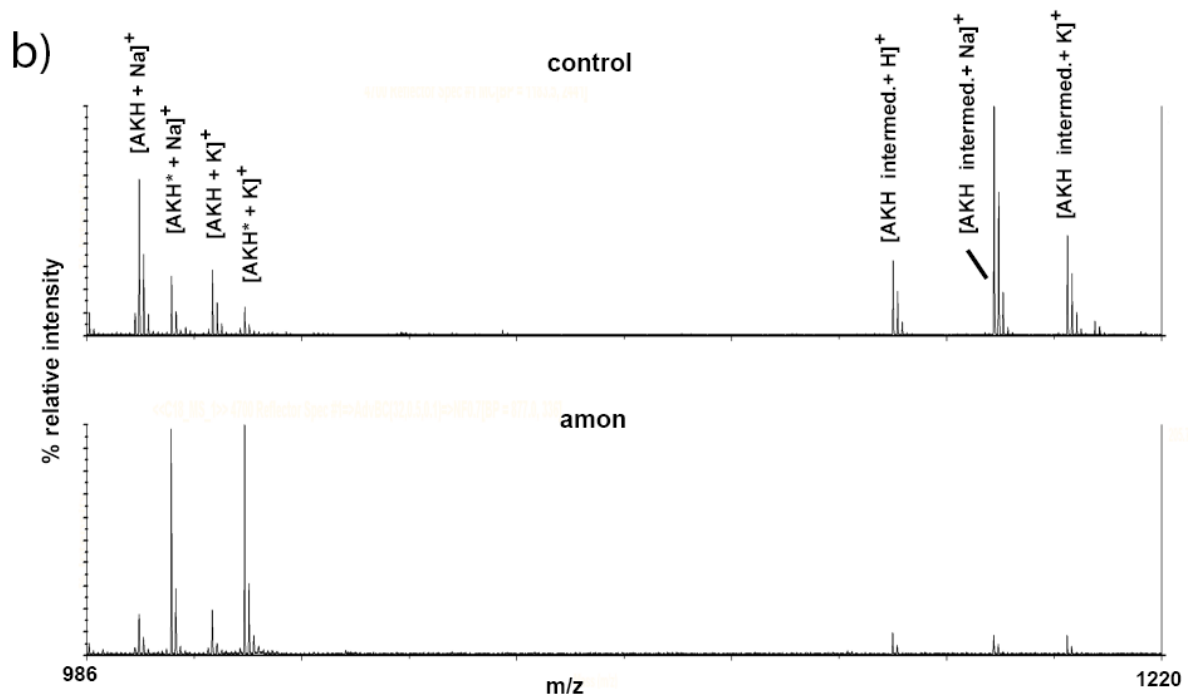
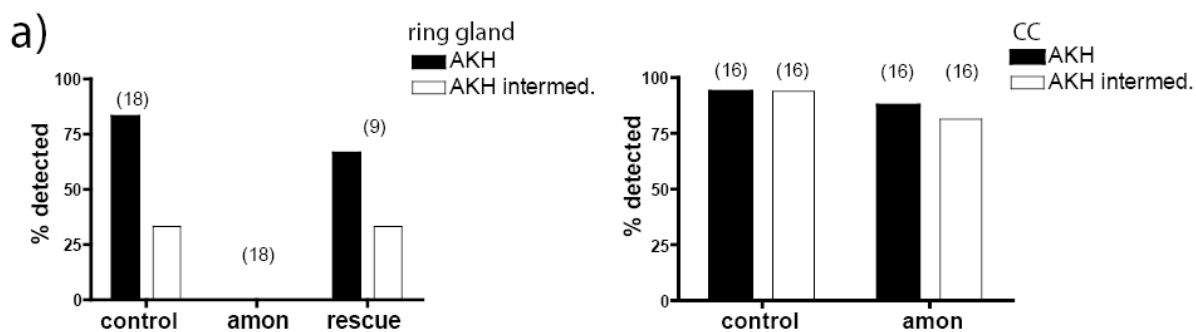


Figure 2.5. Direct peptide profiling of AKH and AKH intermediate in control and *amon* flies. (A) Profiling of the larval ring gland (left) and adult corpora cardiaca (right). While AKH and AKH intermediate were detected in most preparations from control and rescued (continued heatshock once a day) flies, they were not detectable in *amon* flies. (B) Original direct mass profiles from corpora cardiaca of control (above) and *amon* (below) flies. AKH only occurs as characteristic $[M+Na]^+$ and $[M+K]^+$ ion pairs, whereas the AKH intermediate also occurs as $[M+H]^+$. In the control fly, both peptides show higher signal intensities as the stable isotope-labelled standard peptide (AKH*). In the *amon* fly, the signal intensity is clearly higher for AKH* than for the native peptides. (C) Standard curve for adult corpora cardiaca obtained with a dilution series of AKH* added to the matrix salt. The y axis shows the signal intensity ratio of native AKH/AKH*. Error bars are S.E.M. The relationship of AKH/AKH* is linear for AKH* concentrations of 50-500 nM. (D) Peptide quantification with the labeled AKH* standard at 400 nM. The concentrations of both AKH and AKH intermediate are significantly reduced in *amon* flies vs. controls (Mann Whitney test).



CHAPTER 3

THE DROSOPHILA PROPROTEIN PROCESSING PROTEASE AMONTILLADO IS REQUIRED IN THE INSULIN PRODUCING CELLS TO MAINTAIN GLUCOSE HOMEOSTASIS¹

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ABSTRACT

Proteolytic activation of peptide hormones by a family of enzymes referred to as the proprotein convertases (PCs) is an important regulatory step in controlling a variety of processes, including behavior, metabolism, growth, and development. Single precursor molecules can be differentially processed to release distinct sets of peptide hormones, and is dependent on which members of the PC family are present. Thus, understanding how differential expression of the PCs contributes to activation of peptide hormone signals will be important in learning how defects in PCs result in a variety of diseases. In vertebrates, the subtilisin-like proprotein convertases PC1 and PC2 coordinately act to generate the bioactive form of the insulin peptide. Recent studies have demonstrated a remarkable conservation of the insulin signaling pathway in invertebrate systems, raising the possibility that the *Drosophila* insulin-like peptides (DILPs) are proteolytically processed in a manner similar to human insulin. Here, we demonstrate that mutants in the *amontillado* (*amon*) gene, the *Drosophila* homolog of PC2, display a growth defect similar to that seen in larvae lacking insulin producing cells (IPCs). Consistent with a role in DILP processing, the AMON protein co-localizes with insulin-like peptide 2 (DILP2) in the IPCs of the central nervous system. In addition, we demonstrate that *amon* and the *Drosophila* insulin receptor (dInR) genetically interact. Finally, we demonstrate that cell specific reduction of *amon* in the IPCs results in hyperglycemic larvae, a phenotype observed in larvae in which the IPCs have been

ablated. These results suggest that *amon* contributes to insulin signaling in *Drosophila* upstream of the dInR to maintain sugar homeostasis.

Introduction

Endoproteolytic processing of peptide hormone precursors by the PCs is key to regulating hormone signaling for three main reasons: it provides a means by which to create diverse sets of peptides from a single precursor, it ensures active molecules are generated only from tissues in which they are required (Pritchard and White, 2007), and it allows the coupling of external stimuli with the release of active hormone signals (Sanchez et al., 2004). Release of the peptide hormones then regulate other processes, including behavioral, metabolic, growth, and developmental pathways critical to an organism's survival. Thus, understand how expression of PCs influences peptide hormonal signaling will be crucial in understanding how missregulation of these proteases gives rise to various diseases.

The vertebrate insulin signaling pathway has been intensely investigated in efforts to better understand glucose metabolism and the regulation of cell growth (Bohni et al., 1999; Brogiolo et al., 2001; Menon and Sperling, 1996; Rulifson et al., 2002). These topics are not only of fundamental biological interest, but also relevant to disease (e.g. diabetes and cancer). The apparent conservation of the insulin signaling pathway in invertebrate organisms offers a chance to investigate insulin signaling in systems better suited to genetic and experimental manipulation.

In *Drosophila melanogaster*, seven genes encode insulin-like peptides (*dilps*) (Brogiolo et al., 2001). These peptides play a role in maintenance of cell size and number (Brogiolo et al., 2001; Ikeya et al., 2002), and glucose homeostasis (Rulifson et al., 2002). Only three of the seven *dilp* genes, -2, -3, and -5, are expressed in the medial neurosecretory cells of the CNS, often referred to as the insulin producing cells (IPCs) (Brogiolo et al., 2001). Targeted ablation of these cells results in developmental delays, growth defects, and hyperglycemia (Ikeya et al., 2002; Rulifson et al., 2002). Ubiquitous expression of DILP2, the insulin like peptide showing the most homology to human insulin (35% amino acid sequence identity) (Brogiolo et al., 2001), is sufficient to compensate for loss of the IPCs, suggesting that the DILPs produced by these cells are partially redundant (Rulifson et al., 2002).

A key regulatory step in the production of vertebrate insulin is the endoproteolytic cleavage of a precursor molecule to yield the biologically active hormone. Insulin is initially synthesized as a propeptide containing the A and B chains joined by the C (connecting) peptide. This propeptide is processed at dibasic residues located at the A/C and B/C junctions to release mature insulin comprised of the A and B chains joined by disulfide bonds. Insulin processing is achieved by the coordinate action of two subtilisin-like proprotein convertases (PCs), proprotein convertase 1 and 2 (PC1 and PC2), the primary PCs of the neuroendocrine system (Furuta et al., 1998; Zhu et al., 2002). PC1 is believed to act first in the generation of active insulin by recognizing a

dibasic (RR) site at the B/C junction (Smeekens et al., 1992). Cleavage at this site and subsequent trimming of the basic residues results in des-31,32 proinsulin which is then cleaved at the A/C junction, by PC2 to yield the active insulin peptide (Furuta et al., 1998). Mice lacking PC2 display an accumulation of des-31, 32 proinsulin (Furuta et al., 1998) while PC1 null mice accumulate a proinsulin intermediate that has been partially cleaved by PC2 at the A/C junction (Zhu et al., 2002). These results suggest that PC1 and PC2 coordinately act to process proinsulin into its mature form.

The *Drosophila* homolog of PC2 is encoded by the *amontillado* (*amon*) gene (Siekhaus and Fuller, 1999). *amon* mutants and IPC ablated larvae (Rulifson et al., 2002) are both developmentally delayed, suggesting a role for ILPs and AMON in the same pathway. We show here that *amon* mutants exhibit a growth defect, that the AMON protein and *Drosophila* insulin-like peptides co-localize in neurosecretory cells of the insect brain, and that *amon* and *dInR* interact genetically. In addition, we show that cell specific reduction of *amon* in the IPCs results in hyperglycemic larvae. Taken together, these results suggest a role for *amon* in maintaining sugar homeostasis and support the model that one or more of the DILP precursors are a proteolytic target of the AMON proprotein convertase.

Materials and Methods

Immunocytochemistry. Wandering third instar larvae (*Canton S*) were dissected, dehydrated, and rehydrated according to the protocol of (Cao and Brown, 2001).

Tissues were washed in TBS (25 mM Tris, 137 mM NaCl, 0.27 mM KCl) containing 0.5% Triton X-100 (TBST) two times for five minutes each and then blocked in TBST+5% goat serum overnight at 4 °C. All subsequent steps were performed at 4 °C. Primary antiserum was added in TBST+ 2% BSA at a 1:200 dilution and left overnight. Following a wash of at least one hour in TBST+ 2% BSA, the secondary antibody (anti-mouse IgG conjugated to Alexa Fluor 488, 568 or 594, Molecular Probes, Inc.) was added at a dilution of 1:500-1:2000 in TBST+ 2% BSA and tissues were incubated overnight in the dark. Tissues underwent a final wash in TBST and were then mounted on slides in a 1:1 TBST/glycerol solution. Images were captured on an Olympus BX60 microscope using a JVC digital camera (model KYF70BM) and AutoMontage software.

Third instar larval tissues were prepared for co-localization experiments as described above with the following changes. Following overnight incubation in TBST+5% goat serum overnight at 4 °C, tissues were incubated with primary antibody #1 (anti-DILP2) for three hours, washed for one to two hours, incubated in secondary antibody #1 (anti-AMON) for three hours, and washed overnight. The next day, the same procedure was followed for the second primary and secondary antibodies.

Alternatively, tissues were incubated in primary antibody #1 for three hours, washed for one to two hours, incubated in primary antibody #2, washed for one to two hours, and then incubated in both secondary antibodies overnight. The following day, tissues were washed for three hours in TBST, mounted on slides, and visualized.

Quantitation of *amon* mutant growth defect. Approximately 150 virgin *yw; +; Df(3R)*

Tl-X e/TM3 Sb y+ e females and 150 *yw; +; amon^{C241Y} e /TM3 Sb y+ e* males were placed

in an egg collection apparatus and allowed to lay eggs for three days at 25 °C. Five to six hour egg collections were made on grape juice agar egg lay plates and animals were sorted using the presence or absence of the *yellow* marker 36 hours after egg laying

(AEL). *yellow* (*yw; +; amon^{C241Y} e /Df(3R)Tl-X e*) larvae and their wild type siblings were

washed and killed by microwave as described (Rulifson et al., 2002). Length

measurements were made from enlarged print-outs of photographs taken with a digital camera (Hamamatsu 3CCD) mounted to a Leitz dissecting scope.

Rescue of *Inr*-induced overgrowth. To test the effect of *amon* on the previously

described overgrowth phenotype in flies overexpressing the insulin receptor (Brogiolo et al., 2001), approximately 150 virgin females (*UAS-Inr^{wt}*) flies were crossed to

approximately 100 males (*yw; ey-Gal4; amon^{C241Y} /TM3 Sb Ser y+ e*). Flies carrying the

amon mutation were identified based on the absence of the Sb and Ser markers. Eyes of

amon mutants and their siblings were scored individually, and given a numerical value

between 1 and 4 based on morphology: 1=wild-type appearance, 2=large, smooth eyes,

3=large, rough eyes, and 4=large eyes with outgrowths.

Trehalose Measurements. Approximately 50 *w;+;dilp2-gal4* virgin females were crossed

to either 25 control males (*w¹¹¹⁸*) or 25 experimental males (*yw;uas-amon^{28b}*). Flies

were raised on standard fly food and incubated at 29 °C. Larvae were collected as wandering 3rd instar larvae, and glucose and trehalose measurements were performed as described (Rulifson et al., 2002). Briefly, larvae were moved to a siliconized glass slide in groups of three where their cuticles were torn in order to allow the hemolymph to bleed onto the slide. Approximately 0.2 µL of hemolymph were collected using a micro-pipette (Hamilton model #701) with a teflon tip (Hamilton model #84255). Hemolymph samples were combined with 100 µL of Infinity Glucose Hexokinase Liquid Stable Reagent with porcine kidney trehalase at 5 µL per 5 ml. Samples were incubated overnight at 37 °C, during which time trehalose is converted to glucose, and NADH is produced in equal molar proportion to glucose through the enzymatic endpoint reaction involving the coupled hexokinase and glucose-6-phosphate dehydrogenase. Following overnight incubation, total hemolymph concentration was determined by measuring NADH fluorescence at 340 nm (Synergy HT-I). For each experimental trial, standard curves were generated over a 0-2500 mg/dL range. Average concentration values for each hemolymph sample were determined using a linear regression of the standard curve.

Results

***amon* mutants display a larval growth defect.** Homozygous *amon* mutants die between the first and second instar larval with defects in larval molting (Rayburn et al., 2003).

Figure 3.1 shows that *amon*^{C241Y} mutants measured at 36 hrs after egg laying (AEL) are

smaller than their wild-type siblings of the same age. When compared to their wild-type siblings, *amon*^{C241Y} first instar larvae are only 63% as long (average length of wild-type larvae=2.9 mm, average length of *amon*^{C241Y} larvae=1.9 mm), indicating that AMON function is required for normal larval growth. This size reduction is similar to that reported for IPC-ablated animals (Rulifson et al., 2002).

The *amon* protein and DILP2 co-localize to the insulin producing cells. To identify potential targets of the *amon* proprotein convertase, a mouse polyclonal antibody (Rayburn et al., in revision) was used to determine the localization of the protein in the *Drosophila* nervous system. Using this antibody in concert with one against DILP2 (Cao and Brown, 2001), we determined that the *amon* protein and DILP2 co-localize in the IPCs (Figure 3.2), which express *dilp2*, *dilp3*, and *dilp5* (Ikeya et al., 2002; Rulifson et al., 2002). All three of these genes encode peptides that contain the components of the pro-insulin molecule, namely the signal sequence, the A and B chains, and the C peptide, as well as the consensus cleavage sites at the B/C junction, and the A/C junction (Brogiolo et al., 2001). The *amon* protein and DILP2 also co-localize to the IPCs in white pre-pupal and adult brains (data not shown). These results place the *amon* protein in the same cell type as *dilps*-2, -3, and -5.

***amon* and components of the insulin signaling pathway display genetic interactions.**

Targeted overexpression of the *Drosophila* insulin receptor in the eye resulted in

outgrowths of the eye due to an increase in ommatidia number, as well as an increase in cell size. This InR-mediated overgrowth can be suppressed by a deficiency that removes *dilp1-5*, and the suppression can be reversed by ubiquitous expression of *dilp2* (Brogiolo et al., 2001). From this observation, Brogiolo et al. argue that DILP2 is a major ligand of the dInR.

To determine if *amon* function is required upstream of the dInR, we removed one copy of *amon* in the presence of the overexpressed insulin receptor and asked if this was sufficient to suppress the InR overgrowth phenotype. We hypothesized that if *amon* functions in the dInR pathway by proteolytically activating one or more of the *dilps*, then a decrease in *amon* dosage should lead to a decrease in levels of active DILPs, thus triggering a reduction of dInR-induced overgrowth. When the insulin receptor is overexpressed in a background where *amon* is expressed normally, adult eyes are larger than wild-type controls (Figure 3.3 B and C), as has been reported previously (Brogiolo et al., 2001). Reducing the dosage of *amon*, however, resulted in a partial rescue of this overgrowth and restored many eyes to a wild-type appearance (Figure 3.3 D and E). This phenotype was variable between animals and between eyes on a single animal; thus, each eye was scored individually based on morphology (Figure 3.3 F). Those eyes appearing most like wild-type were given a score of 1, while those eyes having severe outgrowths were given a score of 4. Eyes displaying an intermediate morphology were given of score of 2 or 3 depending on their degree of roughness. The peak of the

distribution of scores from animals only overexpressing the insulin receptor was 3, compared to a 2 for *amon* mutants. In addition, the presence of the *amon* mutation prevented the development of outgrowths in the eyes seen in wild-type animals overexpressing dInR. Thus, removing a single copy of *amon* suppresses the overgrowth phenotype induced by increased insulin signaling in the eye. This observation indicates that AMON functions upstream of the dInR.

Reduction of *amon* in the IPCs results in hyperglycemic larvae. Ablation of the IPCs results in small larvae that are developmentally delayed and hyperglycemic (Rulifson et al., 2002). These phenotypes were subsequently rescued through the broad expression of a *hs-dilp2* transgene, confirming IPCs as a key source of insulin for growth and glucose homeostasis (Rulifson et al., 2002). All seven known *dilps* possess optimal predicted basic dipeptide cleavage sites between the A/C and B/C junctions (Brogiolo et al., 2001), suggesting that DILPs are also activated by PCs. If *amon* function is required for the proteolytic activation of one or more of the *dilps*, then we hypothesized that removal of *amon* from the IPCs would result in a similar phenotypes as the IPC ablated larvae. To test this hypothesis, we reduced *amon* expression in the IPCs using an *amon*-RNAi transgene. Unlike IPC ablation, reduction of AMON in these cells did not result in a defect in larval growth or development (data not shown). We did, however, detect a modest increase in circulating carbohydrate levels in *amon*-RNAi larvae as compared

to wild-type controls (Figure 3.4), indicating that AMON activity is required in the IPCs for normal sugar homeostasis.

Discussion

***amon* mutants exhibit a growth defect.** In both vertebrates and invertebrates, the insulin/insulin-like growth factor (IGF) pathways are key regulators of growth and development. In mammals, two related receptors regulate metabolic responses (primarily through the InR) and cell growth responses (primarily through the IGF1 receptor) (Puig and Tjian, 2006). The *Drosophila* genome, however, encodes only a single insulin receptor that has been shown to regulate both growth and metabolism (Puig and Tjian, 2006). Overexpression of the *dilps*, which genetically interact with the dInR, results in an increase in cell size and number, consequently producing larger adult flies (Brogiolo et al., 2001).

In *Drosophila*, both IPC ablated larvae and *amon* mutants are smaller relative to control larvae (Figure 3.1) and developmentally delayed (Rayburn et al, in revision), suggesting that AMON functions in the same pathway as the DILPs to regulate growth. Previous reports have shown that *dilp2*, *dilp3*, and *dilp5* are expressed in the IPCs (Ikeya et al., 2002; Rulifson et al., 2002). All three of these genes encode peptides that contain the components of the pro-insulin molecule, namely the signal sequence, the A and B chains, and the C peptide (Brogiolo et al., 2001). Furthermore, these pro-insulin peptides all contain the consensus cleavage sites at the B chain, C-peptide junction and the A

chain, C-peptide junctions (Brogiolo et al., 2001), suggesting that they are proteolytically processed to generate the mature, active insulin neuropeptide.

Investigations into the maturation of pro-insulin have indicated that two prohormone convertases, PC1 and PC2, participate in this process (Furuta et al., 1998; Rhodes et al., 1992; Smeekens et al., 1992; Zhu et al., 2002). Since no homolog of PC1 has been identified in *Drosophila*, and the other two identified PCs, *fur1* and *fur2* (Roebroek et al., 1992; Roebroek et al., 1991) are homologs of furin, a PC expressed in the constitutive secretory pathway, suggesting that *amon* stands alone in *Drosophila* to proteolytically process DILPs.

The *amon* protein and DILP2 co-localize to the medial neurosecretory cells. Using an antibody raised against a C-terminal peptide of the AMON protein, we were able to determine the localization pattern of the protein in the CNS of larvae, developing pupae, and adults (Rayburn et al., in revision). The localization of AMON to two sets of medial neurosecretory cells in the brain prompted us to ask whether these cells were the same as those previously identified (Cao and Brown, 2001; Rulifson et al., 2002) as those containing insulin-like peptides. Indeed, co-localization experiments demonstrated that AMON and DILP2 are both located in the IPCs of the third instar larval brain (Figure 3.1). These two proteins are also co-localized to the IPCs in the white pre-pupal and adult brains (data not shown). Given that PC2 acts in vertebrate systems to process pro-insulin into its active form, the co-localization of AMON and

DILP2 positions this proprotein convertase as the likely enzyme responsible for insulin-like peptide processing in the IPCs. Our studies, however, do not directly demonstrate such an enzyme/substrate relationship between these two proteins.

***amon* genetically interacts with components of the insulin signaling pathway.** The co-localization of the *amon* protein and DILP2 in the brain prompted us to ask whether a genetic interaction could be demonstrated between the putative pro-insulin processing enzyme and the *dilps* or *inr*. Previously, Brogiolo *et al.* (2001) reported a genetic interaction between *dilp2* and the *inr* using dInR-mediated overgrowth of the eye as an interaction phenotype. Overgrowth was suppressed by removing a single copy of *dilps1-5*. We took advantage of this system and demonstrated that haploinsufficiency of *amon* is also sufficient to suppress dInR induced overgrowth of the eye.

Reduction of *amon* expression in the IPCs results in hyperglycemic larvae. In addition to regulating growth, the insulin signaling pathway also functions in the homeostatic control of circulating glucose levels. We reported previously that *amon* mutant larvae are hypoglycemic (Chapter 2), suggesting that the *Drosophila* glucagon analog adipokinetic hormone (AKH) precursor is a target of *amon* proteolysis. This phenotype is similar to that reported for PC2 knockout mice in which circulating glucagon is undetectable and for a patient found to have mutations in both alleles of PC1 (Jackson *et al.*, 1997). Interestingly, both PC2 knockout mice and a patient lacking

active PC1 also exhibited decreased insulin levels and increased levels of proinsulin and proinsulin processing intermediates (Furuta et al., 1997; Furuta et al., 2001; Jackson et al., 1997). Together, these observations indicate that, while PC1 is required for proinsulin processing, PC2 also contributes to this activation. In contrast, only PC2 appears to be required for the production of glucagon from the pancreas.

Thus, the hypoglycemic defect seen in *amon* mutants may only result from a lack of AKH processing in the corpora cardiaca (CC) cells of the ring gland and loss of *amon* expression from the IPCs would be expected to have no effect on glucose levels in a wild-type background. Reduction of *amon* expression in the IPCs through the targeted expression of an *amon*-RNAi transgene, however, results in hyperglycemic larvae (Figure 3.4) similar to IPC ablated larvae (Rulifson et al., 2002), indicating that *amon* may also be involved in regulating activity of the DILPs and further supporting *amon*'s role as the only endocrine specific PC in *Drosophila*.

In contrast to IPC ablated larvae, reduction of *amon* in the IPCs does not affect larval growth or development (data not shown). While it is possible that *amon* expression has not been reduced to a low enough level to prevent production of active forms of the DILPs sufficient to influence growth, it is interesting to note that expression of a *uas-amon* transgene in the IPCs of an *amon* mutants also showed no effect on the larval growth defect reported here (data not shown). Thus, this data indicates that AMION function in the IPCs is not necessary or sufficient for larval growth, suggesting

that AMON regulation of the DILPs does not contribute to growth control. Even more interesting is the fact that growth of *amon* mutants can be partially rescued by expression of the same *amon* transgene in the AKH cells of an *amon* mutant (Chapter 2). Ablation of the AKH cells does not result in larvae with growth defects (Isabel et al., 2005; Kim and Rulifson, 2004), indicating that the AKH producing cells are not required for normal growth. Our rescue data, however, indicates that AMON function is sufficient in the AKH cells to partially rescue growth, suggesting an alternative pathway independent of the DILPs in regulating growth.

AMON mutants have growth and developmental defects similar to that seen in larvae in which the IPCs have been ablated. In addition AMON and one of the DILPs, DILP2, co-localize to the medial neurosecretory cells in the brain of larval, pupal, and adult animals. This co-localization suggests that AMON could be proteolytically processing DILP2, and potentially other DILPs, in the brain as part of the functionally conserved insulin signaling pathway in *Drosophila*. As further support for the participation of *amon* in insulin signaling, we have demonstrated a genetic interaction between the *inr* and *amon*. Finally, we show that *amon* activity is required in the IPCs in order to prevent the development of hyperglycemia. Together, our results contribute to the growing understanding of insulin signaling in *Drosophila* and implicate the proprotein convertase *amon* in the activation of at least one of the ligands, *dilp2*, of the insulin receptor.

In the future, examining *amon* mutant extracts for the level of DILP processing using Western analysis, HPLC, pulse-chase assays, or heterologous expression of both protein in yeast will be important in solidifying a proteolytic processing role for *amon* in the insulin signaling pathway. As an alternate approach, pro-insulin processing in the presence and absence of AMON can be determined using a tagged pro-DILP2 in *Drosophila* S2 cells expressing *amon* and *d7B2*. Preliminary studies have been able to express tagged pro-DILP2 in S2 cells and to detect this protein using Western analysis. Furthermore, in cells transfected with only DILP2, no significant cleavage products are detected (E. K. LeMosy, personal communication).

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Figure 3.1. *amon* mutants show growth defects. (A) Wild-type siblings (*amon*^{C241Y} or *Tl-X e/TM3 Sb Ser y⁺ e*) and (B) *amon* mutant larvae (*amon*^{C241Y} *e/Tl-X e*) are shown at left. (C) Scatter plot of data from A and B comparing the length of *amon* mutants (filled diamonds) to their wild-type siblings (open diamonds).

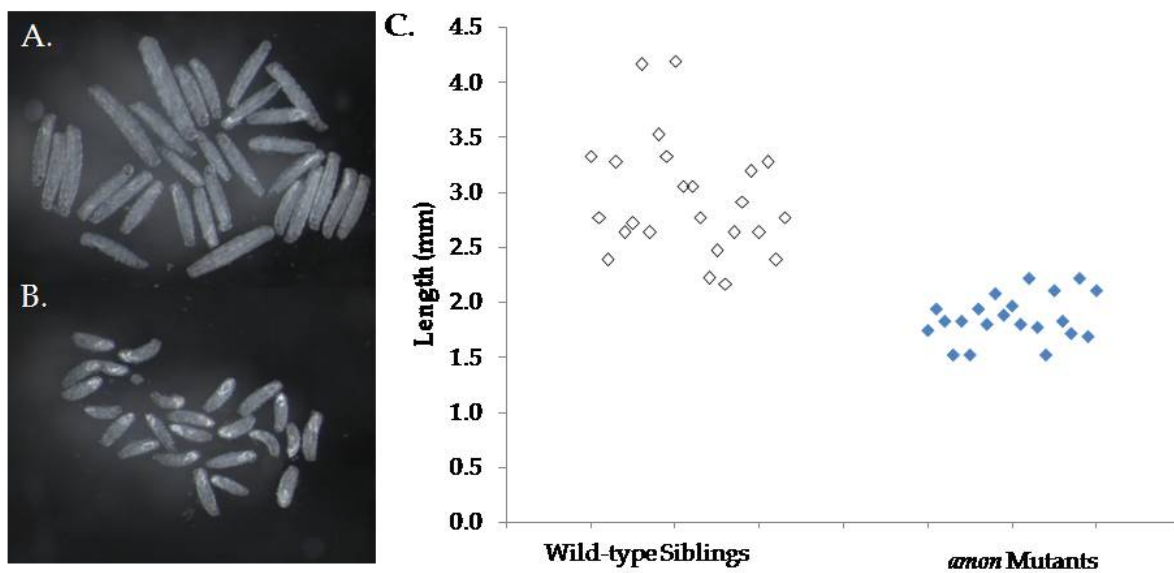


Figure 3.2. AMON and DILP2 co-localize to the medial neurosecretory cells in third instar larvae. Third instar CNS labeled for AMON protein (A) and DILP2 (B). (C) Merged image (10X). (D) A single medial neurosecretory cell (40X magnification) in a third instar larval CNS labeled for AMON. (E) The same cell as in (D) labeled for DILP2. (F) Merged image.

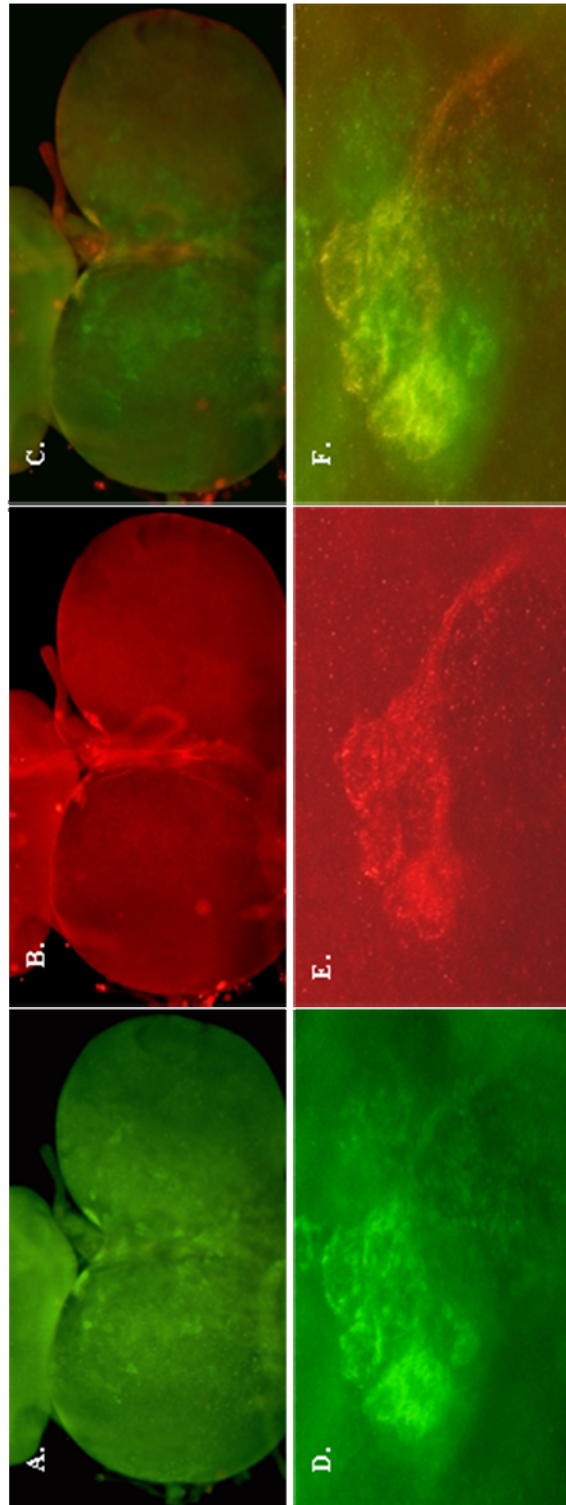


Figure 3.3. *amon* suppresses DInr-induced overgrowth. (A) Wild-type (*UAS-InR^{wt}* or *ey-Gal4/+*) eyes showing a normal smooth, round appearance. (B and C) B shows the most severe phenotype observed for this genotype (*w; UAS-InR^{wt}/ey-Gal4; +/TM3 Sb Ser y+ e*), while C shows the mildest phenotype observed. (D and E) D shows the most severe phenotype observed for *yw; UAS-InR^{wt}/ey-Gal4; amon^{C241Y}/+*, while E represents the mildest phenotype observed. (F) Quantification of the effect of removing one copy of *amon*. Fly eyes were scored on a scale of 1-4 based on appearance. 1=wild-type appearance, 2=large, smooth eyes, 3=large, rough eyes, 4=large eyes with out-growths. Test for Independence, $X^2=9.67$, $df=3$, $p<0.05$.

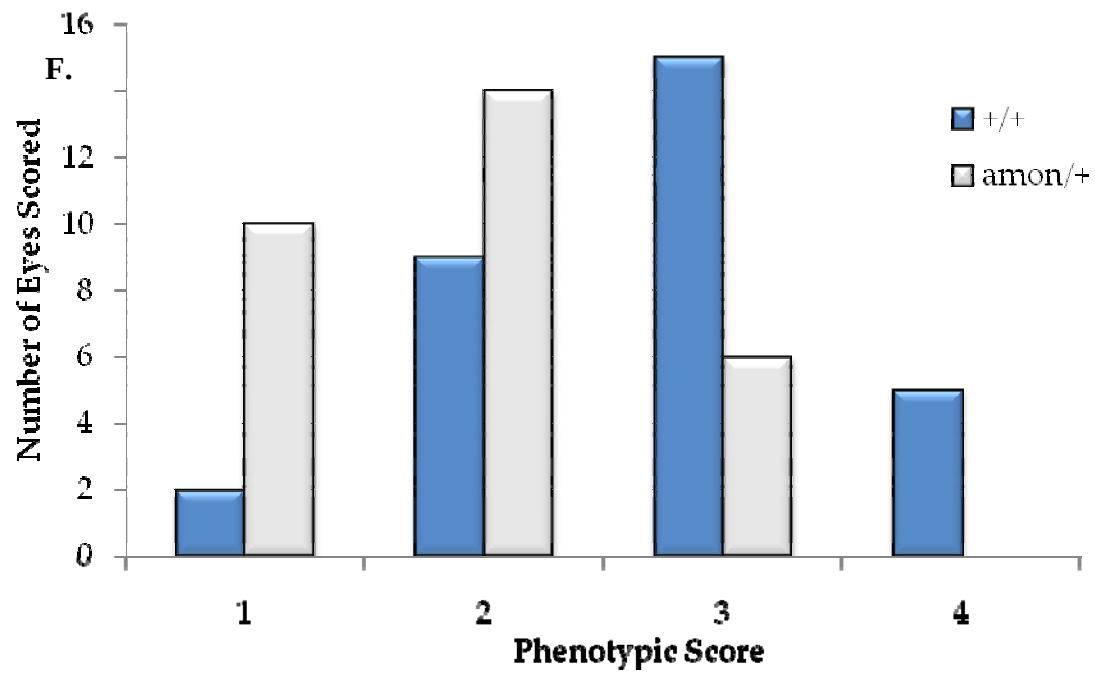
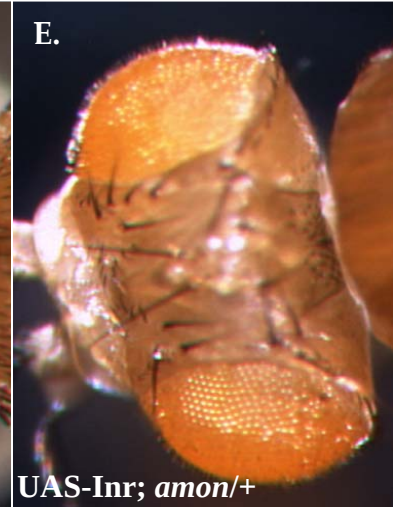
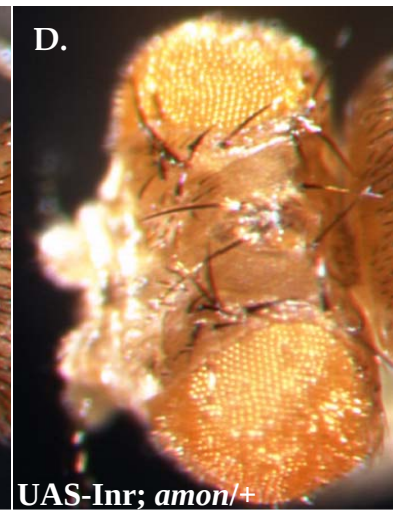
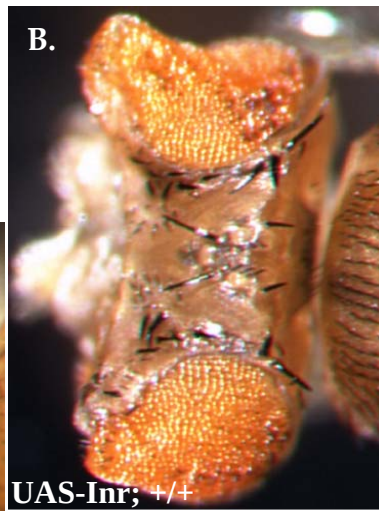
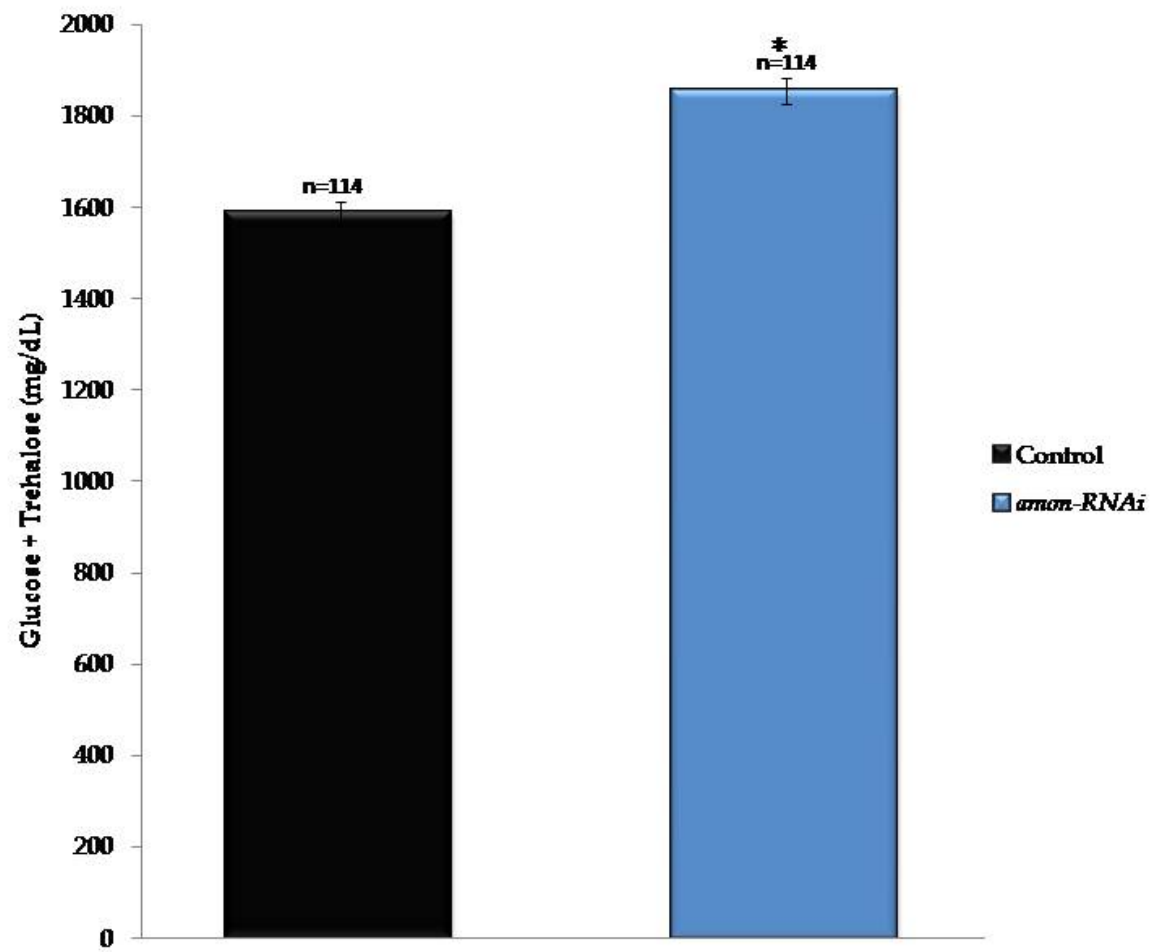


Figure 3.4. *amon* is necessary in the IPCs for normal glucose homeostasis. IPC ablation is known to result in hyperglycemic larvae . Using the *dilp2-gal4* driver to reduce *amon* expression in the IPCs *via* an RNAi transgene, larvae become hyperglycemic (blue bar, 1855 mg/dL, SEM=29.60) compared to wild-type controls (black bar, 1587 mg/dL, SEM=27.24).



CHAPTER 4

CONCLUSION

In the previous chapters, we describe cell specific dissection of *amon* regulation and define a role for *amon* in glucose homeostasis and larval growth using a series of new genetic tools. We investigated cellular requirements of the *amon* proprotein convertase (PC) by using a *uas-amon*-RNAi transgene to reduce *amon* expression in specific cell types. In a complementary approach, we restored *amon* expression in specific subsets of cells in a mutant background using cell specific Gal4 drivers and a *uas-amon* transgene. Use of these tools in *Drosophila* will provide a powerful approach to understanding how PC activity regulates the activation of peptide hormones involved in a wide variety of behavior, biological, and physiological processes. In addition, use of this system can ultimately lead to the identification of new functions for known signals or could identify novel peptide hormones.

Initial investigations into the biochemical properties of the PC family were completed in yeast as well as in cell culture to elucidate the mechanisms behind proteolytic cleavage (Seidah et al., 1998; Steiner, 1998; von Eggelkraut-Gottanka and Beck-Sickinger, 2004). Subsequent studies in mice have begun to reveal target substrates of various members of the mammalian PC family. While studies in PC

knockout mice have provided important insight into potential proteolytic targets of PC activity *via* mass spectrometric analysis of mutant tissues, the specific function of many of these peptides has yet to be identified (Fricker, 2005). The genetic tools available for the model organism *Drosophila* such as the GAL4/UAS system (Brand and Perrimon, 1993) provide the ability to easily reduce proprotein convertase activity in a cell specific fashion to identify functional roles of these proteins. For example RNAi transgenes can be combined with a variety of readily available Gal4 drivers to reduce expression of PCs and assay for phenotypes due to a lack of proteolytic activity. In this same background, additional tools exist for the expression of candidate hormonal signals produced in these cells either through the injection of synthetic peptide hormone or expression of a potential target using the cDNA fused to a heatshock promoter. In Chapter 2, we demonstrate the feasibility of this approach to better understand PC regulation of signaling cascades requiring activation of peptide and neuropeptide hormones.

Glucose serves as a major fuel source for organisms ranging from worms to humans, and as such, mechanisms to maintain a stable concentration of this sugar available to cells have been evolutionarily conserved from *C. elegans* to humans. In mammals, components of the pathway to maintain glucose homeostasis have been extensively characterized, and include the antagonistic action of two peptide hormones: glucagon and insulin. Both of these hormones require proteolytic activation by

members of the PC family; glucagon is liberated from its precursor solely by the action of PC2, while efficient cleavage of insulin requires both PC1 and PC2. Parallel to mammals, glucose homeostasis in *Drosophila* is also regulated by the action of two antagonistically acting hormones: adipokinetic hormone (AKH) and the insulin like peptides (ILPs). AKH is synthesized as a preprohormone (Noyes et al., 1995) and acts to stimulate the release of stored energy reserves in a mechanism similar to mammalian glucagon (Kodrik et al., 2000; Kollisch et al., 2000; Lorenz et al., 2001; Schaffer and Noyes, 1991; Schoofs et al., 1997; Siegert et al., 2000; Stone et al., 1976). A single dibasic processing site is present in the AKH precursor (Noyes et al., 1995), suggesting that activation of AKH may occur in a similar fashion as glucagon. Thus, the identification of *amontillado* (*amon*), the *Drosophila* homolog of mammalian PC2 (Siekhaus and Fuller, 1999), provides the opportunity to establish *Drosophila* as a model system in which to identify peptide hormones requiring proteolytic activation by the PCs.

In Chapter 2 we identify adipokinetic hormone (AKH) as a likely proteolytic target of *amon* activity and show that *amon* activity is required in the AKH cells to maintain glucose homeostasis. Such a requirement for PC activity has also been reported in PC2 null mice (Furuta et al., 1997; Furuta et al., 2001), suggesting that mechanisms to prevent hypoglycemia have been evolutionarily conserved. Similar to vertebrates in which PC2 is expressed in the glucagon-producing pancreatic α -cells (Furuta et al., 1997; Furuta et al., 2001), we show that *amon* co-localizes with AKH in the

corpora cardiaca cells of the ring gland, further supporting our model that AKH expression is regulated by *amon* activity.

In mice, the hypoglycemic defect of PC2 null animals can be rescued by providing a constant supply of glucagon *via* an osmotic pump (Webb et al., 2002). While this result indicates that loss of glucagon is sufficient to correct the missregulation of glucose levels in PC2 null mice, it does not provide a direct correlation between loss of PC2 activity in the glucagon producing cells with decreases in blood glucose levels. Thus, a more powerful approach would be to reduce PC2 expression only in the α -cells of the pancreas and then determine the effect on glucose levels. If these mice are hypoglycemic and can be rescued *via* replacement of glucagon, this would provide a more direct link for PC2 regulation of glucagon. The Cre/Lox system is available for this type of genetic approach, but generation of such mice strains is expensive and time consuming. In *Drosophila*, however, cell specific reduction of gene expression can be easily achieved by expression of uas-RNAi constructs using tissue specific Gal4 expression constructs. Subsequent replacement of hormones can then be accomplished either through direct injection of purified or synthetic hormones, or through broad expression of the cDNA fused to a heatshock promoter. Using this approach, we not only show that *amon* is required in the AKH cells to maintain glucose homeostasis in *Drosophila*, but also that the hypoglycemic defect can be directly attributed to a loss of AKH from these cells.

The power of this technique lies not only in the elegant genetic dissection of cell specific requirements for *amon*, but also in the ability to examine PC requirements in a wide variety of cell types in a relatively short period of time. Numerous Gal4 driver lines have been engineered over the years, and the progeny generated from a simple cross to an RNAi line yields immediate insight into the requirements of PC function in a particular subset of cells. While possible to take a similar approach in mice, it is less feasible as individual mouse strains have to be engineered for each tissue of interest. Thus, performing initial functional studies in *Drosophila* can provide valuable insight into the requirements for PC function in mammals in regulating processes including growth, behavior, development, and metabolism.

Future Directions

In the future it will be imperative to biochemically demonstrate that AMON has the ability to cleave predicted substrates. Previous *in vitro* studies have demonstrated AMON's ability to cleave a fluorogenic substrate, Pyr-E-R-T-K-R-methylcoumarinamide to release free aminomethylcoumarin (Hwang et al., 2000). A similar study was initiated with Dr. Ellen LeMosey, a collaborator at the Medical College of Georgia, to determine if *amon* is capable of processing the DILP2 *in vitro*, and resulted in the creation of *Drosophila* S2 cells that express either *dilp2* alone, or in combination with *amon* and/or 7B2. Preliminary studies using transiently transfected S2 cells by co-expressing *amon* and 7B2 demonstrated the ability to detect AMON-

dependent activity on a fluorogenic substrate in a cell culture system (Ellen LeMosey, personal communication). Ultimately, it will be interesting to determine AMON's ability to process one or more of the DILPs, and to subsequently use this system to assay a variety of other known peptide hormones.

In addition to using cell culture systems to detect processing, it is also possible to generate antibodies that recognize only processed forms of peptide hormones (C. Wegener, personal communication). Antibodies of this type can be generated that recognize hormones that have undergone additional posttranslational modification. C. Wegener, for instance, has generated an antibody to the CAPA peptide that only recognizes the amidated processed hormone. Using these types of antibodies in combination with the Gal4/UAS system will also provide important information on the regulation of peptide hormone synthesis by the proprotein convertases. For example, AMON activity can be specifically reduced in individual subsets of cells that are known to produce a particular hormone. To test the hypothesis that AMON regulates the production of a specific peptide hormone, tissues in which *amon* expression has been reduced can be stained with these antibodies that recognize only the mature form of the hormone. An absence of signal from cells in which *amon* expression has been removed would indicate that AMON is required for the production of a particular signal in a specific tissue.

The strength of using *Drosophila* for such an approach again lies in the ability to easily reduce *amon* expression in subsets of cells and examine the effect in a large number of tissue types in a short period of time. While such an analysis could be performed in mice, currently only animals lacking individual PC activity throughout the whole organism are available. Thus, absence of a mature peptide hormone in a particular tissue cannot be directly correlated with the loss of PC activity in that tissue. For example, PC1 null mice have reduced levels of active growth hormone releasing hormone (GHRH, peptide hormone produced in the hypothalamus) in addition to reduced levels of growth hormone (GH, peptide hormone produced in the pituitary). Because PC1 function is lacking every tissue, however, additional assays have to be performed before creating a model of PC1 function in the regulation of GHRH and GH production. Analysis of mRNA levels reveal that in the presence of reduced GHRH hormone, the levels of GH mRNA are severely reduced, indicating that GHRH regulates the transcription of the GH gene (Scamuffa et al., 2006). Consequently, such an approach cannot provide an answer as to whether GH maturation is also regulated in the pituitary by proteolytic activity of PC1. The creation of individual mouse strains lacking PC activity in a tissue specific manner is costly and time consuming. Thus, identifying specific subsets of cells in which *amon* is required to produce mature peptide hormones will provide insight into proteolytic activation of peptide hormones that can then be applied to other systems such as mammals.

Numerous peptide hormones possessing potential dibasic cleavage sites have been identified in *Drosophila* (Hewes and Taghert, 2001), and GAL4 drivers have already been generated for many of them. This offers the opportunity to examine *amon* function in a cell specific manner by reducing *amon* expression *via* an RNAi transgene. For example, Crustacean cardioactive peptide (CCAP) is a peptide hormone implicated in promoting the transition from pre-ecdysis behaviors to ecdysis behaviors (Park et al., 2003). Ablation of the CCAP neurons results in animals that arrest during pupal development (Park et al., 2003) with phenotypes similar to our *amon* mutants, suggesting that CCAP could be a proteolytic target of *amon*. While ablation of specific cell types can provide insight into the signals produced by these cells, the caveat is that the entire cell has been removed. This eliminates not only all types of signals, produced and released from the cell, but also cell-cell contacts.

Using the tools generated here, it will be possible to determine the consequences of eliminating only those signals dependent on AMON function. To do this, the *amon*-RNAi construct described in Chapters 2 and 3 can be employed to reduce AMON function in a cell-type specific manner *via* Gal4 drivers to determine which cell types require proteolytic activation of target substrates to properly regulate growth, development, metamorphosis, and metabolism. This approach has the power to identify cells in which known signals are used in a novel way and also has the potential to lead to the identification of novel signaling peptides.

As a complementary approach it will also be important to determine cell types in which AMON function is sufficient for development. Using the *uas-amon* rescue construct, we have not yet been able to identify a specific subset of cells in which *amon* expression completely rescues the growth defect, larval lethality, or metamorphic defects associated with an absence of active *amon*. This is most likely due to the fact the multiple peptide hormones produced from a variety of tissue types are known to be required for proper development. Thus, if *amon* expression is required for the regulation of peptide hormones, it is reasonable to speculate that AMON function will be required in multiple cell types for developmental processes. We have identified a subset of cells in the CNS using a *Kurs21-Gal4* driver in which *amon* is sufficient to partially rescue the earlier defects of growth and molting (Appendix A). Not surprisingly, the rescue observed is not equivalent to the degree of rescue seen using the *hs-amon* transgene, indicating that *amon* is required in multiple cell types for normal development. To address this possibility, it will be beneficial to perform rescue experiments with the *uas-amon* construct using a combination of multiple drivers in a single *amon* mutant background.

We have also generated an *amon-gal4* construct that, in preliminary studies, appears to recapitulate *amon* expression observed previously by *in situ* and by immunohistochemistry using a polyclonal AMON antibody. Because this antibody is in limited supply, the creation of the GAL4 driver provides the opportunity to more easily

map when and where *amon* is expressed during all stages of life. Identifying the various cell types that express *amon* can in turn lead to the identification of potential targets of *amon* proteolysis if it localizes to neurosecretory cells known to release specific peptide hormones. In addition, such a reporter also has the potential to lead to the identification of novel secretory cells and/or novel peptide signals. For example, the *amon-gal4* construct drives expression in the *Drosophila* gut endocrine cells where several peptides are known to be expressed. Several of the *Drosophila* genes have homologs in mammals, including neuropeptide F (NPY homolog), somatostatin-like peptide, and drosulfakinin (CCK homolog) (Hewes and Taghert, 2001), thus presenting the opportunity to study the regulation and function of these peptide hormones in a more simple model system.. In addition, our *amon-gal4* construct results in expression of a GFP reporter gene in approximately six cells that surround the foregut/midgut junction that have not yet been characterized. This raises the possibility of identifying novel endocrine cells in the *Drosophila* gut and potentially identifying novel signals released from this organ.

Our work has taken advantage of the evolutionarily conserved nature of the pathways controlling glucose homeostasis to demonstrate the strength and power the tools we have generated will have on dissecting cell specific requirements of PC activity and the subsequent application to mammals. We have shown that specific reduction of *amon* in cells producing AKH and the DILPs results in phenotypes resembling mouse

knockouts of PC1 and PC2, suggesting that mammalian PCs and *Drosophila* AMON function analogously. Future studies using the tools described above will continue to expand our knowledge about how proteolytic processing is an important regulatory mechanism that controls important biological and physiological processes.

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APPENDIX A

AMON FUNCTION IN THE CA-LP1 AND CA-LP2 CELLS OF THE CNS IS
IMPORTANT FOR LARVAL MOLTING AND TIMING OF PUPARIATION¹

¹Appendix A includes experiments that appear in a manuscript currently in revision (Figure A.2 and A.4). Figure A.1 was generated by L.Y.M.R.
Rayburn, L.Y.M., J. Rhea, S. Jocoy, and M. Bender. In revision for *Developmental Biology*.

As in most insects, *Drosophila* larval development is characterized by alternate periods of feeding and molting. During a molt, the old cuticle is shed and replaced with a new one, and this process culminates in a behavioral sequence known as ecdysis. The precise onset of ecdysis is crucial to survival, and requires the integration of behavioral, biosynthetic, physiological, and developmental inputs (Truman and Riddiford, 1970). Progression from one molt to the next requires a carefully orchestrated series of events regulated by the release and degradation of steroid and peptide hormones from the endocrine axis, and is described in detail in Chapter 1.

Phenotypes of *amon* mutants suggest that *amon* may proteolytically activate peptide hormones involved in regulating larval molting, larval growth, head eversion, leg and wing extension, and abdominal differentiation (Rayburn et al., in revision). The genetic tools described in Chapters 2 and 3 provide the opportunity to dissect potential cell specific roles for *amon* in coordinating events required for the successful initiation and completion of molting and metamorphosis.

We started our analysis by focusing on the ring gland of *Drosophila*, a large neuroendocrine gland situated anterior to the larval central nervous system (CNS). The ring gland is comprised of three fused tissues: the corpora cardiaca (CC), the corpus allatum (CA), and the prothoracic gland (PG). This gland is the source of ecdysteroids and juvenile hormone, and serves as the link between neuronal and hormonal systems to control all the major events in larval development. Neuropeptides are also key

players in development and are thought to regulate the release of ecdysteroids and juvenile hormone from the ring gland.

To better understand the neuronal and genetic regulation of *Drosophila* development, Siegmund and Korge (2001) performed enhancer trap screens to identify peptidergic brain neurons that innervate the ring gland of *Drosophila*. One of these lines, Kurs 21, identifies three neurons that innervate the CA: a single corpus allatum innervating neurosecretory neuron of the lateral protocerebrum 1 (CA-LP1) and two corpus allatum innervating neurosecretory neurons of the lateral protocerebrum 2 (CA-LP2). To determine if these cells form synapses on the CA, neurons were labeled by crossing the Kurs 21 to a UAS-GFP reporter line and then staining the tissues with a synapse-specific antibody (SAP47). The three GFP positive neurons are also labeled by SAP47, indicating that the CA-LP1 and CA-LP2 cells form in the CA. No other non-GFP synapses were identified in the CA, indicating these are the only neurons that innervate the CA, the site of JH release (Siegmund and Korge, 2001).

amon expression has been identified in CA-LP1 and CA-LP2 cells (Figure A.1), suggesting the possibility that *amon* may process peptide hormones that are released at the synapses in the CA. Such candidate peptide hormones include allatostatins and allatotropins which regulate synthesis and release of JH (Nijhout, 2003). At least three structurally different types of allatostatins are known, and can function in myoinhibition, neuromodulation, and inhibition of JH release (Siegmund and Korge,

2001). Allatotropins are stimulatory peptide hormones isolated from the moth (Bogus and Scheller, 1996), crickets and locusts (Applebaum et al., 1990; Lehmberg et al., 1992; Lorenz et al., 1999). *amon* mutant animals rescued to the late third instar larval stage using a *hs-amon* transgene die during pupal development with a lack of abdominal differentiation. This phenotype resembles animals treated in the late third instar larvae and prepupae stage with excess JH (Ashburner, 1970; Madhavan, 1973). Thus, it is tempting to speculate that *amon* may proteolytically activate an allatostatin peptide hormone that regulates JH release from the CA. Although this question is not addressed here, in the future this might be addressed by indentifying the subset of cells in which knocking down *amon* reproduces the pupal metamorphoic defects. Once these cells are identified, a *hs-allatostatin* could be ubiquitously expressed in the knockdown background to determine if metamorphosis can be rescued.

To examine the functional consequences of restoring *amon* expression in the CA-LP1 and CA-LP2 cells, we expressed a *uas-amon* construct using the enhancer trap line Kurs 21 in an *amon* mutant background. Figure A.2 shows that *amon* function in these cells is sufficient to partially rescue developmental progression. Only 14% of first instar *amon* mutant larvae survive to the second instar larval stage. In contrast, when *amon* function is restored in the CA-LP1 and CA-LP2 neurons in an *amon* mutant background, survival to the second instar larval stage increases to 64% (Figure A.2). In addition, *amon* function in these cells significantly increases the number of larvae surviving to the

third instar larval stage (Figure A.2, 3% of mutants to 18% of rescued larvae) and to pupariation (Figure A.2, 0.5% of mutants to 2% of rescued larvae). Thus, *amon* function in the cells defined by the Kurs 21 enhancer trap line is sufficient to partially rescue developmental progression.

Interestingly, several rescued larvae remained in the third instar larval stage for an extended period without undergoing metamorphosis (Figure A.3). Consistent with normal developmental timing, control animals remained as third instar larvae for approximately two days before forming a puparium (Ashburner et al., 2005). In contrast, two rescued *amon* mutants stayed as third instar larvae for more than 28 days. Initially, these larvae moved around the food, but were not as active as control animals. After 20 days AEL however, these animals became severely lethargic and responded only to a manual stimulus. We determined that these larvae were not eating by monitoring the uptake of food using yeast paste colored with red food coloring. The gut of control animals turned bright red, while the gut of the rescued mutant larvae remained colorless. At 32 days AEL, the mutants were no longer responsive and were dissected. Interestingly, the fat body (the tissue responsible for energy storage) could not be found in the mutant larvae (data not shown). This observation suggests that the rescued larvae survived until their energy stores had been depleted.

In addition to promoting development, *amon* function in the Kurs 21 cells is also sufficient to partially rescue growth (Figure A.4A). Rescued larvae (Figure A.4D) are

intermediate in size compared to *amon* mutants (Figure A.4C) and control sibling larvae (Figure A.4B), suggesting that cells defined by the Kurs 21 driver can regulate growth. Such regulation could occur via release of peptide hormones from these cells.

Restoring *amon* function to a subset of cells in the larval CNS has revealed a function for *amon* in promoting larval growth and molting. Only a small fraction of rescued larvae survived to pupariation, thus preventing us from determining whether or not *amon* function is also important in these cells for metamorphosis. Future experiments, however, could address this possibility using a *hs-amon* transgene to rescue *amon* mutants past the early larval lethality. When *hs-amon* is expressed every twenty-four hours until the mid-third instar stage (108 hrs AEL) the majority of larvae successfully pupariate, but later die during metamorphosis with the pupal defects described above. Using this conditional rescue system in combination with the *kurs21-gal4* driver and *uas-amon* constructs will provide the opportunity to determine if *amon* is sufficient in the CA-LP1 and CA-LP2 cells to rescue other aspects of pupal development.

As a complementary approach to the rescue experiments, we also specifically reduced *amon* expression in the CA-LP1 and CA-LP2 cells by combining the Kurs21-Gal4 driver with the *amon*-RNAi construct. Initial experiments suggested that *amon*-RNAi knockdown larvae were developmentally accelerated as they pupariated approximately twenty-four hours earlier wild-type control larvae (Figure A.5A).

Typically, wild-type larvae pupariate at approximately 120 hrs AEL. When *amon* function is reduced in the Kurs 21 cells, larvae pupariate at 72 hrs AEL, indicating that reduction of *amon* in these cells accelerates development. The spiracles of each larval stage have distinguishing characteristics, allowing us to determine the developmental stage of each larva. *amon* knockdown larvae transition through each of the instar larval stages, indicating that development proceeds normally, but at an accelerated rate.

In addition to pupariating more quickly than wild-type control larvae, *amon*-RNAi animals also eclose more quickly than control animals (8 days AEL vs 10 days AEL). Unlike pupariation, however, metamorphosis does not seem to occur at a faster rate as both knockdown animals and control animals remained as pupae for five days before emerging as adults. Subsequent repeats of this experiment, however, yielded variable results, where four out of seven experiments showed no obvious phenotypic effect of reducing *amon* function in these cells. To verify that we were in fact reducing *amon* expression, we determined *amon* transcript levels using quantitative real time PCR. *amon* is expressed in additional cell types other than those defined by the Kurs 21 driver and this additional expression could potentially prevent us from detecting any decrease in expression of *amon*. Thus, we dissected the CNS from thirty third instar larvae and carefully removed the ventral nerve cord and ring gland so that we isolated RNA only from the brain. We were able to detect a 70-80% reduction in *amon* transcript

levels (Figure A.6), confirming that we were successful in reducing *amon* expression using the Kurs 21 driver.

We have not yet accounted for the differences seen in our *amon*-RNAi inactivation experiments using the Kurs21-Gal4 driver. Possibly, the differences in our results could reflect an unidentified variable in our assay (eg. humidity, differences in food quality, or temperature) or could be due to variations in the expression strength of the driver construct. With respect to the latter, Siegmund and Korge (2001) reported the occasional labeling of a single ventromedial (VM) neuron using the enhancer trap line Kurs 21. This VM cell is known to be the source of the neuropeptide eclosion hormone (EH). In addition, we noted the occasional labeling of four cells located in the posterior tip of the ventral ganglion. While we have not confirmed the identity of these cells, they appear similar to the dorsal lateral abdominal (DLA) cells shown to be immunoreactive to the peptide hormone allatostatin (Bowser and Tobe, 2005). Thus, one explanation for the variable results in our experiments could be explained by the strength of expression in the VM neuron and DLA cells in addition to the CA-LP1 and CA-LP2 cells.

Materials and Methods

Immunohistochemistry. For co-localization with tissues expressing GFP, the UAS-mCD8-GFP line (Lee and Luo, 1999) Kurs-21 (Siegmund and Korge, 2001) to obtain progeny with GFP in the CA-innervating neurons. Wandering third instar larvae were

dissected and then processed for *amon* protein detection. Tissues were washed in TBS (25 mM Tris, 137 mM NaCl, 0.27 mM KCl) containing 0.5% Triton X-100 (TBST) two times for five minutes each and then blocked in TBST+5% goat serum overnight at 4°C. All subsequent steps were performed at 4°C. Primary antiserum was added in TBST+2% BSA at a 1/200 dilution and left overnight. Following a wash of at least one hour in TBST+2% BSA, the secondary antibody (anti-mouse IgG conjugated to Alexa Fluor 488, 568 or 594, Molecular Probes, Inc.) was added at a dilution of 1:500-1:2000 in TBST+2% BSA and tissues were incubated overnight in the dark. Tissues underwent a final wash in TBST and were then mounted on slides in a 1:1 TBST/glycerol solution. Images were captured on an Olympus BX60 microscope using a JVC digital camera (model KYF70BM) and the AutoMontage (Syncroscopy) software.

Rescue of *amon* mutant defects. To determine if *amon* function in specific neuronal cell types is sufficient to rescue *amon* mutant defects, approximately 150 *yw; uas-amon*^{40L}; *Tl-X e/TM3 Sb Ser y+ e* virgin females and 150 *yw; +; Kurs21-gal4, amon*^{C241Y e/TM3 Sb Ser y+ e} males were mated in an egg collection chamber containing a grape juice agar plate with fresh yeast paste at 25°C. As a control cross, 150 *yw; uas-amon*^{40L}; *Tl-X e/TM3 Sb Ser y+ e* virgin females and 150 *yw; +; amon*^{C241Y/TM3 Sb Ser y+ e} males were mated in the same way. A 4 hr egg collection on grape juice agar plates with yeast paste was taken 24 hrs later. At 36 hrs ael *amon* mutant larvae, recognizable by the *yellow* marker, (*yw; uas-amon*^{40L/+}; *Tl-X e/Kurs21-gal4, amon*^{C241Y e}) and wild-type control larvae (*yw;*

uas-amon^{40L/+}; *Tl-X e* or *amon*^{C241Y e/TM3 Sb Ser y+ e}) were selected and moved to fresh grape juice agar plates with yeast paste. Larvae were scored every 24 hrs based on spiracle morphology to determine whether they were first, second, or third instar larvae.

Knockdown of *amon*. Approximately 50 *w*;⁺*kurs21-gal4* virgin females were crossed to either 30 *w*¹¹¹⁸ control males or 30 *yw*; *uas-amon-RNAi*^{28b} experimental males and placed in an egg collection with grape juice plate and fresh yeast paste. Animals were kept at 29°C. Four hour egg collections were performed by replacing the grape juice plate. At 36 hrs AEL, and equal number of control and experimental animals were moved to separate plates to control for crowding. Plates were checked every 24 hours for pupariation and eclosion.

Quantitative Real Time PCR. Approximately 20 *yw*; *UAS-amon-RNAi*^{28b} virgin females were crossed to 10 *kurs21-gal4* males in an egg collection chamber containing an apple juice plate with fresh yeast paste, and maintained at 27°C. As a control, 20 *w*¹¹¹⁸ virgin females were crossed to 10 *kurs21-gal4* males in the same manner. After 48 hours, a four hour egg collection was taken. Beginning at 36 hrs AEL, egg collection plates were heatshocked every 12 hours for 1 hr at 37°C. Total RNA was isolated from 10 whole third instar larvae at 108 hrs AEL with TRIzol reagent (Invitrogen) and treated with DNaseI, and 5 µg were used for reverse transcription using the Transcriptor First

Strand cDNA Synthesis Kit (Roche). The *amon* transcript was quantified using an Applied Biosystems 7500 Real-Time PCR system according to the manufacturer's instructions. PCR reactions consisted of 2 µl of each primer at 6.5 µM, 3.5 µL dH₂O, 12.5 µL of SYBR green mix, and 5 µL of cDNA. Two sets of primers were used, targeting either exon 10 or exon 11: exon 10 forward (5'-GCCGGCGCCATGGT-3') and reverse (5'-ATAGCGCGGTGGCACTGA-3'), and exon 11 forward (5'-TTCAACTCGCCCCAAACAC-3') and reverse (5'-ATGCAGGACCAAGGACCATTC-3'). Ribosomal protein 49 (rp49) was used as an endogenous control.

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Figure A.1. *amon* is expressed in the CA-LP1 and CA-LP2 cells of the CNS. (A) GFP driven expression by the enhancer trap line Kurs 21 shown expression in a single CA-LP1 neuron (arrowhead) and two CA-LP2 neurons (arrow) in a 3rd instar larval CNS. (B) Same CNS stained using an *amon* antibody. (C) Merged image show that AMON and GFP co-localize to three neurons that innervate the CA. Image from Rayburn et al., 2008.

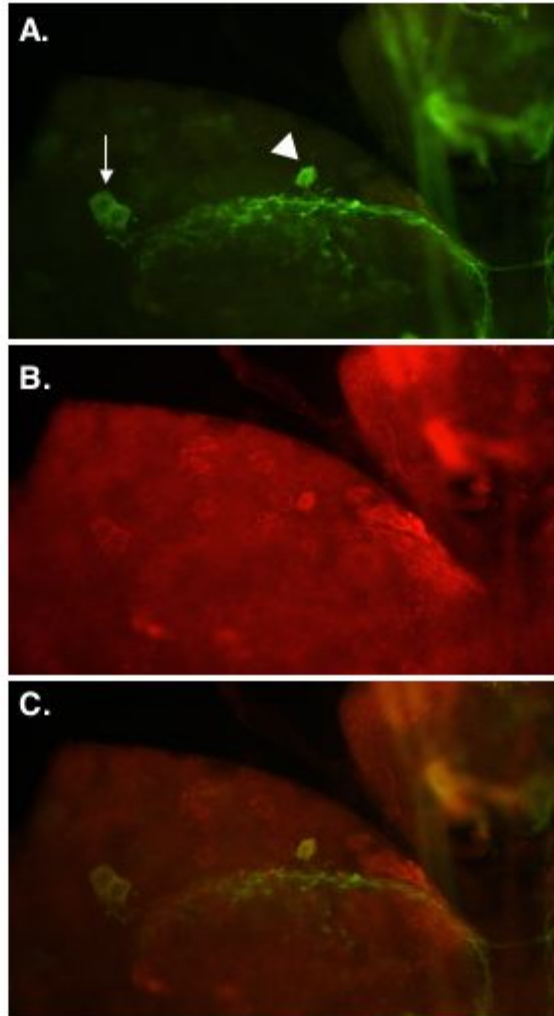


Figure A.2. *amon* expression in the CA-LP 1 and CA-LP 2 cells of an *amon* mutant is sufficient to partially rescue the molting defect seen in *amon* mutants. (A) The majority of *amon* mutants (*yw*; +; *kurs21-gal4*, *amon*^{C241Y} *e*/*Tl-X e*) die during the transition from the first to second instar larval stage as compared to their wild-type siblings (*yw*; +; *kurs21-gal4*, *amon*^{C241Y} or *Tl-X e*/TM3 *Sb Ser y+ e*). When *amon* is expressed using the *kurs-gal4* driver (*yw*; *uas-amon*/+; *kurs21-gal4*, *amon*^{C241Y}/*Tl-X e*), an increase of survival to later developmental stages is observed.

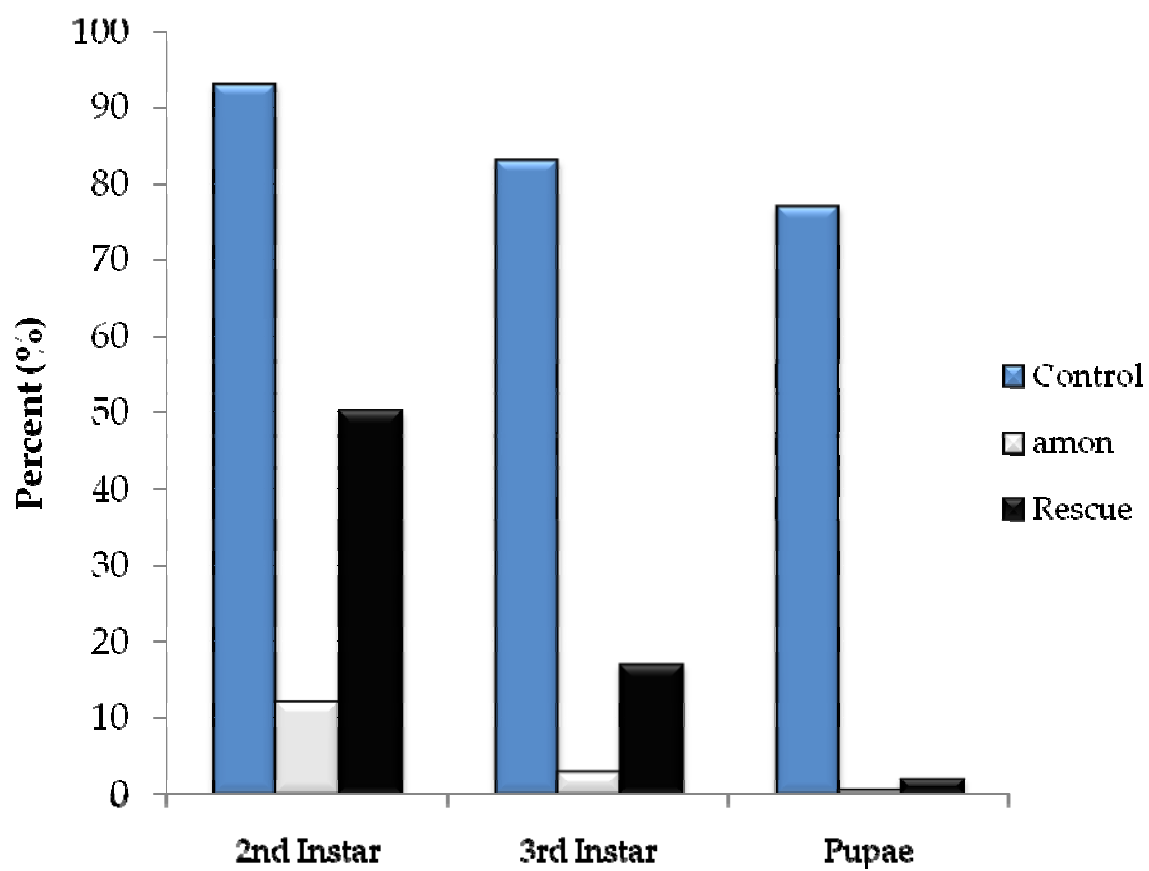


Figure A.3. A couple rescued larvae remain in the third instar larval stage for an extended period. (A) Wild-type third instar siblings (108 hrs AEL, *yw*; +; *kurs21-gal4*, *amon*^{C241Y} or *Tl-X e/TM3 Sb Ser y+ e*) are large and active and typically pupariate at 120 hrs AEL. (B) Some rescued larvae (*yw*; *uas-amon*/+; *kurs21-gal4*, *amon*^{C241Y}/*Tl-X e*) remained as third instar larvae for more than 30 days AEL, but were significantly lethargic. Photo taken at 28 days AEL.



Figure A.4. Expression of *amon* in the CA-LP1 and CA-LP2 cells partially rescues

growth. (A) Rescued first instar larvae (*yw; uas-amon/+; kurs21-gal4, amon^{C241Y}/Tl-X e*)

are significantly larger than *amon* mutants (*yw; +; kurs21-gal4, amon^{C241Y}/Tl-X e*). (B)

Wild-type siblings (*yw; +; kurs21-gal4, amon^{C241Y} or Tl-X e/TM3 Sb Ser y+ e*) at 36 hrs AEL.

(C) *amon* mutant and (D) rescued larvae at 36 hrs AEL.

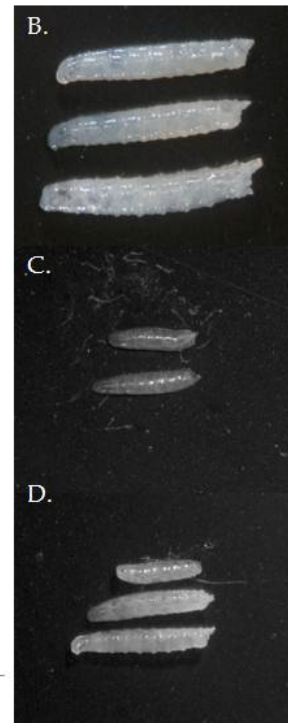
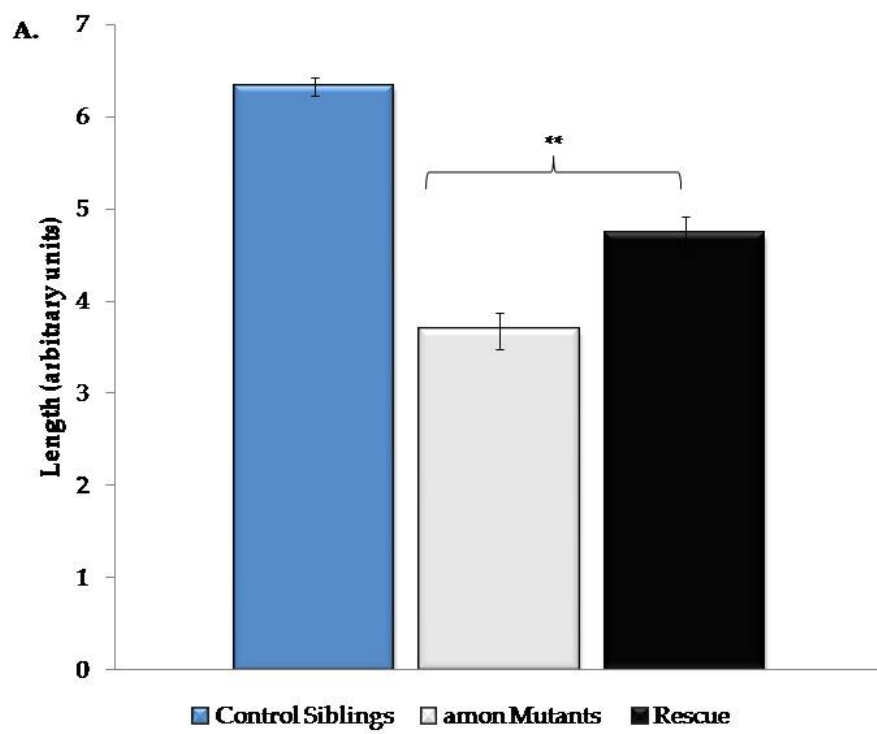


Figure A.5. Reduction of *amon* expression in CA-LP1 and CA-LP2 cells accelerates larval development. (A) Black bars represent the percent of wild-type control larvae (*w*; +; *kurs21-gal4/+*) that pupariated, while blue bars represent *amon*-RNAi larvae (*w*; *uas-amon-RNAi^{28b}/+*). (B) Black bars represent the percent of wild-type controls that eclosed while blue bars represent the *amon* knockdown animals. n=100.

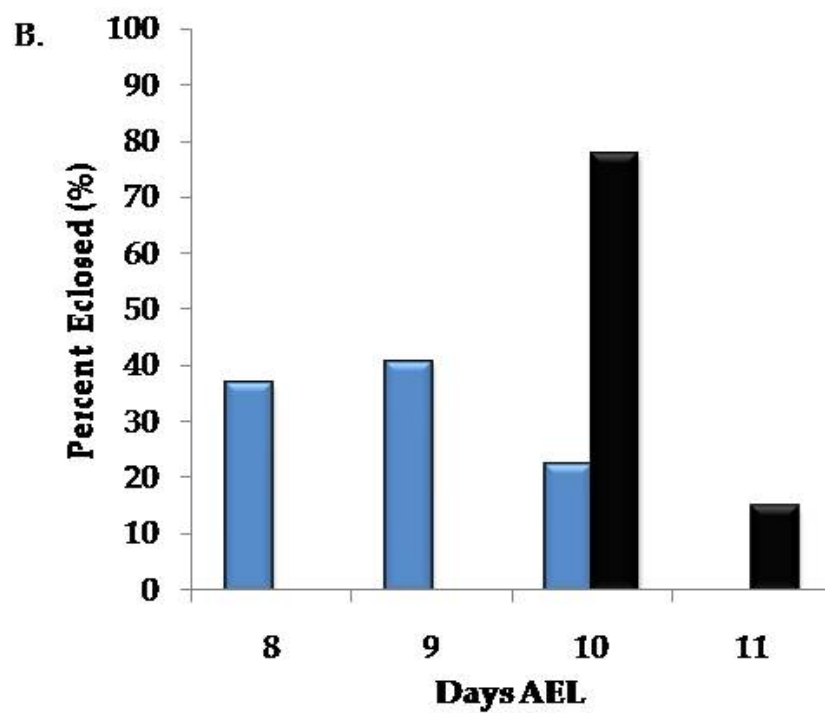
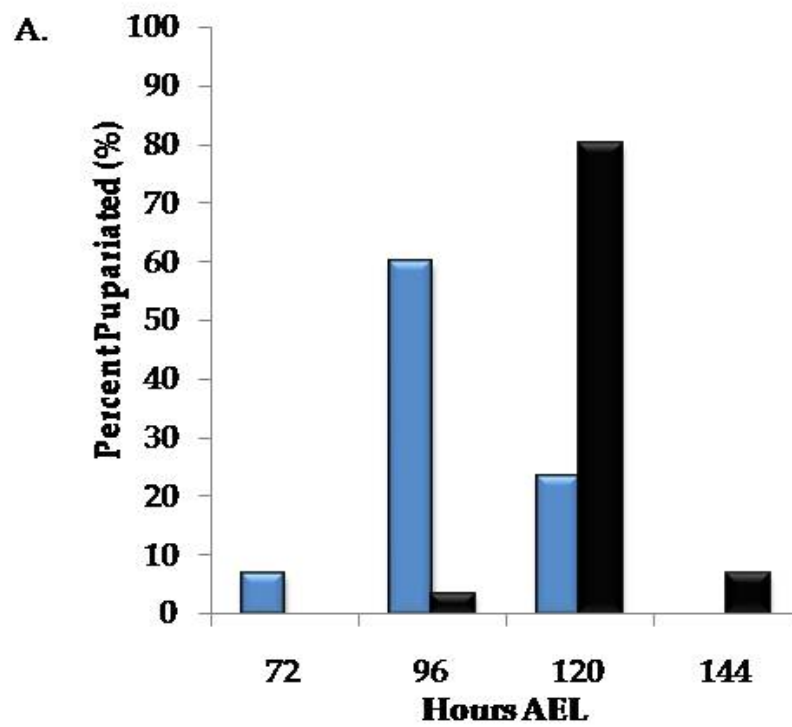
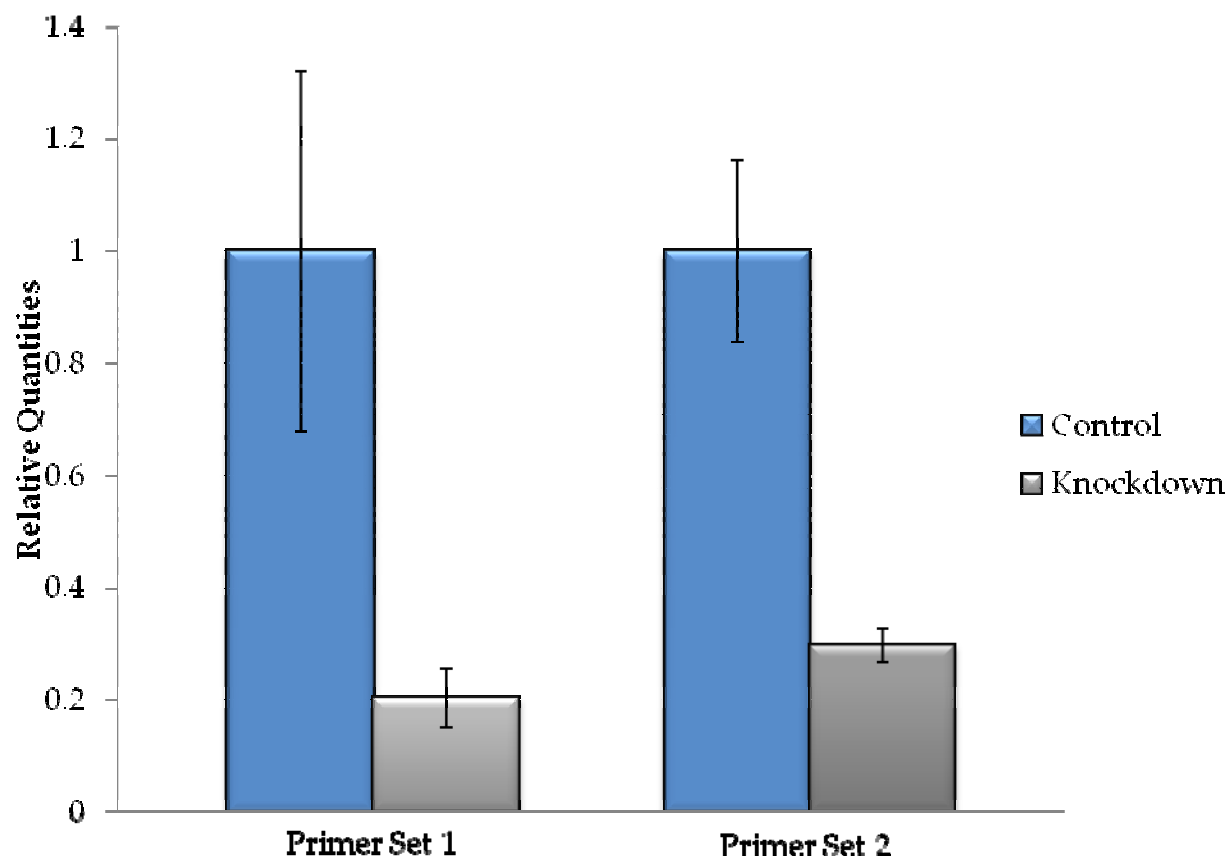


Figure A.6. *amon*-RNAi expression using the Kurs 21 driver reduces *amon* expression in the larval brain. The brain of animals in which *amon* has been reduced by RNAi show a significant reduction in *amon* transcript levels using two separate primer sets when assayed by quantitative real time PCR



APPENDIX B

AMON ACTS UPSTREAM OF ECDYSONE SIGNALING DURING METAMORPHOSIS

Ecdysone is a steroid hormone that regulates embryonic development, larval molting, and the larval to adult transition known as metamorphosis (Richards, 1981; Riddiford, 1993). Ecdysone is synthesized in and released from the prothoracic gland (PG) in response to signaling by a peptide hormone, prothoracicotropic hormone (PTTH), released from brain neurosecretory cells (Henrich et al., 1987; Zitnan et al., 1993). Pulses of ecdysone are released immediately prior to molting, pupariation, and eclosion, and during metamorphosis. *amon* mutants die during the transition from the first to second instar larval stage from a failure to molt. When rescued to later developmental stages via a *hs-amon* transgene, *amon* mutants are unable to complete metamorphosis and display a variety of phenotypes including failure to evert the head, extend the appendages, or complete abdominal differentiation (Rayburn et al., in revision). These pupae resemble animals mutant for components in the ecdysone signaling pathway. To determine if *amon* mutant defects in molting, pupariation and metamorphosis are attributable to an ecdysteroid deficiency, we fed yeast paste supplemented with 20-hydroxyecdysone (20E) at a final concentration of 0.5 mg/ml to *amon* mutant larvae and looked for increased survival to later developmental stages. This concentration has been shown to rescue animals with mutations in components of the ecdysone signaling pathway (Bialecki et al., 2002).

Initially, we sought to determine *amon's* role with respect to ecdysone in pupal formation. *amon* mutant larvae were rescued to the early third instar larval stage (96 hrs

AEL), and transferred to food either with or without 20E. Interestingly, 70% of larvae on the 20E supplemented food were able to successfully pupariate compared to 28% of mutant larvae placed on food lacking 20E (Figure B.1). It should be noted, however, that while the *amon* mutants placed on 20E supplemented food were able to complete pupal formation, they were typically delayed more than 36 hours relative to control siblings. Nevertheless, this rescue of pupariation suggests that *amon* functions upstream of ecdysone prior to the onset of pupal formation.

Typically, *amon* mutant larvae rescued to the late third instar larval stage via a heatshock *amon* transgene show severe developmental defects during metamorphosis, including failure to undergo head eversion, wing and leg extension, and abdominal differentiation (Rayburn et al., 2008). These phenotypes resemble animals with mutations in ecdysone inducible genes: *E74*, *DHR3*, *Broad Complex (BR-C)*, and *βFTZ-1* (Broadus et al., 1999; Fletcher et al., 1995; Hewes et al., 2000; Kiss et al., 1988; Lam et al., 1999), again suggesting that the ecdysone pathway is defective in *amon* mutants. Previous studies have demonstrated that pupal phenotypes resulting from mutations in ecdysone inducible genes can be rescued by feeding animals 20E. Thus, we hypothesized that if the defects seen during metamorphosis in *amon* mutants are due to a lack of ecdysone signaling, it may also be possible to rescue some of these phenotypes by feeding animals 20E.

Figure B.2A shows the distribution of phenotypes seen in *amon* mutants either with (blue bars) or without (black bars) 20E. The most severe of these phenotypes is scored as no differentiation. The majority of these animals have a gas bubble in the abdomen that typically forms after the epidermis contracts from the cuticle of the third instar larva. No evidence of adult structures is present in these animals, and this lack of differentiation makes it difficult to dissect the animal from the pupal case and obtain a picture. It does not appear however, that feeding ecdysone to *amon* mutants has any affect on the subset of animals arresting at this stage during metamorphosis.

At the time of pupation, imaginal discs evert to form the basic outline of the adult fly; the head, however, remains tucked inside the body cavity. Following a pulse of ecdysone approximately 12 hrs after pupariation, head eversion is initiated by a series of abdominal muscle contractions which push an air bubble up to the anterior portion of the pupa to make room for the head to evert. Some animals carrying mutations in components of the ecdysone signaling pathway, are cryptocephalic (Fletcher et al., 1995). In cryptocephalic animals, such as *E74* mutants, the head fails to evert from within the thorax (Figure B2.B) (Fletcher et al., 1995). A subset of *amon* mutants also show this phenotype (Figure B.2B). We found that addition of ecdysone to *amon* mutant food is able to rescue the subset of pupae that arrest at this stage of development (Figure B.2A, approximately 55% of *amon* mutants lacking 20E to 45% of *amon* mutants fed 20E), suggesting that *amon* is required upstream of ecdysone to

initiate head eversion. Supporting this hypothesis is the finding that approximately 5% more mutants that have been fed 20E are categorized as pinhead (Figure B.2C, smaller head than wild-type), indicating that in the presence of 20E more *amon* mutants can complete head eversion.

Finally, approximately 22% of *amon* mutants given 20E developed into a normal appearing pharate adult (Figure B2.D) with proper head eversion, abdominal differentiation, and leg and wing extension as compared to only 8% of those larvae not fed 20E (Figure B2.A). Together, these data suggest a requirement for *amon* upstream of ecdysone to initiate pupal formation and undergo metamorphic events. In the future, it will be interesting to determine whether or not expression of the ecdysone-inducible genes is inhibited in *amon* mutants. This can be determined either through Northern Blots or by the use of quantitative real time PCR to assess gene levels at the time of puparium formation. Failure to see expression of these genes would further implicate *amon* as a regulator of ecdysone release. It will also be interesting to determine if *amon* functions upstream of PTTH to regulate ecdysone synthesis and/or release.

We also determined whether or not 20E had the ability to rescue the early *amon* mutant lethality. When first instar larvae (36 hours AEL) were placed on food supplemented with 20E, no rescue of molting was observed. These animals arrested between the first to second instar larval transition, suggesting that *amon* is required either downstream of ecdysone or in a separate pathway for the proper execution of

ecdysis. Such proteolytic targets may include eclosion hormone (EH), ecdysis triggering hormone (ETH), and crustacean cardioactive peptide (CCAP).

Materials and Methods

Approximately 150 virgin *yw; +; amon^{C241Y}/TM3 Sb Ser y+ e* females were crossed to 100 *yw; hs-amon; Tl-X e/TM3 Sb Ser y+ e* males and placed in an egg collection chamber containing a grape juice plate supplemented with yeast paste. Four hour egg collections were taken, and plates were incubated at 25°C. To determine the effect of 20E on *amon* mutant pupal development, plates were heatshocked every 24 hrs for 45 mins at 37°C beginning 24 hrs AEL and ending at 96 hrs AEL. At 96 hrs AEL larvae were split into four groups: two groups of wildtype siblings (*yw; hs-amon/+; amon^{C241Y} or Tl- X e/TM3 Sb Ser y+ e*) and two groups of mutant larvae (*yw; hs-amon/+; amon^{C241Y}/Tl-X e*). One set of wildtype siblings and *amon* mutants were placed on grape juice plates with yeast paste (50 mg yeast, 95 µl dH₂O, 5 µl of EtOH), while the other sets were placed on grape juice places with yeast paste supplemented with 20E (mg yeast, 95 µl dH₂O, 5 µl 10 mg/mL 20-hydroxyecdysone). Plates were incubated at 25 °C and checked every twenty-four hours for pupal formation. Pupae were transferred to moist filter paper and were monitored for metamorphosis. To determine if 20E can rescue *amon* mutants past the early larval lethality, larvae were split into four groups at 36 hrs AEL as described above and monitored for molting by observing anterior spiracle morphology.

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Figure B.1. *amon* functions upstream of ecdysone to regulate pupariation and eclosion. *amon* mutants (white bars) fail to pupariate or eclose at wildtype control levels (black bars). When 20E is provided in the food of *amon* mutants (blue bars), however, a greater than 50% increase in survival is seen. Each treatment began with 100 first instar larvae.

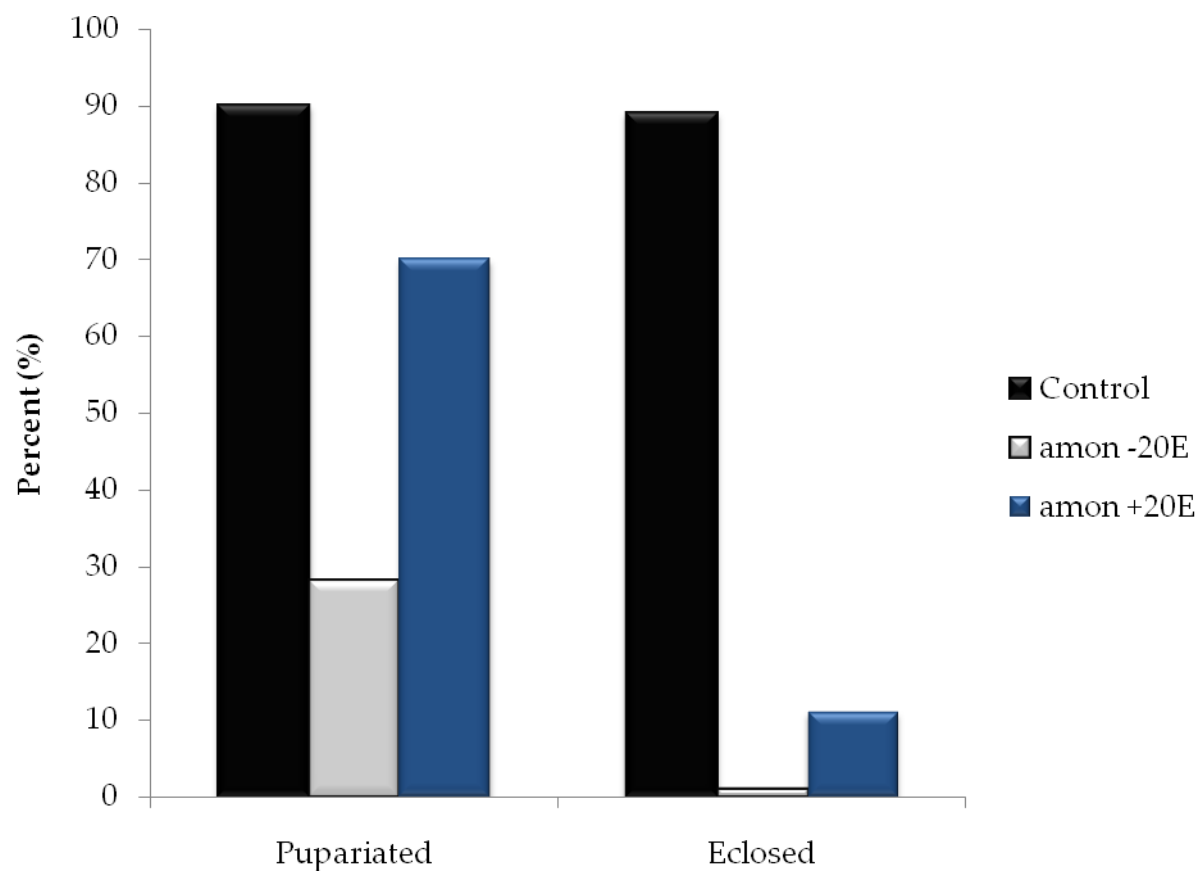


Figure B.2. 20E partially rescues the head eversion defect seen in *amon* mutants. A

majority of *amon* mutants die with a failure to undergo head eversion. The presence of 20E in the food, however, results in a significant increase in the percent of animals that develop to normal appearing pharate adults.

