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Analysis of Peptidergic Neural Networks Regulating Ecdysis in the Fruitfly, *Drosophila melanogaster*

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DEDICATION

To my wife, Hyeonsuk Jang

And mother in law, Musoon Guen and my friend, Jaehoon Shin in Heaven

ABSTRACT OF THE DISSERTATION

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At the end of each developmental stage, insects undergo dramatic changes to shed old cuticle, a process called ecdysis. Ecdysis triggering hormone (ETH) released from epitracheal gland Inka cells is a master controller of events during ecdysis through its direct action on the central nervous system (CNS). ETH receptor neurons in the CNS comprise a peptide signaling cascade that initiates and schedules physiological and behavioral changes during the ecdysis sequence.

Among the physiological events regulated by ETH during ecdysis is tracheal inflation, which is the first visible change that occurs following release of ETH. I investigated the role of kinin neurons in the ecdysis sequence and found that impairment of ETH, kinin, or kinin receptor signaling elicits defects in tracheal inflation. Ablation of kinin neurons produced larvae exhibiting the buttoned-up phenotype and inability to remove residual fragments of old trachea during ecdysis These findings suggest that kinin signaling is involved in both tracheal inflation and ecdysis behavior.

In order to analyze further the signaling cascade involving ETHR peptidergic ensembles in the ecdysis behavioral sequence, I investigated changes in pupal ecdysis behaviors of transgenic flies with targeted ablations of specific ETHR-A neurons. Varying degrees of behavioral defects were detected following ablation of ETHR neurons expressing eclosion hormone (EH), FMRFamide, kinin, crustacean cardioactive peptide (CCAP), myoinhibitory peptide (MIP), burs and bursicon. I determined that kinin neurons are critical regulators of pre-ecdysis and also discovered that bursicon neurons likely are involved in regulation of both ecdysis and post-ecdysis. To verify a previous sequential activation model for ecdysis regulation, I monitored calcium dynamics in two ETHR ensembles (kinin and bursicon) in the same animal elicited by ETH exposure. I found that kinin and bursicon neurons have distinct activation patterns and that they are activated sequentially. To explain the sequential activation of these ETHR ensembles, I tested a differential sensitivity model by analyzing changes in ecdysis scheduling following modification of ETH receptor density. Over-expression or suppression of ETH receptors in targeted neurons resulted in changes in onset timing during pupal ecdysis, supporting the hypothesis that differential sensitivity of ETHR ensembles to their sequential activation by ETH.

In summary, my data demonstrates that differential sensitivity of ETHR peptidergic ensembles can explain how the master hormone ETH schedules changes in physiology and behavior during the ecdysis sequence.

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CHAPTER I

General Introduction

Arthropods are surrounded by a hard cuticle, which is one of the main reasons for their evolutionary success. The cuticle functions as an exoskeleton, providing a kind of body-armor for protection against predators and pathogens. The wax layer of the cuticle limits water loss, providing an added advantage for insects in terrestrial environments. The cuticle layer, however, also places constraints on growth. This is overcome by molting and ecdysis. Molting in insects begins with apolysis, whereby the epidermal cells detach from the old cuticle and begin synthesis of new cuticle, and ends with ecdysis, the shedding of old cuticle. As the final step of the molt, ecdysis is a vital process in insect development that recurs throughout ontogeny from egg to adult. An understanding of ecdysis is important for a number of reasons. From a basic science perspective, ecdysis serves as a model for hormonal scheduling of developmental sequences and behaviors. From an applied standpoint, ecdysis is a vulnerable process that could serve as a target for future insect control strategies. Since the cuticle covers all surfaces of the body, including the outer surface, foregut, hindgut and trachea, ecdysis is very complex behavior that is hard-wired for precise performance. Because failure of ecdysis timing and scheduling can have dire consequences, its precise regulation is critical to survival.

Hormonal regulation of molting was first described by Kopec in 1922 (Kopeć, 1922). His experiments demonstrated that a blood-borne factor (hormone) controls each

step of development. At the beginning of the molt, preparation of new body inside of the old cuticle starts with release of prothoracicotropic hormone (PTTH), which acts on prothoracic glands to release the steroid hormone ecdysone into the hemolymph. Elevation of 20-hydroxyecdysone (20-HE) is the key hormonal signal for molt initiation, which directs changes in epidermal cells and expression of genes appropriate for the next stage. The surge in steroid levels causes cessation of feeding and apolysis.

The role of a novel brain hormone in regulation of eclosion (adult ecdysis) was first suggested in 1970 (Truman and Riddiford, 1970). Truman and colleagues eventually identified eclosion hormone (EH) from pharate adults. EH also induces ecdysis behavior in pharate larvae and pupae (Truman et al., 1981). After this discovery, eclosion hormone was believed to be the sole endocrine signal mediating ecdysis behavior. Beginning in 1996, Zitnan, Adams, and colleagues reported the existence of additional peptide hormones from Inka cells that are regulators of ecdysis (Zitnan and Adams, 2005; Zitnan et al., 1996). The discovery of Inka cells was a real surprise, because most insect hormones previously described originated in the central nervous system (CNS). Inka cells, on the other hand, are present in epitracheal glands situated on trachea and act directly on the CNS to elicit ecdysis behaviors.

In this chapter, I will review several aspects of molting and ecdysis, including: 1) identification of peptide hormones from Inka cells and their roles in ecdysis, 2) regulation of the synthesis and release of ETH during development, 3) identification of ETH receptors in the CNS and their functional roles, 4) previous models for orchestration of ecdysis, 5) specific aims and the organization of this dissertation.

1. Identification of Peptides with Ecdysis-Triggering Activities from Inka Cells

The presence of segmentally distributed epitracheal glands in insects was discovered about a hundred years ago (Ikeda, 1913), but their functions remained unknown until recently. In moths, nine pairs of epitracheal glands occur on major ventrolateral tracheal trunks near the spiracles. Zitnan et al. (1996) first showed that each epitracheal gland contains a peptidergic "Inka cell", which increases in volume as ecdysis approaches and shrinks after ecdysis, suggesting release of its contents during ecdysis. This observation led to investigations of functional roles of Inka cells during ecdysis. Injection of Inka cell extracts into the hemocoel of pharate larvae, pupae, and adults triggers ecdysis within minutes at all stages. The bioactive component of Inka cells was purified by reversed-phase liquid chromatography (RPLC) and identified as a 26-mer polypeptide having an amidated C-terminus, Mas-ETH (*Manduca sexta* ecdysis triggering hormone) (Zitnan et al., 1996). During the search for the gene for ETH in *Manduca sexta*, two other mature peptides, PETH (pre-ecdysis triggering hormone) and ETH-AP (ecdysis triggering hormone-associated peptide) were discovered (Zitnan et al., 1999). PETH induces only pre-ecdysis behavior in *Manduca*, but ETH-AP did not show any obvious biological function. Similar Inka cell peptides were also identified in other insect species and their functions are conserved in various insects (Dai and Adams, 2009; Park et al., 2002; Park et al., 1999; Zitnan et al., 2002; Zitnan et al., 2007; Zitnan et al., 2003).

2. Regulation Mechanism of the Synthesis and Release of ETH

2.1. High Steroid Level for Synthesis of ETH

20-hydroxyecdysone is a master hormone for molting cycle process (Hiruma and Riddiford, 2009; Spindler et al., 2009). During the rise of ecdysteroid levels, Inka cells increase in size and the amount of fully processed PETH and ETH also is increased. The presence of an ecdysone receptor response element in the ETH gene suggests that the steroid up-regulates the ETH gene through the activation of the ecdysone receptor (EcR) (Zitnan et al., 1999). Ecdysteroid - induced ETH expression was proved by the injection of 20-HE into ligated *Manduca* larvae and the measurement of increased PETH and ETH in Inka cells. Block of PETH and ETH elevation by the transcription inhibitor of ecdysteroid, actinomycin D suggested that ecdysteroids induce ETH gene expression by transcriptional activation of the ETH gene.

2.2. Regulation of ETH Release

Release of ETH is critical for completion of the molt, since failure of its timely release can cause fatal entrapment in the old cuticle. Ecdysteroids are involved in the release of ETH. Zitnan et al. (1999) found that, following the surge of ecdysteroids that initiates the molt, a decline of ecdysteroid levels is required for ETH release. If the ecdysteroid decline is delayed by the injection of 20-HE, release of ETH and onset of the ecdysis sequence are also delayed until ecdysteroids decline to low levels.

Eclosion hormone, originally believed to be the triggering hormone for ecdysis, was found to control ETH release through a positive-feedback signaling loop (Ewer et al., 1997; Kingan et al., 2001; Kingan et al., 1997). Direct exposure of *Manduca* epitrcheal

glands *in vitro* to eclosion hormone causes ETH release. The secretory action of ETH can be mimicked by cGMP, suggesting that ETH acts through cGMP as second messenger (Kingan et al., 1997). The EH receptor in Inka cells was identified later as a receptor guanylyl cyclase BdmGC-1 (Chang et al., 2009). ETH, in turn, directly acts on the CNS to evoke release of EH. Ewer et al. (1997) showed that ETH acts on *Manduca* VM neurons, resulting in EH release. The presence of ETH receptors in EH neurons was confirmed in *Manduca* and *Drosophila* (Kim et al., 2006a; Kim et al., 2006b).

Corazonin (CRZ) is another regulator of ETH release. Corazonin is produced by lateral brain neurosecretory cells and its receptors were detected in Inka cells (Kim et al., 2004). The discovery of corazonin receptors (CRZR) in Inka cells and release of CRZ just before pre-ecdysis onset provided a new perspective about the physiological functions of CRZ. Kim et al. (2004) found that concentrations as low as 25 pM CRZ causes ETH release from Inka cells *in vitro*. It is interesting that CRZ elicits a graded release of ETH, while EH gives an all-or-nothing response in the same experiment. It therefore appears that CRZ initiates a low level release of ETH at the beginning of ecdysis that then acts on VM neurons to cause EH release, which in turn causes massive release of ETH through positive feedback.

3. Identification and Functional Analysis of ETH Receptor Neurons in the CNS

ETH acts on the CNS directly to drive diverse behavioral and physiological changes underlying the ecdysis sequence. However, mechanisms underlying CNS control of ecdysis remain to be elucidated. The first breakthrough was identification of the ecdysis

triggering hormone receptor (ETHR) gene in *Drosophila melanogaster*, enabling elucidation of primary cellular targets for ETH (Iversen et al., 2002; Park et al., 2003b). The ETHR gene encodes two subtypes of G protein-coupled receptors (ETHR–A, ETHR-B) through alternative splicing of 3-prime exons. Subsequently, ETH receptors were described in the tobacco hawkmoth, *Manduca sexta* (Kim et al., 2006a).

Following discovery of the ETH receptor gene in flies, attention was focused on the identities of neurons that express ETH receptors and their functional roles. The two ETHR subtypes show differences in ligand sensitivity and specificity and are expressed in separate populations of central neurons, suggesting that they have distinctive roles in ETH signaling. Kim et al. (2006) identified some of neurons that express the ETHR-A subtype in *Drosophila* and *Manduca* by double labeling with *in situ* probes and antibody staining. In *Manduca*, a subset of neuropeptide F neurons co-express both subtypes of ETHRs. This is the only known instance of ETHR-A and ETHR-B co-localization identified until now (Kim et al., 2006a). One of the interesting aspects of ETHR-A neurons described thus far is that they are all peptidergic neurons. ETHR-A neurons are, therefore, referred as "peptidergic ensembles", which express a range of different neuropeptides, including kinins, CRF-like diuretic hormone (DH), eclosion hormone (EH), crustacean cardioactive peptide (CCAP), myoinhibitory peptide(MIP), burs, bursicon, neuropeptide F (NPF), and short neuropeptide F (sNPF). Locations of these peptidergic ensembles in pharate pupal CNS were shown in Figure 1.1.

3.1. Regulation of Behavior by EH

Eclosion hormone is a peptide hormone composed of 62-amino-acids. It was first described as a blood-borne factor that controls circadian-mediated eclosion (Truman and Riddiford, 1970).

It was found that EH is sufficient to initiate premature ecdysis behavior in all developmental stages of the moth (Truman et al., 1981). Subsequently, it was found that EH is involved in the release of ETH via a positive feed-back signaling loop. Bath application of EH on the isolated, de-sheathed CNS induces the fictive ecdysis motor pattern in some preparations but no pre-ecdysis patterns (Gammie and Truman, 1999). In *Drosophila*, EH is released from a single pair of neurons in the ventromedial region of brain. Interestingly, targeted cell-killing (CK) of these neurons did not produce complete mortality in affected animals. One third of EH-CK animals developed successfully, which indicates that EH signaling is not an absolute requirement for ecdysis (McNabb et al., 1997). However, EH is important in coordinating the ecdysis sequence. For example, EH-CK flies show loss of a stereotypic lights-on response. EH-CK animals also exhibit a delay in tracheal inflation during the larval ecdysis (McNabb et al., 1997).

3.2. Regulation of Behavior by Kinin

Neuropeptide kinins (from the Greek *kinin* "move") belong to a large family of peptides found in insects, crustaceans, and mollusks, which show the common C-terminal amino acid sequence motif FX^1X^2WG-amide (Torfs et al., 1999). Kinins are well known for their myotropic and diuretic activity. Leukokinin, the first member of the kinin family to be discovered, was isolated from the CNS of the cockroach *Leucophaea madera.* Eight

isoforms occur in this cockroach species (Holman, 1986; Holman et al., 1987). Leucokinin originally was isolated on the basis of its ability to stimulate visceral muscles of the isolated hindgut. Shortly thereafter, it was shown that leukokinin induces fluid secretion in isolated Malpighian tubules of the yellow fever mosquito *Aedes aegypti* (Hayes et al., 1989). Likewise, locustakinin from the locust *Locusta migratoria* increases fluid secretion in Malpighian tubules, but also stimulates water re-absorption by the rectum (Coast et al., 1999). The mode of diuretic action of kinins has been studied intensively in Malpighian tubules from fruitfly *Drosophila* and mosquito *Aedes aegypti*. Kinins increase transepithelial secretion of Cl^-, thereby increasing the transepithelial movement of Na^+, K^+ and water (Thompson et al., 1995).

Kinins act through production of inositol triphosphate (IP_3) to mobilize Ca^{2+} from IP_3-sensitive stores, leading to increased transepithelial Cl^- conductance (Cady and Hagedorn, 1999; O'Donnell et al., 1998; Pollock et al., 2003). In *Drosophila*, drosokinin receptors are expressed in stellate cells, where elevated intracellular [Ca^{2+}] was observed following drosokinin exposure (Terhzaz et al., 1999). However, the presence of stellate cells is not absolutely necessary for kinin-induced diuresis, since the effects of leucokinin were similar in *A. aegypti* Malpighian tubule segments lacking stellate cells (Yu and Beyenbach, 2004). The precise mechanism of the Cl^- shunt in mosquito is disputed.

The first kinin receptor in insects was identified in *Drosophila* following a genome-wide scan for G-protein coupled receptors (Radford et al., 2002). Kinin receptors were located in stellate cells and in the brain by immunocytochemistry with an

anti-DLKR antibody, and in hindgut, testes and ovary using reverse transcription-PCR. The presence of kinin receptors in testes and ovary indicates that kinin could have additional, as yet undescribed functions in these organs. The involvement of kinin in ecdysis was first demonstrated in *Manduca* (Kim et al., 2006a) and *Drosophila* (Kim et al., 2006b). These authors found that kinin neurons express ETHR-A and are therefore likely to be primary targets of ETH. They also showed that exposure of the isolated *Manduca* CNS to a mixture of kinin and directic hormone leads to fictive pre-ecdysis similar in burst frequency and duration to the natural motor patterns observed during pre-ecdysis.

3.3. Role of Neurons 27/704

Following the identification of EH, it was found that this peptide causes an increase in cGMP in a distinct subset of neurons in the *Manduca* CNS (Ewer et al., 1994). These neurons, called cells 27 and 704, release several peptide hormones that have specific functions (Taghert and Truman, 1982). These peptide hormones are CCAP, MIP, burs and bursicon (burs and pburs). A homologous set of neurons was found subsequently in *Drosophila* and named *Drosophila* neurons 27/704 because they are similar in anatomy, peptide expression, and functional roles.

3.3.1. Regulation of Behavior by CCAP

Crustacean cardioactive peptide (CCAP) was first identified in pericardial organs of the shore crab *Carcinus maenas* based on its stimulatory actions on the heart (Stangier et al.,

1987). The identical peptide was found subsequently in the moth *Manduca* (Cheung et al., 1992). The involvement of CCAP in ecdysis was proposed when Ewer et al. found an EH-induced cGMP elevation CCAP neurons (1994; 1997). The firing of EH releasing VM neurons induces an increase of cGMP in neuron 27/704. Direct evidence of the function of CCAP in ecdysis was demonstrated by applying CCAP directly on the desheathed *Manduca* CNS. Application of 10^{-8} M CCAP induced a rhythmic pattern of bursting that is similar in frequency and duration to natural ecdysis behavior (Gammie and Truman, 1999). The action of CCAP on the CNS was reversible; the ecdysis motor pattern ceased as soon as CCAP removed from the bath. The motor burst was again initiated upon re-application of CCAP. These observations indicate that CCAP is a proximal signal for induction of ecdysis behavior. However in *Drosophila*, CCAP cell-killing (CK) does produce lethality during larval stages, suggesting that other signaling pathways also mediate larval ecdysis (Park et al., 2003a).

3.3.2. Regulation of Behavior Role by Myoinhibitory Peptide (MIP)

The first myoinhibitory peptide (MIP) was found in cerebral nervous tissue of *Locusta migratoria* as a novel peptide that suppresses spontaneous hindgut activity. Later, two MIPs were identified from the ventral nerve cord of *Manduca*: Mas-MIP I and Mas-MIP II. In *Manduca*, MIP is expressed in brain, and in interneuron 704 in abdominal ganglia (AG) 1-7 and AG 8. The expression of ETHR-A in MIP neurons provided new insights for the role of MIP as a modulatory hormone during ecdysis (Kim et al., 2006a). Careful recordings of motor patterns from the isolated *Manduca* CNS revealed that application of

CCAP alone induces ecdysis motor pattern only in anterior abdominal ganglia. However a cocktail of CCAP and MIP, elicited characteristic ecdysis bursts from all ganglia, suggesting MIP is another key hormone in the ecdysis behavioral sequence (Kim et al., 2006a).

3.3.3. Regulation of Behavior by Bursicon

An important peptide expressed by certain ETHR-A neurons is bursicon (from the Greek bursikos, meaning tanners). Although this hormone was described more than 40 years ago in association with tanning in neck-ligated flies (Fraenkel and Hsiao, 1962; Fraenkel et al., 1966), its molecular nature was only recently elucidated as a heterodimer consisting of *burs* and *pburs* partner of burs) (Dewey et al., 2004; Luo et al., 2005). In *Manduca*, bursicon is co-expressed in cell 27 with CCAP. In *Drosophila*, cell 27 in abdominal neuromeres (AN) 1-4 expresses bursicon together with CCAP and MIP. Interestingly, pburs is co-expressed with burs in these four ANs. However, thoracic neuromeres 3 and posterior abdominal neuromeres express burs only (Luo et al., 2005). The function of burs alone in these cells remains unclear. After the finding of ETHR-A in neurons that release bursicon, it was suggested that bursicon neurons are involved in the last step of ecdysis, because tanning happens at the end of the ecdysis (Kim et al., 2006a). The bursicon receptor was discovered in mutant flies displaying a phenotype called "*rickets*". These flies fail to perform sclerotization and melanization even though they have normal levels of bursicon (Baker and Truman, 2002). The *rickets* gene encodes a G protein-coupled receptor DLGR2 belonging to the glycoprotein hormone

receptor family (Luo et al., 2005; Mendive et al., 2005). The distribution of bursicon receptors remains unknown.

3.4. Relevance of the Present Work on Kinin Signaling

The neuropeptide kinin has multiple functions, including hindgut contraction, transepithelial secretion in Malpighian tubules, and ecdysis. During the course of the present work, I observed tracheal inflation defects following kinin cell-killing (CK) and in flies having a mutation in the kinin receptor (Chapter II). These findings indicate that ETH mediates tracheal inflation during ecdysis through downstream kinin signaling. Furthermore, I propose that epithelial Na^+ channels (ENaCs) are possible targets of kinin (Chapter II). Bursicon has been known to be involved in tanning, the last step of ecdysis. In this dissertation (Chapter III), I provide evidence for a new functional role for bursicon in ecdysis.

4. Development of a Model for Orchestration of Ecdysis via ETH Receptor Neurons

4.1. Sequential Activation Model

Since ETH acts directly on the nervous system, establishment of identity and organization of ETH receptor neurons has been a primary objective for understanding the neural basis of ecdysis. Discovery of ETH receptor genes in *Drosophila* and *Manduca* allowed for identification of neurons that express ETHR-A as well as peptides released by these cells. This information led to hypotheses about the organization of ETHR neurons and their possible functional roles in orchestration of the ecdysis sequence. In

Manduca, bath application of hormones that are released from ETHR neurons induced fictive motor patterns in the isolated desheathed CNS. For instance, a cocktail of kinin and diuretic hormone (DH) elicited a pre-ecdysis I-like motor pattern, suggesting that neurons $L_{3/4}$ producing kinin/DH are involved in pre-ecdysis. Application of peptide hormones CCAP and MIP that are expressed in neurons IN704 induced an ecdysis-like motor pattern (Kim et al., 2006a). In *Drosophila*, calcium imaging in live preparations was used to follow activation of these neurons in response to ETH application. Using different lines of transgenic flies, Kim et al. (2006b) observed the timing of ETH-induced calcium mobilization patterns in subsets of ETHR neurons. For example, Tv neurons that express ETH-A and FMRFamide are activated soon (~8 min) after exposure to 600 nM ETH. On the other hand, subsets of segmentally repeated neurons 27/704 that release CCAP, MIP or bursicon show distinct but consistent activation patterns. Following exposure to 600 nM ETH, neurons in TN3 and AN8,9 become active in ~15-20 min. However, activation of neurons in AN1-4 is delayed. Based on information from *Manduca* and *Drosophila*, Kim et al. proposed the "Sequential Activation Model": multiple peptidergic neurons are recruited sequentially and they are involved in different steps in ecdysis behavioral sequence. For example, in *Drosophila*, FMRFamide neurons are involved in all ecdysis steps; EH, TN3, and AN8,9 neurons are critical for ecdysis, and AN1-4 neurons are involved in post-ecdysis.

4.2. Role of Inhibition in Scheduling of the Ecdysis

One of the explanations for sequential activation of peptidergic ensembles by ETH is the presence of descending inhibitory neurons from the cephalic and thoracic ganglia that delay onset of abdominal circuits that are involved in behavioral steps during the ecdysis sequence. Zitnan and colleagues (Zitnan and Adams, 2000) found that the neural network for ecdysis behavior is activated by ETH during early pre-ecdysis, as evidenced by elevation of cGMP. However electrical activity in these neurons was suppressed for a time, suggesting that an inhibitory factor delays the onset of ecdysis. They demonstrated that ligatures applied posterior to cephalic or thoracic ganglia following ETH injection in *Manduca* larvae resulted in a premature switch to ecdysis. This suggests that inhibitory signals originating from cephalic and thoracic ganglia delay ecdysis onset until the end of pre-ecdysis. The effect of desending inhibitory input was also found in *Drosophila* eclosion. Neck ligations applied during pre-eclosion behavior induces premature transition to ecdysis (Baker et al., 1999). Interestingly, Kim and colleagues showed that pretreatment of Ca^{2+} channel blocker, omega-Aga-IVA, in *Drosophila* CNS accelerated activation of EH and CCAP neurons after ETH exposure in their calcium imaging study (Kim et al., 2006b). This preliminary experiment suggests that inhibition of transmitter release from inhibitory neurons by this toxin allows ecdysis to proceed prematurely. ETHR-B neurons are suspected of being these inhibitory neurons (Kim et al., 2006b).

4.3. Relevance of the Present Work

As mentioned above, ecdysis is a controlled by a highly organized physiological and behavioral sequence of events, which are triggered by the hormone ETH. This hormone

appears to activate myriad downstream signaling pathways via sequential activation of ETHR-A neurons in the CNS. The Sequential Activation Model provides insight into peptidergic regulation of ecdysis behavior. In this dissertation (Chapter III), I present new evidence supporting a modification of this model by behavioral analysis of targeted CK flies and calcium imaging study. I provide the first evidence implicating kinin neurons as pre-ecdysis modulators, which is consistent with previous evidence from studies of pre-ecdysis in *Manduca*. However, I also found unexpected evidence that bursicon neurons, previously proposed as post-ecdysis initiators, may also be involved in initiation of ecdysis behavior, Finally, I address the question: How does ETH activate peptidergic ensembles with precisely timed delays? To answer this question, I propose a "Differential Sensitivity Theory" involving ETHR-A peptidergic ensembles and provided evidences that different sensitivity to ETH in different ETHR ensembles is one of the reasons for sequential activation.

5. Specific Aims and the Organization of This Dissertation

The goal of this study is to provide a better understanding of neuronal organization of innate behavior, such as ecdysis. To accomplish this aim, I characterized the functional organization of ETH receptor neurons by combining molecular biology, transgenic techniques, behavioral analysis and calcium imaging techniques using *Drosophila* as a model organism. In Chapter II, I describe a newly recognized function for kinin in tracheal inflation, which is the first visible change during larval ecdysis. Using molecular and transgenic techniques, I show that kinin controls tracheal inflation through ENaC in

tracheal epithelial cells. In Chapter III, I provide phenotypic evidence for behavioral roles of ETHR ensembles. I selectively killed ETHR cells by targeted ablation using overexpression of apoptosis genes *reaper* and *hid* and analyzed resulting changes in ecdysis behavior. To understand the functional organization of ecdysis, I generated a combination Gal4 fly line in order to express the calcium indicator GCaMP in two different ETHR neurons expressing either kinin or bursicon. By monitoring sequential neural activities of these ETHR neurons following exposure to different ETH concentrations, I verified that ETHR neurons are activated sequentially. I also propose differential sensitivity theory as a possible explanation sequential activation. Using kinin and bursicon as example, I provide evidence that differential ETH receptor densities in different ETHR neurons may cause distinct pattern of activation timing.

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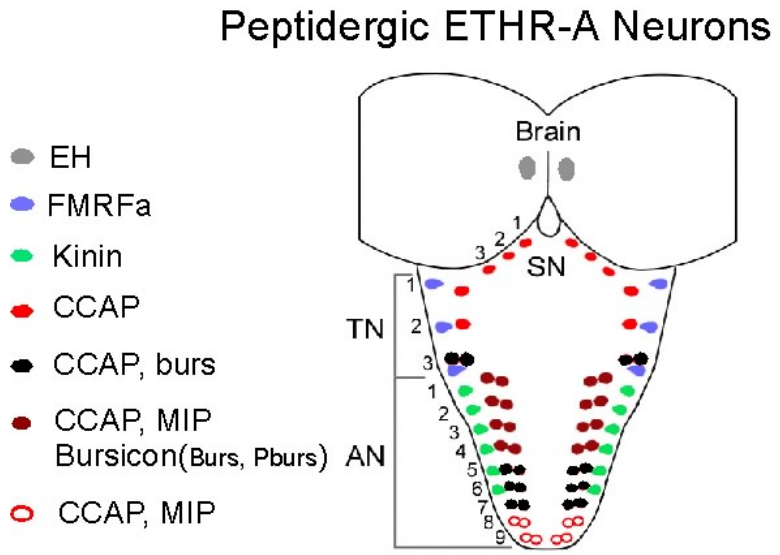

Figure 1.1. Schematic diagram showing peptidergic ETHR-A neurons in the CNS of pharate pupal *Drosophila*. The schematic diagram depicts locations of known ETHR-A subtype neurons in the pharate pupal CNS. Note that CCAP neurons are subdivided into four subgroups on the basis of co-transmitter expression: CCAP in SNs, TN1 - 2; CCAP / Burs in TN3,AN1-7; CCAP / MIP in AN1-4, AN8,9; CCAP / MIP / bursicon (burs and pburs) in AN1-4. SN: subesophageal neuromeres; TN: thoracic neuromeres; AN: Abdominal neuromeres.

CHAPTER II

The Role of Neuropeptide Kinin in Tracheal Inflation and Ecdysis Behavior

ABSTRACT

Neuropeptide kinins occur widely in invertebrates and are associated with myotropic and diuretic functions. Recently, the presence of ecdysis triggering hormone receptors (ETHR) in kinin neurons implicated this peptide in scheduling of the ecdysis behavioral sequence of moths (*Manduca*) and flies (*Drosophila*). Here we describe the involvement of kinins in tracheal inflation in flies. Flies bearing targeted ablations of kinin neurons (kinin-CK) exhibit tracheal inflation phenotypes in all larval stages. Tracheal inflation defects also are observed when kinin signaling is impaired by expression of an inwardly rectifying potassium channel (Kir), kinin-RNAi, and ETHR-RNAi. In order to verify the role of kinin in tracheal inflation, the phenotype of a hypomorphic kinin receptor mutant fly line (Lkr^{f02594}) having a *piggyBac* insertion in exon1 was investigated. Lkr^{f02594} flies exhibited significant, but less severe tracheal inflation defects in all larval stages. The contribution of the receptor mutation to tracheal inflation defects was confirmed by complementation tests and rescue. The characteristic buttoned-up phenotype and retained fragments of old tracheal trunks of experimental flies suggest the presence of ecdysis behavioral defects in these animals. Tracheal inflation defects also result from the expression of kinin receptor RNAi in pickpocket cells of tracheal epidermal cells, suggesting that kinin targets these cells directly for

tracheal inflation. Our findings suggest a new function for kinin in tracheal inflation and provide further evidence that kinin signaling is crucial to events in the ecdysis behavioral sequence.

INTRODUCTION

Ecdysis is the process of shedding old cuticle. Since the cuticle covers not just the outer surface of the animal, but also the inner linings of tracheae, foregut, and hindgut, complex physiological processes along with behavioral changes are required to perform complete cuticle shedding. Tracheal tubules are breathing organs for insects. During the molting process, a new and larger tracheal system, which is filled with liquid, is generated around the smaller old trachea. The collapse of old trachea and inflation of new trachea are essential events for successful ecdysis. These events occur immediately prior to the start of the ecdysis behavioral sequence. Ecdysis triggering hormone is a master hormone in the process of tracheal inflation; deletion of the ETH gene delays tracheal inflation significantly (Park et al., 2002). In *Drosophila*, the primary ion channel known to be involved in tracheal inflation is known as pickpocket (ppk), a member of the Degenerin /epithelial Na^+ channel (DEG/ENaC) family (Liu et al., 2003). Nine different pickpocket genes are expressed in the *Drosophila* tracheal system. Pickpocket cells also express the transmembrane protein wurst, which is essential for endocytosis during tracheal inflation (Behr et al., 2007). However, downstream signaling mechanisms underlying ETH-induced tracheal inflation have remained obscure, because of the lack of information about ETH receptor neurons.

Expression of the ecdysis triggering hormone receptor (ETHR) in kinin neurons of the moth *Manduca* and the fruitfly *Drosophila* suggest novel functions for this peptide hormone in ecdysis (Kim et al., 2006a; Kim et al., 2006b). Furthermore, the fact that kinin is involved in pre-ecdysis behavior in *Manduca* suggests the possibility that this peptide is involved in early events during ecdysis. Functions for kinin previously established can be classified into two categories: myotropic functions in muscles and water transport in Malpighian tubules and rectum. Here, we propose a new function for kinin in tracheal inflation. Using genetic manipulation techniques in *Drosophila*, we implicate kinin as part of the signaling cascade responsible for tracheal inflation during ecdysis. We also provide evidence that kinin is involved in pre-ecdysis behavior in *Drosophila*.

MATERIALS and METHODS

Fly Strains. All flies were raised at 25˚C on standard cornmeal-agar media under a 12 hr light/dark regimen. *UAS-rpr,hid* flies were provided by Paul Taghert (Washington University, St Louis, MI). *UAS-Dicer2* was provided by Barry Dickson (IMP, Vienna, Austria). *UAS- Kir3.1* flies were provided by Giovanni Galizia (Univ. of Konstanz, Konstanz, Germany). *Kinin-Gal4(2), kinin-Gal4(3),* and *UAS-ETHR-RNAi* were generated from our lab. Doubly homozygous *kinin-Gal4(2,3), UAS-kinin-RNAi;UAS-Dicer2,* and *UAS-ETHR-RNAi:UAS-Dicer2* were generated by normal fly genetics. Kinin receptor mutant flies (*PBac{WH}Lkr*^{f02594}) were obtained from Exelixis (Harvard University, Boston, MA) and homozygote flies were collected for experiment. Other

flies were obtained from Bloomington stock center (Indiana University, Bloomington, IN); *UAS-rpr* (stock number, 5824), *w^{1118}; P{Tub-PBac}* (stock number, 8285), *UAS-mCD8-GFP* (stock number, 5137), *Df(3L)Exel6105* (stock number, 7584), *Df(3L)ZN47* (stock number, 3096), *ActGFP* (stock number, 4534) *breathless-Gal4* (stock number, 8807) and *w^{1118}* (stock number, 5905). All Gal4 or UAS fly lines used in our experiment were homozygous lines.

Immunohistochemistry. We crossed *kinin-Gal4* (Kim et al., 2006b) transgenic flies with *UAS-mCD8-GFP* flies to produce progeny expressing GFP in kinin neurons and used them for GFP and anti-kinin immunohistochemical staining. To confirm the death of kinin neurons, Gal4 transgenic flies were crossed to *UAS-rpr,hid*; *UAS-mCD8-GFP* (*UAS-RH;GFP*) fly lines for expression of apoptosis genes *rpr, hid* and expression of GFP. Progeny resulting from these crosses were used for double staining with GFP and kinin antibodies.

Test of Defects in Tracheal Inflation. 1st, 2nd and 3rd instar larvae exhibiting defects in kinin signaling were positioned in a drop of tap water on a glass slide with thin supports on both sides; a coverslip was placed over the preparation. Shapes of trachea were observed with a compound microscope. Larvae were rolled over to view detailed abnormality of tracheal tubes. The degree of defects observed were defined according to three levels: total failure, partial failure, and normal. Larvae showing a complete absence of air in the tracheal system were scored as total failure. Tracheal inflation defects were

photographed with a Sony digital camera to illustrate morphological characters of tracheal defects.

Real Time Quantitative PCR (qPCR). To clarify suppression of receptor expression in the kinin receptor mutant line, Lkr^{f02594}, mRNA levels were measured by quantitative PCR. Gene-specific primers for kinin receptor were designed using the exon 6 and exon 7 region. Two housekeeping genes, RP49 and actin, were used as references. Gradient PCRs followed by electrophoresis on 1% agarose with ethidium bromide gels were first used to check for specificities of primers and optimal annealing temperatures. Serial dilutions of cDNA were then used with qPCR to generate standard curves to evaluate amplification efficiency and to further check specificity of the primers. QPCR was carried out in 25 µl reaction volumes containing 12.5 µl of SYBR Green Supermix (Bio-Rad, USA), 2 µl of cDNA template, primers at the appropriate concentrations, and water. The Bio-Rad CFX96 Real Time PCR Dectection System (Bio-Rad, USA) was used according to the manufacturer's specifications. Optimized kinin receptor gene primers used for qPCR analysis were as follows: Forward 5′-ACGCACAGGATTCAC GGGAC-3′, Reverse 5′-CAGCCAATCGCAGCAAAAC-3′used at a concentration of 200 nM with an expected DNA size of 192bp. Optimized RP49 primers were Forward 5′-CCAAGA TCGTGAAGAAGCGCACCAA-3′, Reverse 5′-GTTGGGCTACAGATACTG TCCCTTG-3′at a concentration of 200 nM with an expected DNA size of 143 bp. Optimized actin primers were Forward 5′-CATCCACGAGACCACCTACA-3′, Reverse 5′-TTGGAGATCCACATC TGCTG-3′at

a concentration of 200 nM with an expected DNA size of 258 bp. For each experimental replicate, cDNA (diluted 10-fold) from separate samples was run in triplicate on the same plate. All control reactions also were run in triplicate on the same plate with experimental reactions. The cycling parameters were 3 min at 95˚C, followed by 45 cycles consisting of 10s at 94˚C, 20s at 60˚C and 30s at 72˚C. The reaction carried out without DNA sample was used as a negative control. A melting curve was generated for every PCR product to confirm the specificity of the assays. Threshold cycle (C_t) values were determined by exponential product amplification and subsequent increased fluorescence intensity above background. Relative gene expression data were normalized against C_t values for the house-keeping genes (RP49 and actin), and fold changes ($2^{-\Delta\Delta Ct}$) were determined by comparison to the average expression levels for the control samples with the index defined as 1.0 using Pfaffl equation (Pfaffl, 2001).

Statistics. Changes in kinin receptor transcript levels were compared with those of controls using Student's *t-test* at the 99% significant level ($p < 0.01$).

Complementation Test. To confirm that tracheal inflation defects in the Lkr^{f02594} line stem from piggyBac insertion in the kinin receptor gene, we performed test crosses between Lkr^{f02594} and GFP labeled 7584 and 3096 that are heterozygote deficiency lines in which kinin receptor gene loci are missing on one chromosome [*Df(3L)Exel6105* (7584, breakpoints: 64D1;64D6)-GFP and *Df(3L)ZN47*(3096, Breakpoints:64C;65C)-GFP] and the other normal chromosome is labeled as actin GFP (Figure 2.6). GFP

labeled 7584 and 3096 were generated by a genetic cross with the ActGFP line 4534, which also has *Sb* and *Ser* markers. Tracheal inflation defects of F1 progeny were scored in non-GFP larvae. If the kinin receptor indeed is involved in tracheal inflation, non-GFP larvae (*Df/PBac*) should retain the tracheal inflation phenotype. If the kinin receptor mutation is NOT related to tracheal inflation defects, *Df/PBac* should be normal because two different chromosomes complement each other.

Rescue Test. For precise excision of the *piggyBac* insertion in Lkr^{f02594}, flip-out *piggyBac* transposase [w^{1118}; *P{Tub-PBac}*] flies were crossed to Lkr^{f02594}. Among F1 progenies, white eye flies were selected by normal genetic methods. To confirm precise excision of *piggyBac* constructs, we designed specific primers for the *piggyBac* insertion region: forward primer 1 (F1) and reverse primer 1 (R1) was designed from the insertion point of the kinin receptor gene; F2 and R3 were designed from the *piggyBac* vector sequence; as a control, F3 and R2 primers were designed from another region of kinin receptor gene, exon 8 (Figure 2.8). The presence or absence of the *piggyBac* vector was verified by the difference in polymerase chain reaction (PCR) products from Lkr^{f02594} and rescued Lkr^{f02594}. PCR products were also sequenced to verify the precise excision.

Survival Rate of Kinin-CK flies. To quantify the survival rate of flies, we collected eggs from each cross in 2-hour time intervals and placed them in a small 35mm Ø plate with apple juice-agar media. Animals were counted and transferred to new plates in

every developmental stage. Failure of ecdysis and other developmental defects were registered with a Sony digital camera.

RESULTS

Impairment of Kinin Signaling Causes Tracheal Inflation Defects. Kinin cells were ablated (kinin-cell killing; kinin CK) using the Gal4/UAS method for cell-specific expression of apoptosis genes (Brand and Perrimon, 1993). This method makes use of a cell-specific promoter to drive expression of the yeast transcriptional activator Gal4. The expressed Gal4 protein activates transcription of the upstream activation sequence (UAS). For our experiments, we used *kinin-Gal4* lines that were generated in our lab. To generate kinin targeted cell-killing fly lines, *kinin-Gal4* lines were crossed to fly lines carrying apoptosis genes (*rpr* only or *hid*, *rpr*) under the control of UAS.

Kinin cell-specific expression of Gal4 was verified by crossing *kinin-Gal4* to *UAS-mCD8-GFP*, followed by double immunohistochemical staining with antisera directed against GFP and kinin (Figure 2.1, A). Three groups of kinin-expressing neurons occur in the brain, subesophageal neuromeres, and abdominal neuromeres, consistent with previous reports (de Haro et al., 2009; Herrero et al., 2007). Targeted ablation of kinin neurons was confirmed by absence of double immunohistochemical staining in kinin-CK flies, which was apparent already during the 1st instar (Figure 2.1, B).

Kinin-CK flies exhibited pronounced tracheal inflation defects during all three larval instars (Figure 2.2). The percentage failure was variable, depending on the fly line.

For example, 1st instar larvae of *Kinin-Gal4(3)* X *UAS-rpr,hid* exhibited 45% complete failure and 35% partial failure, while *Kinin-Gal4(2)* X *UAS-rpr,hid* showed 2.8% complete failure and 75% partial failure. Sums of complete and partial failures were similar (76.6% and 79.4%, respectively). When the cell-killing process was accelerated by doubling the gene dosage, [*Kinin-Gal4(2,3)*], we observed 100% tracheal inflation defects in 1st instar larvae. No tracheal inflation defects were observed in control flies. To confirm that kinin neurons are involved in tracheal inflation, we inhibited kinin signaling through expression of an inward rectifier K^+ channel (Kir3.1), kinin gene RNAi or ETH receptor gene RNAi (Figure 2.3). In all cases, only experimental groups showed tracheal inflation defects, although the percentage of defects was lower than that of kinin CK flies.

***PiggyBac*-insertional Mutation of the Kinin Receptor Gene Causes Tracheal Inflation Defects.** To verify involvement of kinin signaling in tracheal inflation, kinin receptor mutant flies (Lkr^{f02594}) carrying a *piggyBac* insertion in exon 1 of the kinin receptor gene (Figure 2.4) were obtained and homozygous progeny were collected to check for tracheal inflation defects. Lkr^{f02594} flies also showed tracheal inflation defects, but they were comparatively less severe than those observed in kinin-CK flies (Figure 2.7). Nevertheless, significant numbers of larvae showed partial defects in tracheal inflation during all three larval stages.

Real Time Quantitative PCR Confirms a Reduction in Kinin Receptor Gene Expression in in the *Lkr^{f02594}* Line. Phenotypic evidence from *Lkr^{f02594}* suggests this mutantion is hypomorphic. To check the level of reduction in kinin receptor expression, qPCR was performed with two housekeeping genes used as references: RP49 and actin. *Lkr^{f02594}* showed a ~25% reduction in expression level compared to control flies (w^{1118}). Reduction of expression was statistically significant at $P < 0.01$.

Complementation Tests and Rescue of *Lkr^{f02594}* Confirms that *PiggyBac* Insertion in Kinin Receptor Mutant Causes Tracheal Inflation Defects. To verify whether the tracheal inflation defect exhibited by *Lkr^{f02594}* flies is a consequence of an insertional mutation in the kinin receptor gene, *Lkr^{f02594}* was crossed to *Df(3L)Exel6105* (7584)-GFP and *Df(3L)ZN47*(3096)-GFP lines, which carry a deletion of the kinin receptor site and a GFP-labeled normal chromosome (Figure 2.6). The total percentages of tracheal inflation defects in F1 progenies were 94.5% and 92.1% (Figure 2.7). In 1st instar larvae, these defects are much more severe than that of *Lkr^{f02594}* itself (22.6%), suggesting that the *Lkr^{f02594}* is a hypomorphic mutation. To determine if the tracheal inflation defect could be rescued by precise excision of the *piggyBac* insertion, *Lkr^{f02594}* was crossed with *piggyBac* transposase flies and progeny were selected. Rescued flies were completely devoid of tracheal inflation defects (Figure 2.7). To confirm precise excision of *piggyBac,* a series of PCR reactions with specially designed primers was performed (figure 2.8). Prior to precise excision, PCR reactions using F1- R1 primers produced no PCR products from *Lkr^{f02594}* *g*DNA, because the size of the *piggyBac* vector is quite large (10.7 kb). In

contrast, F1 - R3 primers or F2 - R1 primers produced PCR products because of the presence of F2 and R3 vector sequences. This was also true for flies generated by complementation tests (*Lkr*^{f0259} X *Df(3L)Exel610, 7584*). After precise excision, PCR reactions using F1- R1 primers produced PCR products, while F1-R3 or F2-R1 produced no products from rescued *Lkr*^{f02594} *g*DNA, because of the absence of the vector. PCR reactions using F3 and R2 primers produced PCR products from all fly lines. Precise excision of the TTAA *piggyBac* recognition site by transposase was confirmed by sequencing of PCR products.

Impairment in Kinin Signaling Causes Low Survival Rate. To quantify the effects of kinin signaling impairment during development, the number of flies that survived to each developmental stage was assessed in experimental and control flies. Flies having defects in kinin signaling showed significant decreases in survival rate during development (Figure 2.9, A). In *kinin-Gal4(3)* X *UAS-rpr* crosses, we observed only 37.1% survival to the prepupal stage. The survival rate further decreased when two apoptosis genes, *rpr* and *hid,* were employed. When the copy number of the Gal4 driver was doubled by crossing *kinin-Gal4(2,3)* either with *UAS-Kir3.1* or *UAS-rpr,hid*, survival to the pupal stage was abolished completely (Figure 2.9, A, B left). Kinin-RNAi or ETHR-RNAi expression also caused severe decreases in survival rate. In controls, more than 90% of eggs developed normally to the adult stage.

Defects in Kinin Signaling Produce Buttoned-up Phenotypes and Incomplete Removal of Old Trachea. Disruption of kinin signaling produced not only tracheal inflation phenotypes, but also defects in ecdysis behaviors. During larval stages, we observed many instances of lethality associated with the buttoned-up phenotype (Figure 2.9, B top right), which is evidence of characteristic ecdysis behavioral defects (Park et al., 2002). To verify whether larvae failed ecdysis because of abnormal ecdysis-related behaviors or because of respiratory problems, we collected kinin-CK 1^{st} instar larvae that have normal trachea. When we monitored their ecdysis behaviors, we found that 3 out of 8 animals died of the buttoned-up phenotype. Failure of ecdysis behavior also occurred during pupal ecdysis (Figure 2.9, B bottom right). In all, 8 out of 31 animals failed pupal ecdysis. During tests for tracheal inflation defects, we observed interesting features during ecdysis from 2^{nd} to 3^{rd} instar larva. We found parts of old tracheal tubes remained inside of new trachea of 2^{nd} and 3^{rd} instar larvae (Figure 2.10). In some cases, new tracheae were distorted or broken where the old trachea remained. Remaining old tracheae and breaks in new tracheae suggests that tracheal inflation associated with larval ecdysis is abnormal in these fly lines.

Kinin Controls Tracheal Inflation through DEG/ENaC Channels. It is possible that kinin mediates tracheal inflation through activation of receptors expressed in tracheal epidermal cells. To investigate this possibility, we checked for tracheal inflation phenotypes following RNAi silencing of kinin receptor in all trachea epithelial cells using the trachea-specific *breathless (btl)-Gal4* (Figure 2.11). Some 52% of progeny

from these crosses showed tracheal inflation defects, suggesting presence of kinin receptors in tracheal cells. One consequence of kinin receptor activation in these cells is modulation of epithelial Na^+ channels (ENaC), which are known to be important in respiratory inflation in both *Drosophila* and humans (Behr et al., 2007; Liu et al., 2003). ENaC sodium channels are known to be expressed in pickpocket cells (Figure 2.11). When kinin receptor RNAi was performed in several different pickpocket lines, we also observed tracheal inflation defects. Five different *pickpocket (ppk)-Gal4* lines were used and tracheal inflation defects were obsesrved in 3% to 15% of animals observed. We also observed an increase in tracheal inflation defects (28%) following use of doubly homozygous *ppk10;ppk11-Gal4*.

DISCUSSION

ETH release at the end of the molting process regulates physiological and behavioral changes associated with ecdysis. Park et al. (2002) described in detail the sequential events that occur during larval ecdysis in *Drosophila*. Old tracheae collapse shortly after ETH release and new tracheae are inflated. Old trachea are broken apart and pulled out through spiracular pits during ecdysis behavior. To gain a better understanding of mechanisms underlying ETH control of tracheal inflation and ecdysis behaviors, we investigated the possible roles of kinin neurons, which express ETHR-A. Previous studies showed that kinin and diuretic hormone are likely downstream co-regulators of pre-ecdysis in *Manduca*. Our results indicate that kinin plays important roles both in regulation of tracheal inflation and pre-ecdysis behavior in *Drosophila* (see Chapter III).

ETH Controls Tracheal Inflation Through Downstream Kinin Signaling. Kinin is a peptide hormone involved in ion and water balance in malphigian tubules (Cady and Hagedorn, 1999; Yu and Beyenbach, 2002). A more recent report documented the presence of ETHR-A in kinin neurons of *Manduca* and *Drosophila* (Kim et al., 2006a; Kim et al., 2006b). Based on these findings, we hypothesized that kinin could be involved in tracheal inflation, which is the earliest event that occurs during the ecdysis sequence in flies (Park et al., 2002).

When we performed ablation of kinin neurons, animals showed a high rate of tracheal inflation defects. Defects in trachea also were observed when kinin neurons were inactivated through expression of Kir3.1 or when kinin transcript levels were suppressed by kinin gene-specific RNAi. The percentage of tracheal inflation defects varied between different fly lines, however, increasing the copy number of *kinin-Gal4* caused a higher total percentage of tracheal inflation defects. Tracheal inflation defects also were found when we expressed ETH receptor RNAi in kinin neurons, which suggests that ETH regulates tracheal inflation through downstream kinin signaling.

To further investigate the role of kinin in regulation of tracheal inflation, we searched fly databases for kinin receptor mutants and their phenotypes. Examination of the *PBac* insertional kinin receptor mutant, Lkr^{f02594} revealed tracheal inflation defects, even though the severity was much lower than kinin-CK. Quantitative PCR and complementation tests suggest that the insertional mutation in kinin receptor gene accounts for the defects and also suggests that Lkr^{f02594} is a hypomorphic mutation,

explaining the difference in severity. In the *piggyBac* transposon, the transposase executes precise cleavage at the TTAA site and transfers the transposable element (Fraser et al., 1996). Using this feature, we excised the *PBac* insertion and showed that the excision of *PBac* rescues tracheal inflation defects. Taken together, these findings provide strong evidence that kinin signaling is involved in tracheal inflation during larval and pupal ecdysis.

Kinin May Have Role in Ecdysis Behavioral Sequence. In our study, we observed that many experimental animals could not perform normal ecdysis behavior. Many normal-looking experimental larvae could not complete ecdysis and died from buttoned-up phenotype. We also found that some parts of old trachea remained in 2^{nd} and 3^{rd} instar kinin-CK larvae, which indicates that larvae could not perform normal ecdysis behavior. Ecdysis failure also was observed during pupal ecdysis. In *Manduca*, application of kinin/DH mimicked the preecdysis-like motor pattern in the isolated CNS (Kim et al., 2006a). Our observations suggest that kinin signaling also may regulate pre-ecdysis in *Drosophila*, which is the first step in the behavioral sequence. When larvae perform pre-ecdysis behavior, old trachea are loosened; this assists in subsequent pulling-out of old trachea through spiracular during ecdysis. The remaining old tracheae in animals with impaired kinin signaling suggests pre-ecdysis is abnormal.

DEG/ENaC Channels Are Possible Targets of Kinin in Trachea. To investigate events downstream of kinin signaling, we checked for the presence of kinin receptors in

tracheal epidermal cells. Although Radford et al. (2002) showed the presence of kinin receptor in Malpighian tubule, hindgut, brain, testis and ovary, no mention was made of kinin receptors in the tracheal system. Here we provide indirect evidence that tracheal cells also express kinin-receptors using kinin receptor-RNAi under the control of *breathless-Gal4*, which is trachea-specific Gal4. We next tried *ppk-Gal4*, because it is known that in *Drosophila*, as in humans, the Degenerin /epithelial Na^+ channel (DEG/ENaC) family is involved in tracheal inflation (Liu et al., 2003). Observations of five different *ppk-Gal4* lines revealed tracheal inflation defects, although none showed as high a percentage of defects as *btl-Gal4* (52%). When we targeted multiple pickpocket genes using *ppk10/ppk11-Gal4*, the percentage of tracheal inflation defect grew to 28%. This was higher than ppk10 (15%) or ppk11 (4%) alone. These data suggest that different pickpocket cells respond to kinin and work together to accomplish tracheal inflation. It is known that different *ppk* genes have distinct spatial expression patterns in the larval tracheal system. *Ppk4* and *ppk11* show relatively widespread expression, but the other *ppk* genes are expressed in restricted regions (Liu et al., 2003). Therefore, we suggest that the signaling cascade regulating tracheal inflation starts with ETH, which acts through kinin signaling to target tracheal epidermal cells to activate epithelial Na^+ channels (Figure 2.12).

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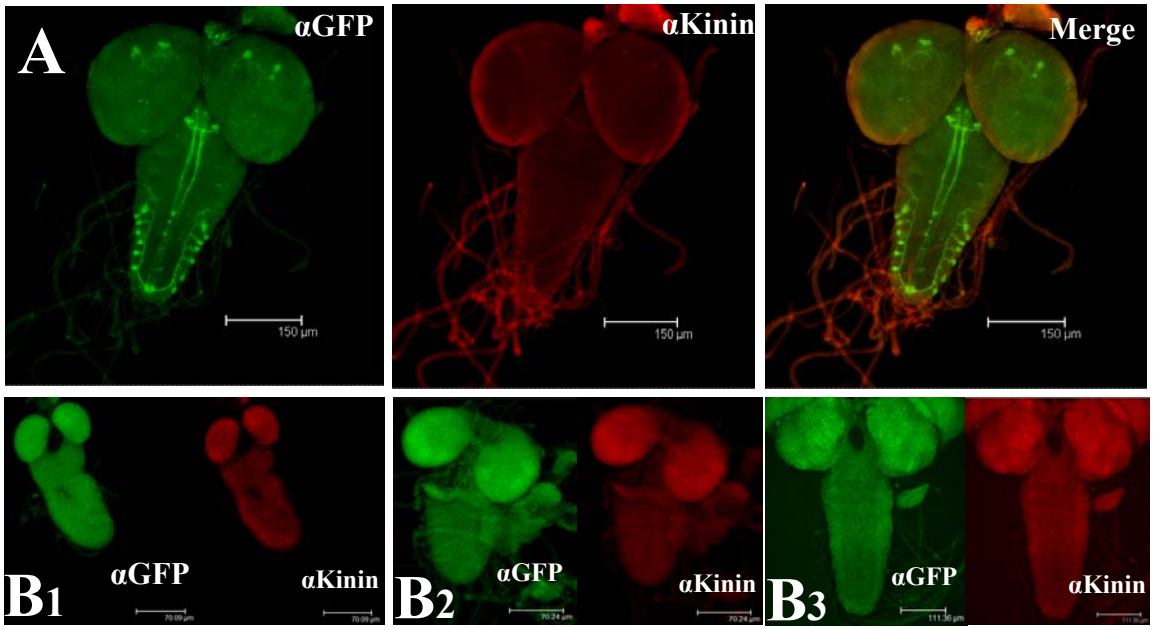


Figure 2.1. Immunohistochemical staining confirms targeted cell-killing (CK) of kinin neurons.

(A) Double labeling of anti-GFP (left), anti-kinin (center) and merge (right). Specific expression of Gal4 in kinin neurons was verified using homozygous *kinin-Gal4* crossed with homozygous *UAS-mCD8-GFP* flies. Co-expression of GFP and kinin was observed in 7 pairs of kinin neurons in abdominal neuromere, 4 pairs in brain and 3 pairs in subesophageal neuromere.

(B) Targeted kinin cell-killing (CK). To verify the targeted ablation of kinin neurons, flies bearing *kinin-Gal4* and *UAS-rpr,hid;UAS-GFP* were generated and immunohistochemistry was performed with antisera against GFP and kinin. Kinin neurons are completely ablated in kinin-CK flies ($B_{1\text{-}3}$) but not in wild type flies (A). A: 3rd instar, B_1: 1st instar, B_2: 2nd instar, B_3: 3rd instar CNS.

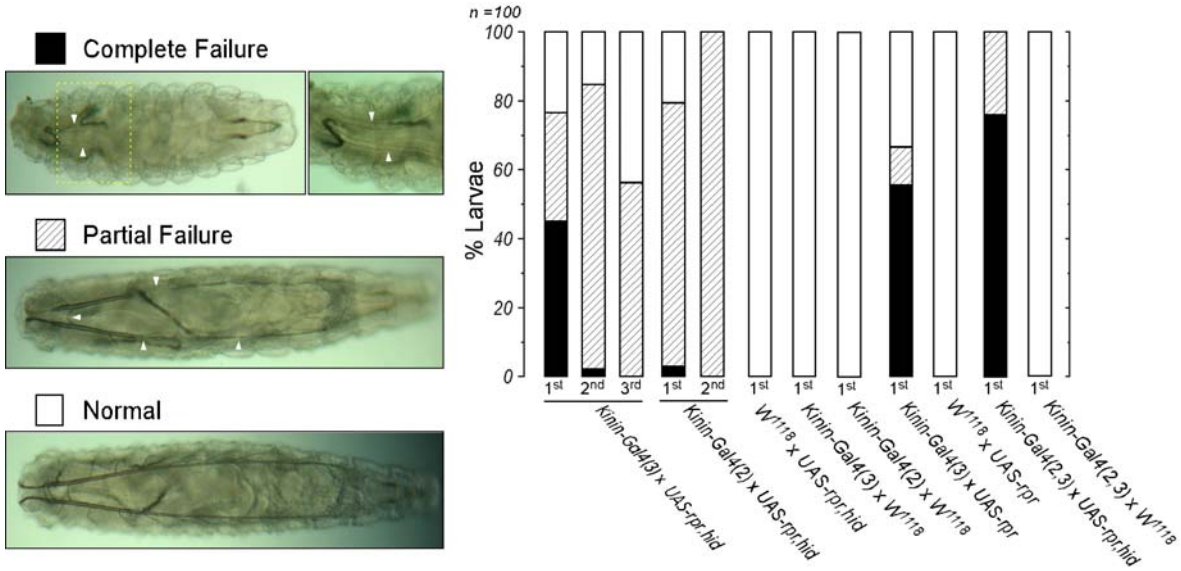


Figure 2.2. Kinin-CK flies showed tracheal inflation defects. Percentages of tracheal inflation defects in kinin cell-killing (CK) flies were displayed. The larvae were counted as complete failure (black) when the tracheal system has no air at all. Other levels of defects were counted as partial failure (hatched). Different Gal4 lines and UAS lines showed different levels of defects. All Gal4 or UAS fly lines used were homozygous lines.

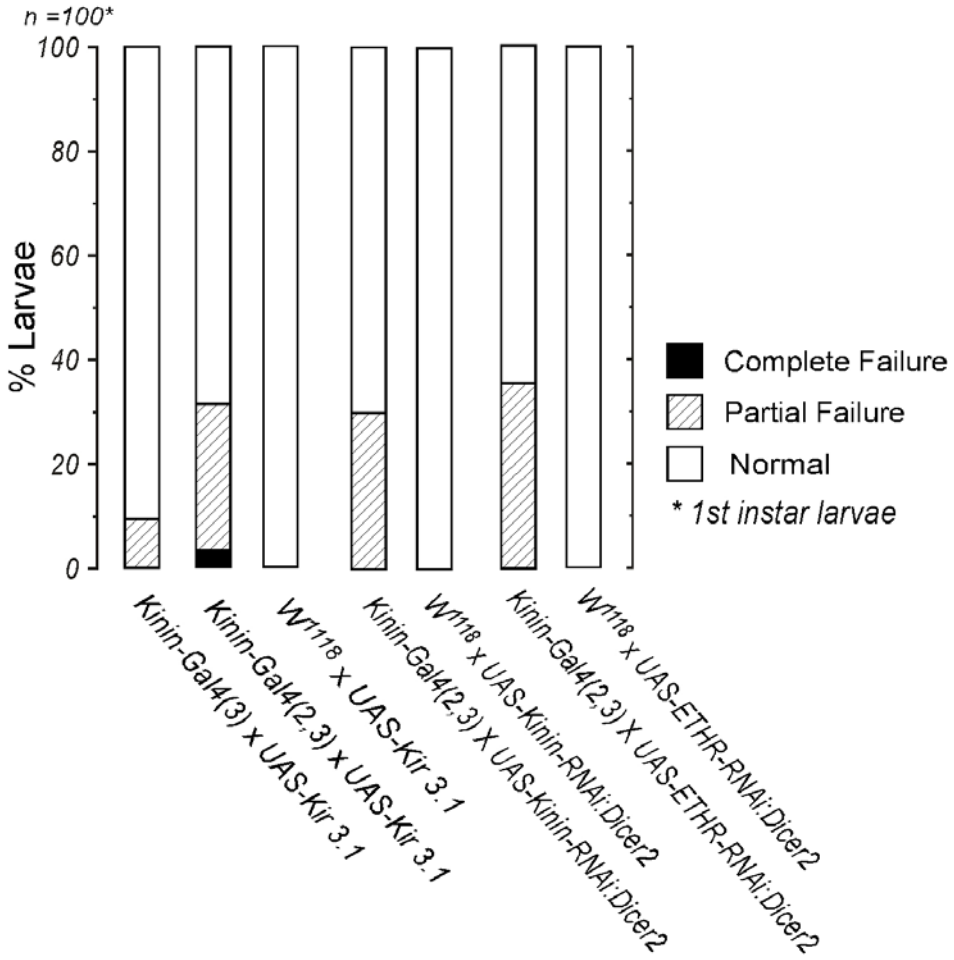


Figure 2.3. Impairment of kinin signaling showed tracheal inflation defects. Tracheal inflation defects were observed when kinin signaling was impaired by the expression of the inward rectifying K^+ channel (Kir3.1), kinin-RNAi, or ETH receptor-RNAi in kinin neurons. Double the Gal4 number caused higher inflation defects in *kinin-Gal4(2,3)* X *UAS-Kir3.1*. All Gal4 or UAS fly lines used were homozygous lines.

Figure 2.4. *PiggyBac* insertion locus in kinin receptor mutant (*Lkr*^{f02594}). Schematic drawing of the kinin receptor gene structure in region 64E of chromosome *3L* and *piggyBac* location is shown. Blue boxes are exons for kinin receptor gene. Orange boxes are open reading frames (ORF).

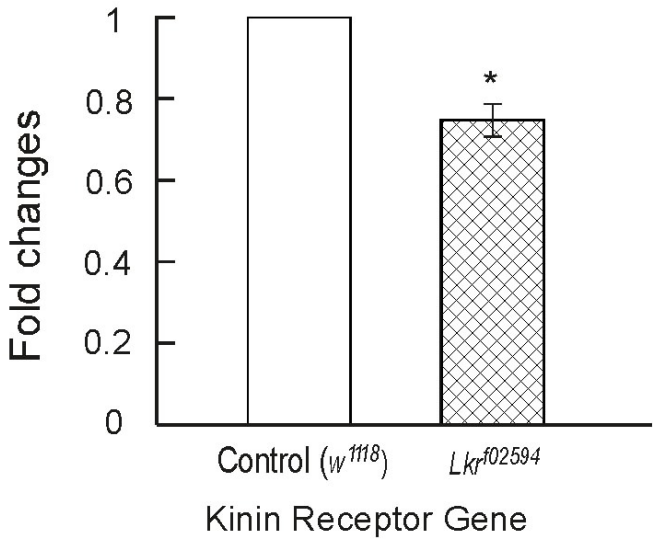


Figure 2.5. Relative expression ratio of kinin receptor genes in control and Lkr^{f0259}. *PiggyBac* insertional kinin receptor mutant Lkr^{f02594} showed significant reduction in gene expression level as 74.8% of normal level. Error-bars represent standard error of mean (S.E.M). Student's t-test was used to evaluate statistical significance with * $P < 0.01$.

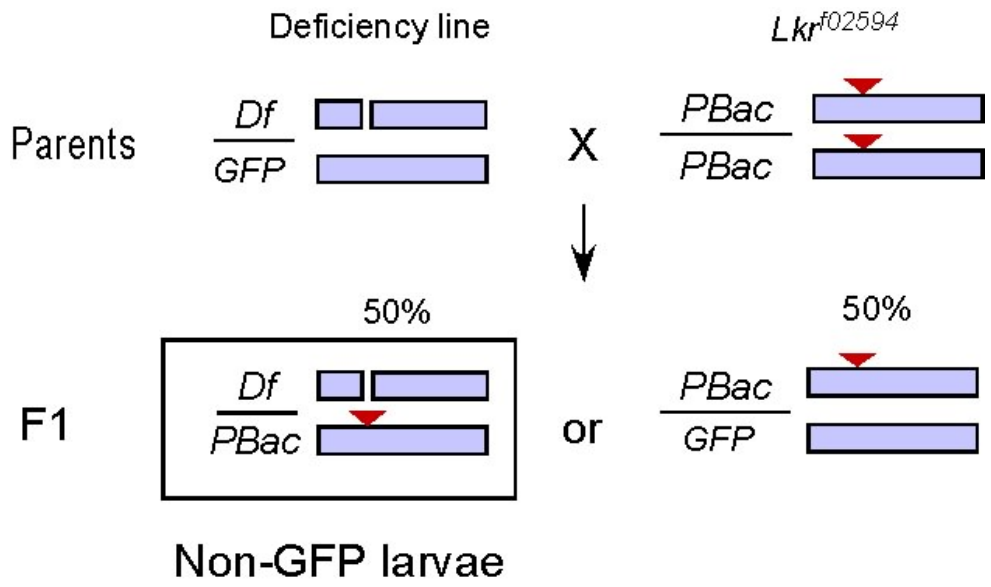


Figure 2.6. Complementation test scheme for kinin receptor mutant (*Lkr*^{f02594}). Heterozygote deficiency lines that kinin receptor gene loci are missing were crossed to homozygous *Lkr*^{f02594}. Among two F1 genotypes, 50% of larvae that express GFP (*PBac/*GFP) should have normal trachea. When the mutation of *Lkr*^{f02594} is the reason of tracheal inflation defects, the other 50% (*Df/PBac*) should retain the phenotype. When the mutation is NOT related to the tracheal inflation defects, *Df/PBac* should be normal because two different chromosomes complement each other. After the crossing, we found that they do not complement the tracheal inflation defects and even showed more severe defects (see Figure 2.7).

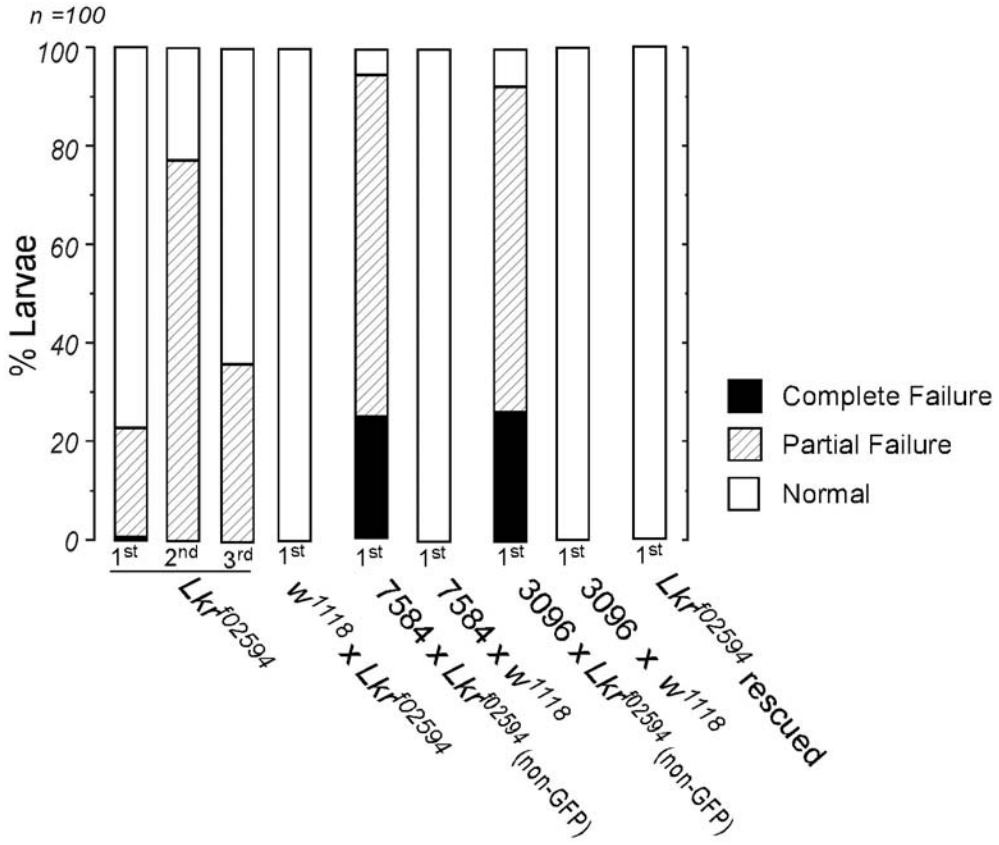
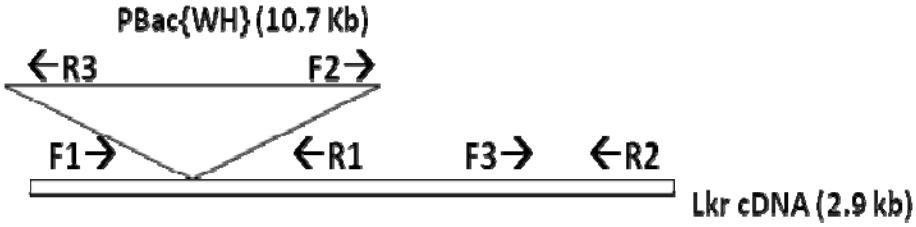
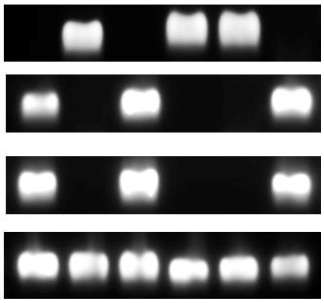


Figure 2.7. Complementation test and rescue of *Lkr*^{f02594} confirmed that *piggyBac* insertion in kinin receptor mutant causes tracheal inflation defects. Homozygous kinin receptor mutant *Lkr*^{f02594} showed tracheal inflation defects in all larval stages. Its degree of defects was highly increased by complementation test (94.5% in 7584 X *Lkr*^{f02594} and 92.1% in 3096 X *Lkr*^{f02594}) (see Figure 2.6 for detail). Precise excision of *piggyBac* insertion from *Lkr*^{f02594} rescued tracheal inflation defect.



PCR	Size (bp)	*PBac/Df* (*Lkr*^{f02594} x *7584*)	*+/Df* (7584)	*PBac/PBac* (*Lkr*^{f02594})	*+/+* (*w*^{1118})	*Lkr*^{f02594} Rescued	*Lkr*^{f02594} Not Rescued
F1-R1	307	-	+	-	+*	+*	-
F1-R3	297	+	-	+*	-	-	+
F2-R1	513	+	-	+*	-	-	+
F3-R2	437	+	+	+	+	+	+



* Sequenced

Figure 2.8. Polymerase chain reaction (PCR) test to confirm the precise excision of *piggyBac* insertion from kinin receptor mutant (*Lkr*^{f02594}). Different PCR sets were designed to test the precise excision after *PiggyBac* insertion of *Lkr*^{f02594} was excised using *piggyBac* transposase fly line. The PCR products were sequenced to confirm precise excision of *piggyBac* construct. The fly lines of complementation test also tested to confirm their genotypes. Rescued flies completely restored their ability to inflate their trachea (see Figure 2.7).

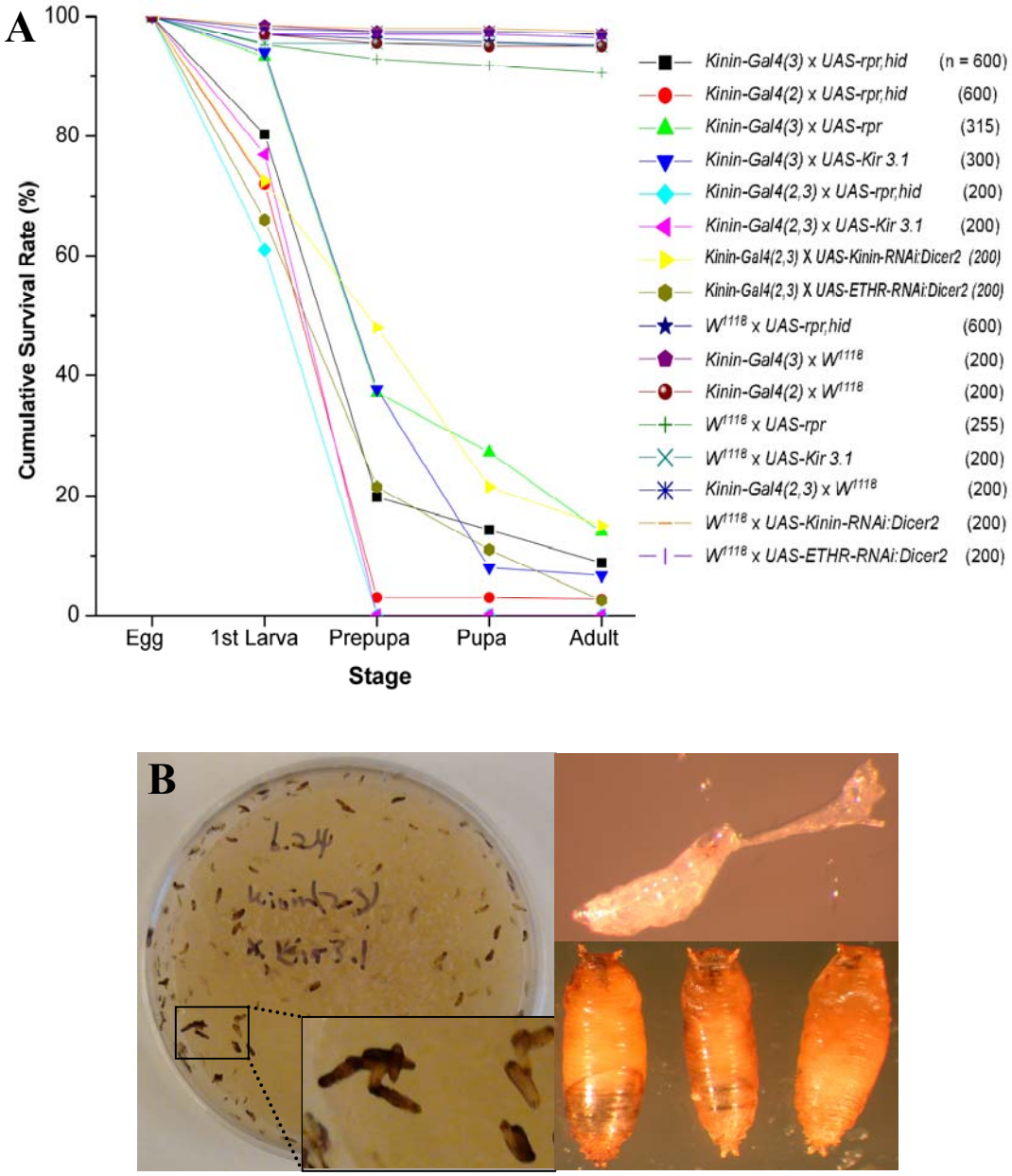


Figure 2.9. Impairment of kinin signaling cause low survival rate and buttoned-up phenotype.

(A) Cumulative survival rates of kinin cell killing, Kir, RNAi and control flies. The treatment animals showed mortality during all developmental stages.

(B) Pictures of dead animals. All animals died during eggs and larval stages when kinin neurons are inactivated by Kir expression (left). During larval ecdysis, animals did abnormal behavior and showed typical buttoned-up phenotype (top right). Behavioral defects also found during pupal ecdysis (bottom right).

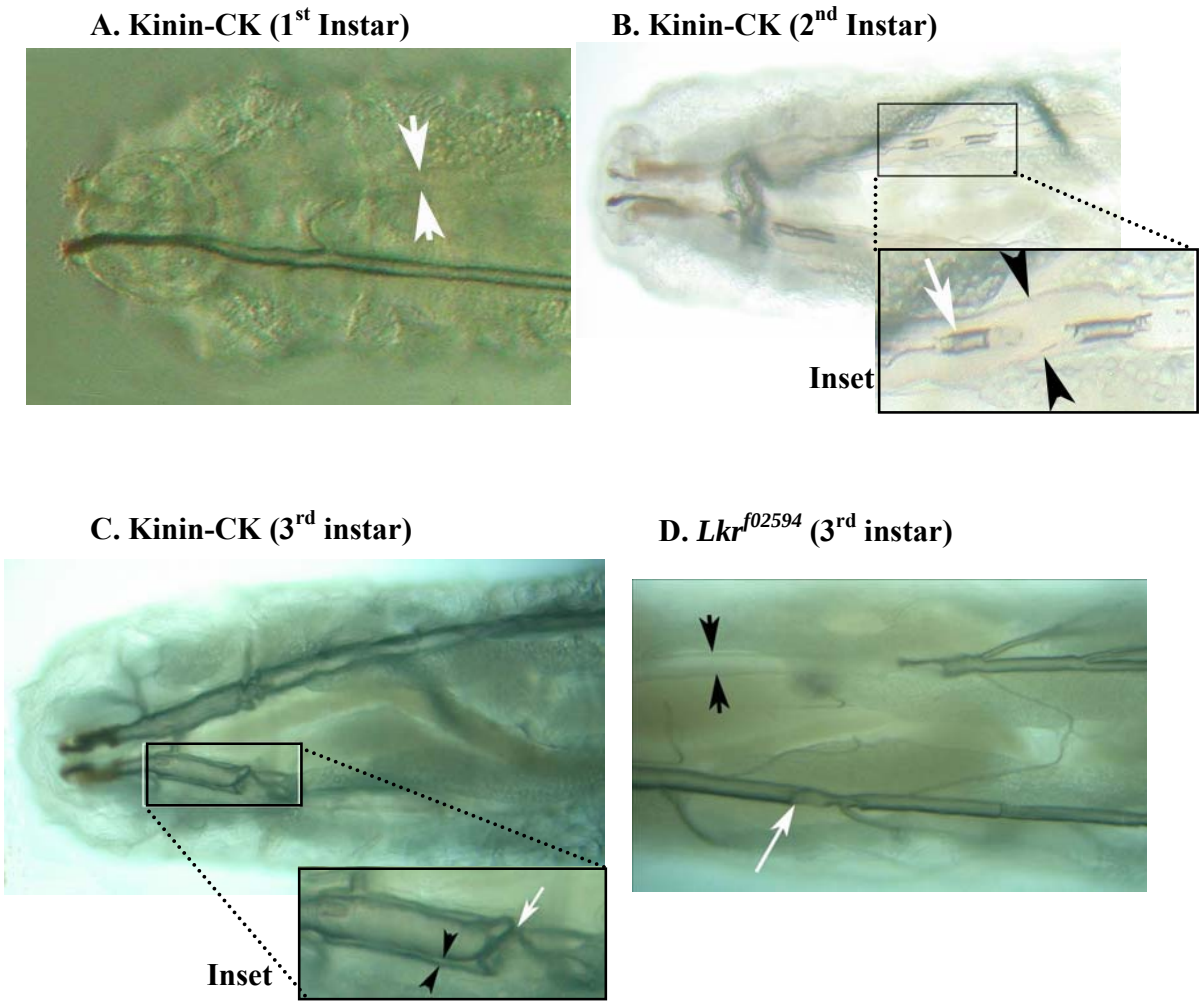


Figure 2.10. Impairment of kinin signaling causes incomplete removal of old trachea and breaks in new trachea in addition to tracheal inflation defects. (A) Tracheal inflation defects in kinin-CK 1st instar larva. (B) Old trachea (white arrow) inside of new trachea (black arrow head) in kinin-CK 2nd instar larva. (C) Old and new trachea (black arrow head) and break (white arrow) in kinin-CK 3rd instar larva. (D) break (white arrow) and tracheal inflation defects in *Lkr*^{f02594} 3rd instar larva. Some of 2nd and 3rd instar larvae of kinin-CK or *Lkr*^{f02594} failed complete removal of old trachea and have breaks in new trachea which implies defects in ecdysis behavior.

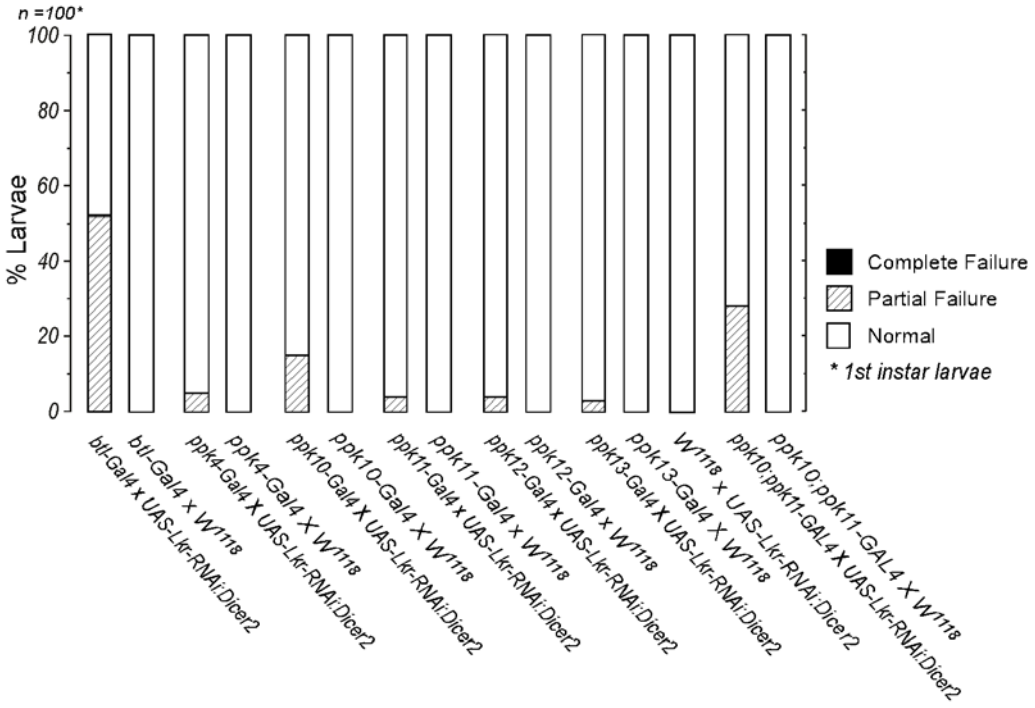


Figure 2.11. ENaC family pickpocket cells in trachea are involved in kinin signaling to regulate tracheal inflation. *Kinin receptor (Lkr)-RNAi* expression in trachea using *breathless(btl)-Gal4* showed tracheal inflation defect which implies the presence of kinin receptor in tracheal system. Defects in tracheal inflation by the expression of Lkr-RNAi in pickpocket (ppk) cells suggest that kinin regulates tracheal inflation through epithelial Na^+ channel (ENaC). All Gal4 or UAS fly lines used were homozygous lines.

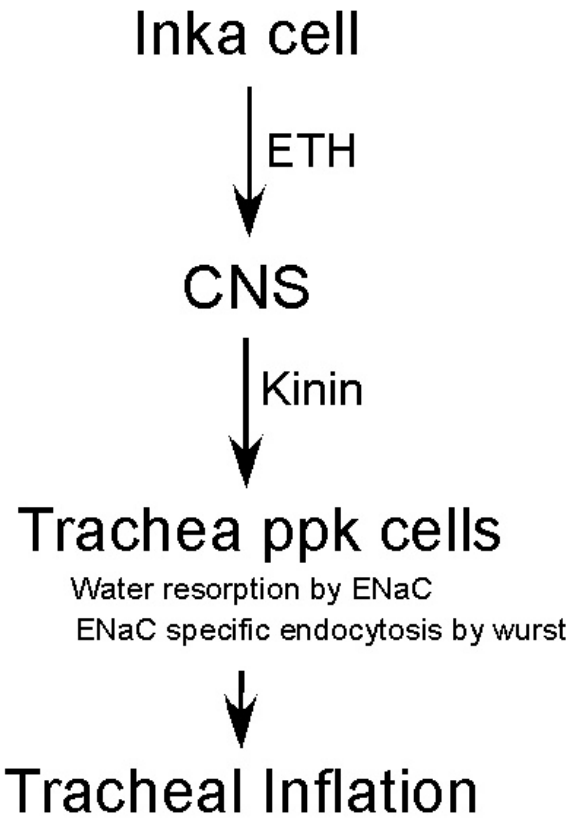


Figure 2.12. Suggested model for signaling cascade during tracheal inflation. Peptidergic ecdysis triggering hormone (ETH) released from Inka cell activates kinin neurons in central nervous system (CNS) to release another peptidergic kinin hormone into the body. The binding of kinin into kinin receptors in pickpocket cells in trachea induces water resorption and endocytosis in new trachea.

CHAPTER III

Analysis of an Innate Behavioral Sequence by Genetic Manipulation of a Peptidergic Signaling Cascade

ABSTRACT

Insect ecdysis is an example of an innate behavioral sequence performed as a fixed action pattern. The release of ecdysis-triggering-hormone (ETH) from endocrine Inka cells drives the stereotypical sequence of ecdysis-related behaviors through direct and sequential activation of peptidergic ETH receptor ensembles in the central nervous system (CNS). Here we show that targeted ablation of ETH receptor (ETHR) neurons, including those that express eclosion hormone (EH), FMRFamide, kinin, crustacean cardioactive peptide (CCAP), myoinhibitory peptide (MIP), burs, and bursicon (burs and pburs) alters scheduling of the ecdysis sequence. The results demonstrate that bursicon neurons are necessary for termination of pre-ecdysis and the onset of ecdysis, and that kinin is involved in scheduling of pre-ecdysis. Concurrent monitoring of neuronal activity by calcium imaging in kinin and bursicon neurons provides compelling evidence for sequential activation of ETH receptor neurons and functional roles of these neurons in ecdysis. To explain the sequential activation of ETHR neurons, a differential sensitivity model is proposed: variable sensitivity to ETH causes differences in activation timing of ETHR ensembles. Evidence supporting this model is provided through over-expression or silencing of ETHR in kinin and bursicon neurons, and by

observations of changes in behavioral scheduling that result from these manipulations. Our findings provide new evidence for functional roles of peptidergic ETHR neurons and mechanisms of sequential activation of peptidergic ensembles to schedule steps of the stereotypic ecdysis behavioral sequence.

INTRODUCTION

Innate behaviors, which result from coordinated activity of genetically determined circuits in the central nervous system, provide useful models for explaining complex brain functions. Most behaviors performed by insects are innate, including courtship, migration, foraging, and ecdysis. Among these behaviors, ecdysis represents a "chemically-coded" behavior triggered by the hormone ETH, which initiates and schedules behavioral steps through direct action on the CNS (Zitnan et al., 1996; Zitnan et al., 1999). Analysis of ecdysis behavior can provide a more thorough understanding of how hormones assemble and regulate behavioral circuitry.

ETH is synthesized and released by large peptidergic Inka cells under the precise control of the steroid hormone 20-hydroxyecdysone. The presence of Inka cells in more than 40 species of arthropods, along with the sequence similarity of ETH peptides in different insect groups, suggests that ETH signaling is highly conserved in insects. Identification of the Ecdysis Triggering Hormone receptor (ETHR) gene in *Drosophila melanogaster* provided a breakthrough, enabling elucidation of a complex downstream signaling cascade triggered by ETH (Park et al., 2003b). The ETHR gene encodes two functionally distinct subtypes of G protein coupled receptors (ETHR-A and -B) through

alternative splicing. The presence of two ETH receptor subtypes has been observed in all species thus far examined (Dai and Adams, 2009; Kim et al., 2006a; Park et al., 2003b). The two subtypes receptor show differences in ligand sensitivity and specificity and are expressed in separate populations of central neurons, suggesting that they have distinctive roles in ETH signaling.

A diversity of ETHR neurons in the moth *Manduca sexta* and fruitfly *Drosophila melanogaster* has been identified (Kim et al., 2006a; Kim et al., 2006b). One of the most striking properties of ETHR-A neurons is that they are virtually all peptidergic. We refer to groups of ETHR-A neurons as "peptidergic ensembles", which express a range of different neuropeptides, including kinins, diuretic hormone (DH), eclosion hormone (EH), FMFRamide hormone, crustacean cardioactive peptide (CCAP), myoinhibitory peptides (MIPs), bursicon (burs and pburs), neuropeptide F (NPF), and short neuropeptide F (sNPF). The release of ETH acts directly on the CNS to activate these peptidergic neurons for control of specific central pattern generator circuits that elicit stereotyped ecdysis behaviors.

Likely functions of certain ETHR-A peptidergic ensembles have been elucidated (Kim et al., 2006a). For example, serially homologous $L_{3/4}$ neurons of abdominal ganglia in *Manduca* express a cocktail of kinins and diuretic hormones. Co-application of these peptides elicits a fictive pre-ecdysis I-like motor pattern in the isolated CNS. Similarly, the IN704 peptidergic ensemble that co-expresses CCAP and MIPs is implicated in initiation of ecdysis behavior. Homologous peptidergic ensembles also were described in *Drosophila* (Kim et al., 2006b). ETH-induced activity of these ensembles was observed

through calcium imaging studies. As in *Manduca*, CCAP/MIP neurons in *Drosophila* appear to be involved in ecdysis, although the role of kinin neurons remained obscure. In calcium imaging studies, different ETHR ensembles became active at characteristic times following application of ETH. A sequential activation model was proposed to explain how different neuronal groups are involved in stepwise innate behavioral sequence.

Here we provide additional evidence for roles of ETHR-A ensembles in scheduling the ecdysis behavioral sequence, and propose a modified sequential activation model illustrating how these neurons regulate a stepwise sequence of behaviors.

MATERIALS and METHODS

Fly Strains. All flies were raised at 25˚C on standard cornmeal-agar media under a 12 hr light/dark regimen. *FMFRa(Tv)-Gal4* (Kim et al., 2006b) and *UAS-rpr,hid* flies were provided by Paul Taghert (Washington University, St Louis, MI). *UAS-GCaMP* (Wang et al., 2003) was obtained from Richard Axel (Howard Hughes Medical Institute, Columbia University, New York, NY). *UAS-ETHR-A-RNAi* and *UAS-Dicer2* was provided by Barry Dickson (IMP, Vienna) *CCAP-Gal4* flies (Kim et al., 2006b) were obtained from J. Ewer (Cornell University, Ithaca, NY). *Burs-Gal4*, and *pburs-Gal4* were provided by Jea-Hyun Park (Tufts University School of Medicine, Boston, MA). *kinin-Gal4*, *MIP-Gal4* and *UAS-ETHR-RNAi* were generated from our lab. Pburs;kinin combination Gal4 was generated from *pburs-Gal4* and *kinin-Gal4* by normal fly genetics.

Kinin receptor mutant flies (*PBac{WH}Lkr*^{f02594}) were obtained from Exelixis (Harvard University, Boston, MA). Other flies were obtained from Bloomington stock center (Indiana University, Bloomington, IN); *EHups-Gal4* (stock number, 6310), *UAS-rpr* (stock number, 5824), *UAS-mCD8-GFP* (stock number, 5137), w^{1118} (stock number, 5905). All Gal4 and UAS fly lines used in our experiments were homozygous lines except *UAS-ETHR-A-RNAi*, which has a GFP marker on the partner chromosome. After the crossing with Gal4 lines, non-GFP flies selected during larval stage and used for experiments.

RNA silencing of the ETH receptor was accomplished using two constructs, one directed specifically at ETHR-A (*UAS-ETHR-A-RNAi*) and another using a sequence that is common to both ETHR-A and ETHR-B subtypes (*UAS-ETHR-RNAi*).

Immunohistochemistry. We crossed the following strains of Gal4 transgenic flies with *UAS-mCD8-GFP* flies to produce progeny expressing GFP in peptidergic neurons and used them for GFP immunohistochemical staining: for eclosion hormone (EH), *EHups-Gal4* (McNabb et al., 1997); for FMRFamide, *FMFRa(Tv)-Gal4* (Kim et al., 2006b); for kinin, *kinin-Gal4* (Kim et al., 2006b); for crustacean cardioactive peptide (CCAP), *CCAP-Gal4* (Park et al., 2003a); for myoinhibitory peptide (MIP), *MIP-Gal4* (Kim et al., 2006b); for burs, *burs-Gal4*; for bursicon which is heterodimeric hormone with burs and partner of burs (pburs), *pburs-Gal4*. To generate cell-killing(CK) in specific target neurons, Gal4 transgenic flies were crossed to *UAS-rpr,hid*; *UAS-mCD8-GFP* (*UAS-*

RH;GFP) for the expression of apoptosis genes *rpr,hid* to kill target cells and the expression of GFP to confirm the death of target neurons.

The CNS Preparation for *In Vitro* Ca^{2+} Imaging. Calcium imaging experiments performed *in vitro* were performed in the pharate pupae of triply homozygous flies carrying *UAS-GCaMP, pburs-Gal4*, and *kinin-Gal4* in X, 2nd, and 3rd chromosome respectively. Correlation between sex and Ca^{2+} signal intensities have not been investigated. The pharate pupae (3~5 hr before the pupation) were used for all imaging studies. For the correct measurement of the stage of prepupae, None-buoyant prepupae [stage P3 (Bainbridge and Bownes, 1981)] were selected and buoyancy was tested every hour to select the prepupae that newly became buoyant as minus 6~5 hr pharate pupae. The CNS was dissected in fly saline under minimal light exposure, and placed in a chamber containing 300 μl of bathing media (fly saline). The test chamber consisted of a metal frame slide with 9 mm hole in the center. The floor of the chamber was provided by glass cover slip, which was discarded after each experiment. In previous study (Kim et al., 2006b), low-melting agarose was used to immobilize the isolated CNS. We recently found that the CNS sticks well to the new cover glass installed prior to each experiment. We therefore used this agarose-free strategy, which eliminated delay arising from slow diffusion of ETH peptides through agarose. CNS preparations showing spontaneous activity were discarded. Such preparations usually resulted from damaged to the CNS that occurred during dissection; this occurred in less than 15% of the preparations.

Imaging Setup and Calcium Imaging. We used an imaging setup (TILL Photonics) consisting of a xenon lamp and a monochromator as light sources and a CCD camera. The microscope (Olympus) was equipped with a 40 X W NA 0.8 objective. Binning on the chip (8 X 8) was set to give a spatial sampling rate of 1 μm/pixel (image size 172 X 130 pixels, corresponding to 172 μm and 130 μm). Images were taken at a rate of 1 Hz. The excitation wavelength was 488 nm, and exposure time was 25 msec. Fluorescent light passing an excitation filter (370-510 nm) was directed onto a 500 nm DCLP mirror followed by a 515 LP emission filter for EGFP. 1 hr long continuous images were acquired from each CNS preparation and ETH was applied into a bathing media ~5 min after imaging onset. The volume of applied ETH was 3.6 μl. We used cocktail of ETH1 and ETH2 for all experiment. 300nM ETH (300nM of ETH1 and ETH2 each) or 600nM ETH was applied on the bathing media with micropipette. Fluorescence intensity was calculated as ΔF/F; the mean fluorescence over the entire 100 frames was taken, for each pixel, as an estimate for F.

Behavioral Analysis of Pupal Ecdysis. Transgenic flies that have targeted ablation in subset of ETH receptor neurons were generated by crossing various Gal4 lines to *UAS-RH;GFP*. Their genotypes are *UAS-rpr,hid/+*; *EH-Gal4/UAS-mCD8-GFP* (for EH-CK), *UAS-rpr,hid/+*; *FMFRa(Tv)-Gal4/UAS-mCD8-GFP* (for FMFRa-CK), *UAS-rpr,hid/+; UAS-mCD8-GFP/+; kinin-Gal4/+* (for kinin-CK), *UAS-rpr,hid/+*; *CCAP-Gal4/UAS-mCD8-GFP* (for CCAP-CK), *UAS-rpr,hid/+*; *MIP-Gal4/UAS-mCD8-GFP* (for MIP-CK),

UAS-rpr,hid/+; *burs-Gal4/UAS-mCD8-GFP* (for burs-CK), *UAS-rpr,hid/+*; *pburs-Gal4/UAS-mCD8-GFP* (for pburs-CK). The heterozygous flies carrying Gal4 only or carrying *UAS-rpr,hid/+; UAS-mCD8-GFP/+*were produced as controls. For the behavioral analysis of each fly line, we collected buoyant pharate pupae (~ 2 hr before pupal ecdysis) [late stage P4(i) (Bainbridge and Bownes, 1981)] and placed 5~8 pharate pupae ventral-side up in a small recording chamber containing wet filter-paper strips. Ecdysis recordings were done at normal speed under dissection microscope (Wild Heerbrogg) using an ExwaveHad digital color video-camera (Sony) and HITACHI Kokusai electric color CCD camera attached to RD-XS34SU or RD-XS35SU video recorder (Toshiba).

Statistics. Changes in pre-ecdysis duration observed in EH-CK flies were compared with those of control flies using Student's *t-test* ($p < 0.01$). Statistical analyses performed on ETHR over-expression and suppression data sets were assessed using the non-parametric Mann-Whitney test. In instances where the number of replicates was low, a series of Mann-Whitney Rank Sum tests were performed to compare changes in pre-ecdysis duration in test groups to control groups at the 95% significance level ($p < 0.05$).

RESULTS

Targeted Ablation of ETH Receptor Neurons Alters the Ecdysis Behavioral Sequence. Scheduling of behavioral steps in the ecdysis sequence observed in whole animals is correlated with sequential activation of ETH receptor neurons in the CNS.

Characteristic activity patterns exhibited by central ETHR peptidergic ensembles reported previously (Kim et al., 2006b) suggests that each ensemble drives a particular behavioral step during the ecdysis sequence. To verify the importance of each peptidergic ensemble in the ecdysis sequence, we investigated pupal ecdysis behaviors of transgenic flies that have targeted ablation of specific ETHR-A peptidergic ensemble using the binary expression system Gal4/UAS. For specific cell-killing, we used *FMFRa(Tv)-Gal4*, *EH-Gal4*, *kinin-Gal4*, *CCAP-Gal4*, *MIP-Gal4*, *burs-Gal4*, and *pburs-Gal4*. Locations of these peptidergic ensembles in pharate pupal CNS are shown in Figure 1.1. These flies were crossed to *UAS-rpr,hid; UAS-GFP* (*UAS-RH;GFP*) for expression of apoptosis genes *rpr* and *hid* in targeted neurons; disappearance of GFP in these neurons provided verification of cell death. We also checked for persistence of targeted neurons by immunohistochemical staining in the prepupal CNS (Figure 3.1). Death of targeted cells was confirmed by comparison with control flies having GFP-expression in eclosion hormone (EH), FMRFamide, kinin, crustacean cardioactive peptide (CCAP), myoinhibitory peptide (MIP), burs and pburs. Since *FMRFa(Tv)-Gal4* is expressed only in three pairs of thoracic ventrolateral neurons, other FMRFamide neurons were not observed in control flies.

Changes in scheduling of the pupal ecdysis behavioral sequence in each ETHR-CK fly line were monitored to ascertain their possible roles in scheduling of the ecdysis sequence (Figure 3.2). In control flies carrying only Gal4 or *UAS-RH;GFP*, pupal ecdysis consisted of three distinctive sequential behaviors: pre-ecdysis (~10 min), ecdysis (~12 min) and post-ecdysis (~1 hr) as described previously (Kim et al., 2006b).

FMRFa(Tv)-CK flies showed no measurable abnormalities. Since *FMRFa(Tv)-Gal4* drives expression of Gal4 only in three pairs of thoracic Tv neurons, FMRFa(Tv)-CK flies lost only Tv neurons (Kim et al., 2006b). Transgenic flies bearing targeted cell-killing of EH neurons (EH-CK) showed a significant delay (6.0 min delay, $p < 0.01$) in ecdysis onset with quite high variation, leading to increased duration of pre-ecdysis. Despite the delay in ecdysis onset, EH-CK flies completed pupal ecdysis and did not show any severe defects in development. No other abnormalities in pupal ecdysis were observed.

Defects in Kinin Signaling Alters the Ecdysis Behavioral Schedule. Kinin-CK flies exhibited several interesting defects in pre-ecdysis and ecdysis behaviors (Figure 3.2, Figure 3.3). First of all, kinin-CK flies showed very weak pre-ecdysis and ecdysis behaviors. Pre-ecdysis and ecdysis contraction frequencies in kinin-CK flies were significantly lower (~ 0.8 and 1.9 times per min, respectively) than those of controls (~ 3.6 and 2.3 times per min, respectively). Kinin-CK flies also showed highly variable pre-ecdysis duration (SD: ± 5.47 min). During ecdysis, animals spend more time engaged in rhythmic contractions before the head eversion (3.16 min in kinin-CK vs. 1.20 min in control) and ~25% of individuals failed head eversion completely (HE, 8 animals out of 31). Pupae that failed to perform head eversion exhibited either no pre-ecdysis or very long pre-ecdysis.

To further explore the possible involvement of kinin signaling in regulation of the ecdysis sequence, we examined kinin receptor mutant flies (Lkr^{f02594}) carrying a

piggyBac insertion in exon 1 of kinin receptor gene. We found that Lkr^{f02594} is hypomorphic mutant (see Chapter II). Ecdysis behavioral sequences and individual pre-ecdysis durations of kinin-CK and Lkr^{f02594} are illustrated in Fig. 3.4. In kinin-CK flies, many animals skipped pre-ecdysis and initiated ecdysis behaviors abruptly (no pre-ecdysis: 8 animals). Other animals showed variable pre-ecdysis duration from 2.8 min to 21.9 min. Pre-ecdysis durations for kinin receptor mutant flies also were highly variable, even though the variability (SD: ± 4.76 min) was lower than for kinin-CK (SD: ± 5.47 min). One of the kinin receptor mutants failed to perform head eversion and died. Behavioral comparisons of kinin-CK and kinin receptor mutants led us to conclude that high variability of pre-ecdysis was an actual characteristic phenotype caused by a defect in kinin signaling.

Bursicon Neurons (AN1-4) are Necessary for Termination of Pre-ecdysis and Onset of Ecdysis and Post-ecdysis. CCAP neurons that express ETHR-A are known to be critical for ecdysis behavior (Kim et al., 2006a; Kim et al., 2006b). To pinpoint functional roles for CCAP neurons or subsets thereof in ecdysis behavior, we performed selective cell-killing using the following Gal4 lines: *CCAP-Gal4, MIP-Gal4, burs-Gal4, pburs-Gal4*. We observed remarkable defects in behavioral sequences of CCAP-CK, MIP-CK, burs-CK, pburs-CK flies. All CK flies failed to initiate ecdysis and post-ecdysis behaviors and head eversion. All CK flies also failed to terminate pre-ecdysis behavior at the normal time, resulting in prolonged pre-ecdysis duration. After performing pre-ecdysis contractions for about 10 min, contractions gradually decreased

in intensity and eventually ceased altogether. Prolonged pre-ecdysis was followed by weak, random contractions that bore no resemblance to normal ecdysis and post-ecdysis behaviors. Each CK group showed a different pre-ecdysis duration: 23.1 ± 4.76 min in CCAP-CK, 18.2 ± 3.31 min in MIP-CK, 24.4 ± 4.97 min in Burs-CK, 26.1 ± 5.27 min in pburs-CK. However, all showed complete absence of ecdysis and post-ecdysis behaviors. Failure of normal pre-ecdysis termination and absence of ecdysis and post-ecdysis behaviors in pburs-CK flies (AN1-4) indicate that this group of neurons is the key to the signaling that underlies the transition from pre-ecdysis to ecdysis (Figure 3.2).

Activation Timing of Two ETHR Ensembles Monitored by Ca^{2+} Imaging.

Expression of ETHR-A occurs in several distinct classes of peptidergic neurons. These primary targets of ETH comprise a downsteam peptidergic signaling cascade that activates central pattern generators sequentially to schedule the ecdysis behavioral sequence. In a previous study, ETH-induced calcium dynamics were monitored in several subgroups of ETHR neurons . Results of these investigations indicated that ETHR groups become active following variable delays (Kim et al., 2006b). A sequential activation model was proposed to explain how activity in diverse ETHR-A neurons is organized to schedule each behavioral step. This model was based on average delays established in separate fly lines, each expressing the calcium reporter in a single peptidergic ensemble. In this study, I sought to verify the sequential activation model by monitoring calcium dynamics in two distinct ETHR-A ensembles within the same fly line.

To accomplish this, I generated transgenic flies that express Gal4 in two different ETHR groups – bursicon and kinin – to monitor calcium dynamics in both sets of neurons. A *pburs* gene-specific promoter line (*pburs-Gal4*) was used to drive expression of GCaMP (*UAS-GCaMP*) in bursicon neurons in AN1-4 (Luo et al., 2005). *Kinin-Gal4* line was used to drive expression of GCaMP in kinin neurons in AN1-7. Gal4 expression in both kinin and bursicon neurons was confirmed by immunohistochemical staining (Figure 3.4). Besides observing staining in cell bodies of these distinct peptidergic ensembles, projections of kinin neurons into the terminal plexus (TP) in AN9 were visualized (Santos et al., 2007).

We monitored calcium dynamics in bursicon and kinin neurons by driving expression of the GFP-based Ca^{2+} sensor, G-CaMP in these neurons using the Gal4/UAS system (Duffy, 2002; McGuire et al., 2004). G-CaMP is a circularly permutated EGFP having the M13 fragment of myosin light chain kinase on the N-terminus and the calcium binding motif of calmodulin on the C-terminus (Nakai et al., 2001; Pologruto et al., 2004; Tian et al., 2009). Calcium elevation in the cell induces a conformational change of G-CaMP through binding of calmodulin to the target site M13 fragment, resulting in increased GFP fluorescent intensity. Through measurement of fluorescence changes, it is possible to monitor changes in calcium levels in targeted ETHR neurons following exposure to ETH. Since the peptides ETH1 and ETH2 are released from Inka cells under natural conditions, (Park et al., 2002), we used a cocktail of ETH1 and ETH2 (300 nM or 600 nM each) in all *in vitro* experiments.

ETH Induces Calcium Dynamics in Kinin and Bursicon Neurons Sequentially. To monitor $[Ca^{2+}]i$ levels in bursicon and kinin neurons, we generated transgenic flies bearing *pburs-Gal4*, *kinin-Gal4* and *UAS-GCaMP* transgenes. The CNS dissected from pharate pupal flies of this line 3-5 hr prior to ecdysis onset showed moderate levels of G-CaMP fluorescence in cell bodies and TP (Figure 3.5, B1). Following exposure to 300 nM or 600 nM ETH, cell bodies and TP showed a robust increase in fluorescent intensity, indicating an increase in cytoplasmic $[Ca^{2+}]i$ levels (Figure 3.5; Figure 3.7). More interestingly, kinin and bursicon neurons were activated sequentially; kinin neurons became active first and bursicon neurons became active almost immediately after termination of kinin neuron activity.

Upon exposure to 300 nM ETH, all kinin neurons became active simultaneously (average ± standard deviation, SD: 8.4 ± 1.43 min) and showed characteristic in-phase spike-like activity patterns (Figure 3.5; Figure 3.6). Calcium dynamics in all kinin neurons were highly synchronized, suggesting that they are electrically coupled. Transient increases and decreases of calcium levels in cell bodies and TP were synchronous, although intensities varied. In addition, transient increases in $[Ca^{2+}]i$ were observed first in the TP several seconds before increases in cell bodies occurred. Synchronized spike-like activities of kinin neurons lasted for 9.1 ± 3.64 (n=10) min, and activity in all neurons terminated simultaneously (17.5 ± 2.94 min) (Figure 3.5, A, B2; Figure 3.6). In our experimental setup, $\Delta F/F$ values reached up to 20.5 ± 7.34 % in response to 300 nM ETH over a base line noise level of less than 3%. Activation of bursicon neurons followed by termination of kinin activity within 3 minutes. Onset of

calcium dynamics in bursicon neurons was generally synchronized (20.0 ± 2.24 min), but not as tightly as that observed in kinin neurons. In bursicon neurons, ΔF/F reached up to 28.9 ± 8.17 % over a base line noise level of less than 3%. Unlike kinin neurons, ETH-induced calcium dynamics in bursicon neurons remained high for more than 1 hr.

Following exposure to higher ETH1 and ETH2 (600 nM each), activation patterns of both neurons were accelerated (Figure 3.7). Kinin neurons became active within 6.0 ± 1.39 min (duration: 5.0 ± 2.41 min) and bursicon neurons initiated activity within 12.0 ± 3.34 min (Figure 3.7, B). Compared to kinin neurons, calcium dynamics in bursicon neurons showed a larger range of intensity. Increasing ETH concentration from 300 nM to 600 nM decreased the latency to calcium mobilization from 11.6 min to 6.0 min. Even though higher ETH concentrations had a larger effect on onset timing in bursicon neurons, sequential activity in kinin and bursicon neurons was maintained. Exposure to 600 nM ETH elicited the same pattern of synchronized spike-like activity in kinin neurons as was observed at the lower ETH concentration (300 nM), but intervals between the peaks were reduced (Figure 3.7, A). Even though neurons in the brain and SOG express kinin (de Haro et al., 2009; Herrero et al., 2007), these neurons did not respond to ETH (data not shown). In our experimental setup, ΔF/F values reached up to 23.6 ± 7.33 % in response to 600 nM ETH over a base line noise level of less than 3%. Bursicon neurons also showed a similar activity pattern and duration as that observed following exposure to 300 nM ETH. Although some bursicon neurons showed activity before the end of kinin activity (example: AN3L in Figure 3.7, A), most bursicon

neurons became active only after termination of kinin neuron activity. In bursicon neurons, ΔF/F reached up to 24.2 ± 7.47% over a base line noise level of less than 3%.

Altered ETH Receptor Expression Levels in Kinin Neurons Change the Timing of Pre-ecdysis Onset. To further investigate the underlying mechanisms for sequential activation of ETHR neurons, we hypothesized that there are differences in sensitivity to ETH in different ETHR ensembles. To test this hypothesis, we tested changes in ecdysis behavioral sequence by either overexpression or suppression of ETHR in kinin neurons, which are involved in onset of pre-ecdysis. We made transgenic flies carrying *kinin-Gal4* and *UAS-ETHR-A* to over-express ETHR and flies carrying *kinin-Gal4, UAS-Dicer2* and *UAS-ETHR-A-RNAi* or *UAS-ETHR-RNAi* to suppress ETHR expression. Since kinin is involved in pre-ecdysis regulation, changes in pre-ecdysis onset timing by ETHR over-expression or suppression are shown as changes in pre-ecdysis duration (Figure 3.9). Pre-ecdysis durations were longer than normal in flies bearing *kinin-Gal4* (3) and *UAS-ETHR-A*(1) or *UAS-ETHR-A*(3) (12.4 ± 0.28 min and 10.8 ± 0.26 min S.E.M). Changes are more dramatic when the copy number of *kinin-Gal4* is doubled by combining expression on chromosomes 2 and 3 [*kinin-Gal4(2,3)*] (13.1 ± 0.27 min S.E.M).

The effect of suppressing ETHR by driving ETHR-RNAi in kinin neurons was apparent as a shortening of pre-ecdysis duration using double *kinin-Gal4(2,3)* and *UAS-Dicer2;UAS-ETHR-RNAi* (8.6 ± 0.21 min S.E.M). Pre-ecdysis durations in control groups ranged from 9.5 to 9.7 min. The means of test groups were significantly different

from controls at $p < 0.05$. Average contraction numbers during pre-ecdysis were similar in all groups (from 3.6 ± 0.05 min to 3.8 ± 0.07 min S.E.M).

Changes of ETH Receptor Expression Level in Bursicon Neurons Cause Alterations of the Timing in Ecdysis Onset. I next asked whether changes in ETHR density in bursicon neurons influences scheduling of the ecdysis sequence. To answer this question, ETHR was over-expressed in flies carrying *pburs-Gal4* and *UAS-ETHR-A*. Expression of ETHR was suppressed in flies carrying *pburs-Gal4* and either *UAS-Dicer2* and *UAS-ETHR-A-RNAi* or *UAS-Dicer2* and *UAS-ETHR-RNAi* to suppress ETHR expression. Changes in ecdysis onset timing by ETHR over-expression or suppression were measured as changes in pre-ecdysis duration (Figure 3.10).

In flies that over-express ETHR, pre-ecdysis duration is shortened. Mean pre-ecdysis duration in one *UAS-ETHR-A* line was 6.3 min ± 0.27 (S.E.M) and 6.7 (±1.95 S.E.M) min in a second line.

One the other hand, RNA silencing of ETHR in bursicon neurons resulted in prolonged pre-ecdysis behavior. When ETHR-A was suppressed selectively, (*pburs-Gal4/UAS-ETHR-A-RNAi*), mean pre-ecdysis duration 12.3 ± 0.37 min. In a second line carrying an RNAi construct designed to suppress both ETHR-A and ETHR-B, (*pburs-Gal4/UAS-ETHR-RNAi*), mean pre-ecdysis duration was 12.0 ± 0.38 min. These mean values for pre-ecdysis duration were significantly different that those in the control fly lines (9.2 ± 0.31 min to 9.7 ± 0.16 min). The means of test groups are significantly different from controls at $p < 0.05$. Average contraction numbers during pre-ecdysis were similar in all groups (from 3.5 ± 0.05 min to 3.7 ± 0.07 min S.E.M).

DISCUSSION

Ecdysis triggering hormone (ETH) acts directly on the CNS to initiate and schedule the ecdysis behavioral sequence. Through targeted ablation of specific ETHR subgroups, we provided phenotypic evidence showing that different ETHR neurons are involved in different behavioral steps. To understand the organization of ETH signaling in the CNS, we demonstrated sequential activation of two ensembles of ETH receptor neurons that express either kinin or bursicon, not by the comparison of relative activation times in different animals, but by direct comparison of sequential activities in the same animals. Finally, we modified ETH receptor density in different ETHR neurons and observed changes in behavioral scheduling, which demonstrates that different sensitivities to ETH in different ETHR ensembles could account for sequential activation of these neurons.

Targeted Ablations ETHR-A Peptidergic Ensembles Causes Distinct Behavioral Defects. To investigate functional roles of ETHR neurons in ecdysis behavior, we selectively ablated peptidergic ensembles and observed changes in scheduling of the behavioral sequence. In FMRFa-CK flies, we found no measurable alterations of ecdysis scheduling. On the other hand, EH-CK flies exhibited a significant delay in ecdysis onset, but this did not result in ecdysis failure or lethality. We found more severe defects in behavior or even lethality when kinin or subsets of CCAP neurons were ablated.

Therefore it is clear that these different ETHR neurons tested have different roles during ecdysis.

Bursicon Neurons (AN1-4) Are Necessary for Pre-ecdysis Termination and Ecdysis Onset. The most striking behavioral defects occurred following targeted ablation of CCAP neurons and subsets thereof. Through a series of cell-ablation experiments in subgroups of CCAP neurons, we found that pburs-CK flies fail to terminate pre-ecdysis at the proper time and failed to initiate ecdysis and post ecdysis behavior. These defects occurred even though many CCAP and MIP neurons were unaffected. We therefore conclude that bursicon neurons in AN1-4 are key to the transition between pre-ecdysis and ecdysis. This is surprising because it is generally assumed that bursicon is a postecdysis signal critical for cuticle plasticization and sclerotization (Barvara et al., 1995; Peabody et al., 2008; Taghert and Truman, 1982). Bursicon (from the Greek *bursikos*, meaning tanners) was described more than 40 years ago in association with tanning in neck-ligated flies (Fraenkel and Hsiao, 1962; Fraenkel et al., 1966), but its molecular nature was only recently elucidated (Dai et al., 2008; Dewey et al., 2004; Luo et al., 2005). Interestingly, *Drosophila* thoracic neuromeres 3 (TN3) and AN1-7 express burs, which is one of the monomers that, together with pburs, comprise the heterodimeric hormone bursicon. Pburs is, however, co-expressed with burs in only four abdominal neuromeres 1-4, which produce the functional hormone bursicon. Bursicon-CK flies failed to start both ecdysis and post-ecdysis, suggesting that bursicon neurons are critical for initiation and scheduling of both behaviors. Ca^{2+} imaging studies support this

conclusion because, under normal circumstances, bursicon neurons remain active for over one hour, which would correspond to both ecdysis and post-ecdysis.

In *Manduca*, CCAP and MIP are key hormones involved in initiation of ecdysis behavior (Kim et al., 2006a). However, our data from *Drosophila* studies supports the notion that bursicon also is involved in ecdysis initiation. Independent studies showed that knockout of the *pburs* gene show defects in pupal ecdysis similar to those observed here, whereas knockout of the *CCAP* gene did not (John Ewer, Cornell University, unpublished data). Nevertheless, it is also known that defects larval ecdysis defects occur following CCAP cell-killing (Park et al., 2003a). In *Drosophila*, it may be that neurons releasing CCAP, MIP or burs play modulatory roles in ecdysis.

Even though pupal ecdysis defects were severe after killing of bursicon neurons, we observed no mortality during larval stages. This suggests that specific aspects of the hormonal regulation of larval and pupal ecdysis differ. It will be interesting to investigate the specific defects in pupal ecdysis that result from ablation of bursicon neurons.

ETHR neurons are activated sequentially by direct action of ETH. Following discovery of the ETHR gene and identification of peptidergic ETHR-A neurons, calcium imaging experiments demonstrated that distinct peptidergic ensembles became active at different times during the ecdysis behavioral sequence (Kim et al., 2006b). These studies suggested that scheduling of each behavioral step involved activation of different ETHR-A ensembles, which became active after characteristic latencies after ETH

exposure. While this model implicates EH neurons and different groups of CCAP neurons in activation of ecdysis and post-ecdysis, no obvious peptidergic ensemble was connected to activation of pre-ecdysis behavior.

To further test and expand this model, we investigated activity patterns of two different ETHR-A neuronal ensembles: kinin and bursicon. By monitoring calcium dynamics of both ensembles in the same animals, we observed that kinin neurons are invariably activated first and that their activity ceases prior to onset of activity in bursicon neurons. Stepwise activation of kinin and bursicon neurons was observed following exposure of the CNS to 300 nM ETH. At higher ETH concentrations (600 nM), onset of activity was accelerated, but the sequential activation pattern of kinin following by bursicon neurons was maintained. At 600 nM ETH, overlap between kinin neuron and bursicon neuron activities did not occur, even though acceleration of bursicon neuron activity onset was much larger than that of kinin neurons. Instead, activity of kinin neurons stopped earlier, suggesting that bursicon neurons are simultaneously involved in terminating activity in kinin neurons and activation of ecdysis behavior. This hypothesis is supported by the increase in pre-ecdysis duration that occurs in pburs neuron-CK flies.

Activity patterns of kinin and bursicon ensembles are quite different. Kinin neurons are activated in a highly synchronized way. All neurons exhibit synchronous, spike-like patterns of Ca^{2+} dynamics, which return to baseline. Although the intensity of calcium dynamics in each neuron was variable, onset and termination of activity in all kinin neurons were almost identical. These data suggest that kinin neurons are

electrically coupled. Such synchronous activity is reminiscent of synchronized activity patterns in the FMRFa Tv neurons reported previously (Kim et al., 2006b).

In contrast, calcium dynamics in individual bursicon neurons were not synchronous, but average onset and termination times were similar. Activation of bursicon neurons started with an abrupt increase of fluorescence intensity, which resulted in an overall shift of the baseline for up to 60 min, during which time fluorescence gradually returned to baseline values. In contrast, activity in kinin neurons was of relatively short duration (9.1 min in 300 nM ETH and 5.0 min in 600nM ETH). The activation pattern and timing of bursicon neurons were different from those described in a previous study (Kim et al., 2006b). We found that use of agarose to immobilize the CNS by Kim et al. causes delays in ETH action on the CNS (unpublished data). Here, we employed new techniques to hold CNS in place without the use of agarose, which allowed easier access of ETH to the CNS and shorter latencies to neuronal activation.

Calcium Imaging and Targeted Cell-Killing Supports a Role for Kinin Neurons in Regulation of Pre-ecdysis. In kinin-CK and kinin receptor mutant flies, we observed a variety of behavioral defects. Weak pre-ecdysis and ecdysis behaviors and highly variable pre-ecdysis duration suggest that kinin signaling is critical for proper scheduling of the ecdysis behavioral sequence. However, phenotypic evidence did not provide a clear answer for the role of kinin in behavioral scheduling. Abnormal pre-ecdysis duration can be brought about either by variable pre-ecdysis onset or by variable ecdysis onset timing. If kinin is involved only in pre-ecdysis, loss of control over pre-ecdysis

duration in kinin-CK flies would occur because of variable pre-ecdysis onset. Defects in ecdysis (delay in HE and low contraction number) would be an indirect outcome of weak pre-ecdysis behavior. On the other hand, if variable ecdysis onset stems from variable pre-ecdysis duration, kinin would be involved in both pre-ecdysis and ecdysis behavioral sequence. Luckily, we have *in vitro* Ca^{2+} imaging information about activation timings of kinin and bursicon neurons and phenotypic evidence of the role of bursicon. In the Ca^{2+} imaging study, kinin neurons are activated and terminated before the activation of bursicon. And we now know that bursicon is critical for ecdysis onset based on the defects in behavior of pburs-CK. Therefore we can conclude that kinin is actually involved in pre-ecdysis and that variable pre-ecdysis duration of kinin-CK results from variable pre-ecdysis onset (Figure 3.8).

Originally, kinin was associated with myotropic and diuretic functions (Hayes et al., 1989; Holman, 1986). However discovery of ETHR-A expression in kinin neurons and data presented in this work implicates them in regulation of the ecdysis sequence. The likely role of kinin in *Drosophila* pre-ecdysis is further supported by a similar function in *Manduca*, where co-application of kinin and diuretic hormone causes pre-ecdysis-like fictive motor patterns in the isolated CNS (Kim et al., 2006a).

Targeted Cell-killing Provides New Insights in Ca^{2+} imaging data. In *Drosophila* pupal ecdysis, it was suggested that bursicon is involved in postecdysis, based on Ca^{2+} dynamics of neurons 27/704 in abdominal neuromeres 1-4 (AN1-4) (Kim et al., 2006b). However, cell ablation data from the present work indicates that bursicon also is

involved in ecdysis initiation (Figure 3.2). Since now we know the roles of kinin and bursicon, we have new insights in the analysis of Ca^{2+} imaging data. Previously, we compared relative activation time of ETHR ensembles to ecdysis behavioral sequence for prediction of behavioral roles of these neurons. However, the phenotypic evidence and concurrent imaging of kinin and bursicon neurons in the same animal suggests that the relative time between different ETHR groups (kinin and bursicon) is more meaningful than the time from ETH application to activation of neurons.

In cell-killing experiments, we found that bursicon-CK flies failed to terminate pre-ecdysis at the correct time. This suggests that activity in bursicon neurons is involved in termination of pre-ecdysis. This phenotypic evidence helps us understand the lack of overlap in calcium dynamics observed in kinin and bursicon neurons. Whether the interaction between bursicon neurons and kinin neurons is direct or indirect remains to be demonstrated.

Differential Sensitivity of ETHR Neurons to ETH May Contribute to Sequential Activation of Calcium Dynamics and Behavioral Scheduling. We observed that kinin and bursicon neurons have characteristic differences in latency to calcium dynamics following ETH exposure, even though they both express ETHR-A. It was proposed previously that these different latencies might be due to differential sensitivities of the neurons involved (Kim et al., 2006b). In *Manduca* ETH levels in the hemolymph are dynamic, ranging from low at the beginning of Inka cell release and high later (Zitnan, et al., 1999). Based on this, we hypothesized that peptidergic ensembles controlling pre-

ecdysis have higher sensitivity to ETH and hence might be activated earlier. Other peptidergic ensembles controlling ecdysis and post-ecdysis would have lower sensitivity to ETH and be activated later.

To test this differential sensitivity model, we altered the sensitivity of certain peptidergic ensembles by increasing or decreasing ETHR density. Over-expression of ETHR would increase the sensitivity to ETH, whereas ETHR-RNAi would decrease it. Previously in this chapter, we implicated kinin and bursicon neurons in pre-ecdysis and ecdysis behaviors, respectively. By using these two ETHR groups, we investigated the consequences of altering their sensitivities to ETH by making changes in ETHR density (Figure 3.9; Figure 3.10). If the model is correct, changes in ETHR sensitivity of kinin would alter the schedule of pre-ecdysis (Figure 3.11). The higher sensitivity of kinin by ETHR over-expression would lead to early onset of pre-ecdysis, which would make pre-ecdysis longer. Expression of ETHR-RNAi in kinin neuronn would decrease sensitivity to ETH and lead to late onset of pre-ecdysis, which would make pre-ecdysis shorter. On the other hand, increased sensitivity to ETH of bursicon neurons would cause early onset of ecdysis, which would make pre-ecdysis duration shorter. Decreased sensitivity by the expression of ETHR-RNAi would cause late onset of ecdysis, making pre-ecdysis duration longer (Figure 3.12). One critical point here is that, according to our hypothesis, anticipated outcomes of ETHR over-expression and suppression in kinin neurons would be opposite to those resulting from the same manipulations in bursicon neurons. The results obtained match these predictions, and therefore support our hypothesis. When ETHR-A densities in bursicon or kinin neurons were modified, we observed changes in

pre-ecdysis duration as predicted (Figure 3.9; Figure 3.10). The effect of over-expression and suppression of ETHR was confirmed by two different *UAS-ETHR-A* lines and *UAS-ETHR-RNAi* lines. Changes in pre-ecdysis duration were increased by doubling the copy number of *kinin-Gal4* (Figure 3.9). The direction of change in pre-ecdysis duration by ETHR over-expression in kinin was opposite to that of bursicon because they involved different steps in the ecdysis behavioral sequence. This was also true in the case of suppressing ETHR by RNAi expression in kinin and bursicon. The opposite effects from the altered ETHRs in kinin and bursicon support once more different roles of these neurons in ecdysis behavioral sequence. Previously, it was shown that the characteristic delay of ecdysis onset would be controlled by inhibitory input. Here, however, we proposed that timely activation of ecdysis neurons by differences in ETH sensitivity is also important. Since the transition of ecdysis behavioral steps is critical for successful development, insects may use both timely activation of ecdysis signal and inhibitory signals for the precise scheduling of ecdysis behavior.

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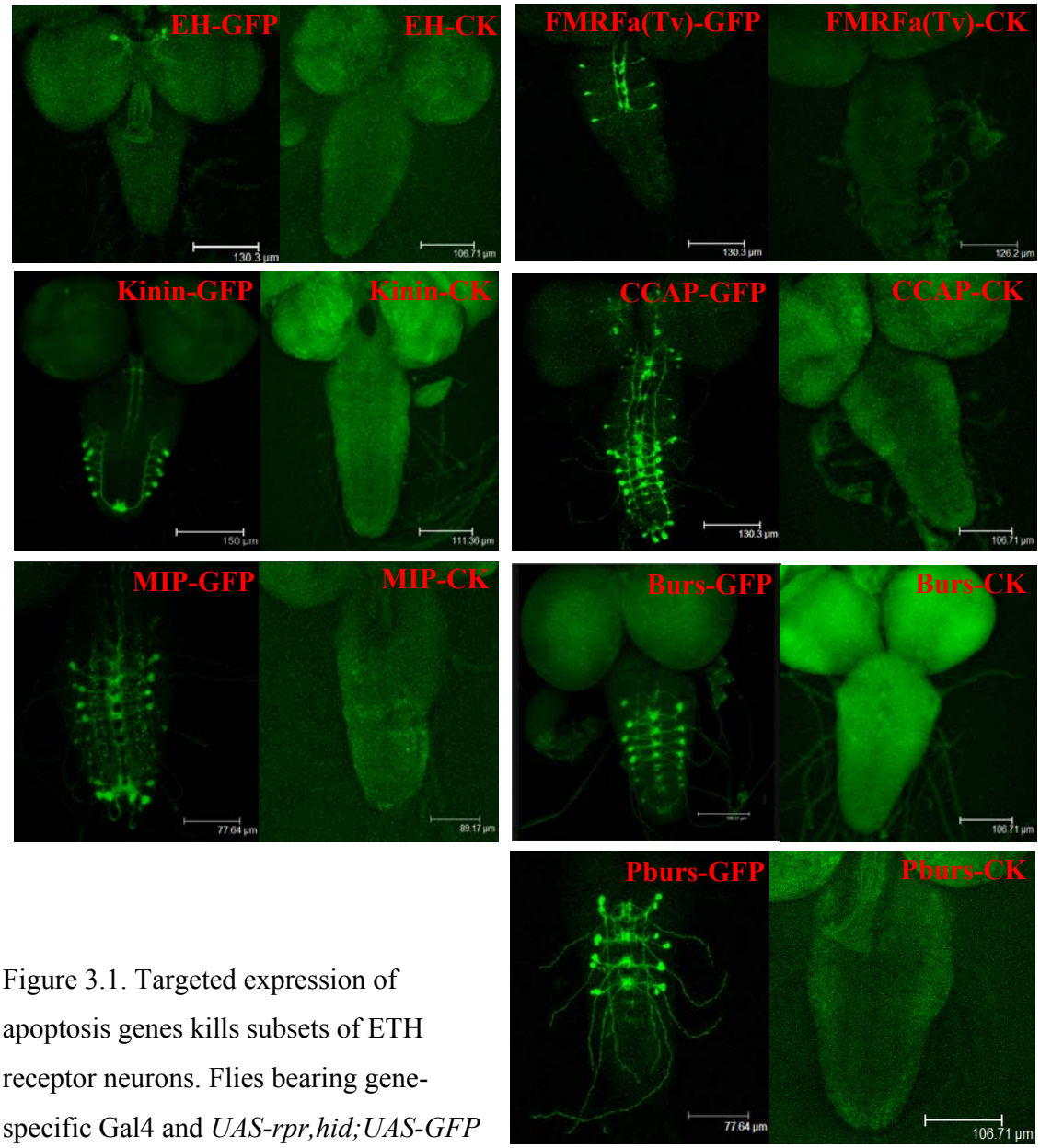

Figure 3.1. Targeted expression of apoptosis genes kills subsets of ETH receptor neurons. Flies bearing gene-specific Gal4 and *UAS-rpr,hid;UAS-GFP* were generated to verify targeted cell-killing (CK). The absences of each peptidergic neurons were confirmed by immunohistochemistry performed with antisera against GFP. The targeted neurons were completely ablated in targeted cell-killing flies but not in wild type. Note that *FMRFa(Tv)-Gal4* is expressed only in Tv neurons of FMRFamide neurosecretory cells.

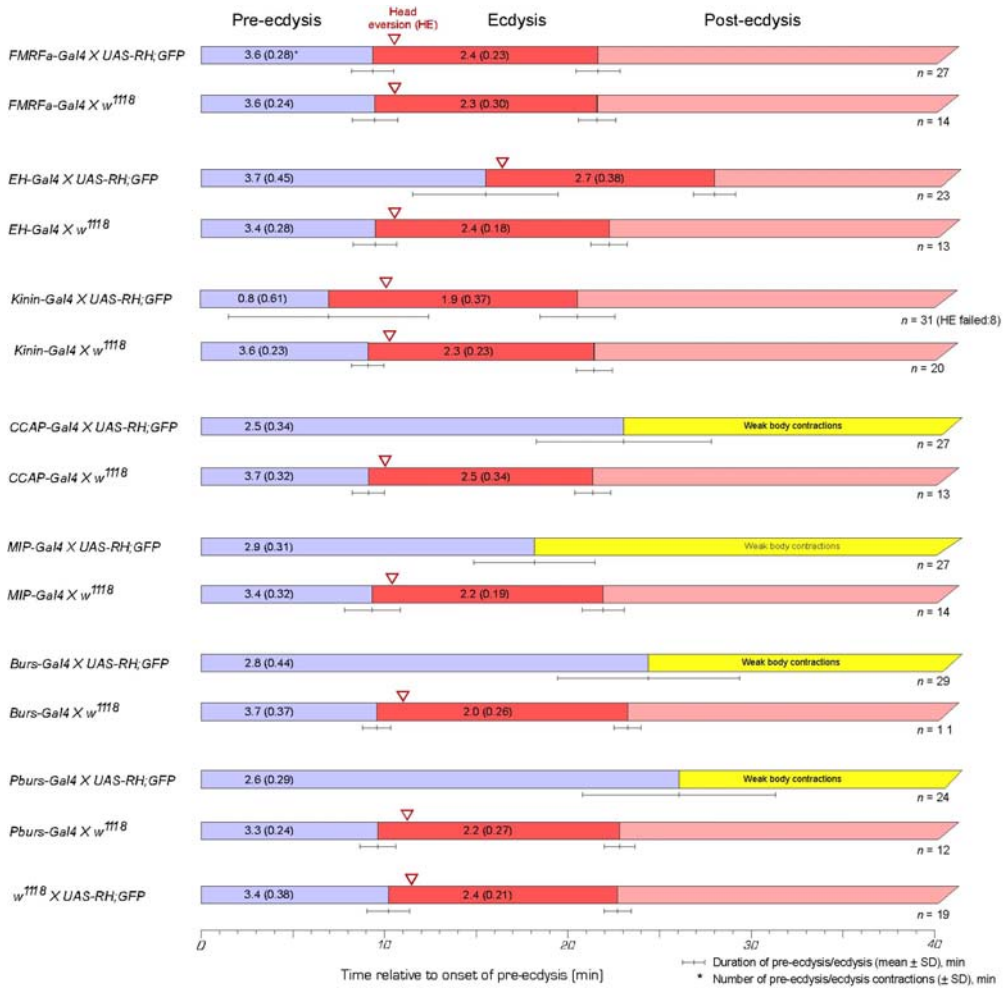

Figure 3.2. Targeted cell-killing of subsets of ETH receptor neurons alters ecdysis behavioral sequence. Cell-killing (CK) flies bearing targeted cell-killing of specific subsets of ETHR neurons revealed various degrees of behavioral defects in pupal ecdysis. FMRFa(Tv)-CK flies showed similar behavioral patterns to that of controls. EH-CK flies showed ~6 min delay in ecdysis onset with high variation. Kinin-CK flies showed very weak pre-ecdysis (~0.8 contractions per min vs. ~3.6 in control) with extremely high variation. CCAP-CK, MIP-CK, burs-CK and pburs-CK flies showed similar behavioral defects in terms of prolonged pre-ecdysis and no ecdysis and post-ecdysis. All Gal4 or UAS fly lines used were homozygous lines. Error bars represent Standard Deviation (SD). The numbers in the pre-ecdysis/ecdysis bar (asterisk) represent number of pre-ecdysis/ecdysis contractions per minute (± SD).

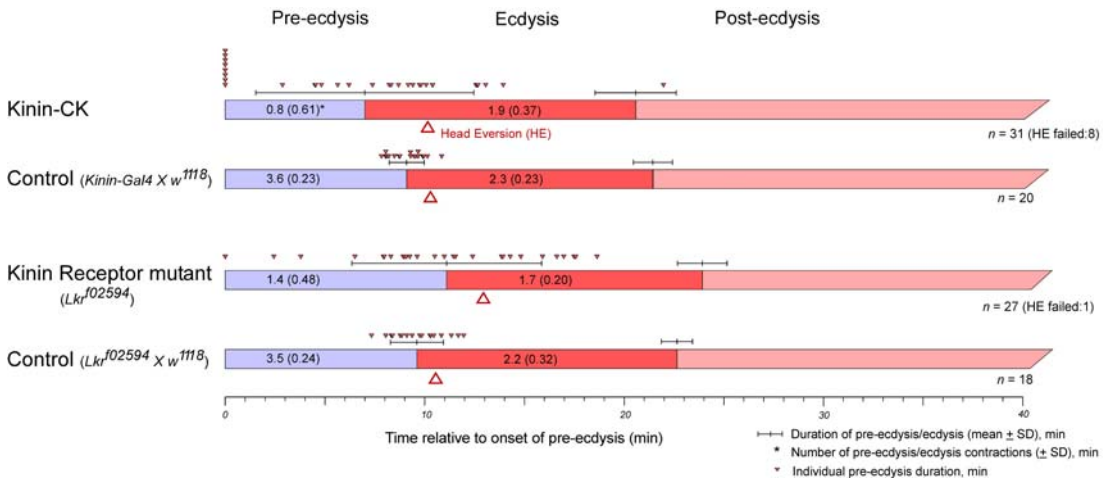

Figure 3.3. Flies with impaired kinin signaling show significant defects in ecdysis behavior. The role of kinin in ecdysis behavioral sequence was investigated by the analysis of behavioral defects in kinin cell-killing (CK) flies and homozygous *piggyBac*-insertional kinin receptor mutant flies. In both flies, contraction numbers during behavior were low and pre-ecdysis durations were highly variable. The small red arrowheads represent pre-ecdysis durations of individual animals.

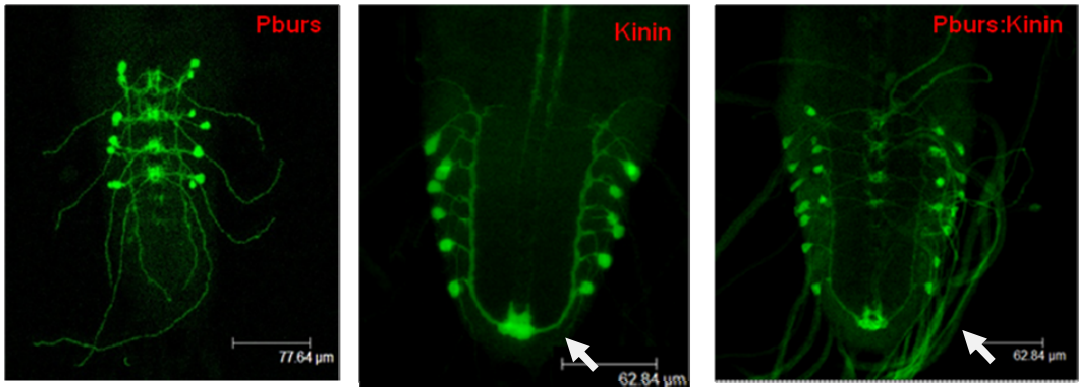

Figure 3.4. Immunohistochemical staining to verify Gal4 Expression in both bursicon and kinin neurons. Flies bearing *pbur-Gal4* and *kinin-Gal4* were generated to monitor bursicon and kinin neurons. Bursicon neurons (abdominal neuromere, AN1-4) release the heterodimeric hormone bursicon (burs and pburs). Bursicon neurons (left), kinin neurons (center) and both of them (right) were labeled by GFP using *UAS-GFP* and *pbur-Gal4*, *kinin-Gal4* or pburs;kinin combination Gal4. Note that kinins have terminal plexus (TP, neuropil) on AN9 (arrow). Kinin cells project axons posteriorly to TP and then turn anteriorly along the ventral midline.

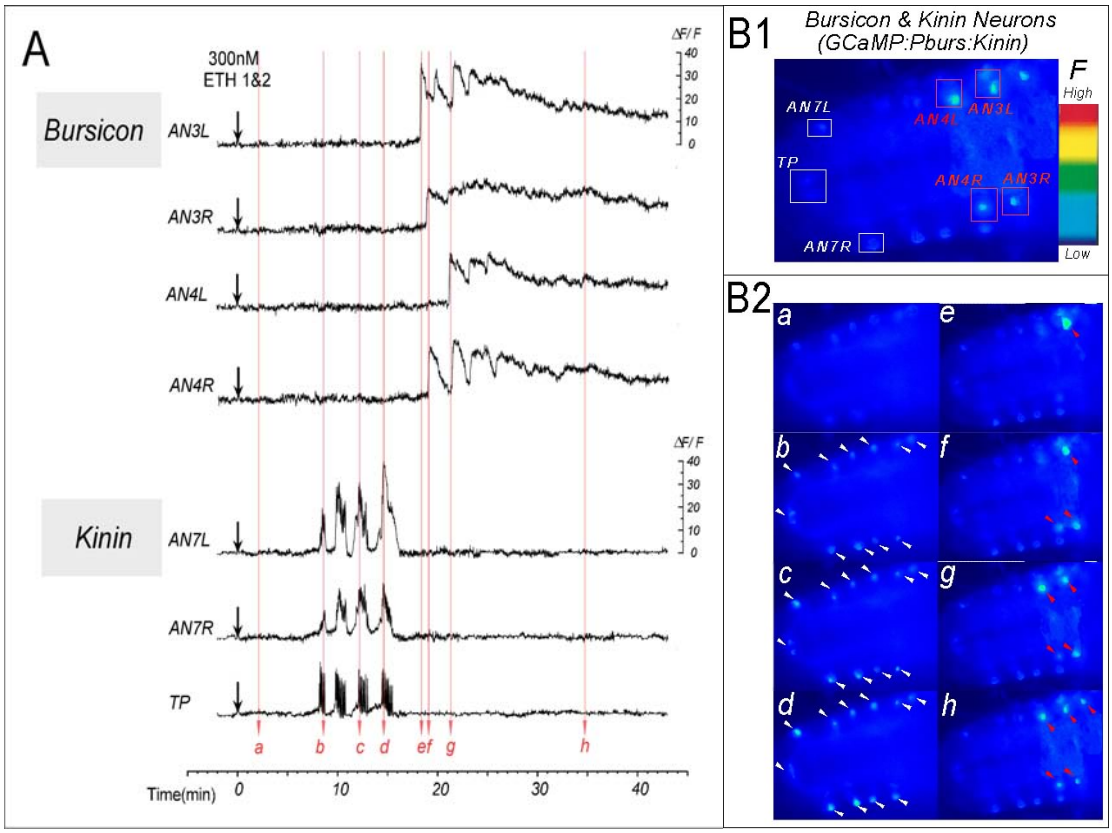

Figure 3.5. ETH-evoked Ca^{2+} dynamics in kinin and bursicon neurons (300 nM ETH). (A) Representative recordings of intracellular Ca^{2+} dynamics in bursicon (AN3, 4) and kinin neurons (AN7, TP) following exposure to ETH 1 & 2 (300 nM each) applied at time 0 (downward arrows). Following ETH application, kinin cell bodies in abdominal neuromeres (AN) 1-7 and in the terminal plexus (TP) showed robust and highly synchronized calcium oscillations after characteristic delays. Bursicon neurons became active shortly after termination of kinin activity. (B1) Video image shows locations of cell bodies and TP where Ca^{2+} activity was recorded in (A). (B2) Time-lapse video images captured during Ca^{2+} responses. The timing of video image recordings (B2: a-h) is indicated by vertical arrows in A (faint red)

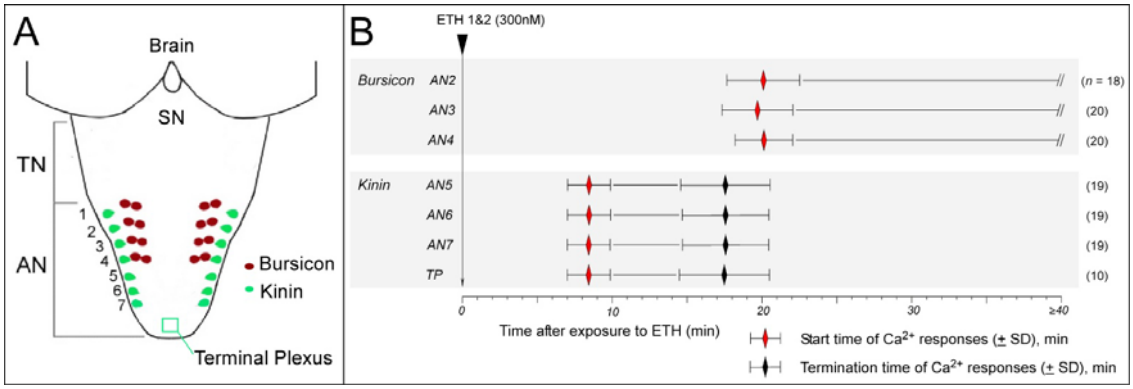

Figure 3.6. Sequential activation of kinin and bursicon neurons following exposure to 300 nM ETH. (A) Schematic diagram of kinin and bursicon neurons in the prepupal CNS. (B) Upon exposure to ETH, kinin and bursicon neurons were activated sequentially at 8.4 min and 20.0 min respectively. The onset (red) or termination (black) of Ca^{2+} responses induced by ETH 1 & 2 (300 nM each) are shown in the right (B). Note that bursicon neurons are active more than 1 hr. SN: subesophageal neuromeres; TN: thoracic neuromeres; AN: Abdominal neuromeres; TP: terminal plexus.

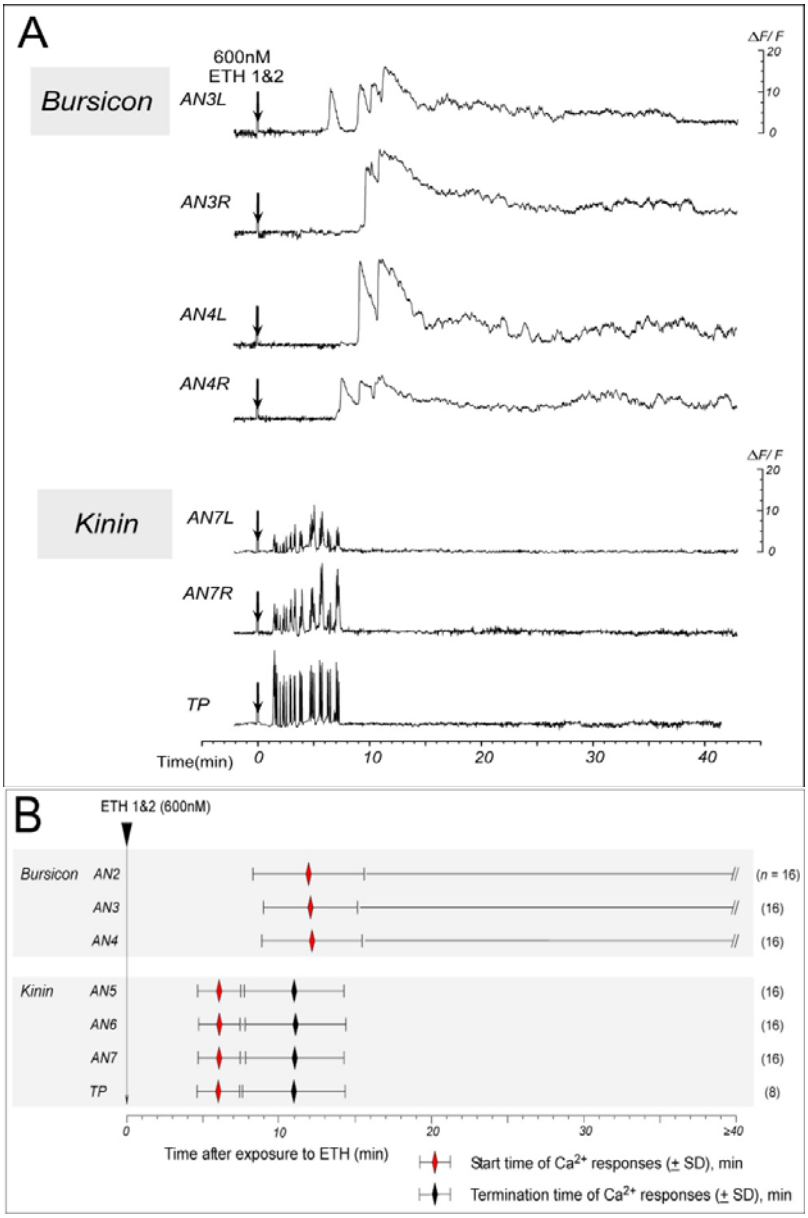

Figure 3.7. Ca^{2+} dynamics in kinin and bursicon neurons by 600 nM ETH. (A) Example of Ca^{2+} dynamics in bursicon (AN3, 4) and kinin neurons (AN7, TP) upon exposure to ETH 1 & 2 (600 nM each). (B) Average activation and termination times of kinin and bursicon neurons. Upon exposure of high ETH, activity in kinin and bursicon neurons was accelerated but remained sequential (6.0min, 12.0min respectively). Some of the bursicon neurons became active before the end of kinin activity (example: AN3L in A). However, most other bursicon neurons started their response after the termination of kinin activity. AN: Abdominal neuromeres; TP: terminal plexus.

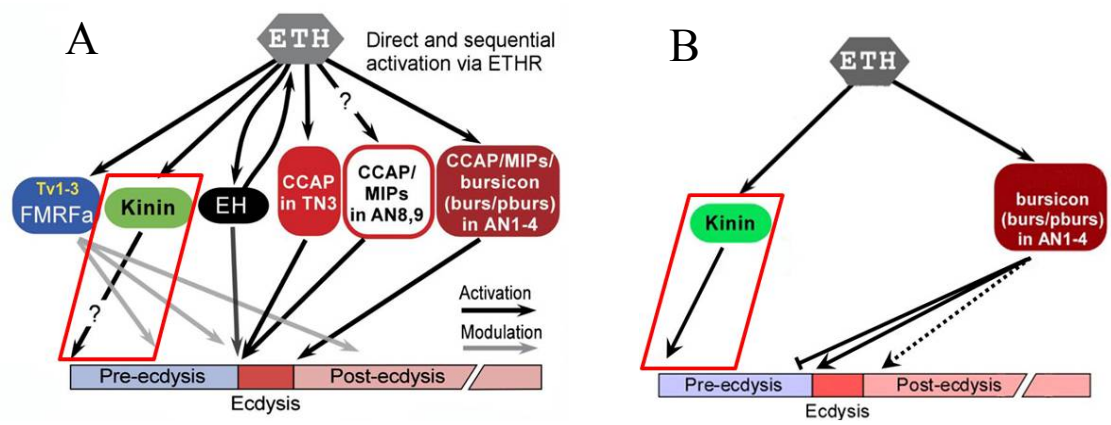

Figure 3.8. Previous model for peptidergic regulation of the ecdysis behavioral sequence (A, from Kim et al., 2006b) and (B) An updated version of the model based on evidence generated in the present study. Note that kinin is involved in pre-ecdysis and bursicon is involved in the termination of pre-ecdysis and the onset of ecdysis and post-ecdysis.

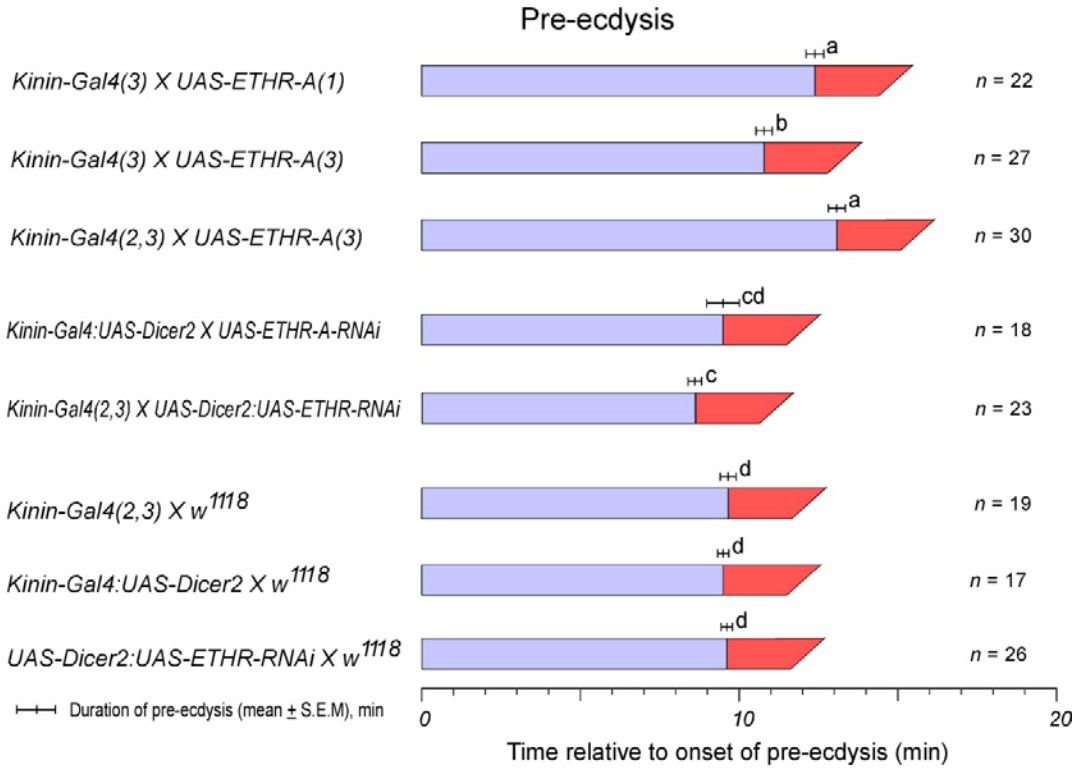

Figure 3.9. Different ETH receptor expression levels in kinin neurons changes scheduling of the ecdysis behavioral sequence. ETHR over-expression in kinin neuron revealed early onset of pre-ecdysis, which gives longer pre-ecdysis duration. Suppression of ETHR by the expression of ETHR-RNAi in kinin decreased pre-ecdysis duration. Error-bars represent standard error of mean (S.E.M). Data was analyzed using Mann-Whitney test at $p < 0.05$. Different letter means different mean of pre-ecdysis duration.

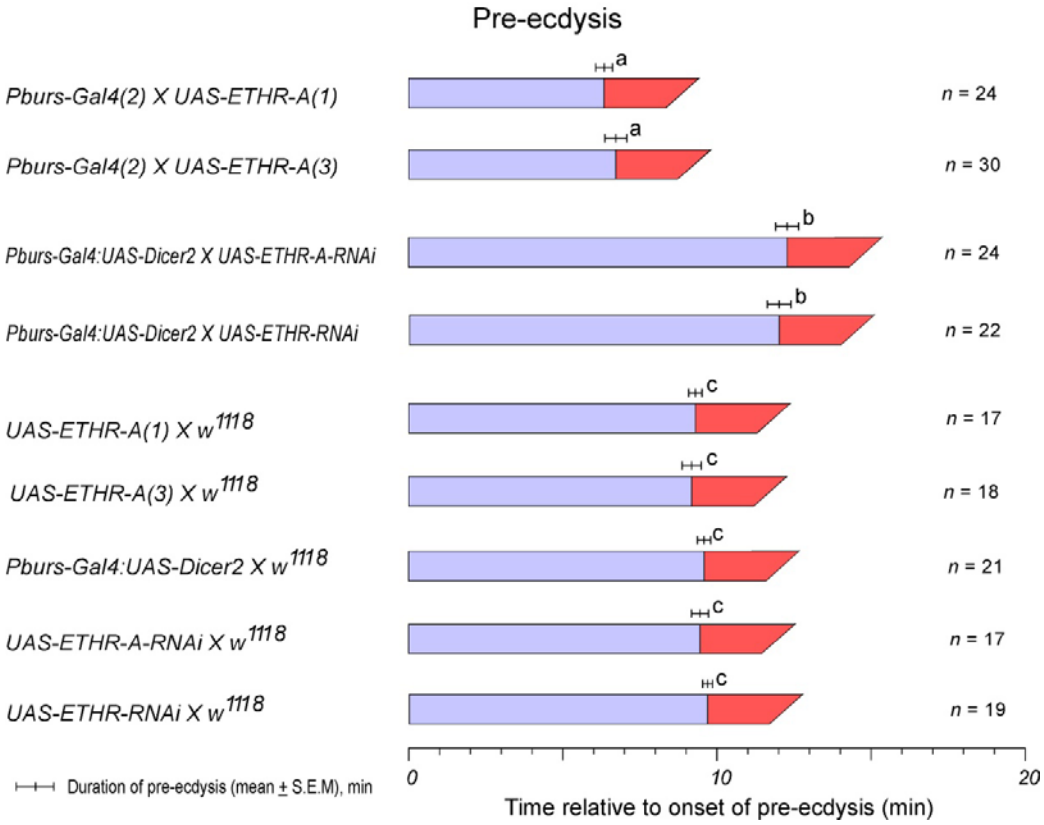

Figure 3.10. Different ETH receptor expression level in bursicon neurons changes scheduling of the ecdysis behavioral sequence. ETHR over-expression in bursicon neurons produces early ecdysis onset, and abbreviated duration pre-ecdysis. Suppression of ETHR by the expression of ETHR-RNAi delays onset of ecdysis, which leads to longer pre-ecdysis duration. Note that the changes in pre-ecdysis duration by increase or decrease of ETH receptor density in bursicon neurons are the opposite direction as in kinin neurons (Figure 3.10). Error-bars represent standard error of mean (S.E.M). Data was analyzed using Mann-Whitney test at $p < 0.05$. Different letter means different mean of pre-ecdysis duration.

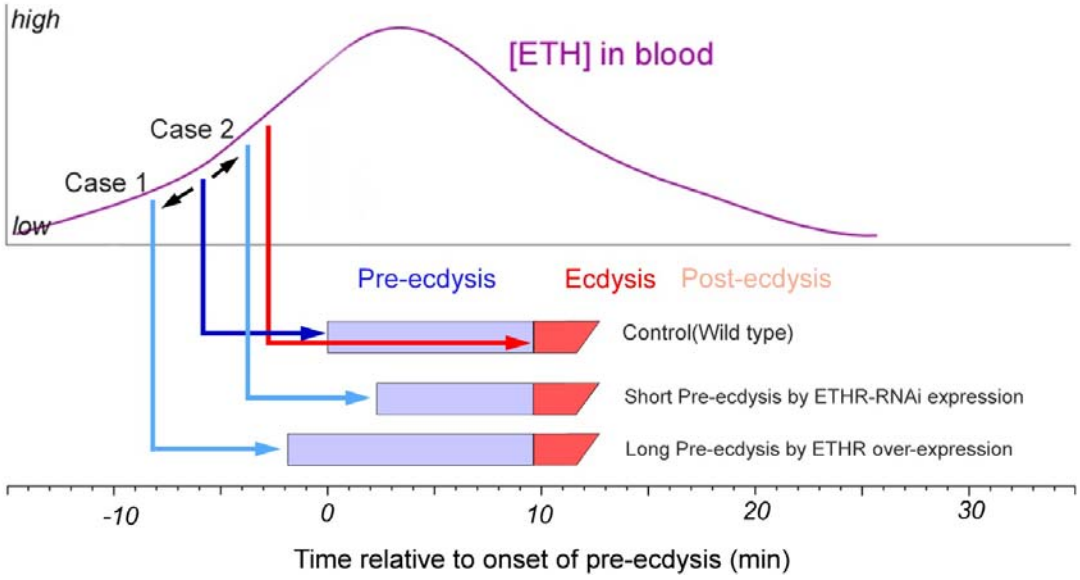

Figure 3.11. A model explaining how different ETH receptor expression levels in kinin neurons can change scheduling of the ecdysis behavioral sequence. Higher receptor density by ETHR-A over-expression in kinin increases sensitivity to ETH and causes early onset of pre-ecdysis, which makes pre-ecdysis longer (case 1). Lower receptor density by RNAi decreases sensitivity and delays the onset of pre-ecdysis, which makes pre-ecdysis shorter (case 2). Note that higher sensitivity of kinin neurons to ETH produces longer pre-ecdysis duration (case 1), while lower sensitivity leads to shorter duration (case 2).

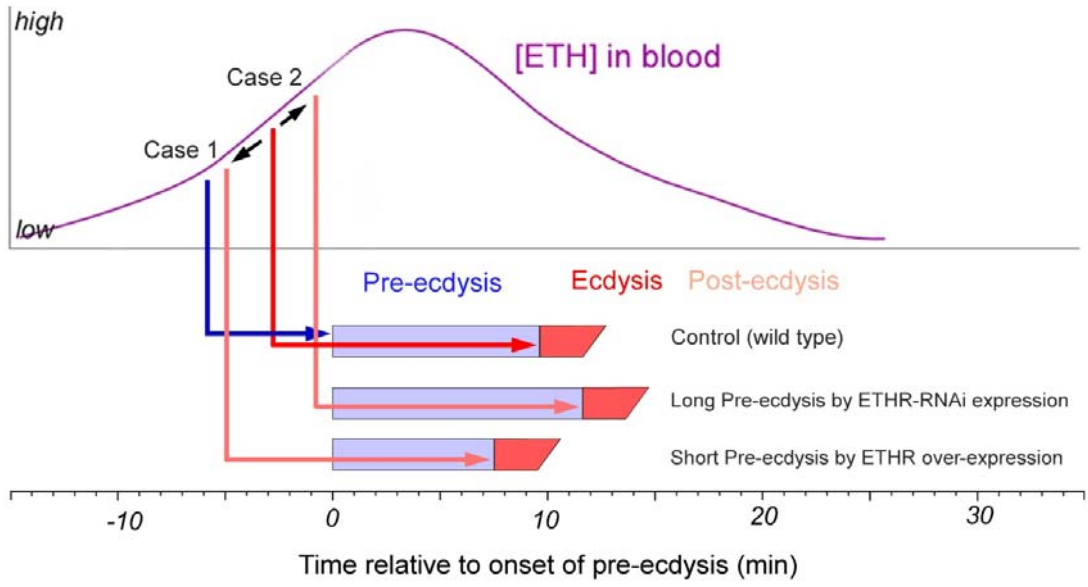

Figure 3.12. A model explaining how different ETH receptor expression levels in bursicon neurons can change the timing of the ecdysis behavioral sequence. Higher receptor density by ETHR-A over-expression in bursicon increases sensitivity to ETH and causes early onset of ecdysis, which reduces the duration of pre-ecdysis (case 1). Lower receptor density by RNAi decreases sensitivity and delays onset of ecdysis, which increases the duration of pre-ecdysis (case 2), which is opposite to that which occurs in kinin neurons.

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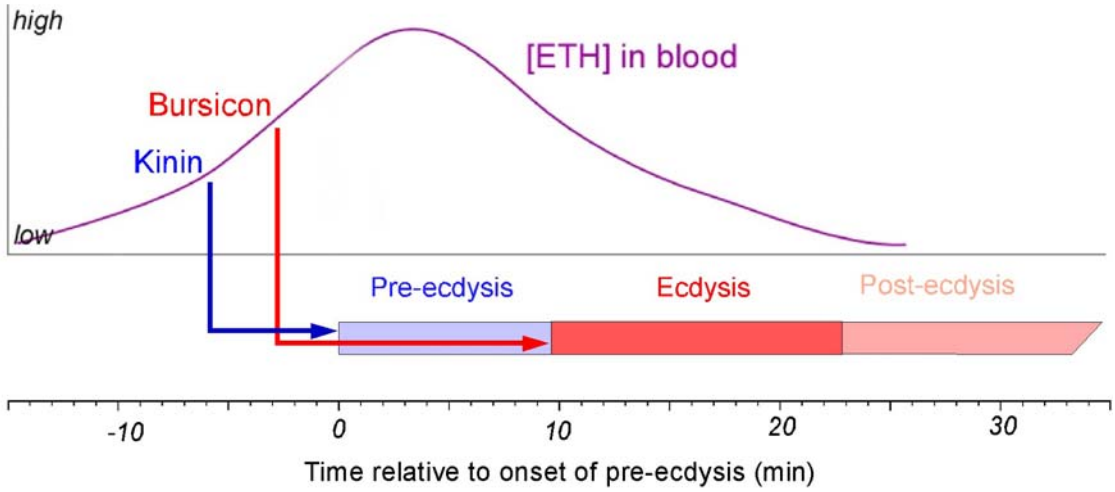

Figure 3.13. A model depicting how different sensitivities to ETH in distinct ETHR peptidergic ensembles control scheduling of the ecdysis behavioral sequence. In this model, ETHR ensembles with higher sensitivity (example: kinin neurons) become active early to control the pre-ecdysis step and those with lower sensitivity (example: bursicon neuron) become active later to control the ecdysis step.

CHAPTER IV

General Conclusions

Ecdysis is a very interesting event in many different aspects. It is a universal process that recently resulted in the naming of a new phylogenetic group, the ecdysozoa (Aguinaldo et al., 2007). It is dramatic event that changes the whole shape of the animal, such as turning a green worm into a beautiful butterfly. It is critical step of development. Ecdysis is involved in many physiological changes such as water and ion balance, tracheal inflation, sclerotization, and melanization (Chung et al., 1999**;** Fraenkel and Hsiao, 1962; Fraenkel et al., 1966; Park et al., 2002). It is also great example of hormonal regulation of behavior. Since ecdysis is elicited by a simple chemical stimulation, the behavioral sequences can be studied easily by the application of ETH. Ecdysis behavior is a good model for behavioral studies because it is a stereotyped behavior, which is identical for all individuals. Ecdysis behavioral sequences are driven by central pattern generators in CNS without sensory input. Changes in motor patterns of the isolated CNS have a direct connection with behavioral output. The simple stimulation and reproducible behavioral sequence are great features for behavior studies. The fact that the central nervous system is the direct target of ETH represents another favorable feature of ETH system: it gives us chance to study the neural organization for behavioral control. The neural circuit is one of the biggest topics in neuroscience. ETHR neurons are activated simply by ETH and the response can be monitored by behavioral output. Identification of diverse peptidergic neurons that express ETH receptors (Kim et

al., 2006a; Kim et al., 2006b) provided a good opportunity to dissect the ecdysis control mechanism.

In this study, I investigated the underlying mechanism of tracheal inflation, which is one of the physiological changes controlled by ETH during the ecdysis sequence. The inflation of new trachea happens right after the release of ETH from Inka cell. ETH injection into *eth* null mutants induces ecdysis behavior starting from tracheal inflation (Park et al., 2002). Since kinin neurons become active right after the ETH exposure, it is reasonable to consider kinin as the first responder to ETH and a controller of tracheal inflation. To verify the function of kinin signaling in tracheal inflation, I searched for tracheal inflation defects in fly lines impaired in kinin release through cell-killing or expression of Kir or RNAi in kinin neurons. All animals showed tracheal inflation defects of different severities. To impair reception of the kinin signal, I tested tracheal inflation defects of a hypomorphic kinin receptor mutant line, Lkr^{f02594}. This fly line showed a certain level of tracheal inflation defect, which was increased when crossed with deficiency lines. The rescue of inflation defects by the excision of *piggyBac* insertion of Lkr^{f02594} confirmed a functional role for kinin in the regulation of tracheal inflation.

It is known that tracheal inflation in *Drosophila* is controlled by one of the epithelial Na^+ channels (ENaC) that is a member of the pickpocket gene family (Liu et al., 2003). The transmembrane protein wurst, which is essential for endocytosis, also works with ENaC to promote tracheal inflation (Behr et al., 2007). To ascertain whether the kinin receptor is present in tracheal epithelial cells, I made transgenic flies that

express kinin receptor RNAi specifically in these cells. About half of the animals showed partial defects in tracheal inflation. The expression of kinin receptor RNAi in the cells that express the *pickpocket* (*ppk)* gene gave low level inflation defects. However, kinin receptor RNAi expression in two ppk populations (ppk10 and 11) yielded synergistic effects, which suggests combinational actions of ppk cells in tracheal inflation.

To understand how innate ecdysis behaviors are controlled by ETH, I analyzed the organization of ETHR neurons. Previously, a sequential activation model was proposed in our lab based on calcium imaging studies of the isolated *Drosophila* CNS combined with information from the moth, *Manduca* moth. This theory hypothesized that ETHR neurons become active at different times to control different behavioral steps in the ecdysis sequence. However, the sequential activation model, based on previous calcium imaging studies, had several drawbacks: it was based on comparison of neuronal activity patterns that occurred in different CNS preparations. There were potential delays of ETH action because the preparation was covered by low-melting agarose. There was little phenotypic evidence to support the model at the behavioral level. Therefore, I tested the sequential activation model using several new methods. I developed a new CNS preparation that does not involve diffusion delays caused by agarose. Using this preparation, I monitored changes of neural activities in two different ETHR neuronal groups, kinin and bursicon, in the same CNS. I found that kinin and bursicon became active at different times following ETH exposure in same preparation, supporting the previous model. During the imaging study, I found that calcium dynamics in kinin and bursicon neurons did not overlap following exposure to ETH at relatively high

concentrations (600 nM); instead, the duration of kinin activity was shortened. Our data suggest that there is either direct or indirect communication between these neurons for the proper sequence of activation. In the CNS of the moth *Manduca*, CCAP actively terminates pre-ecdysis motor bursts and initiates the ecdysis motor pattern (Gammie and Truman, 1999). A similar functional model involving bursicon neurons could be operating in the *Drosophila* CNS.

I then tested the function of ETHR neurons in ecdysis by targeted cell-killing (CK) of a subset of ETHR neurons. Through analysis of ecdysis behaviors in different CK flies, I found that kinin is necessary for regulation of normal pre-ecdysis behavior. Neurons that release only CCAP or CCAP/MIP were not totally necessary for normal ecdysis. This is interesting because CCAP is a key hormone for ecdysis in the moth. Instead of CCAP, neurons that release bursicon (also CCAP and MIP) are critical for initiation of ecdysis behavior. Calcium imaging studies support functional roles for kinin and bursicon in two different steps of the ecdysis sequence. The fact that bursicon neurons are necessary for initiation of ecdysis behavior suggests functions for bursicon in both ecdysis and postecdysis behaviors, including tanning. Interestingly, cell-killing of CCAP/MIP/Bursicon neurons did not appear to compromise larval ecdysis, consistent with a previous report (Park et al., 2003). EH-CK flies also did not exhibit differences in ecdysis success during larval stages. Non-lethal changes in pupal ecdysis were apparent, even though EH elicits ecdysis behaviors in moth *in vivo* and *in vitro* (Kim et al., 2006a; Truman et al., 1981). It is not yet clear at what level such interspecies differences arise.

My data provide further support for the sequential activation model. I tried to go further in order to find a mechanistic basis for sequential activation among different ETHR neurons. I hypothesized that differences in sensitivity to ETH caused by different ETHR expression level could account for sequential activation of ETHR neurons. ETH is released from epitracheal glands and circulates in hemolymph. The concentration of ETH in the hemolymph changes over time. To test the hypothesis, I altered ETHR density in two different peptidergic ensembles that express either kinin or bursicon; these ensembles are implicated in pre-ecdysis and ecdysis, respectively. Through expression of ETHR-A or ETH-RNAi specifically in these neurons, I altered the sensitivities of these neurons to ETH and monitored changes in the pupal ecdysis behavioral sequence. Higher ETHR density by the expression of ETHR could make the neuron become active in lower concentration of ETH, which cause earlier onset of behavior. On the other hand, lower ETHR density by the expression of ETH-RNAi could cause later onset of behavior. Using kinin and bursicon specific Gal4 flies, I showed that both pre-ecdysis and ecdysis can occur earlier or later, influenced by changes in ETHR density in these neurons, which supports our theory.

In this dissertation, I analyzed the neural organization of ETHRs for the control of ecdysis using diverse techniques such as molecular biology, imaging, transgenesis, and behavioral analysis. These methods supported each other to give general view of ecdysis process, which starts from the release of ETH from Inka cells, and then goes to sequential activations of ETHR neurons based on differential sensitivity and ends with the generation of stereotypic pattern of behavior. Calcium imaging studies of the

Drosophila CNS provides information about radical changes of neural activities. Currently, diverse reporters for different second messengers such as calcium, cGMP, and cAMP are available for imaging studies (Grewe and Helmchen, 2009; Niino et al., 2009; Niino et al., 2010). Imaging studies using different second messengers will provide a better understanding of the neural activities of diverse ETHR neurons. Transgenesis and analysis of phenotypes in ecdysis can provide detailed information about the behavioral and physiological roles of specific neurons.

Here, I showed that kinin has multiple functions in regulation of tracheal inflation and behavior. ETH is involved in physiological changes, such as water movement, cardioactivity, and melanization (Mykles et al., 2010). This suggests that many other as yet undescribed hormones are also involved in the physiological regulation of ecdysis. This also suggests that many hormones may have multiple functions. Diverse approaches to find physiological and behavioral changes during ecdysis can provide multiple options for eludication of new candidates for regulatory hormones.

I believe neural activity patterns revealed by imaging studes and their functional analysis by transgenic manipulation of behavioral and physiological processes can be used as a basic framework to dissect signaling cascades for the control of ecdysis. I hope that my work helps to further our understanding of signaling cascades operating during ecdysis and, in a larger sense, to reveal general aspects of cellular and molecular regulation of behavior in animals and humans.

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