A Novel Method for Investigation of Postembryonic Neural Reorganization in the Tobacco Hornworm, *Manduca sexta*

by

Kelly McNamara Patterson

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The tobacco hornworm, *Manduca sexta*, provides a model system for examining the reorganization of neural circuits during metamorphosis. The larval tobacco hornworm exhibits a pre-ecdysis behavior which serves to loosen the old cuticle at the culmination of each molt before the old cuticle is shed at ecdysis. These insects undergo behavioral changes during metamorphosis involving reorganization of the neural circuits that produce the pre-ecdysis behavior, decreasing the strength of the pre-ecdysis behavior during the molt from the larval to pupal life stages. The methodology used in the past for experiments investigating this important model system was extremely complex, relying on precise staging of the insects as well as lengthy dissections and methods for nervous system preparation. This study examined the effectiveness of exposure of isolated abdominal nerve cords to synthetic Pre-Ecdysis Triggering Hormone in initiating the motor pattern that produces the pre-ecdysis behavior.

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INTRODUCTION

The nervous system is comprised of vast numbers of **neurons**,* cells which carry signals in the form of electrical currents (**action potentials**) through the nervous system and between the nervous system and the body. It is through **neural circuits**, or pathways of interconnected neurons, that messages are sent to accomplish actions and bodily processes such as limb movements and breathing. The inner workings of the nervous system are complex, and the use of model systems for research provides important opportunities to discover the mechanisms of neural function. A **model system** is an experimental system that exemplifies the characteristics of more complicated systems while maintaining a level of simplicity. For example, it is easier to study neural circuitry in animals with numerically simpler nervous systems than in mammals. The principles learned in studying model systems can be applied to understanding the corresponding systems within more complex organisms, including humans. *Manduca sexta*, the hawkmoth, provides a system in which to study neuronal changes during postembryonic development. For this study, *Manduca* was used to examine hormonally-triggered changes in neural circuitry during **metamorphosis**, the transformation between larval (caterpillar) and adult (moth) body forms.

The postembryonic **life stages** of *Manduca* include periods as larva, pupa, and adult moth. See Figure 1 for a drawing of *Manduca* at each life stage. Insects **molt** as larvae and between each life stage, shedding their outer protective layer. These insects molt as larvae to allow growth, and each stage as larvae is called an **instar**. At a later point in development they undergo metamorphosis by molting as larvae to become pupae

* Terminology in bold is defined in the Glossary of Terms.

and again to become adults. At each molt a new **cuticle**, or outer protective covering, is formed and the old one is shed. The process of shedding the old cuticle is called **ecdysis** (Miles and Weeks 1991; Novicki and Weeks 1993, 1995, 2000). This process is similar to a snake shedding its outer layer of skin to allow growth.

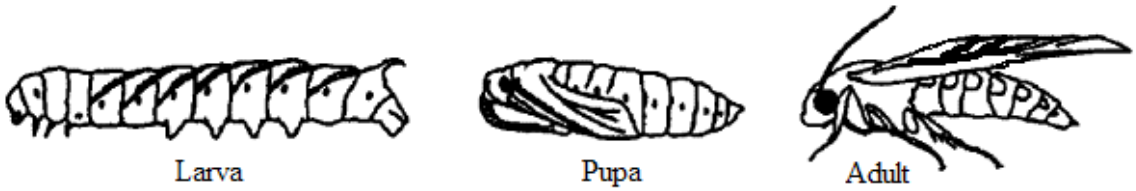

Figure 1: The three life stages of *Manduca*. From Weeks (2003).

This study investigated a specific behavior in *Manduca*, called the **pre-ecdysis behavior**, which serves to loosen the cuticle prior to ecdysis. See Figure 2 for a drawing of this behavior. The aspect of the pre-ecdysis behavior this project investigated consists of rhythmic (synchronized in time) **dorso-ventral** (top-to-bottom) compressions of the abdominal segments of these insects (Miles and Weeks 1991, Novicki and Weeks 1993, 1995, 2000). The abdominal contractions begin weakly and strengthen over time until they become clearly visible in the larval insect (Miles and Weeks 1991). This behavior continues for approximately 60 to 90 minutes before the onset of ecdysis (Miles and Weeks 1991). This behavior is triggered by complex interactions between **steroid** hormones and **peptide** (protein) hormones (Novicki and

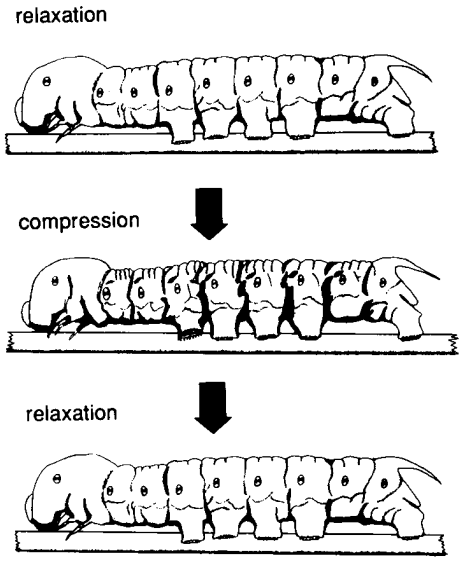

Figure 2: The compression behavior associated with larval pre-ecdysis: successive relaxation, top-to-bottom compression, and relaxation of the abdominal segments of the insect. This cycle takes approximately 5 seconds to complete. From Miles and Weeks (1991).

Weeks 1993, reviewed in Weeks 2003; Knittel and Kent 2005). **Hormones** are chemical messengers that are secreted from **gland cells** or **neurosecretory cells** in the nervous system. One peptide hormone, **Pre-Ecdysis Triggering Hormone (PETH)**, initiates the neuronal signaling that ultimately generates the muscle contractions that produce pre-ecdysis behavior (Zitnan *et al*. 1999).

NEUROBIOLOGY REVIEW

A neuron has distinct regions that receive, carry, and pass on action potentials. The **dendrites** are branched processes that receive signals from other neurons. The signals pass through the cell body to the **axon.** The action potential is propagated along the axon to the synaptic terminal of the neuron. The **terminal** is the portion of the cell that contacts other neurons. Two neurons communicate through a connection, a **synapse**, between the terminal of the preceding cell and the dendrites of the next cell in the pathway (the **postsynaptic cell**). The signal is transmitted through the synapse using electrical messages or chemical messengers called **neurotransmitters**.

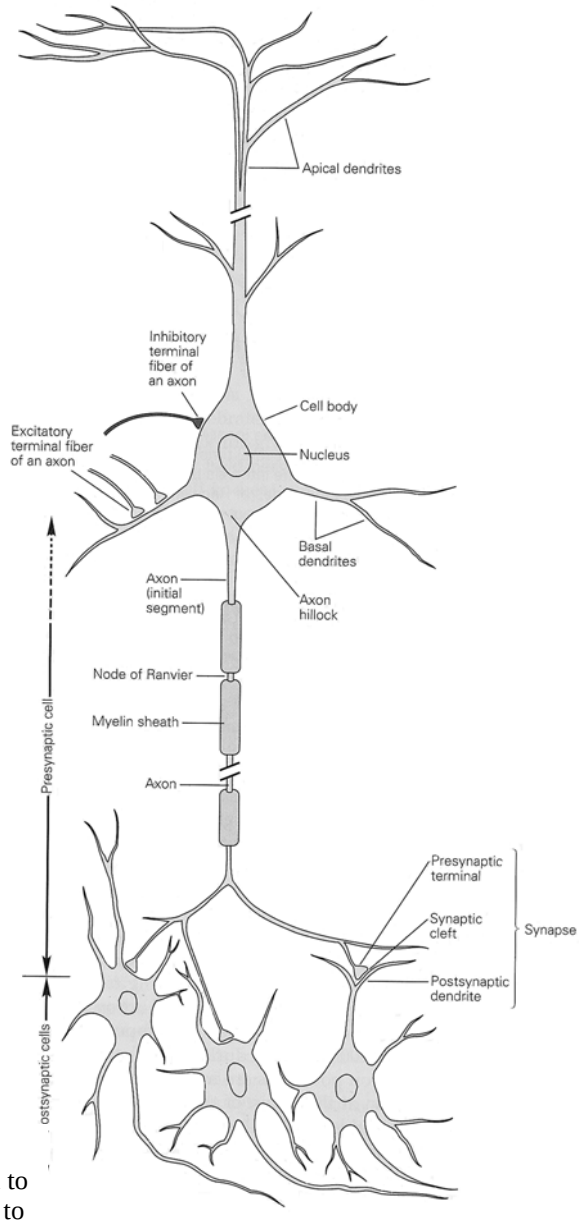

Figure 3: The anatomy of a neuron. A signal is received at the dendrites and passed down the cell to the terminal, where it is passed through a synapse to other neurons. From Kandel *et al* 2000.

The central nervous system (CNS) of *Manduca* includes the **ventral nerve cord**, a collection of neurons and bundles of axons that stretches along the lower surface of the insect (see Figure 4). This ventral nerve cord contains **ganglia**, which are aggregations of interconnected neurons. In *Manduca*, there is one ganglion in each **thoracic** and **abdominal** body segment, plus additional ganglia and a brain in the head. The ganglion in the terminal abdominal segment is a **compound ganglion**, composed of a fusion of multiple ganglia.

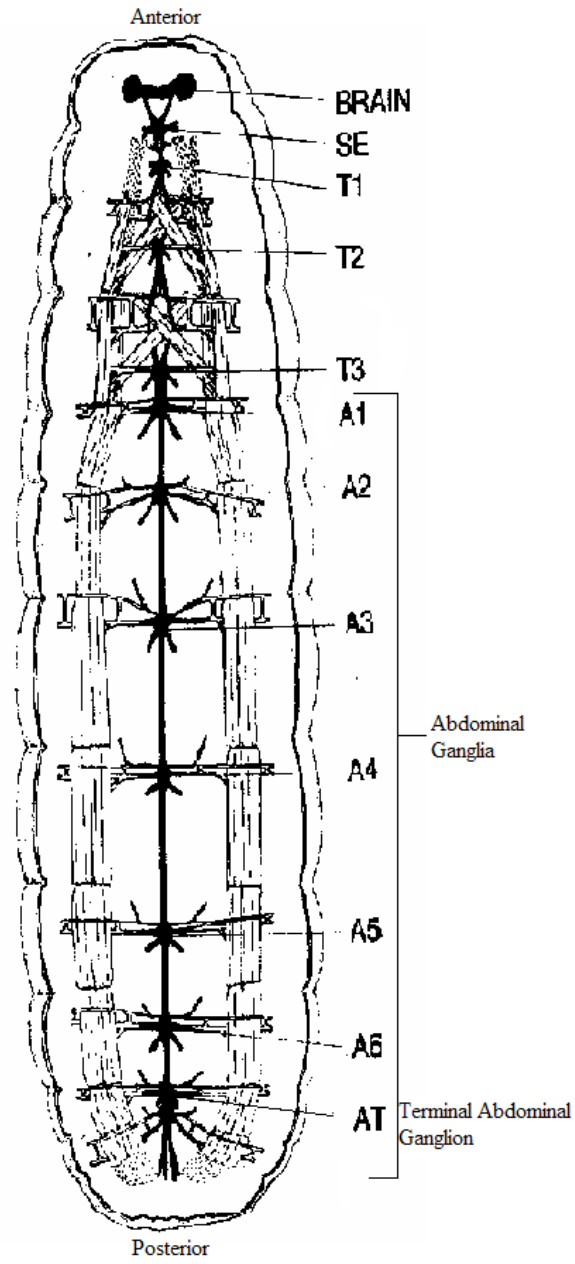

Figure 4: The central nervous system of a butterfly that has the same CNS structure as *Manduca*. From Heywood (1965).

A great deal of research has been done into the composition and organization of the neural circuit that produces the pre-ecdysis behavior. Two motor neurons, motor neuron 2 and motor neuron 3 (MN-2 and MN-3) (Levine and Truman 1985) in each abdominal ganglion, have been identified as the motor neurons that produce the pre-ecdysis compression behavior by

instructing the appropriate abdominal muscles to contract (Miles and Weeks 1991). **Motor neurons** pass a signal in the form of a **burst** of action potentials traveling in rapid succession to muscle cells instructing them to contract to produce the desired muscle movements. Two MN-2 neurons and two MN-3 neurons are present in each **abdominal ganglion**, with one of each type **innervating** the muscles on each side of the body (Levine and Truman, 1985). These motor neurons transmit the rhythmic bursts of action potentials that result in the regular contractions of these muscles.

There are also important neurons in this system that transport signals to MN-2 and MN-3. A pair of interneurons in this neural circuit, Interneuron 402s (IN-402s), has been identified (Novicki and Weeks 1993, 1995). **Interneurons** are the most abundant type of neuron in the CNS and provide an extensive network of interconnections among neurons. The axons of interneurons may be extremely long and carry signals over great distances. For example, in humans a single interneuron may carry signals from the base of the spinal cord to the brain (Kandel *et al*. 2000). The IN-402s have been found to project from the **terminal abdominal ganglion**, located at the **posterior** (tail end) of the insect, forward to synapse onto the MN-2 and MN-3 cells in each abdominal ganglion (Novicki and Weeks 1993, 1995). These interneurons **excite** each MN-2 and MN-3, thereby causing the motor neurons to stimulate muscle contraction during pre-ecdysis behavior.

The last portion of this neural pathway consists of the **central pattern generator**, a set of as-yet unidentified interneurons that synapse onto the IN-402s. See Figure 5 for a pictorial summary of this neural circuit. The central pattern generator is located in the terminal abdominal ganglion, the same ganglion that houses the cell bodies and dendrites of the IN-402s (Novicki and Weeks 1993, 1995). The rhythmic neural activity that

generates the pre-ecdysis **motor pattern** originates from the central pattern generator (Novicki and Weeks 1995). Thus, this activity originates in the central pattern generator and passes to the pair of IN-402s, which then signal MN-2 and MN-3 to stimulate the muscle contractions that produce the pre-ecdysis behavior.

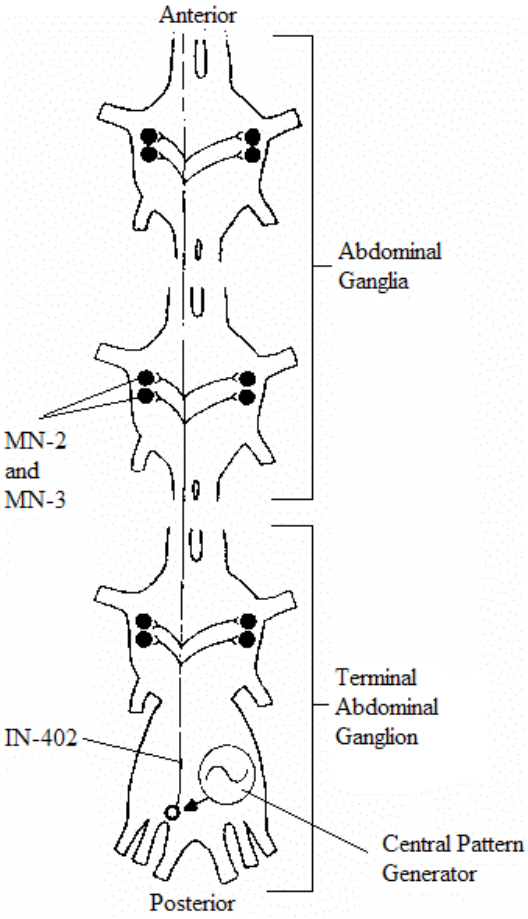

Figure 5: The neural circuit that produces the pre-ecdysis motor pattern. Only one IN-402 is shown, but there is a second IN-402 located on the right side of the ganglion that also synapses onto the four motor neurons within each ganglion. From Novicki and Weeks (2000).

At each transition between life stages, major **morphological** (body structure) and behavioral changes occur. During metamorphosis, the nervous system of the insect is also rewired. Neural circuits associated with obsolete body structures or behaviors are replaced by new neural circuits for structures or behaviors of the new life stage (Jacobs and Weeks 1990; Miles and Weeks 1991). This **neural reorganization** causes the pre-ecdysis behavior to be weakened during the larval to pupal transformation and to be lost completely in the transformation to the adult stage (Novicki and Weeks 2000). In larvae, the compressions of the abdominal segments are strong; however, at the molt between the larval and pupal stages, the synaptic inputs to the motor neurons are greatly reduced. Weak dorso-ventral compressions occur only in the **anterior** body segments, those closest to the head (Miles

and Weeks 1991, Novicki and Weeks 1993, 1995). Figure 6 shows the pupal pre-ecdysis behavior. This weakened behavior provides evidence that the pre-ecdysis neural circuit must be modified during the larval-pupal transformation (Novicki and Weeks 1993).

Figure 6: The pupal pre-ecdysis behavior. The dashed lines show the extent of the dorso-ventral movements of the head and thorax, indicated by the arrowheads. From Miles and Weeks (1991).

Thus, studying how this neural circuit is weakened during postembryonic development may provide broader insight into the mechanisms of postembryonic change in neural circuitry.

The overarching goal of this research is to examine the effects of hormones on neural circuits during the metamorphosis from the larval to pupal life stages in *Manduca*. Understanding changes in neural circuits within the *Manduca* model system contributes to the understanding of potential causes of human neurodegenerative disorders such as Parkinson's and Alzheimer's diseases (reviewed in Weeks 2003). A better understanding of neural circuit reorganization could influence stroke recovery as well. For example, a person may have a paralyzed or weakened arm following a stroke. Immobilizing the unaffected arm and forcing use of the weak arm can often greatly improve the patient's ability to use the affected limb. This change occurs due to increased muscular strength as well as reorganization of neural circuits. This reorganization is accomplished by recruitment of additional neurons to take over control of the weakened limb (Liepert *et al*. 1998; Miltner *et al*. 1999). Studying systems such as those of *Manduca* reveals additional information about the **plasticity** (flexibility) of the postembryonic nervous system and ways in which hormones regulate changes in neural circuits.

Previous research has been conducted to locate the site or sites of changes in the pre-ecdysis neural circuit during the larval-pupal transformation. It has been determined that the weakening of the behavior is not due to degeneration of the muscles responsible for the abdominal contractions (Miles and Weeks 1991). The ability of MN-2 and MN-3 to receive signals (Miles and Weeks 1991, Novicki and Weeks 2000) and IN-402 to transmit signals to MN-2 and MN-3 is also unchanged during this neural reorganization (Novicki and Weeks 2000). Thus, the weakening of the pre-ecdysis motor pattern is likely due to changes in IN-402 or central pattern generator interneurons, localizing the change to the terminal abdominal ganglion.

A new methodology would be beneficial in allowing further study of this neural circuit and in completing further experiments in a more efficient and effective manner. The currently established methods are laborious and rely heavily on precise staging of insects for experimentation, thereby making these experiments difficult. These methods depended upon the insects releasing their own **endogenous** hormones, making the staging and timing of dissection critical for the success of these experiments. The present study examined the hypothesis that addition of **exogenous** synthetic PETH to a completely **isolated nerve cord** (removed from the insect via dissection) from larval and **prepupal** insects would successfully induce production of the motor patterns associated with the pre-ecdysis behavior.

This study showed that synthetic PETH successfully initiated the pre-ecdysis motor pattern in isolated abdominal nerve cords from larval and prepupal insects. Exposure of the isolated terminal abdominal ganglion from larval insects also resulted in initiation of the motor pattern. The use of synthetic PETH to initiate the pre-ecdysis

motor pattern is an effective method for studying this neural circuit in larval and prepupal nerve cords.

MATERIALS AND METHODS

This experiment involved recording the electrical signals transmitted through the pre-ecdysis neural circuit. These recordings allowed visualization of the action potentials comprising the motor pattern initiated by synthetic PETH. Recordings were made from larval and prepupal nerve cords in order to compare the activity of motor neurons in the larval pre-ecdysis motor pattern with their activity in the prepupal pre-ecdysis motor pattern.

Manduca larvae were raised individually on an artificial diet (modified from Bell and Joachim 1976) at the colony at the University of Oregon. The insects were kept in a regulated chamber with a temperature cycle of 27 ºC in the daytime and 25 ºC in the nighttime with a photoperiod of 17 hours of light and 7 hours of dark. Data from insects of both sexes in the larval and prepupal developmental stages were compared. Insects designated as larval were in the 4^{th} larval instar, preparing to enter the 5^{th} (final) larval instar. Cuticular markers were used to **stage insects** prior to ecdysis from the 4^{th} to 5^{th} larval instar. Larvae were selected at either the 'liquid-filled brown mandibles' or the 'air-filled head capsule' stage, which occurs within five hours of the onset of normal pre-ecdysis behavior (Copenhaver and Truman 1982). Insects designated as prepupae were near the completion of the 5^{th} instar, preparing to enter the pupal life stage. When these insects initiated metamorphosis, marked by **wandering** behavior, the 5^{th} instar larvae were placed in holes bored into wooden blocks. The holes were then covered with wooden slats, secured with rubber bands, and allowed to complete the molt from the larval to pupal life stage. The prepupae were selected when 'dorsal bars' were present and prior to the 'posterior shrink' stage. The 'posterior shrink' stage occurs

approximately one hour before pre-ecdysis behavior begins (Novicki and Weeks 2000, Truman *et al*. 1980).

Larvae and prepupae were anesthetized by immersion in water for 10 min and then dissected to remove the abdominal CNS. The abdominal nerve cord is made up of a series of unfused ganglia that are identified by their segmental location (A1 through A6) and a compound terminal ganglion (AT) (Novicki and Weeks 2000). For the dissection, the insects were bathed in physiological saline (Trimmer and Weeks 1989). An incision was made along the dorsal length of the insect, and the gut was removed, as was fat body in prepupae. This exposed the abdominal nerve cord, which was isolated by severing the ganglionic nerves and **trachea** and then severing the nerve cord itself anterior to A1 and posterior to AT (Miles and Weeks 1991). In experiments examining pattern initiation in larval **preparations** containing AT alone, the abdominal nerve cord was severed anterior and posterior to AT. Once isolated, the nervous system was pinned out in a dish lined with **Sylgard** (Dow Corning, Midland, MI) and bathed in physiological saline.

After the nerve cord was isolated, the ganglia were then desheathed. This process involved exposing the **sheath** surrounding the ganglia to 3 % collagenase-dispase (Roche Diagnostics Corporation, Indianapolis, IN) for 2 min. This **enzyme** served to break down the tissue composing the sheath. After this treatment, fine **forceps** were used to tear a hole in the sheath surrounding each ganglion (Weeks and Jacobs 1987). This allowed the exogenous synthetic hormone to enter the ganglia and affect the neurons. The exogenous synthetic PETH treatment consisted of addition of 1µL PETH at a concentration of 600µM in dH_2O to the physiological saline. This gave a final PETH concentration of approximately 200nM in a 6mL volume of saline solution. Professor Michael Adams at

the University of California, Riverside, very kindly provided the synthetic PETH used for these experiments. The PETH was frozen at -80 ºC until used.

Glass-tipped **suction electrodes** were used to make extracellular recordings from **ganglionic nerves** containing the axons of MN-2 and MN-3. The axons of MN-2 and MN-3 are the only motor neuron axons in the anterior branch of the dorsal nerve (DN_a) (Levine and Truman 1985, Weeks and Truman 1984). This allowed unambiguous identification of the source of action potentials recorded from DN_a. Simultaneous recordings from two nerves were made to allow comparison of activity bilaterally and between segments. The recordings were amplified using AC-coupled preamplifiers (Grass Instruments, Quincy, MA). The electrical currents were then passed to an **oscilloscope**, which created two-dimensional graphs representing the action potentials transmitted through DN_a. A PC-based analysis system (AxoScope, Axon Instruments Inc., Foster City, CA) was used to collect and analyze these recordings.

Recordings were made immediately before the synthetic PETH was added to the physiological saline surrounding the abdominal nerve cord, in order to establish a record of baseline motor neuron activity. Further recordings were made following the addition of synthetic PETH and the subsequent initiation of the pre-ecdysis motor pattern in some preparations. Pre-ecdysis motor bursts were identified in the recordings by the presence of a series of regular bursts of synchronous activity in MN-2 and MN-3 characteristic of the pre-ecdysis motor pattern (Miles and Weeks 1991). Recordings from larvae and prepupae were compared to determine differences between the two patterns. The cycle period of pre-ecdysis bursts was measured for 10 larval nerve cords (A1 to AT), 7 larval terminal abdominal ganglia, and 9 prepupal nerve cords (A1-AT). The **burst period** was

analyzed only in recordings in which the pre-ecdysis motor pattern was successfully initiated and was measured in the part of each recording when the bursts were most regular. Burst period was measured as the time interval between the midpoints of consecutive bursts in the same nerve (Miles and Weeks 1991). The commencement and termination of each burst was clearly evident even when background action potentials were present. The burst periods for recordings from larval nerve cords, larval terminal ganglia, and prepupal nerve cords were averaged, and t-tests were performed to test for statistically significant differences between means.

RESULTS

The purpose of this study was to develop a new methodology for the completion of experiments examining the pre-ecdysis neural circuit in *Manduca*. Application of synthetic PETH to isolated larval abdominal nerve cords resulted in successful initiation of the pre-ecdysis motor pattern. Exposure of the isolated larval terminal ganglion similarly resulted in successful initiation of the pre-ecdysis motor pattern in the majority of preparations examined. Prepupal abdominal nerve cords initiated a clear pre-ecdysis motor pattern less reliably than did larval preparations.

Extracellular recordings were made from the ganglionic nerves in isolated abdominal nervous systems of 5^{th} instar larvae and prepupae. These nerve cords were treated with synthetic PETH in order to determine the success of the hormone in initiating the pre-ecdysis motor pattern. Initially, the ganglionic nerves showed a relatively low and steady level of activity. In successful trials, this was followed by the initiation of neural bursting activity of the pre-ecdysis motor pattern. The pre-ecdysis motor pattern was identified by the presence of regular time intervals separating the bursts of synchronous activity in MN-2 and MN-3.

Larval Nerve Cord (A1 to AT)

This study examined the reaction of the larval abdominal central nervous system, consisting of AT through A1, to the synthetic PETH in a total of 8 larval insects. The simultaneous recordings typically consisted of a ganglionic nerve from an anterior segment of the insect and one from a more posterior segment. From these 8 insects, a total of 15 recordings were made from various DN_as. Of these recordings, 80 % (12 of

15) clearly showed rhythmic motor neuron activity identifiable as the larval pre-ecdysis motor pattern. The bursts of activity were synchronous and of approximately equal strength in different body segments and on both sides of the nerve cord. Figure 7 shows examples of the pre-ecdysis motor pattern recorded from DN_as in nerve cords from larval insects. Of the 15 recordings, 12 showed clear synchronous bursting in both ganglionic nerves, consistent with the characteristic pre-ecdysis motor pattern. Of these 12 recordings showing synchronicity, 1 displayed synchronous bursts between segments, 3 exhibited synchronous activity between sides of the nerve cord, and the remaining 8 showed synchronous activity between both sides and segments of the body. Segmental synchronicity was recorded as far apart as A2 and A5 within a single preparation. One of the 15 recordings showed bursting in only one electrode, with no detectable activity in the other nerve. Two of the 15 recordings showed asynchronous bursting between

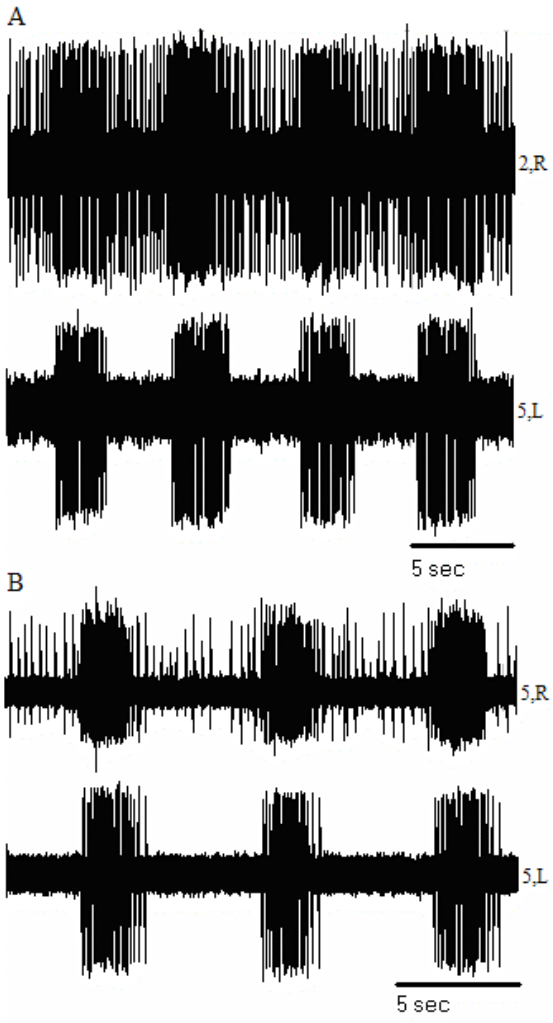

Figure 7: The larval pre-ecdysis motor patterns initiated by synthetic PETH in sets of 2 simultaneous extracellular recordings from DN_a in 2 isolated nerve cord preparations. Each spike represents an action potential from MN-2 or MN-3. The bursts of signals, shown by the denser regions of spikes, would correspond with the contractions of the muscles to produce the pre-ecdysis behavior (if the muscles were present). The numbers and letters indicate the abdominal segment and side of the body where the recordings were made. **A** This recording shows synchronous bursts in different segments and body sides. **B** This set of recordings show synchronous bursts on both sides of 1 segment.

segments and sides of the body, perhaps due to damage during the dissection (see Discussion). When the pre-ecdysis motor pattern was successfully initiated in larval nerve cords, it generally began as fairly robust and regular bursting soon after addition of the exogenous PETH. See Figure 8 for recordings of neuronal activity before addition of PETH, during pattern initiation, during regular bursting, and as the pattern degraded. The mean cycle period was determined in 10 larval preparations (8 bursts per preparation) and was found to be 6.8 ± 0.31 (SEM) seconds (N=10). See Table 1 for this data and related statistics.

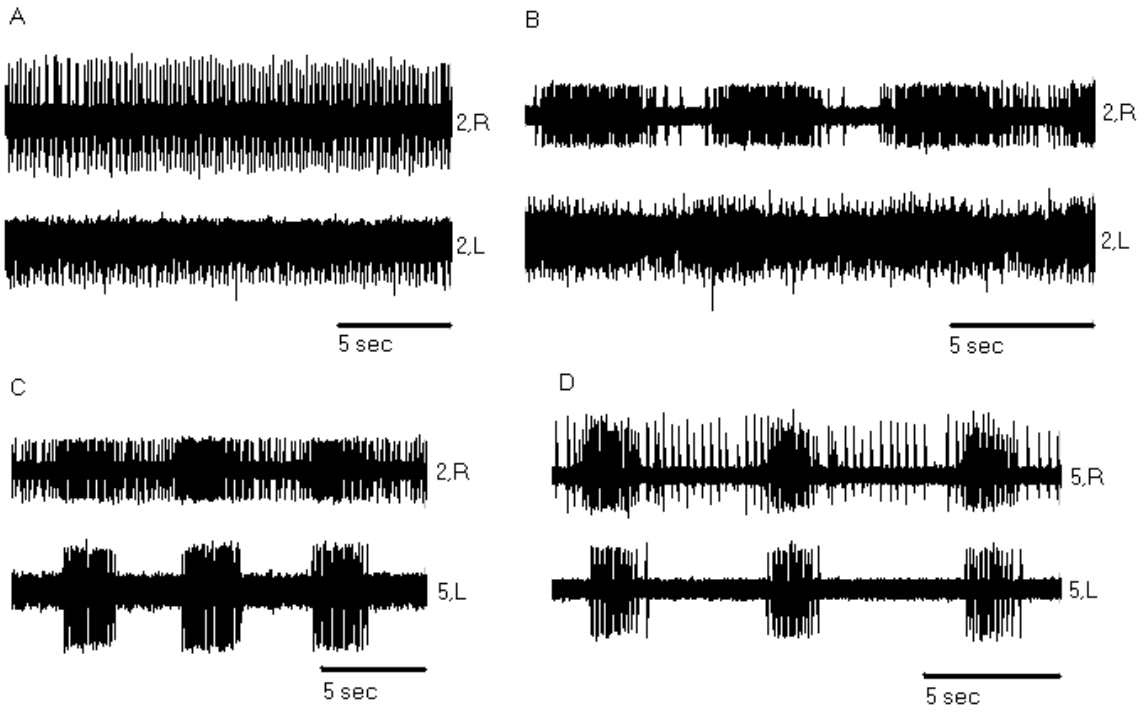

Figure 8A, B, C, D: The larval pre-ecdysis motor pattern. **A** The neuronal activity approximately 4 min before addition of exogenous PETH. **B** The activity as the motor pattern was being initiated, approximately 5 min following addition of PETH. **C** The synchronous bursting of the regular motor pattern, approximately 20 min following addition of PETH. **D** The motor pattern after it began to degrade and the bursts had become weaker, approximately 35 min following addition of exogenous PETH.

Table 1: The mean cycle periods and statistical analyses in larval nerve cord, larval terminal ganglion, and prepupal nerve cord preparations.

Preparation	N	Mean	SEM (means' standard error)	NS*
Larval Nerve Cord (A1-AT)	10	6.78	0.31	*
Larval Terminal Ganglion	7	7.53	0.46	*
Prepupal Nerve Cord (A1-AT)	9	8.55	0.94	*

*NS (not significant) indicates that none of the means were statistically significant

Larval Terminal Ganglion

Experiments were completed in a total of 10 larval insects to examine initiation of the pre-ecdysis pattern in the terminal ganglion (AT) alone. The purpose of this phase of the experiment was to determine the most reduced preparation that would successfully produce the larval pre-ecdysis motor pattern in response to PETH. From the 10 insects studied, a total of 10 paired recordings were made from the DN_as on the left and right side of AT. Of these recordings, 67 % (6 of 9) clearly showed the motor pattern associated with the pre-ecdysis behavior. One recording was excluded from this study as the terminal ganglion was visibly damaged during desheathing. Six of the remaining 9 recordings showed synchronous bursting occurring in both nerves. Of these, 3 showed weak bursting prior to addition of the synthetic PETH. In 1 of these cases, weak bursting was seen in both ganglionic nerves in which activity was being recorded; while in the other 2 recordings, bursting was seen in only one nerve prior to addition of the hormone. In all three of these cases, addition of synthetic PETH resulted in an increased strength of the bursts already present and initiation of synchronous bursting in the previously quiet nerve. The mean burst period for these preparations was 7.5 ± 0.46 (SEM) seconds (N=7) which was not significantly different than that of the larval abdominal nerve cord burst period of 6.8 seconds. See Table 1 for this data. Of the 9 recordings made, 2

showed asynchronous bursting between the two sides of the terminal abdominal ganglion, and both of these preparations showed some bursting in one of the ganglionic nerves prior to addition of the synthetic PETH. One of the 9 preparations failed to initiate any bursting pattern.

Prepupal Nerve Cord (A1 to AT)

The initiation of pupal pre-ecdysis behavior was also examined in the prepupal abdominal central nervous system in a total of 15 preparations. Two of these preparations were excluded from this study due to visible damage to the nerve cord during the desheathing process. From the 13 prepupal preparations studied, a total of 48 recordings were made from a combination of two DN_a branches. Of the prepupal recordings made, 23 % (11 of 48) successfully showed the bursting activity of the rhythmic pre-ecdysis pattern following application of the synthetic PETH. Often, these motor bursts began sporadically following the hormone application and gradually became more regular. This was in contrast to the larval preparations which generally began the motor pattern with robust and regular bursts. The bursts were generally weaker than those seen in larvae, and were also much more variable in intensity and burst duration. The bursts seen in posterior segments were generally weaker than those in anterior segments.

Of the 48 recordings made, 11 showed clear bursting in both ganglionic nerves characteristic of the pupal pre-ecdysis motor pattern. Of these, 5 exhibited synchronicity between sides of the nerve cord, 1 exhibited synchronous bursting between different body segments, and 5 showed synchronous bursts between both segments and sides of the

nerve cord. The mean cycle period was determined in 9 prepupal preparation (8 bursts per recording) and was found to be 8.6 ± 0.94 (SEM) seconds (N=9). This is not a statistically significant difference from the mean burst period of 6.8 seconds measured for larval preparations. See Table 1 for this data. In addition to these 11 recordings, 2 showed synchronous bursting between nerves but had an average burst period of 81.3 ± 10.19 (SEM) seconds (N=3), which was approximately 12 times longer than that found for the larval pre-ecdysis motor pattern. Both of these recordings were made from a single preparation, and this was likely a result of damage during dissection or desheathing. In 22 of the 48 recordings made, bursting was recorded in only one of the nerves. Thus, although the bursting appeared consistent with the pre-ecdysis behavior, synchronicity could not be confirmed and thus this activity could not be concretely identified as part of the pre-ecdysis motor pattern. Including these as successful pattern initiations would result in 46 % of the preparations successfully initiating the pre-ecdysis motor pattern in response to PETH exposure.

Thirteen of the recordings showed no bursting initiated in either nerve. Of these recordings showing no bursting, 69 % consisted of both nerves originating from the posterior region of the nerve cord (A5-AT) in which the bursts are expected to be extremely weak. Thus, this may have accounted in part for the lack of bursting seen in some of these recordings. As with the larvae, when bursts were seen in both ganglionic nerves at the same time, the bursts were synchronous between segments and sides of the abdominal central nervous system. See Figure 9 for examples of the pre-ecdysis motor pattern recorded from prepupal preparations.

This study produced a high success rate of pattern initiation in larval nerve cords and larval terminal ganglia of 80 % and 67 %, respectively. It is therefore most likely that the preparations in which bursts were not initiated or were asynchronous had been damaged during the dissection or desheathing process. This is also likely to be the case for prepupal nerve cord preparations in which bursts were seen in only one nerve, as the pattern was initiated successfully 23 % of the time in these preparations.

Exposure of the isolated nerve cord from larval *Manduca* to synthetic PETH clearly initiates the pre-ecdysis motor pattern. Synthetic PETH successfully initiated the motor pattern associated with the rhythmic series of larval pre-ecdysis muscle contractions in isolated abdominal nerve cords and isolated terminal abdominal ganglia. The ability

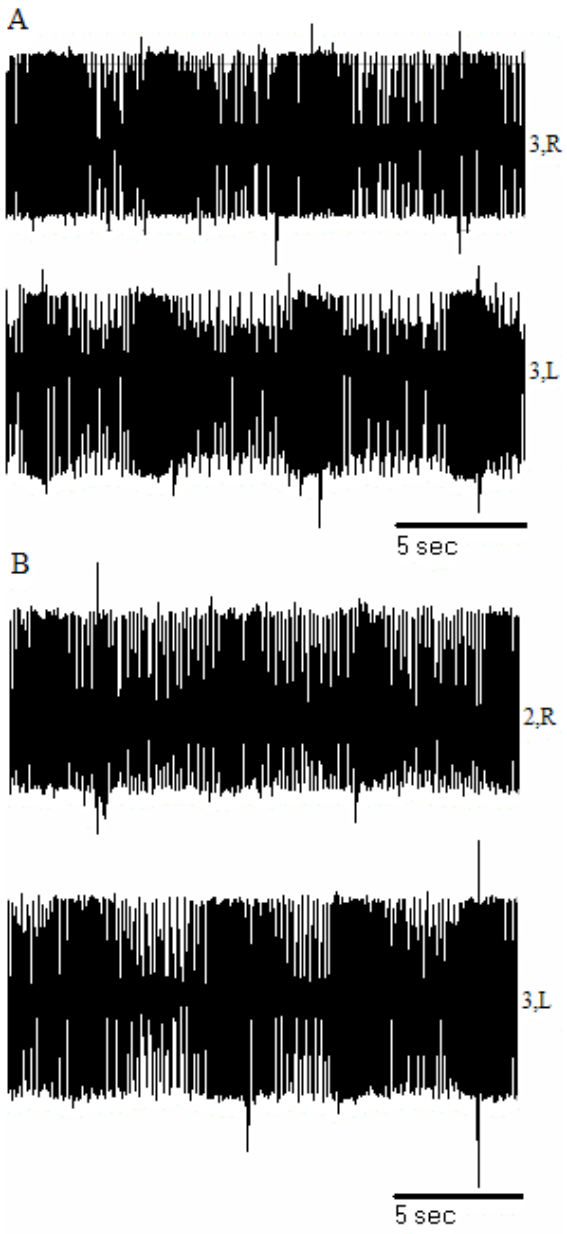

Figure 9: The prepupal pre-ecdysis motor pattern from sets of 2 simultaneous extracellular recordings from DN_a in 2 isolated nerve cord preparations. The numbers and letters indicate the abdominal segment and side of the body from which the recording was made. **A** These recordings show synchronous bursts on both sides of the nerve cord. **B** These recordings shows synchronous bursts in different segments and body sides.

for synthetic PETH to initiate the pre-ecdysis motor pattern in prepupal isolated nerve cords was less reliable.

DISCUSSION

This study examined the ability of synthetic PETH to trigger the pre-ecdysis motor pattern within isolated nerve cords. Synthetic PETH was able to successfully initiate the pre-ecdysis motor pattern in isolated larval and prepupal nerve cords and in the isolated larval terminal abdominal ganglion. These results replicated previous experiments showing pre-ecdysis motor pattern initiation following addition of synthetic PETH to the isolated larval CNS (Zitnan *et al*. 1999). This study further demonstrated the ability of exogenous synthetic PETH to initiate the pre-ecdysis motor pattern within the isolated larval terminal ganglion and the isolated prepupal CNS. The response to the hormone was stronger in larvae than in prepupae, resulting in a higher success rate for initiation of the motor pattern. The motor patterns initiated were indistinguishable from those expected based on prior work with different hormones and in incompletely isolated nervous systems (Miles and Weeks 1991, Zitnan *et al*. 1999).

In larval abdominal nerve cords, successful initiation of the pre-ecdysis motor pattern occurred 80 % of the time. Thus, this is a highly reliable mode of pattern initiation. The failure to initiate the pre-ecdysis motor pattern, or the initiation of asynchronous bursting, within the remaining recordings was most likely due to damage to the axons running through the **connectives** between ganglia which may have occurred during the dissection or desheathing. This can be confidently assumed, as the remainder of the recordings showed a very robust response to the synthetic PETH. Exposure of isolated larval nerve cords to synthetic PETH will be a valuable method for further examination of the neural circuitry involved in the larval pre-ecdysis motor pattern.

The pre-ecdysis motor pattern was successfully initiated in prepupal abdominal nerve cords 23 % of the time. This success rate is sufficient to conclude that addition of exogenous PETH is effective for completion of further experiments using this method. This decreased success rate is consistent with the observation that the motor pattern is greatly weakened and generally more variable in prepupal insects (Miles and Weeks 1991). The difficulty in the desheathing process is likely to cause a greater effect on the already weakened activity of this neural circuit than on the robust activity of the larval neural circuit, even when visible damage is not evident. This is in all likelihood the cause of bursting observed in one of the DN_as, while the other nerve branch remained silent (passing no action potentials) or maintained only low levels of **tonic activity** (a constant level of activity). Less success of pattern initiation was also seen in the posterior segments of the prepupal nerve cord preparations. This result is consistent with the fact that the bursts of action potentials are weaker in the posterior segments at this stage (Miles and Weeks 1991). It is possible that the motor pattern simply was not activated in these more posterior segments. Bursting was seen in posterior segments in several recordings, however, indicating that exogenous PETH is likely capable of pattern initiation in the absence of complication by other factors. Further experiments will be necessary to determine if the success rate of pattern initiation can be improved in prepupal abdominal nerve cords with more practice and thus greater accuracy within the dissection and desheathing process.

Another aim of this study was to determine the shortest length of abdominal nerve cord that could successfully initiate the pre-ecdysis motor pattern when exposed to synthetic PETH. In abdominal terminal ganglia, the pattern was successfully initiated

67 % of the time. Thus, the motor pattern can be reliably monitored in the terminal abdominal ganglion alone. Use of this preparation allows vast simplification of the dissection involved and lessens the chance for damage to occur, as AT is the only ganglion that needs to be isolated and desheathed for study. Further research will need to be completed to determine the shortest prepupal nerve cord preparation in which synthetic PETH can successfully initiate the pre-ecdysis motor pattern.

The results of this study also provide support for the belief that the weakening of the motor pattern in the molt from the larval to pupal life stage is due to changes in the central nervous system (Novicki and Weeks 2000). The motor pattern initiated in the isolated prepupal nerve cords showed weaker bursts, especially in the posterior segments, and a lower rate of successful initiation. The motor pattern in isolated larval nerve cords, however, was more easily initiated, and the bursts of activity were of equal strength between segments. This result indicates that the change in neural circuitry occurs within the abdominal nervous system, since the nerve cord itself was the only source of the pattern observed. Further investigation of the motor pattern produced in the prepupal terminal abdominal ganglion alone could also shed light upon the location of the changes. If the motor pattern were seen to be weakened normally in the isolated prepupal terminal ganglion following exposure to synthetic PETH, this result would provide support for the idea that the neural changes are localized within the terminal ganglion itself.

The ability of synthetic PETH to initiate the pre-ecdysis motor pattern in the isolated abdominal nerve cord of *Manduca* will allow further research to be completed within this model system. This mode of pattern initiation allows greater flexibility in the staging of insects to be used for experimentation. The larval CNS becomes responsive to

PETH approximately 30 hours prior to ecdysis, allowing addition of exogenous PETH over a wide window of time (Zitnan *et al*. 1999). More research into the minimum length of nerve cord necessary to observe the pre-ecdysis motor pattern initiated in prepupae will be helpful for potentially simplifying the dissection and preventing damage to ganglia and connectives. Although the bursts in the anterior segments of prepupae are stronger, it is possible that bursting might be observed in more posterior ganglia with a shorter nerve cord. Answers to these questions will be beneficial for proceeding with further research utilizing this method. This study indicates that the use of exogenous synthetic PETH to initiate the pre-ecdysis motor pattern in isolated nerve cords will be an effective method for investigating the postembryonic neural reorganizations during metamorphosis in *Manduca*. Further study into these neural changes may one day provide insight into mechanisms of neural reorganization within humans.

GLOSSARY OF TERMS

Abdominal—pertaining to the posterior segment of the body, located behind the thorax

Abdominal ganglion—the collection of neurons located within one of the insects' abdominal segments

Action potential—a rapid electrical signal sent down an axon to the terminal of the neuron and then communicated to the postsynaptic neuron

Anterior—toward the head of the insect

Axon—the long portion of the neuron that carries the signal through the neuron, often stretching over long distances

Burst—a rapid succession of action potentials occurring more frequently than the baseline rate of neuronal firing

Burst period—the period of time elapsed between bursts in the motor pattern

Central pattern generator—an interconnected set of interneurons that generates the rhythmic pre-ecdysis motor pattern and relays it to the IN-402s

Compound ganglion—a ganglion composed of multiple ganglia fused together

Connectives—the bundles of axons running between the ganglia in the central nervous system

Cuticle—the outer protective layer that surrounds the body of the insect

Dendrites—the regions of the neuron that receive signals from other neurons

Dorsal—the upper side of the animal, or their back

Dorso-ventral—dorsal to ventral, or from top to bottom of the insect (upper side to the lower side)

Ecdysis—the behavior during which the old cuticle is shed during a molt

Endogenous—hormones originating and being released naturally from the insect

Enzymes—proteins that often serve to produce chemical changes in organic substances (digestion for example)

Excite—to provide input onto the dendrites that result in an action potential in the postsynaptic cell

Exogenous—hormones being added artificially from outside of the insect

Forceps—like tweezers but with very fine tips

Ganglion—a collection of interconnected neurons located along the central nervous system

Ganglionic nerves—the nerves projecting from the ganglia that were recorded from extracellularly for this study (MN-2 and MN-3)

Gland cells—cells in the nervous system that secrete, or release, chemicals such as steroid hormones into the blood

Hormones—chemical messengers secreted inside the body that diffuse throughout the body via the blood and affect the functions of specific tissues within the body

Innervating—growing into; i.e. the nerves grow into the muscles and synapse onto muscle cells, providing the electrical stimulation that causes them to contract

Instar—a larval life stage (between larval molts)

Interneurons—a class of neurons that forms networks of synaptic connections between many other neurons throughout the nervous system

Isolated nerve cord—the abdominal nervous system that has been removed from the insect via dissection; consisting of only the abdominal ganglia and connectives (containing axons) running between them

Life stages—larval, pupal, and adult forms of the insect make up their three life stages

Metamorphosis—the transformation between larval and adult body forms and behaviors

Model system—a simple system that can be studied to make discoveries related to more complex organisms such as humans

Molt—the production of a new cuticle in order to allow growth and metamorphosis

Morphological—pertaining to the body structure or organization

Motor neurons—the type of neuron that passes a signal to muscle cells, instructing them to contract in order to produce the muscle movements required for a certain action

Motor pattern—the pattern of signals traveling through the pre-ecdysis neural circuit, resulting in the muscle contractions that make up the pre-ecdysis behavior

Neural circuits—pathways of neurons through which signals are transmitted from a site in the body to the signal generating center, such as the brain, or from the brain to a site in the body

Neural reorganization—the alteration of neural circuits, involving the deconstruction, weakening, and reconstruction of certain neural circuits

Neurons—the cells comprising the nervous system that carry signals throughout the body in the form of electrical currents.

Neurosecretory cells—cells in the nervous system that secrete, or release, chemicals such as peptide hormones into the nervous system

Neurotransmitters—the chemical messengers used for the vast majority of communications between neurons within synapses

Oscilloscope—an instrument that creates a two-dimensional graph of the electrical signals passing through the neurons

Peptide—a protein or organic molecule; made up of amino acids and comprising most of the mass of an organism

Plasticity—flexibility, or the ability to be modified or altered

Posterior—toward the tail of the insect

Postsynaptic cell—the cell that a signal is being transferred to through a synapse

Pre-ecdysis behavior—the behavior that loosens the old cuticle prior to shedding the cuticle at the culmination of a molt

Pre-Ecdysis Triggering Hormone (PETH)—the peptide hormone that initiates the pre-ecdysis motor pattern

Preparations—the isolated abdominal nerve cord that has been pinned in a Sylgard plate to allow recordings to be made from the ganglionic nerves

Prepupal—insects preparing to enter the transition from the larval to the pupal life stage

Sheath—a layer of tissue surrounding each ganglion and the axons and connectives that run between the ganglia within the nerve cord

Staging Insects—the process of staging insects involves determining the approximate time until they undergo the process of ecdysis, using physiological markers

Steroid—a particular type of organic chemical compound that has a specific physiological action (example: sex hormones such as testosterone)

Suction electrodes—a small probe that picks up electrical currents passing through the axons of neurons which have been placed inside the glass tip using suction

Sylgard—a type of clear silicone polymer that holds in place the pins securing the isolated nerve cord within the Sylgard-lined dish

Synapse—the connections through which two neurons communicate signals using chemical or electrical messages

Terminal—the portion of the neuron at the end of the axon that communicates with other neurons

Terminal abdominal ganglion—the collection of neurons located within the most posterior body segments of the insect

Thoracic—pertaining to the portion of the body of the insect located between the head and the abdomen

Tonic activity—action potentials are passed along the axon at a fairly constant level, with little change in the spike rate, and there is a lack of bursting or other changes in activity

Trachea—the respiratory tubing which delivers oxygen throughout the insect's body

Ventral—the lower side of the animal, or their stomach

Ventral nerve cord—a collection of neurons and bundles of axons that stretches along the lower (ventral) surface of the insect

Wandering—a behavior that marks the beginning of metamorphosis and involves the late 5^{th} larval instar insects stirring up their frass (feces) with their diet by increased locomotion as well as cessation of feeding

LITERATURE CITED

Bell, R.A. and F.A. Joachim (1976). "Techniques for rearing laboratory colonies of tobacco hornworms and pink bollworms." *Annals of the Entomological Society of America* 69: 365-373.

Copenhaver, P.F. and J.W. Truman (1982). "The role of eclosion hormone in the larval ecdyses of *Manduca sexta*." *Journal of Insect Physiology* 28: 695-701.

Heywood, R.B. (1965). "Changes occurring in the central nervous system of *Pieris brassicae* L. (Lepidoptera) during Metamorphosis." *Journal of Insect Physiology* 11: 413-430.

Jacobs, Gwen A. and Janis C. Weeks (1990). "Postsynaptic Changes at a Sensory-to-Motoneuron Synapse Contribute to the Developmental Loss of a Reflex Behavior During Insect Metamorphosis." *The Journal of Neuroscience* 10(4): 1341-1356.

Kandel, Eric R., James H. Schwartz, and Thomas M. Jessell. *Principles of Neural Science*. New York: McGraw-Hill Companies, 2000.

Knittel, Larua M. and Karla S. Kent (2005). "Remodeling of an Identified Motoneuron during Metamorphosis: Hormonal Influences on the Growth of Dendrites and Axon Terminals." *Journal of Neurobiology* 63(2): 106-125.

Levine, R.B. and J.W. Truman (1985). "Dendritic Reorganization of Abdominal Motoneurons during Metamorphosis of the Moth, *Manduca sexta*." *The Journal of Neuroscience* 5(9): 2424-2431.

Liepert, J., W.H.R Miltner, H. Bauder, M. Sommer, C. Dettmers, E. Taub, and C. Weiller (1998). "Motor cortex plasticity during constraint-induced movement therapy in stroke patients." *Neuroscience Letters* 250(1): 5-8.

Miles, C., and J.C. Weeks (1991). "Developmental attenuation of the pre-ecdysis motor pattern in the tobacco hornworm, *Manduca sexta*." *Journal of Comparative Physiology [A]* 168: 179-190.

Miltner, W.H.R., H. Bauder, M. Sommer, C. Dettmers, and E. Taub (1999). "Effects of Constraint-Induced Movement Therapy on Patients With Chronic Motor Deficits After Stroke: A Replication." *Stroke* 30: 586-592.

Novicki, A., and J.C. Weeks (1993). "Organization of the larval pre-ecdysis motor pattern in the tobacco hornworm, *Manduca sexta*." *Journal of Comparative Physiology [A]* 173: 151-162.

Novicki, A. and J.C. Weeks (1995). "A single pair of interneurons controls motor neuron activity during pre-ecdysis compression behavior in larval *Manduca sexta*." *Journal of Comparative Physiology [A]* 176: 45-54.

Novicki, A. and J.C. Weeks (2000). "Developmental attenuation of *Manduca* pre-ecdysis behavior involves neural changes upstream of motoneurons and relay interneurons." *Journal of Comparative Physiology [A]* 186: 69-79.

Trimmer, B.A., and J.C. Weeks (1989). "Effects of nicotinic and muscarinic agents on an identified motoneurone and its direct afferent inputs in larval *Manduca sexta*." *Journal of Experimental Biology* 144: 303-337.

Truman, James W, Paul H. Taghert, and Stuart E. Reynolds (1980). "Physiology of Pupal Ecdysis in the Tobacco Hornworm, Manduca sexta I. Evidence for Control by Eclosion Hormone." *Journal of Experimental Biology* 88: 327-337.

Weeks, Janis C. (2003). "Thinking globally, acting locally: steroid hormone regulation of the dendritic architecture, synaptic connectivity and death of an individual neuron." *Progress in Neurobiology* 70: 421-442.

Weeks, J.C. and G.A. Jacobs (1987). "A reflex behavior mediated by monosynaptic connections between hair afferents and motoneurons in the larval tobacco hornworm, *Manduca sexta*." *Journal of Comparative Physiology [A]* 160: 315-329.

Weeks, J.C. and J.W. Truman (1984). "Neural organization of peptide-activated ecdysis behaviors during the metamorphosis of *Manduca sexta*. I. Conservation of the peristalsis motor pattern at the larval-pupal transformation." *Journal of Comparative Physiology [A]* 155: 407-422.

Zitnan, D., L.S. Ross, I. Zitnanova, J.L. Hermesman, S.S. Gill and M.E. Adams (1999). "Steroid Induction of a Peptide Hormone Gene Leads to Orchestration of a Defined Behavioral Sequence." *Neuron* 23: 523-535.