THE DROSOPHILA AND MANDUCA HEARTS AS MODELS FOR STUDYING
THE ROLE OF INNERVATION IN CARDIAC FUNCTION

by

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DEDICATION

This dissertation is dedicated to my children, Matteo and Marco Boassa-Dulcis.
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ABSTRACT

Cardiac activity of *Drosophila melanogaster* and *Manduca sexta* changes during metamorphosis. The larval heart has only anterograde contractions. Adult heart activity becomes a cyclic alternation of anterograde and retrograde contractions originated by putative anterograde and retrograde pacemakers.

During the development of Manduca, the larval skeletal muscle motoneuron-1 in abdominal segments 7-8 becomes respecified to innervate the terminal cardiac chamber of adults and undergoes morphological and physiological reorganization. MNs-1 activate and sustain the anterograde pacemaker activity of the terminal chamber.

The innervation of the adult abdominal heart of *Drosophila melanogaster* was studied to determine whether the adult heart receives neuronal input or whether its complex activity must be considered independent from the nervous system. The larval heart lacks innervation suggesting a myogenic cardiac impulse. At metamorphosis, neural processes grow onto the myocardium. A pair of glutamatergic transverse nerves innervates bilaterally each cardiac chamber. In addition, CCAP-immunoreactive fibers originating from peripheral, bipolar neurons (BpNs) fasciculate with the transverse nerve projections and terminate segmentally throughout the abdominal heart.

To determine the role of this innervation in cardiac function, a novel optical technique based on the movement of GFP-labeled nerve terminals was developed to monitor heartbeat in intact preparations. Simultaneous monitoring of adjacent cardiac chambers revealed the direction of contractions and allowed correlation with volume
changes. Intracellular recordings from the first abdominal cardiac chamber, the conical chamber, revealed pacemaker action potentials and the excitatory effect of local glutamate application. Bath-applied glutamate initiated retrograde contractions in semi-intact preparations. Similarly, electrical stimulation of the transverse nerve that serves the conical chamber caused a chronotropic effect and initiation of retrograde contractions. This effect is distinct from that of peripheral CCAP-immunoreactive neurons, which potentiate the anterograde beat. Cardiac reversal was evoked pharmacologically by sequentially applying CCAP and glutamate to the heart.

The role of the neuropeptide, Crustacean Cardioactive Peptide (CCAP) in adult *Drosophila melanogaster* cardiac function was studied by RNA interference (RNAi) and targeted cell ablation. CCAP has a cardioacceleratory effect when it is applied *in vitro*. Lack of CCAP-innervation in CCAP knock-out flies altered one cardiac phase, the anterograde beat, without preventing the cyclic cardiac reversal.
CHAPTER 1. BACKGROUND AND LITERATURE REVIEW

1.1 BLOOD FLOW REGULATION IN OPEN AND CLOSED CIRCULATORY SYSTEMS

The blood contributes to the maintenance of the homeostasis in complex organisms only as long as it stays in motion. When stationary, the blood loses its important functions as nutrients and gas gradients become exhausted and/or saturated quickly. Circulatory systems fall into two categories depending on how blood circulation is organized.

Closed circulatory systems, such as those found in humans, are cardiovascular systems that transport blood in a closed circuit. The blood exits and returns from and to the heart flowing within arteries, capillaries and veins but never leaves the vessels, although nutrients and waste products are exchanged. In contrast, the blood vessels of open circulatory systems in invertebrates, such as insects, are reduced to a single dorsal vessel. The hemolymph flows through the body cavity, or hemocoel (reviewed by Jones, 1977; Miller, 1985; Wasserthal, 1998). The dorsal vessel of insects, which has contractile properties (Brazeau and Campan, 1970; Markou and Theophilidis, 2000), is a tube-like heart containing highly specialized structures, such as localized pacemaker tissue, a conducting system, valves and chambers (McCann, 1970; Sanger and McCann, 1968; Hessel, 1969; Rizki, 1978; Angioy et al., 1999). It is divided into the thoraco-cephalic aorta and the abdominal heart (reviewed by Jones, 1977; Miller, 1985).

The hemolymph contained in the insect abdominal hemocoel enters the heart flowing through cardiac valves and is subsequently pumped into the aorta (Figure 1.1;
Wigglesworth, 1972). The latter, which has more of a conduction function (Angioy et al., 1999) opens in front of the brain. From the head capsule, the blood has to flow passively through the thoracic and abdominal cavities in order to re-enter the heart.

The maintenance of a relatively constant internal environment (homeostasis) is a complex task that both closed and open circulatory systems must accomplish. Insects lack respiratory pigments, the oxygen being transported by tracheae directly to the tissues, however the hemolymph maintains most of the functions of vertebrate blood, such as transport of CO₂, nutrients, catabolites, hormones, phagocytic cells and thermoregulation.

Although open and closed circulatory systems have different vascular organization, they share the need to regulate blood flow according to the dynamic conditions of the organism. Holometabolous insects, which reach the adult stage by going through complete metamorphosis, can shunt hemolymph flow from the head/thorax compartment to the abdominal hemocoel and vice versa through a phenomenon called cardiac reversal (Figure 1.2; Dulcis et al., 2001). Cardiac activity in adult lepidopterans and dipterans is a complex pattern characterized by cyclic alternation of anterograde (Figure 1.2, lower trace in blue) and retrograde (Figure 1.2, lower trace in red) phases of contraction; reversal of these phases results in reversal of the direction of hemolymph flow (Tenney 1953; Queinnec and Campan 1972; Wasserthal 1976; Ichikawa and Ito 1999; Smits et al. 2000, Dulcis et al., 2001). That these two phases of contraction correspond to reversal of hemolymph direction was recently demonstrated convincingly in the fly Calliphora vicina by using thermocouples (Wasserthal, 1999). This phenomenon indicates that contractions of the dorsal vessel can originate either at the
posterior end of the heart or at the anterior end of the aorta and, thus, that there is an anterograde pacemaker associated with the terminal chamber of the heart and a retrograde pacemaker, probably near the anterior end of the aorta. An alternative hypothesis was suggested by Rizki (1978) in Drosophila. The author suggested that the first abdominal chamber, named the conical chamber for its shape and located at the junction of the heart and aorta, might represent the location where the retrograde beat, thus cardiac reversal, originates. This is in agreement with what has been described for larger flies, such as *Calliphora vicina* (Wasserthal, 1999). During the anterograde beat of adult Calliphora the hemolymph flows into the abdominal heart through lateral incurrent ostia and is pumped forward into the aorta. During this phase, which represents the systolic phase of a multi-chambered heart, the hemolymph exits the anterior aorta and flows back accumulating in the thorax (Fig. 1.3A). When the retrograde beat starts, the hemolymph is aspirated from the thorax into the first cardiac chamber through the first pair of incurrent ostia. Then, it is pumped backward to fill the posterior chambers (diastolic phase) and accumulates in the abdominal hemocoel through the posterior excurrent opening (Fig. 1.3B). However, the specific location of these putative pacemakers has not yet been demonstrated in either Lepidoptera or Diptera. Cardiac reversal does not occur in the larval stage in which the hemolymph is merely pumped in the anterograde direction. The anterograde beat therefore does not require cardiac innervation in the larval stage.

In resting adult insects, cardiac reversal occurs spontaneously at a regular frequency. The control of heart reversal has remained an intriguing but poorly understood phenomenon. The involvement of the nervous system in cardiac reversal has been
questioned because even isolated heart chambers can exhibit different contraction rates and directions (Gerould 1938; Tenney 1953; McCann 1970). Insect hearts have propagated myogenic contractions (reviewed by McCann 1970), and, therefore, under experimental conditions contraction may be initiated at any point on the dorsal vessel. In the intact insect, however, it is very likely that cardiac contractions usually originate only at the anterior and posterior ends of the dorsal vessel or of the abdominal heart, as suggested by Rizki (1978).

Cardiac reversal in Lepidoptera and Diptera can also be triggered as a response to a variety of sensory stimuli (reviewed by Kuwasawa et al. 1999), and this response has the characteristics of a neural reflex. The cardiac reversal reflex has been used in several studies of the neuronal modulation of heart activity. In Diptera the reflex can be initiated by changes in illumination (Campan 1972), by motor responses to visual stimuli (Thon 1982), and by feeding activity (Angioy 1988). In Lepidoptera the reversal can be initiated by mechanical and olfactory stimuli (Ai and Kuwasawa 1995; Angioy et al. 1998, Dulcis et al., 2001), by calling behavior (Ichikawa and Ito 1999), and by an increase in thoracic temperature resulting from flight activity (Heinrich 1970). These studies suggest that the physiological significance of cardiac reversal is related to the onset of motor activity associated with various types of behavior. A requirement common to these activities is a need for an increase in the rate of exchange of respiratory gases. Cardiac reversal results in pressure changes that produce tracheal ventilation (Wasserthal 1981, 1996). Thus, the nervous system must be modified during metamorphosis to provide the circuitry for both the sensory-evoked and the spontaneous cardiac reversal.
1.2 DUAL INNERVATION OF THE HEART AS A GENERAL CONTROL MECHANISM OF CARDIAC PACEMAKERS

One of the mechanisms by which the mammalian circulatory system changes blood flow is by modulating cardiac contractility (heart rate and force of ventricular contraction). These cardiac parameters are influenced by the autonomic nervous system. Dual innervation by the sympathetic and parasympathetic components greatly affects the default pacemaker activity of the heart. Sympathetic innervation increases heart rate by releasing noradrenaline and activating adrenergic receptors on the myocardium. Conversely the vagal parasympathetic innervation inhibits heartbeat by releasing acetylcholine, which activates muscarinic receptors. Furthermore a collection of local neurons form the intrinsic ganglionated plexus, which is localized within fat pads of the interventricular and interatrial septum (Armour, 1991). The role of these intrinsic cardiac neurons is not completely understood, although they contain a variety of cardioactive peptides, such as neuropeptide Y, atrial natriuretic peptide, neurotensin, and substance P (Galoyan et al., 2001). The intrinsic cardiac nervous system remodels itself after cardiac transplantation (Murphy et al., 2000) but direct assessment of extracardiac and intrinsic cardiac neuronal behavior is required to fully understand cardiac control after transplantation. The process of innervation of larval myogenic hearts during postembryonic insect development may provide a useful model to study general mechanisms of neuronal cardiac control and its development, applicable to mammalian systems.

Dual innervation and neuropeptides are general mechanisms of controlling
cardiac function. Examples of dual innervation, comprising motor and neurosecretory innervation, have been described in many insects, such as moths Caligo and Sphinx (Wasserthal, 1977; 1980), flies Glossina and Protophormia (Anderson, 1978; Angioy et al., 1999), cockroaches (Miller, 1973; Sinakevitch et al., 1996) and locusts (Stevenson and Pflueger, 1995; Duch et al., 1999). The ultrastructure of synapses on the alary muscles and myocardium of the terminal chamber in Sphinx ligustri, a moth related to Manduca, has been described by Wasserthal and Wasserthal (1977). These synapses contain both dense-core and lucent vesicles. The lucent vesicles release their contents into the synaptic cleft. These vesicles probably contain a classical transmitter substance, but it has not been identified. As at other synapses, the dense-core vesicles probably have a modulatory function and may account for the crustacean cardio-active peptide (CCAP) immunoreactivity of these synaptic endings, as noted by Davis et al. (2001) in Manduca.

A recent study of heart innervation in Manduca has shown distinct differences between the larval and adult stages (Davis et al., 2001). The larval heart lacks innervation, other than neurohemal endings on the alary muscles, and the myogenic activity of the larval heart appears to be modulated by circulating hormones. In the adult, the terminal chamber of the heart is innervated by branches of the seventh and eighth segmental dorsal nerves (Figure 1.4; Davis et al., 2001). Identified pairs of bilateral motor neurons (MNs-1) located in the terminal abdominal ganglion project through these nerves to terminate on the terminal heart chamber. These neurons and their terminals are labeled by an antiserum to crustacean cardioactive peptide (Davis et al., 2001). This peptide is known to stimulate heart contractions in Manduca (Cheung et al., 1992; Lehman et al., 1993).
Because MN-1 innervates a skeletal muscle in the larval stage, it may also release glutamate at cardiac synapses, as glutamate is the excitatory neurotransmitter released at neuromuscular junctions in insects.

A physiological study of the role of cardiac innervation in Manduca (Dulcis et al., 2001) clarified how the central nervous system exerts cardiac control in holometabolous insects. Neuronal blockage, produced by TTX in adults, resulted in elimination of cardiac reversal and, notably, the elimination of the anterograde phase of contraction. As the heart became stabilized, the contractions were slow, regular, and retrograde. These results suggest that neuronal control of the moth heart is exerted through regulation of the anterograde phase of contraction. Although the anterograde beat in larvae does not require cardiac innervation, once the anterograde/retrograde cardiac reversal develops in adults, the posterior pacemaker may require innervation to reinitiate the anterograde beat. Based on these results the authors proposed a model by which cardiac reversal is controlled in adult Manduca. When the anterograde pacemaker receives input from MN-1, this pacemaker is dominant and initiates a phase of rapid-anterograde contractions. After a period of time MN-1 becomes inactive; this change results in the inactivation of the anterograde pacemaker and the anterograde contractions cease. The retrograde pacemaker then initiates a phase of retrograde contractions. After a second period of time, MN-1 becomes reactivated to initiate another series of anterograde beats. Thus, the regular cardiac reversal cycle may be controlled by the periods of activity and inactivity of MN-1, although this must be confirmed and the mechanism controlling the cyclic activity of these cells remains to be determined. Similarly, it is not known how MN-1 is
modified structurally and functionally as it is remodeled from a skeletal muscle motoneuron in the larval stage, to control the anterograde cardiac pacemaker in the adults.

Insect myocardial cells express pacemaker potentials (reviewed by McCann 1970; Ebara et al. 1990; Markou and Theophilidis 2000), but they have not been studied in myocardial cells from regions of the dorsal vessel that serve as pacemakers in the heart. Dulcis et al. (2001) hypothesized that the pacemaker potentials of the myocardial cells of the anterior aorta of adult Manduca have a steeper slope than other myocardial cells; this feature would confer to the anterior aorta the properties of a retrograde pacemaker.

The anterograde pacemaker is activated by bursts of impulses from MN-1 (Dulcis et al., 2001). Thus, MN-1 may be driven by a central pattern generator or express intrinsic burst-generating properties. Alternatively, the cyclic reversal of contraction phases in the heart of Manduca may result from feedback between the heart pacemaker system and neurons in the terminal abdominal ganglion. Clearly, further study of various aspects of the control of heart reversal in insects is needed, and this system may prove be a useful model for the study of pacemaker activity and the production of rhythmic motor patterns.
1.3 MORPHOLOGICAL AND PHYSIOLOGICAL CHANGES OF DROSOPHILA MELANOGASTER CIRCULATORY SYSTEM DURING METAMORPHOSIS

The contractile dorsal vessel of Drosophila extends the entire body length, and consists of a distinct abdominal heart and a thoraco-cephalic aorta which communicate through the cardio-vascular valve (Rizki, 1978). Ultrastructural differences between aorta and abdominal heart myocardium of the adult dipteran P. terraenovae suggest that the abdominal heart may represent the pump of this open circulatory system and the aorta may function more as a weak contractile vessel conducting the hemolymph to the head capsule (Angioy et al., 1999). As is typical of other insects, the heart in Drosophila consists of a series of segmental chambers marked by incurrent ostia and sets of alary muscles (Rizki, 1978; Curtis et al., 1999). During diastole, dilation of each cardiac chamber by the coupled alary muscle contractions results in hemolymph inflow through the lateral ostia. The contraction of intrinsic cardiac muscles of each chamber during systole causes ostia closure and hemolymph movement into the adjacent chamber (Rizki, 1978).

The larval heart undergoes dramatic morphological and physiological changes during adult development. Anterior to the cardio-vascular valve, a conical chamber forms "de novo" as a product of differentiation of the proximal stem of the aorta (Rizki, 1978). In addition, an outer layer of longitudinal muscle fibers develops along the ventral surface of the heart (Miller, 1950; Molina et al., 2001). The myofibers of this ventral muscle layer are distributed symmetrically along the conical chamber and become distorted posterior to it (Rizki, 1978).
Before the adult emerges from the puparium, cyclic alternation of anterograde and retrograde phases of contractions, referred to as cardiac reversal, replaces the constant anterograde larval heartbeat (Rizki, 1978). Because the isolated heart shows two pulsatile regions, one at the caudal end and another at the junction of the heart and aorta, Rizki (1978) hypothesized that the conical chamber might represent the location where the retrograde beat, thus cardiac reversal, originates.

*In vitro* experiments have shown that neurotransmitters (Johnson et al., 1997; Zornik et al., 1999) and neuropeptides (Nichols et al., 1999a) influence the heart rate of Drosophila at any stage throughout metamorphosis. Because the literature fails to describe any cardiac innervation, it was concluded by Johnson et al. (1997) that the heart of Drosophila is myogenic, with neurotransmitters and neuropeptides in the hemolymph acting as modulators of the heartbeat. Immunolocalization of the cardioinhibitory peptide SDNFMRFamide in the anterior region of the adult aorta (Nichols et al., 1999b) suggested, however, that the adult heart of Drosophila receives neuronal input at specific regions.

During the larval stage and for the first day after pupation, the heartbeat of Drosophila is myogenic (Dowse et al., 1995) and originates in a pacemaker that is probably located in the most caudal region of the heart. Because both cardiac remodeling and heartbeat reversal occur later in adult development, cardiac innervation also might develop during this stage so as to control adult heartbeat. The presence of segmental innervation of the abdominal heart has already been described in adults of other fly species, such as *Glossina morsitans* (Anderson and Finlayson, 1978). However, perhaps
because of the small size of Drosophila, classical neural tracing techniques have failed to demonstrate any innervation of the adult abdominal heart. The presence of a nerve observed in histological sections of the conical chamber (Miller, 1950) is, until now, the only evidence of innervation in the adult abdominal heart. Rizki (1978) suggested that the origin of the cardiac impulse in this chamber might be neurogenic. Clearly, further examination of the adult heart, and the possibility of innervation, is warranted.

In general the embryological origins as well as gene expression patterns are similar in Drosophila and vertebrate hearts. For example, the homeobox gene tinman (Bodmer, 1993), which is expressed in the embryonic heart of Drosophila and is necessary for its development, has mouse, frog and fish homologs with considerable sequence and functional similarity (Bodmer, 1995). Therefore, insect cardiac innervation could represent a novel experimental model to study the development of cardiac innervation. The natural process of cardiac innervation during insect metamorphosis, together with the availability of powerful genetic tools in Drosophila, may reveal general mechanisms applicable to re-innervation of transplanted mammalian hearts (Murphy et al., 2000). Similarly, studying the role of neuropeptides and neurotransmitters in insect cardiac function may illuminate mechanisms by which peptidergic intrinsic cardiac neurons in humans exert their activity in both normal and clinical scenarios. Finally, the understanding of the physiological differences between myogenic and neurogenic hearts may allow the Drosophila adult heart to serve as a new pharmacological model for testing new drugs that act directly on the myocardium or at cardiac synapses.
1.4 SPECIFIC AIMS

Despite the morphological differences between open and closed circulatory systems, neuronal control of cardiac activity is a common mechanism shared by vertebrates and invertebrates to guarantee homeostasis in the face of dynamic conditions during different types of behavior.

Holometabolous insects, such as the lepidopteran Manduca sexta and the dipteran Drosophila melanogaster, are good systems in which to study neuronal control of cardiac function because this mechanism is acquired during post-embryonic development, when the myogenic larval heart comes under the control of the adult nervous system. Thus, the physiology of the heart can be compared before and after innervation has developed. Detailed knowledge of the organization of the central nervous system in Manduca allowed the study of cardiac control at the level of single identified neurons. In parallel, Drosophila mutants were used to distinguish the distinct physiological roles of cardiac neurons utilizing a classical neurotransmitter and a neuropeptide in the control of the adult heart.

My first specific aim was to determine whether an identified larval skeletal muscle motoneuron (MN-1) modifies its morphological and physiological properties during respecification to innervate the heart in adult moths (Manduca sexta). I compared the morphology of MN-1 at larval and adult stages using retrograde dye fills and intracellular dye injections to determine whether its respecification was accompanied by remodeling of the dendritic tree. I recorded MN-1 activity intracellularly with sharp electrodes and extracellularly with suction electrodes. The experiments described in
Chapter 2 were performed to define MN-1 pattern of activity *in vitro* and *in vivo*, to compare its larval and adult intrinsic properties, and to determine if a central pattern generator produces the adult MN-1 output. I monitored heart activity using isometric transducers and performed simultaneous intracellular and extracellular recordings of MN-1 activity to define its function in cardiac regulation in the adult. Stimulation of sensory structures (chordotonal organs, antennae, etc.) and simultaneous extracellular and/or intracellular recording of MN-1 determined the role of MN-1 during reflex responses such as cardiac reversal.

My second specific aim was to describe the nature of cardiac innervation in adult fly *Drosophila melanogaster*. I used ELAV-GAL5/UAS-GFP transgenic flies to directly image adult cardiac innervation and its development. In Chapter 3 I defined immunocytochemically the nature of cardiac synapses and identified the neurotransmitters and neuropeptides expressed by central and peripheral cardiac neurons. I have also employed specific molecular markers to identify active zones and postsynaptic receptors.

My third specific aim was to characterize heart function of adult *Drosophila melanogaster*. Photo-detection of myocardial contractions in intact wild-type adult flies was the technique I designed to describe *in vivo* cardiac activity at rest and define diastolic/systolic phases of this multi-chambered heart. Real time recording of *in vitro* and *in vivo* heartbeat, which are described in Chapter 3, were obtained with a CCD camera that allowed to visualize volume changes, dynamics, and direction of propagation of cardiac contractions.
Finally, my fourth specific aim was to determine the consequences of dual innervation for cardiac activity in adult fly *Drosophila melanogaster*. To describe the specific role of glutamatergic innervation, I performed intracellular recordings of epsps and cardiac action potentials following glutamate bath application to determine the effect on the myocardium. In addition to the latter experiment, Chapter 4 includes optical measurements of cardiac contractions to monitor the effect of bath-applied glutamate on the direction of cardiac contractions. Extracellular stimulation was applied to the transverse nerves that serve the conical chamber to determine whether its activity was sufficient to produce the retrograde beat.

The experiments described in Chapter 5 were designed to examine the role of peptidergic (CCAP) innervation. *In vivo* recordings of cardiac activity in transgenic flies that had CCAP-releasing neurons knocked out or knocked down were performed to define how the heartbeat is altered when CCAP neuromodulation is abolished or reduced. Finally, bath application of CCAP determined its pharmacological effect on cardiac activity.
Figure 1.1: Diagrammatic representation of the blood circulation in an intact insect with fully developed circulatory system. Arrows indicate the course of circulation. Hemolymph enters the abdominal heart through ostia located bilaterally in each abdominal cardiac chamber and then pumped forward into the aorta and the head capsule during the anterograde beat. Circulation inverts direction during the retrograde beat.

Figure 1.2: Cardiac cycle of adult Manduca sexta. Monopolar, extracellular recording showing three repetitive cardiac cycles of the heart of adult M. sexta. The trace between dashed lines shows a complete cardiac cycle at expanded time scale. The arrows indicate the relative duration of each cardiac phases.
Figure 1.3: Blood circulation in *Calliphora vicina*. Schematic drawings showing position of ostia, caudal opening, and abdominal air sacs. O1-O5, incumbent ostia of abdominal segments 1-5; EO, caudal excurrent opening. Arrows indicate the direction of hemolymph flow during the anterograde (A) and the retrograde (B) beats. The first cardiac chamber is situated anteriorly to the large air sacs (Wasserthal, 1999).
Figure 1.4: Innervation of the terminal cardiac chamber in *Manduca sexta*. Depiction of the dorsal nerves (DN7, DN8) that extend from the terminal abdominal ganglion (TAG) to their caudal cardiac branches (CCN7, CCN8) to innervate the terminal chamber (TCh) of the heart and its alary muscles (AM). The dorsal nerves extend over the spiracular chambers (SpCh) of each segment, and transverse nerve-8 (TN8) is almost completely incorporated into dorsal nerve-8. The basal region of transverse nerve-7 (TN7) and its link nerve (LN) can be distinguished.
CHAPTER 2. REMODELING OF LARVAL SKELETAL MUSCLE MOTONEURON TO DRIVE THE POSTERIOR CARDIAC PACEMAKER IN THE ADULT MOTH, *MANDUCA SEXTA*

2.1 INTRODUCTION

The circulatory systems of holometabolous insects undergo dramatic changes during metamorphosis as the larval myogenic heart becomes innervated (Davis et al., 2001; Dulcis and Levine, 2003). This phenomenon offers a unique opportunity to address both the developmental mechanisms that regulate the formation of cardiac innervation and the role of heart innervation in cardiac function. In the moth, *Manduca sexta*, the neuron that will innervate the adult heart is already present during the larval stage as a skeletal muscle motoneuron (Davis et al., 2001). This study investigates whether the larval motoneuron acquires new structural and functional properties to accommodate its new role in controlling the anterograde cardiac pacemaker in the adult.

Hemolymph circulation in adult Lepidoptera is mediated by a tubular multichambered heart located underneath the dorsal midline of the body wall. The activity of this relatively simple heart is characterized by cyclic alternation of anterograde and retrograde beats. This reversal has the physiological role of periodically reversing the direction of hemolymph flow (Tenney, 1953; Wasserthal, 1976; Smits et al., 2000; Dulcis et al., 2001). Although cardiac reversal is a spontaneously-occurring phenomenon that participates in the regular cardiac cycle of a resting moth, it can also be initiated by a variety of visual, mechanical and olfactory stimuli (reviewed by Kuwasawa, 1999). Interestingly, cyclic reversal is coupled to other systems, such as tracheal ventilation (Wasserthal, 1981, 1996) and neurosecretion (Ichikawa and Okada, 2002) and is
correlated with behaviors such as female calling (Ichikawa and Ito, 1999).

Cardiac reversal has the characteristics of a fast neural reflex that can be abolished by TTX blockade of neuronal activity (Dulcis et al., 2001). In Manduca sexta the 7th and 8th dorsal nerves (DN7 and DN8) innervate the caudal chamber of the adult abdominal heart (Davis et al., 2001). Surgical transection of DN8 causes the loss of the anterograde beat in both in vitro and in vivo preparations (Dulcis et al., 2001). Although the myocardium possesses myogenic properties, the anterograde beat always requires neuronal activity in order to be initiated and sustained (Dulcis et al, 2001). The retrograde beat represents the default cardiac function in denervated adult hearts. DN8 stimulation in such preparations evokes cardiac reversal by initiating the anterograde beat in the caudal chamber (Dulcis et al., 2001). Using neuronal tracing techniques and immunocytochemistry, Davis et al. (2001) discovered that two pairs of CCAP-immunoreactive motoneurons (MNs-17 and MNs-18), with somata located bilaterally in the seventh and eighth neuromeres of the terminal ganglion, innervate the caudal chamber of adult moths.

Motoneuron-1 (MN-1) is present in every abdominal segment (Taylor and Truman, 1974). In most segments it innervates a skeletal muscle (dorsal external oblique muscle-2) in larvae. During metamorphosis this muscle degenerates and, in most segments, MN-1 becomes respecified to innervate a new adult skeletal muscle (dorsal external muscle-4) and undergoes extensive dendritic remodeling (Levine and Truman, 1985). In the 7th and 8th segments of adults, however, MN-1 becomes respecified to innervate the caudal cardiac chamber of the adult heart (Davis et al., 2001). Neural
tracing techniques and electrophysiology were used in the present study to determine whether this unique target respecification from a skeletal muscle to a visceral organ, was accompanied by structural and functional remodeling of MN-1. We found that MN-1 acquires new morphological and physiological properties to control the anterograde cardiac pacemaker of adult moths.

2. MATERIALS AND METHODS

(1) Experimental animals. Larvae and adults of the tobacco hawkmoth, *Manduca sexta*, were obtained from our laboratory colony. The larvae were reared at 25°C, 50-60% relative humidity, under a long-day photoperiod regimen (17/7 h light/dark), and on an artificial diet adapted from that of Bell and Joachim (1976). Day-1 or -2, fifth-instar larvae and adults were used for most experiments. All experiments were conducted at an ambient temperature range of 23-25°C. The adult and larval specimens were anesthetized on crushed ice for about 20 minutes before dissection. A physiological saline solution formulated for *M. sexta* (Trimmer and Weeks, 1989) was perfused for dissections and recordings.

(2) Neuronal tracing techniques. Motoneuron somata were traced by retrograde filling of Rhodamine Dextran 3000 (Molecular Probes, Eugene, OR) through dorsal nerve stumps. To visualize fine dendritic branches the incubation period was 2-3 days at about 4°C. To keep the CNS alive, the vaseline pool containing the dye was built inside the restrained body of the animal and the cuticle sealed with vaseline (Sherwood, St. Louis, MO). After
filling, the material was fixed in 4% paraformaldehyde in 0.1 M phosphate buffer solution (PBS) for 12h at room temperature, washed 3 times for 10 minutes each in PBS (pH 7.4), dehydrated in Ethanol series (50%, 70%, 95%, 100%) for 10 minutes each, cleared in methyl salycilate (Sigma, St. Louis, MO), and mounted in Permount (Fisher, Fair Lawn, NJ). Fourteen larval DN8, fifteen adult DN8, seven adult DN7, and three adult DN6 preparations were analyzed.

To visualize the somata of the sensory neurons of the chordotonal organ (CO), orthograde Rhodamine Dextran 3000 fills of dorsal nerve stumps were incubated overnight at about 4°C. Terminals in the TAG neuropil of CO sensory neurons were stained by retrograde Rhodamine Dextran 3000 fills of the hemisected CO. After fixation the preparations were fixed, dehydrated, cleared and mounted as described above.

(3) Intracellular staining techniques. Five adult MNs-1 preparations that where successfully backfilled for 2-3 days with Rhodamine Dextran 3000 were co-labeled with Lucifer Yellow (Sigma) in LiCl2 solution that was injected intracellularly. The tips of thin-walled borosilicate electrodes (resistance, 25-30 MΩ) were filled with Lucifer Yellow. After intracellular penetration and identification of the orthodromic spike of MN-1 in the dorsal nerve, dye was injected iontophoretically by applying a constant hyperpolarizing current (5nA) for 10 to 30 minutes. After injection the preparations were fixed, dehydrated, cleared and mounted as previously described.

(4) Laser scanning confocal microscopy. Digital images of filled neurons were collected
on a Nikon PCM 2000 laser-scanning confocal microscope equipped with green He/Ne (543 nm), red He/Ne (633 nm) and argon (457 nm) lasers. Rhodamine Dextran fluorescence was detected with the green He/Ne laser line and using a band-pass filter at 565 nm. Scanning with an argon laser and using a band-pass filter at 510 nm detected Lucifer Yellow fluorescence. Stacks of digitized images were merged by using Simple PCI (Compix Inc., Cranberry Township, PA) as image acquisition software. Corel Draw and Corel Photopaint (Corel Corp., Ottawa, Ontario, Canada) were used to enhance contrast and provide color when needed. Prints were made with a Tektronix Xerox Phaser 6200 printer.

(5) Recording of cardiac activity. Contractions of the heart were monitored in vitro and in vivo by an isometric transducer (Model 602297, Harvard Apparatus, South Natick, MA) connected to a tungsten wire the tip of which rested on the heart wall (Fig. 2.6D). The isometric transducer was set to a 0-0.5 g range, and the signals were acquired and displayed on the computer screen using the Axoscope 9.0 software (Axon Instruments, Foster City, CA).

(6) Intracellular and extracellular recordings. An axoclamp 2B amplifier (Axon Instruments) was used for intracellular recordings. Action potentials were recorded in bridge mode with thin-walled borosilicate electrodes (resistance, 25-30 MΩ) filled with 1M potassium acetate.

A differential AC amplifier (Model 1700, A-M Systems Inc., Everett, WA) was
used for extracellular recordings. Extracellular recordings from dorsal nerves were made with suction electrodes fabricated from microhematocrit capillary tubing (Scientific Products, McGaw Park, IL). The tips were made with a vertical pipette puller (Model 700C, David Kopf Instruments, Tujunga, CA) and then cut with forceps to the correct size under a dissecting microscope. The nerve stumps were sucked into the tip of the suction electrode by applying negative pressure with a syringe. To improve signal/noise ratio, fat tissue was sometime sucked in along with the nerve. Bilateral recordings from DNs8 in intact adult preparations were made by lifting the DNs through small incisions in the cuticle with small hooks. A loop of the intact DN was then sucked into the suction electrode tip.

Intracellular and extracellular signals were acquired with Clampex 9.0 (Axon Instruments). Datapac III (Laguna Hills, CA) and Clampfit 9.0 (Axon Instruments) were the software used to analyze the instantaneous spike frequency of MN-1 by selecting the event detection mode and the spike threshold option. Statistical analysis and Student’s t-test were performed with Microsoft Excel (Microsoft Corporation, Redmond, WA).

(7) Electrical stimulation of dorsal nerves. Stimulation of DNs7-8 was performed to observe the effect on contralateral MN-1 activity recorded extracellularly. In this preparation, the TAG was isolated by cutting all of the segmental nerves and connectives to the VNC. The recorded and the stimulated DNs were cut as far distally as possible. The proximal stumps were drawn into the tip of a glass suction electrode connected to a stimulator (GRASS Instruments, S48), and a train of pulses (5V, 2s, 20 Hz) was applied.
(8) Tactile stimulation of the antenna. The cardiac reversal reflex of adults was evoked by applying mechanical stimuli at the beginning of the anterograde beat in in vivo preparations. The stimulus was made by touching an antenna with a pin mounted on a micromanipulator. The tactile stimulation of the antenna was applied similarly in an isolated VNC preparation, as shown in figure 2.12C.

2.3 RESULTS

Dextran-rhodamine retrograde backfills of dorsal nerve-8 (DN8) made it possible to visualize and compare the branching patterns of the dendrites in larvae and adults. Among the several neurons that can be visualized by DN8 backfills of larval preparations, motoneurons-1, -2 and -3 are the only identified motoneurons that have their somata located contralaterally to the filled nerve (Fig. 2.1A). MN-1s can be easily distinguished from the others because its cell body has the most anterior position in the eighth abdominal ganglion and its axon makes a characteristic twist past the ganglion midline. No dendritic branches are apparent in the neuropil region ipsilateral to the MN-1, -2 and -3 somata (Fig. 2.1A) similar to the more anterior larval segments (Levine and Truman, 1985). By contrast, dendrites extending posteriorly in the neuropil contralateral to the somata are always apparent (Fig. 2.1A). During development, some of the larval neurons undergo cell death, consequently only a few neurons (Fig. 2.1B) are visualized by backfilling the adult DN8 to the terminal abdominal ganglion (TAG). MN-2 degenerates 2-3 days after adult eclosion (Taylor and Truman, 1974) and its swollen axon can sometimes still be identified in the adult TAG if the backfill was performed earlier (Fig.
2.1B, arrow). MN-1 and MN-3 are the only surviving motoneurons that have their somata located contralateral to the filled nerve. New dendritic arbors extending bilaterally and anteriorly in the TAG neuropil develop during metamorphosis. A distinctive feature of MN-1 are the long branches ipsilateral to the cell body that originate from its main horizontal process and extend longitudinally, mostly unramified, to terminate anteriorly in the 6th neuromere (Fig. 2.1B, arrowheads). Depending on the preparation, MN-1 showed one or two of these ipsilateral branches. The retrograde backfill technique was often not sufficient to determine for certain if these newly developed dendrites belonged to MN-1. Therefore, Lucifer Yellow dye was injected iontophoretically into the MN-1 cell body (Fig. 2.2B) in previously backfilled adult TAG preparations (Fig. 2.2A). Merged confocal images of the two dyes demonstrated that the ipsilateral dendrites indeed originated from MN-1 (Fig. 2.2C, arrow).

Both MN-17 and MN-18 innervate the caudal cardiac chamber (Davis et al., 2001) and have a functional role in controlling cardiac reversal in adult moths (Dulcis et al., 2001). Double backfills of DN7 and DN8 with dextran-fluorescein and dextran-rhodamine respectively (Fig. 2.2D) showed that these cardiac motoneurons undergo similar dendritic remodeling during development. They both develop one or two longitudinal processes that are ipsilateral to the somata and extend anteriorly towards the 6th neuromere (Fig. 2.2D-E). Often these long dendritic processes from the two MNs-1 intermingle with each other so tightly that there is virtual colocalization when the confocal images of the two dyes are merged (Fig. 2.2E, arrowheads).

Triple retrograde backfills of adult DN6,7,8 (Fig. 2.2F) made it possible to compare
the dendritic tree of MN-1_6, which innervates a dorsal skeletal muscle in abdominal segment-6, to MN-1_7,8 arbors. In contrast to MN-1_7,8, most of the processes of the extensive dendritic arbor that develops ipsilaterally to the MN-1_6 cell body extend posteriorly towards the more caudal neuromeres (Fig. 2.2G). Analogous dendritic reorganization has been described previously for the homologous MNs-1 that are located in the more rostral abdominal segments, all of which serve dorsal skeletal muscles (Levine and Truman, 1985). No ipsilateral ascending dendrites were visible in 3 backfills of MN-1_6.

In both larva and adult, action potentials recorded intracellularly from the MN-1_8 cell body were correlated one-to-one with spikes recorded extracellularly in DN_8 (Fig. 2.3A-B). Following intracellular dye injection (Fig. 2.2B-C) the recorded cell could be identified as MN-1_8, based on its soma location and dendritic/axon morphological pattern. Larval and adult MN-1_8 activity recorded from isolated ganglia showed consistent differences in the spike frequency (Fig. 2.3C), duration and shape of the action potentials (Fig. 2.3D-E). Although spike frequency depends on the membrane potential, at similar membrane potentials (about 40 mV in the representative traces shown in figure 2.3C) the larval MN-1_8 had a consistently lower spontaneous spike frequency (10.2±2.3 Hz, mean±Std.Dev, n=10) that was significantly different (p < 0.0001 by Student’s t-test for unpaired data) from the adult MN-1_8 activity (27.3±3.9 Hz, mean±Std.Dev, n=10). In addition, the larval soma action potentials were each followed by membrane hyperpolarization (Fig. 2.3D, arrows), in contrast to the adult soma spikes. Another significant difference (p < 0.0001 by Student’s t-test for unpaired data) was that the
duration of the adult action potentials was 10±2 msec (mean±Std.Dev, n=7), which is more than twice that of the larval MN-1₈ (4.4±0.5 msec, mean±Std.Dev, n=7), with a slower repolarizing phase (Fig. 2.3E).

Simultaneous intracellular and extracellular recordings made it possible to identify the unit corresponding to MN-1₈. Extracellular traces recorded from DN₈ of isolated adult TAGs showed only two spontaneously firing units that were easy to distinguish on the basis of their amplitude and frequency in most of the preparations; a large, slow-firing rate unit and a small, high-firing rate unit (Fig. 2.4A and 2.5B, top traces). The intracellular spikes recorded from the MN-1₈ soma correlated consistently with the smaller extracellular unit recorded from DN₈ (Fig. 2.4A). Injections of negative current into the MN-1₈ soma, which caused it to cease firing (Fig. 2.4C, lower trace), caused the small extracellular unit to disappear (Fig. 2.4B).

Differential extracellular recordings from DN₈ (Fig. 2.5A) were performed to demonstrate that the smaller extracellular unit is indeed the cardiac unit serving the caudal chamber of the adult heart. The extracellular trace recorded by the electrode placed close to the TAG detects at least two units (Fig. 2.5B, top trace). By contrast, only the small unit is detected by the suction electrode located distally in the thin cardiac branch innervating the caudal heart chamber (Fig. 2.5B, lower trace). The same record at expanded time scale (Fig. 2.5C) shows that the small amplitude units recorded proximally always precedes the spikes detected distally, confirming that the small amplitude unit represents an efferent neuron.

Heart contractions and DN₈ activity were monitored simultaneously to define the
correlation, if any, between the MN-1\textsubscript{s} activity and heartbeat (Fig. 2.6). Spontaneous cardiac reversals still occur in semi-intact adult preparations that have the connectives anterior to the TAG cut and all nerves intact to the periphery except for one of the DN\textsubscript{s}\textsubscript{8} (Fig. 2.6D). The instantaneous firing frequency of MN-1\textsubscript{s} was measured before, during and after cardiac reversal (Fig. 2.6A). During the retrograde beat (1.5 Hz) MN-1\textsubscript{s} did not fire action potentials, but as soon as the anterograde beat (3-4 Hz) began the instantaneous frequency of MN-1 rose from zero to 27±3 Hz (mean±Std.Dev, n=3) initially, then gradually decreased to 22±2 Hz (mean±Std.Dev, n=3) and was maintained at this level for the duration of the anterograde beat. MN-1\textsubscript{s} instantaneous frequency rapidly decreased to zero soon after a new retrograde beat was initiated. The first MN-1\textsubscript{s} action potentials (Fig. 2.6B, arrowheads) always preceded the first anterograde cardiac contraction (Fig. 2.6B arrow). Although the instantaneous frequency of MN-1 had decreased by 75% when the first retrograde contraction occurred (Fig. 2.6C, arrow), the last MN-1\textsubscript{s} action potentials (Fig. 2.6C, arrowheads) occurred a few seconds after the end of the anterograde beat.

Because the anterograde beat in adult moths occurs regularly and cyclically, extracellular activity was recorded simultaneously from both left and right DN\textsubscript{s}\textsubscript{8} to determine if MN-1\textsubscript{s} was active cyclically as well. Our results from \textit{in vivo} preparations demonstrate that both left and right MNs-1\textsubscript{s} fire bursts of activity in a cyclic manner (Fig. 2.7A). Furthermore, the averaged duration of these bursts of action potentials is 159±7 seconds (Table 2.1), which is close to the average duration (108±23) of the anterograde beat of intact adult preparations (Dulcis et al., 2001). Moreover, the cyclic activities of
MNs-1₈ are bilaterally coupled since the right and the left extracellular small units stopped and started firing at similar times (Fig. 2.7A-B, arrowheads). Although a detailed analysis of right/left synchrony of MNs-1 in neuromeres where this motoneuron innervates skeletal muscles was not performed, the regular bursting activity that is typical of MN-1₈ was not observed (Levine and Truman, 1985; D. Dulcis, unpublished observations), suggesting that this pattern is a distinctive feature of MNs-1 that are respecified to innervate the heart.

To understand whether the MN-1₈ cyclic pattern of activity is caused by a central pattern generator or is dependent upon interactions with the periphery, we compared MN-1₈ bursting properties in intact preparations to adult CNS preparations that were transected at different levels (Fig. 2.8, Table 2.1). To avoid injury effects, we waited at least 30 minutes following nerve transection. Unilateral transection of one of the DNs₈ reduced almost to 50% the burst frequency, but did not affect the maximum instantaneous firing frequency of MN-1₈ (Fig. 2.8C). The lower frequency was accompanied by an increase in both burst (Fig. 2.8D) and interburst duration. In the absence of bilateral and segmental sensory inputs, but in the presence of descending inputs from more anterior ganglia, MN-1₈ activity recorded from isolated ventral nerve cords showed a decrease in burst duration (Fig. 2.8E-F). To maintain burst frequency similar to the intact preparations, the shortening of the burst durations was accompanied by an increase in interburst duration (Table 2.1). Finally, MN-1₈ activity recorded from isolated TAGs showed the most dramatic changes as compared to in vivo preparations. Both instantaneous spike rate and burst duration decreased by 50% (Fig. 2.8G-H) but the
ability of MN-1₈ to produce a bursting pattern of activity remained.

Extracellular stimulation of distal DN₈ was performed to investigate the effect of sensory input on MN-1₈ activity. Stimulation of the left distal DN₈, with the attachment to the heart cut, caused a prolonged inhibition of the MN-1₈ recorded extracellularly from the right DN₈ in isolated TAGs (Fig. 2.9A). The motoneuron stopped firing soon after the stimulation began (Fig. 2.9B) and always took several minutes to resume its regular pattern of activity. The inhibitory effect was restricted to MN-1₈, since the other extracellular unit in DN MN-1₈ responded by slightly increasing its spike rate (Fig. 2.5A). The same effect on MN-1₈ was observed when the contralateral or ipsilateral DN₇ was stimulated (data not shown).

Detailed dissections were performed to identify distal sensory structures with axons entering the TAG via the dorsal nerves. A previously undescribed chordotonal organ (CO) was identified bilaterally, adjacent to the terminal cardiac chamber (Fig. 2.10). The CO is composed of a globular portion (Fig. 2.10A, arrow) that attaches to the dorsal abdominal cuticle via a thick attachment strand (Fig. 2.10A, arrowheads), and connects to a branch of the DN₈. A thin tissue layer, which forms a delicate membrane attached to the CO (Fig. 2.10B, arrowheads), connects this sensory organ to alary muscles of the terminal cardiac chamber. Retrograde dextran-rhodamine backfills of this branch toward the CO made it possible to visualize the sensory neuron somata (Fig. 2.10B, arrow). The somata of at least four sensory neurons lie within the CO (Fig. 2.10C). Their uniterminal dendrites (Fig. 2.10C, arrow) enter the base of the attachment of the ligament and attach into a terminal cap (Fig. 2.10C, arrowhead). As described for
COs of other insects (Field and Matheson, 2002), the dendritic inner segment gives rise to two much narrower cilia (Fig. 2.10D, arrow) and ciliary dilations (Fig. 2.10D, arrowhead).

Bilateral dextran-rhodamine orthograde backfills from hemisected COs showed that the axons of the CO sensory neurons run within the DN8 to ramify in the eighth neuromere neuropil and terminate in regions that are related spatially to the location of MN-l8 dendrites (Fig. 2.10 F, arrowhead). Some or all of the sensory axons then enter the connectives to terminate in more anterior ganglia (Fig. 2.10E).

As previously described (Dulcis et al., 2001), the cardiac cycle is composed of an anterograde beat in alternation with a retrograde beat (Fig. 2.11A), which are associated with forward and backward hemolymph flow, respectively. The spontaneous interruption of the anterograde beat, which is referred to as cardiac reversal, leads to a short period of cardiac inactivity called the isoelectric period (Fig. 2.11A, arrows). The cardiac reversal can be evoked repeatedly by tactile stimulation of the abdominal cuticle or of the antennae (Fig. 2.11A, S1-S8). Repetitive stimulation always caused inhibition of the anterograde beat (Fig. 2.11B, arrowheads) but the retrograde beat did not follow the brief isoelectric periods. Complete cardiac reversal was evoked by increasing the strength of the mechanical stimulus (Fig. 2.11B, arrows). A regular cardiac cycle started as soon as the stimulation was stopped, but the timing of the anterograde beat was altered compared to that expected had no stimulation been applied (Fig. 2.11A, second trace). In this way, strong stimuli reset the cardiac cycle by inhibiting the anterograde beat and allowing a retrograde beat to occur.
To determine whether tactile stimulation also affects MN-18 activity during evoked cardiac reversal, we recorded extracellularly and bilaterally from DNs8 of isolated ventral nerve cords during antennal displacement (Fig. 2.12 C, double arrow). Following mechanical stimulation MNs-18 stopped firing or dramatically decreased their spike rates (Fig. 2.12A, S1-S3). Inhibition and recovery in left and right MN-18 occurred at similar times and always briefly (Fig. 2.12B, S1).

2.4 DISCUSSION

The first goal of the present study was to determine whether MNs-17,8 undergo anatomical and physiological remodeling during metamorphosis. New dendritic arbors, ipsilateral to MN-17,8 somata, extend anteriorly in the TAG neuropil (Fig. 2.1), rather than posteriorly as has been described for MN-1 of more anterior abdominal segments (Levine and Truman, 1985). The anteriorly-directed dendritic extension seems to be a specific characteristic that distinguishes the remodeling of those MNs-1 that will become cardiac neurons.

Our findings support the hypothesis that MN-17,8 cardiac neurons require distinct synaptic inputs in order to produce the pattern of activity that controls heart reversal. It is not surprising that MNs-17,8 have dendrites that are tightly intermingled, given their synergistic effect on the caudal chamber to produce reversal. Stimulation of DN8 causes cardiac reversal from retrograde to anterograde beat, whereas stimulation of DN7 causes an increase in the force of contraction of the terminal chamber without increasing the rate of the anterograde heart contractions (Dulcis et al., 2001). Thus, MN-18 initiates the
anterograde beat and MN-17 increases the force of anterograde cardiac contractions (Dulcis et al., 2001), suggesting the need for synchronized synaptic inputs and coupled activity.

Intracellular and extracellular recordings of MN-18 activity at larval and adult stages revealed physiological changes that accompany the development of cardiac innervation. There are developmental differences in firing rate, duration of the action potentials, and pattern of activity of MN-18. Adult MNs-18 always had higher firing rates and broader action potentials than larval MNs-18 at similar resting membrane potentials (Fig. 2.3). An analogous postembryonic physiological remodeling has been described for MN-5, a larval body-wall skeletal muscle motoneuron that becomes respecified to innervate a flight muscle in adult moths (Duch and Levine, 2000). The authors demonstrated that changes in the duration and size of action potentials were due to changes in voltage-gated potassium and calcium currents in the motoneuron during metamorphosis. Changes in the input resistance and firing threshold were reported to influence the adult MN-5 spiking rate. Further electrophysiological experiments need to be done to determine whether the higher firing rate displayed by MN-18 during respecification is due to changes in intrinsic properties or synaptic drive.

Identification of the MN-18 unit recorded extracellularly from DN8 (Figs. 2.4 and 2.5) made it possible to determine its firing pattern and correlate it to the cardiac activity in almost intact adult preparations. The most profound physiological change in MN-18 during metamorphosis is the acquisition of a bursting activity pattern (Fig. 2.7). Each burst correlates with an anterograde cardiac phase that is originated at the caudal chamber
(Fig. 2.6). The anterograde pacemaker, putatively located in the caudal chamber, requires neuronal activity to initiate reversal as well as to maintain the anterograde beat (Dulcis et al., 2001). Thus, the anterograde phase is a neurogenic property. Neurogenic hearts have been well-described in other arthropods, particularly in crustacean circulatory systems where heart contractions occur exclusively as a result of neuronal activation by a cardiac central pattern generator (Cooke, 2002). The adult heart in *M. sexta* could represent a hybrid between absolute neurogenic and myogenic hearts. Its activity is composed of a myogenic retrograde beat that is in alternation with a neurogenic anterograde beat (Dulcis et al., 2001). A detailed physiological study of the cardiac synapse could elucidate the mechanisms by which MN-18 activity overrides the ongoing contractions that are initiated by the retrograde myogenic pacemaker.

The periodic reversal of heart beat is derived from the characteristic bursting pattern of MN-18 in the adult (Dulcis et al., 2001; present results). There are at least two possible mechanisms that might account for the acquired bursting activity of adult MN-18. The first scenario involves sensory feedback, such as the detection of hemolymph pressure in the abdominal hemocoel, which could sculpt MN-18 activity directly or through interneurons. A second scenario relies on a central pattern generator to produce the MN-18 activity pattern. The latter scenario might involve a neuronal circuit that dictates MN-18 output and controls the anterograde cardiac pacemaker of the caudal chamber. Alternatively MN-18 itself might acquire physiological properties that allow it to produce the cyclic bursting pattern intrinsically. In either case some mechanism of bilateral coordination between MNs-18 would be necessary.
MN-1₈ cyclic bursting was not prevented by removing sensory feedback, as well as descending input from the brain and more anterior ganglia (Fig. 2.8). MN-1₈ showed cyclic bursts of activity in isolated terminal abdominal ganglia, suggesting that a neuronal cardiac pacemaker is located there. Nevertheless, burst duration and frequency, as well as spike rate, were all influenced by sensory inputs from segmental neurons and higher centers. Thus, sensory feedback and a central pattern generating network may both participate in producing the physiological pattern of activity that ultimately controls the cyclic activation of the cardiac pacemaker in intact organisms.

Although MN-1₈ bursting activity occurs independently of sensory inputs, previous studies (Tenney, 1953; Wasserthal, 1976; Smits et al., 2000; Dulcis et al., 2001) have shown that a variety of sensory stimuli can evoke cardiac reversal by ending the anterograde beat.

In the attempt to understand how sensory inputs affect MN-1₈ output we discovered a novel CO adjacent to the caudal cardiac chamber (Fig. 2.10), with axons that enter the TAG in DN₈. Stimulation of DN₈ caused a dramatic inhibition of MN-1₈ activity, perhaps mimicking the sensory input that the COs furnish to the TAG neuropil. Although further study needs to be done to define the specific functional role of these sensory structures, based on their attachments to the dorsal cuticle of segments 7-8 and their morphological similarities to previously described COs (Field and Matheson, 1988), we predict that they detect body wall movements. Telescopic movements of abdominal segments during calling behavior of the female moth stop the anterograde beat (Ichikawa and Ito, 1999). The CO may detect movement of the abdominal tergites and feed back to
the CNS to inhibit MN-1₈ activity.

Repetitive tactile stimulation of the antenna causes cardiac reversal by early blockage of the anterograde beat in intact moths (Fig. 2.11). Tactile stimulation of the antenna attached to the otherwise isolated nerve cord demonstrated that sensory-evoked reversal is mediated by a descending pathway that ultimately inhibits MN-1₈ firing in the terminal ganglion. Thus, sensory-evoked cardiac reversals are caused by strong inhibition of MN-1₈ activity.

Based on our findings and published data, we suggest the following functional models to distinguish spontaneous versus sensory-evoked cardiac reversals. The spontaneous reversal is probably produced by a neuronal pacemaker that sums central pattern generator activity and modulatory sensory input to provide the drive to MN-1₈. By contrast, evoked cardiac reversal is mediated by abrupt inhibitory sensory inputs that override and reset the central pattern generator to cause an early offset of the MN-1₈ burst and, consequently, of the anterograde beat. The spontaneous reversal would guarantee the regular diastolic/systolic cardiac cycle in resting moths. The evoked reversal might represent the preparatory mechanism of the circulatory system for specific behaviors that require a sudden shunt of hemolymph flow.

In conclusion, the morphological and physiological remodeling of MN-1₈ during the development of cardiac innervation places this cardiac neuron into a functionally new neuronal circuit that is specialized to produce the pattern of activity that controls adult heartbeat. Moreover, this circuit is designed to receive the modulatory sensory feedback that allows the neural pacemaker to accommodate its control to the dynamic needs of the
circulatory system.
Figure 2.1: Morphological remodeling of MN-1. Dextran-rhodamine retrograde backfills to the terminal abdominal ganglion from near the proximal end of dorsal nerve-8 (DN-8) of larval (A) and adult (B) preparations. A: ventral view of the larval seventh and eight abdominal ganglia showing the location of the somata of motoneurons-1, 2, and 3 (MN-1,2,3) which are contralateral to the DN-8 filled. Most of the dendrites are ipsilateral to the DN-8 filled. B: ventral view of the adult terminal abdominal ganglion, backfilled at 12 hrs after emergence, showing the location of the somata of MN-1 and MN-3 after fusion of the 6th, 7th, and 8th neuromeres. MN-2, which dies within 48 hrs of emergence, shows a residual fluorescence (arrow). New dendritic processes (arrowheads) extending anteriorly develop contralaterally to the filled DN-8. Scale bars = 50 μm.
Figure 2.2: Dendritic remodeling of cardiac MNs-1. See next page for details.
Figure 2.2: Dendritic remodeling of cardiac MNs-1. Laser-scanning confocal microscope images of the terminal abdominal ganglion of adult preparations in which one (A-C) or multiple (D-G) dorsal nerves have been filled. In all of the images motoneuron-1 (MN-1) is indicated with an arrow. A: dextran-rhodamine retrograde backfill of dorsal nerve-8 (DN-8). B: same preparation showing Lucifer Yellow intracellular fill of MN-18. C: same images shown in A and B merged together and at higher magnification. Regions of co-localization (yellow/green) show that the newly developed dendrites ipsilateral to the soma originate from MN-18. D: double backfill of dorsal nerve-7 (DN-7; filled with dextran-rhodamine and visualized in red) and DN-8 (filled with dextran-fluorescein and visualized in green) showing the respective location of MN-17 and MN-18 somata (arrows) and their dendritic arbors. E: different confocal stack of same preparation in D showing details of dendritic processes in the box in D. Some of the MN-17 and MN-18 dendrites that are ipsilateral to the somata and run anteriorly in the ganglion intermingle tightly with each other (arrowheads). F: triple backfill of dorsal nerve-6 (DN-6) and DN-8 (both filled with dextran-rhodamine and visualized in red) and DN-7 (filled with dextran-fluorescein and visualized in green). G: Montage of confocal optical sections of the triple backfill shown in F. The elaborate dendritic trees of both MN-16 (arrow) and MN-36 (arrowhead) descend posteriorly in the ganglion neuropil unlike those of MN-17, and MN-18. Scale bars = 200 μm in F, 100 μm in A-B,D, 50 μm in C,G, 10 μm in E.
Figure 2.3: Physiological properties of larval and adult MN-1. See next page for details.
Figure 2.3: Physiological properties of larval and adult MN-1. Intracellular recordings from larval and adult MN-1g in isolated ganglion preparations. A: Representative dextran-rhodamine backfill of larval DN8 showing the location of the intracellular and suction electrodes in the isolated terminal ganglion. Scale bar = 100 μm. B: correlation of spontaneous intracellular action potentials recorded from the larval MN-1g cell body (top trace) and extracellular spikes recorded from DN8 (lower trace). The traces contain 15 superimposed sweeps. C: representative intracellular recordings (resting potential, 40 mV) of spontaneous activity recorded from the cell body of larval (top trace) and adult (lower trace) MN-1g. D: same recordings shown in C at expanded time scale. The arrows indicate the hyperpolarization of the membrane potential that follows each larval MN-1g spike. E: difference between larval (solid line) and adult (dotted line) spontaneous action potentials. Each trace represents a signal average of 10 spikes taken from a representative recording.
Figure 2.4: Correlation of intracellular/extracellular spikes in the isolated TAG. A: spontaneous activity recorded simultaneously from MN-I₈ cell body (lower trace) and extracellularly from DN₈ (top trace). B: effect of MN-I₈ hyperpolarization on the pattern of the extracellular recording from DN₈. C: same recording shown in B at expanded time scale. The small extracellular units always correlate with the intracellular spikes and disappear as MN-I₈ membrane potential is hyperpolarized by injection of negative current (-I) into the cell body.
Figure 2.5: Identification of extracellular cardiac motor unit. Differential recording from dorsal nerve-8 (DN₈) in semi-intact adult preparations. A: schematic drawing of the innervation of the terminal cardiac chamber (TCh) showing the location of the proximal and distal suction electrodes on the DN-8. Alary muscles (AM), dorsal nerve-7 (DN₇), terminal abdominal ganglion (TAG), transverse nerve-7 (TN-7). B: simultaneous extracellular recording from the proximal DN-8 (top trace) and the distal cardiac branch (lower trace). C: traces between dashed lines shown in B at expanded time scale to show that the small amplitude units recorded proximally always precede the small units detected distally.
Figure 2.6: Correlation of DN₉ activity with the anterograde beat. See next page for details.
Figure 2.6: Correlation of DN₈ activity with the anterograde beat. Correlation of dorsal nerve-8 (DN₈) activity and anterograde cardiac beat in semi-intact adult preparation. A: extracellular recording of DN₈ activity and cardiac mechanogram (Heart) during cardiac reversal. In the top graph the instantaneous spike frequency of the small amplitude unit (MN-lg; black circles) and of the cardiac contractions (Heart, gray circles) are plotted over time. The direction of heartbeat is shown at the bottom. B: traces between the dashed lines shown in A (labeled B) at expanded time scale show that the small unit (arrowheads) starts firing right before the first anterograde cardiac contraction occurs (arrow). C: traces between the dashed lines shown in A (labeled C) at expanded time scale show that the small unit (arrowheads) stops firing soon after the retrograde beat begins (arrow). D: schematic drawing of the electrophysiological setup showing the location of both the isometric transducer to measure cardiac activity and the suction electrode to record DN₈ activity.
Figure 2.7: Bursting activity of MNs-1. Bilateral extracellular recording from dorsal nerve-8 (DN₈) in *in vivo* adult preparation. **A**: simultaneous recording from right (R) DN₈ (top trace) and left (L) DN₈ (lower trace) showing bursting activity of the small amplitude unit. The arrowheads indicate the start and stop times of the MN-1 unit. **B**: the same traces between dashed lines in A at expanded time scale show that the right and left small units stop and start firing action potentials at similar times.
Figure 2.8: Bursting patterns of activity of MN-1. See next page for details.
Figure 2.8: Bursting patterns of activity of MN-1. Instantaneous spike frequency analysis of spontaneous MN-1 activity for adult preparations transected at different levels. For each preparation the analysis of about 20 minutes (1200 sec) of MN-1 activity is shown (A-C-E-G). The graphs on the right (B-D-F-H) show instantaneous spike frequency for a representative burst of MN-1 activity at expanded time scale in each experimental condition. A-B: intact animal (in vivo). C-D: unilateral transection of dorsal nerve-8 (DN8). E-F: isolated ventral nerve cord (VNC). G-H: isolated terminal abdominal ganglion (TAG).
Figure 2.9: Effect of DN₈ stimulation on MN-1 activity. Effect of contralateral dorsal nerve-8 (DN₈) electrical stimulation on MN-1₈ activity in isolated terminal abdominal ganglion preparations of adult *M. sexta*. The thick black vertical bars represent the stimulus artifact. A: extracellular recording from the right (R) DN₈ showing the prolonged inhibitory effect of left (L) DN₈ stimulation (5V, 2s, 20 Hz) on MN-1 activity. B: the same trace between dashed lines in A at expanded time scale shows that MN-1₈ action potentials stop occurring soon after the electrical stimulation begins.
Figure 2.10: Chordotonal organ. See next page for details.
Figure 2.10: Chordotonal organ. Light microscope (A) and confocal microscope (B-F) micrographs of the chordotonal organ adjacent to the terminal cardiac chamber and its terminal processes in the CNS of the adult moth. A: dissection of the peripheral nervous system showing the chordotonal organ (arrow), its ligament (arrowheads) and its connection to the dorsal nerve-8 DN8. B: dextran-rhodamine orthograde backfill to the chordotonal organ (arrow). The somata of the sensory neurons and the membranous tissue (arrowheads) are visible. C: same preparation in B at higher magnification showing the dendrites (arrow) of the sensory neurons entering at the base of the ligament and terminating with a cap (arrowhead). D: the dendrites of the sensory neurons in C connect to the cap via two thin cilia (arrow) and distal ciliary dilations (arrowhead). E: bilateral dextran-rhodamine retrograde backfills to the terminal abdominal ganglion (TAG) from left and right hemisected chordotonal organs. The sensory processes reaching the TAG neuropil are visible. F: different confocal stack and higher magnification of the same preparation in E showing sensory terminals (arrowhead) in the TAG neuropil. Scale bars = 200 μm in A, 100 μm in B-E, 20 μm in C, 5 μm in D.
Figure 2.11: Evoked cardiac reversal. Effect of tactile stimulation on cardiac activity of the intact moth. A: cardiac mechanogram showing the regular anterograde and retrograde heartbeat reversal (top trace) and the inhibitory effect of repetitive tactile stimulations (S₁-S₅) on the anterograde beat (lower trace). The arrows indicate the isoelectric period. B: the same trace between dashed lines in A at expanded time scale shows that consecutive tactile stimulations cause a temporary inhibition of anterograde contractions (arrowheads) or evoke a cardiac reversal by initiating a short-lasting retrograde beat (arrows).
Figure 2.12: Effect of mechanical antennal stimulation on MN-1 activity. A: extracellular recording from right (top trace) and left (lower trace) dorsal nerve-8 (DN8-R; DN8-L) showing the effect of three repetitive stimulations (S1-S3). B: the same traces between dashed lines in A show the inhibitory response of MN-1 following antennal displacement (S1). Arrowheads pairs indicate the size of MN-1 unit. C: schematic drawing of the isolated VNC preparation showing the location of the suction electrodes and the manner of stimulus application. Abdominal ganglia of segments 3-5 (AG3-5), pterothoracic ganglion (PtG), terminal abdominal ganglion (TAG).
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<td>284 ± 9</td>
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<td>87 ± 3</td>
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<td>Interburst duration (sec)</td>
<td>18± 2</td>
<td>89 ± 11</td>
<td>86 ± 11</td>
<td>49 ± 3</td>
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Table 2.1: Bursting patterns of activity of MN-1. Maximum instantaneous spike rate (Hz), burst frequency (number of bursts/10 minutes), burst duration (sec), and interburst duration (sec) of motoneuron-1 activity recorded extracellularly were compared under different experimental conditions including intact in vivo, unilateral transaction of dorsal nerve-8 (DN₈), isolated ventral nerve cord (VNC) with intact head (i.e. all peripheral nerves cut), and isolated terminal abdominal ganglion (TAG). Values are mean ± standard error.
CHAPTER 3. INNERVATION OF THE HEART OF THE ADULT FRUIT FLY, 
DROSOPHILA MELANOGASTER

3.1 INTRODUCTION

Although insects have an open circulatory system, their relatively high metabolic rate requires efficient circulation of hemolymph. Furthermore, the rich behavioral repertoire of insects necessitates regulation of the circulatory system to accommodate different conditions and metabolic demands. This study investigates the possibility of direct neuronal control of the heart in a widely used model organism, the fruit fly Drosophila melanogaster.

The contractile dorsal vessel of D. melanogaster extends the entire body length, and consists of a distinct abdominal heart and a thoraco-cephalic aorta which communicate through the cardio-vascular valve (Rizki, 1978). The abdominal heart functions as the pump of this open circulatory system; the aorta acts more as a vessel conducting the hemolymph to the head capsule. As is typical of other insects, the heart in D. melanogaster consists of a series of segmental chambers marked by incurrent ostia and sets of alary muscles (Rizki, 1978; Curtis et al., 1999). During diastole, dilation of each cardiac chamber by the coupled alary muscle contractions results in hemolymph inflow through the lateral ostia. The contraction of intrinsic cardiac muscles of each chamber during systole causes ostia closure and hemolymph movement into the adjacent chamber (Rizki, 1978).

The larval heart undergoes dramatic morphological and physiological changes during adult development. Anterior to the cardio-vascular valve, a conical chamber forms
“de novo” as a product of differentiation of the proximal stem of the aorta (Rizki, 1978). In addition, an outer layer of longitudinal muscle fibers develops along the ventral surface of the heart (Miller, 1950; Molina et al., 2001). The myofibers of this ventral muscle layer are distributed symmetrically along the conical chamber and become distorted posterior to it (Rizki, 1978).

Before the adult emerges from the puparium, a cyclic alternation of anterograde and retrograde phases of contractions, which is referred to as cardiac reversal, replaces the constant anterograde larval heartbeat (Rizki, 1978). Because the isolated heart shows two pulsatile regions, one at the caudal end and another at the junction of the heart and aorta, Rizki (1978) hypothesized that the conical chamber might represent the location where the retrograde beat, thus the cardiac reversal, originates. This stereotyped reversal of the hemolymph flow occurs in most adult holometabolous insects, such as the Diptera (Campan, 1972; Wasserthal, 1999) and Lepidoptera (Tenney, 1953; Queinnec and Campan, 1972; Wasserthal, 1976, 1981; Ichikawa and Ito, 1999; Smits et al., 2000; Dulcis et al., 2001). Because even isolated heart chambers of insects can contract at different rates and directions, the involvement of the nervous system in triggering cardiac reversal has been questioned. However, it has been recently demonstrated that the adult cardiac innervation in *Manduca sexta*, which develops during metamorphosis (Davis et al., 2001), originates and controls cardiac reversal by activating the caudal pacemaker (Dulcis et al., 2001). Since the dorsal vessel of holometabolous insects appears to have both anterior and posterior pacemakers, which are alternatively active, it has been suggested that the nervous system might determine when each pacemaker becomes
dominant (Dulcis et al., 2001; Davis et al., 2001).

*In vitro* experiments have shown that neurotransmitters (Johnson et al., 1997; Zornik et al., 1999) and peptides (Nichols et al., 1999a) influence the heart rate of *D. melanogaster* at any stage throughout metamorphosis. Because the literature fails to describe any cardiac innervation, it was concluded by Johnson et al. (1997) that the heart of *D. melanogaster* is myogenic, with neurotransmitters and neuropeptides in the hemolymph acting as modulators of the heartbeat. Immunolocalization of the cardioinhibitory peptide SDNFMRFamide in the anterior region of the adult aorta (Nichols, 1999b) suggested, however, that the adult heart of *D. melanogaster* receives neuronal inputs at specific regions.

During the larval stage and for the first day after pupation, the heartbeat of *D. melanogaster* is myogenic (Dowse et al., 1995) and originates in a pacemaker that is probably located in the most caudal region of the heart. Because both cardiac remodeling and heartbeat reversal occur later in adult development, we hypothesized that cardiac innervation also might develop during this stage so as to control adult heartbeat. The presence of segmental innervation of the abdominal heart has already been described in adults of other fly species such as *Glossina morsitans* (Anderson and Finlayson, 1978). However, perhaps because of the small size of *D. melanogaster*, classical neural tracing techniques have failed to demonstrate any innervation of the adult abdominal heart. The presence of a nerve observed in histological sections of the conical chamber (Miller, 1950) was, until now, the only evidence of the innervation of the adult abdominal heart. Rizki (1978) suggested that the origin of the cardiac impulse in this chamber might be
neurogenic.

Transgenic flies in which all neurons express GFP, and immunocytochemical techniques were used in this study to demonstrate the neurons terminating on the adult myocardium, and the neurotransmitters and peptides they may release at cardiac synapses. Because the conical chamber may have an important role in the control of cardiac function, we investigated its innervation in detail. The results provide clear evidence of at least two distinct sources of heart innervation in adults of *D. melanogaster*.

### 3.2 MATERIALS AND METHODS

1. **D. melanogaster culture.** Flies were raised on medium consisting of instant food, agar, and oatmeal (Condie and Brower, 1989) supplemented with yeast. All stocks were maintained at 25°C under uncrowded conditions. Wild-type Oregon-R and ELAV-GAL4/UAS-GFP transgenic flies were used in this study. The data were collected from late third instar larvae and 2-day old adults. The animals were anesthetized on ice for about 10 minutes then dissected in cold *D. melanogaster* saline (Stewart et al. 1994).

2. **Visualization of the peripheral nervous system.** More than one hundred ELAV-GAL4/UAS-GFP transgenic flies were used to describe the peripheral fibers innervating the heart. In these animals, the expression of the green fluorescence protein (GFP) is controlled by the GAL4/UAS system with the GAL4 transcription factor driven by the pan-neuronal promoter ELAV (Estes et al., 2000). After removal of the ventral abdominal sternites and visceral organs the tissue was fixed with 4% paraformaldehyde.
for two minutes to keep the abdominal segments flat and to stop the heart from beating
during confocal imaging. Because fixation greatly reduces GFP fluorescence and
increases background autofluorescence of the fat bodies, confocal images were also
captured from fresh tissue preparations.

To visualize the pericardial cells in larval preparations, tissues were incubated for
5-10 minutes with coomassie brilliant blue solution (Sigma) made in saline. The dye,
accumulated by the pericardial cells into intracellular granules, is autofluorescent and was
detected by scanning with an argon laser line (488 excitation maximum) and using a
band-pass filter at 510 nm.

(3) Synaptotagmin immunostaining. To examine the nature of cardiac synapse-like
terminals in adult flies, a rabbit polyclonal antiserum to D. melanogaster synaptotagmin
(Littleton et al., 1993; generously provided by Dr. M. Ramaswami) was used in four
preparations. The myocardium was fixed with 4% paraformaldehyde in 0.1 M phosphate
buffer solution (PBS) for 45 minutes on a shaker at room temperature. After rinsing in 25
mM PBS-Glycine and PBS (pH 7.2) for 10 minutes each, the preparations were
incubated for 20 minutes in the blocking solution comprising 2% bovines serum albumin
(BSA, Sigma), 5% normal donkey serum (NDS, Sigma) in PBS with 1.5% Triton-x100
(T). The primary antiserum (1:400) made up in blocking solution was applied for 2 hours
in a moist chamber and subsequently washed out with blocking solution for a total of 30
minutes (3 times for 10 minutes each). The Cy5-conjugated donkey anti-rabbit IgG
(Jackson ImmunoResearch Laboratories, West Grove, PA) was used as the secondary
antibody applied at a dilution of 1:200 for 1 hour at room temperature. The preparations then were washed several times in 1.5% PBST for 30 minutes, in PBS for 10 minutes and mounted in 80% glycerol solution.

To reduce background staining in some of the preparations, the autofluorescent fat bodies were removed by squirting the dissected tissue rapidly with a syringe with fixative diluted in saline.

(4) CCAP immunostaining. Five whole-mounted preparations of the fly abdomen were studied by an immunofluorescence method given by Davis (1993). Dissected tissue was fixed in 4% paraformaldehyde at 4° C overnight and washed in 0.5% triton made in PBS solution for 6 hours (6 times for 1 hour each), last wash at 4° C overnight. Tissues then were treated for about 24 hours at room temperature with rabbit anti-CCAP antiserum (generously provided by Dr. H. Agricola) diluted 1:5,000 in blocking solution made of 10% normal goat serum (NGS, Sigma) in 1% PBST. After being washed in PBST for 4 hours (4 times for 1 hour each), tissues were blocked for 1 hour and soaked overnight at room temperature in a 1:400 dilution of the secondary antibody Cy5-conjugated donkey anti-rabbit IgG. The preparations then were rinsed in PBST and PBS and cleared and stored in 60% - 80% glycerol.

Because the GFP-fluorescence expressed by the neurons of ELAV-GAL4/UAS-GFP transgenic flies is reduced by prolonged fixation, the monoclonal mouse antibody 22C10, developed by Zipursky et al. (1984), and obtained from the Developmental Studies Hybridoma bank, was used in a few preparations to double label the cardiac
innervation. The 22C10 antiserum, which recognizes the Futsch protein localized to the microtubule compartment of neurons (Hummel et al., 2000), was added in a 1:100 dilution together with the other primary antibody and visualized with fluorescein (FITC)-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories).

(5) Glutamate immunocytochemistry. We used rabbit polyclonal anti-glutamate antibodies that recognize the conjugate Glu-G-BSA (Chagnaud et al., 1989; Campistron, 1990; Sinakevitch-Pean et al., 1998, 2001; Sinakevitch et al., 2001). The fixative solution was always prepared fresh and made of 0.1M cacodylate buffer (pH 7.2), 1% gluteraldehyde, 2.5% paraformaldehyde, and 1% sodium metabisulfite (SMB, Sigma). Tissues were fixed for about 8 hours at room temperature, treated with $10^{-2}$ M sodium borohydride (NaBH₄, Sigma) in a solution of 0.05 M Tris-HCl-SMB and washed for 1 hour (4 times for 15 minutes each) in 0.05 M Tris-HCl-SMB and for 30 minutes in SMB 0.5% Triton. After blocking for 1 hour with 2% normal donkey serum (NDS) in a solution of Tris-HCl-SMB with 0.5% Triton, the preparations were incubated for 1-2 days at room temperature with a 1:1,000 diluted polyclonal rabbit anti-glutamate antibody generously provided by Dr. N. Strausfeld. Tissues then were rinsed in Tris-HCl 0.5% Triton for 3 hours (6 times for 30 minutes each) and incubated for 1-2 days with the Texas Red (TR)-conjugated (Jackson ImmunoResearch Laboratories) or Cy5-conjugated donkey anti-rabbit secondary antibodies at a dilution of 1:200. The preparations then were washed several times in Tris-HCl 0.5% Triton and Tris-HCl and cleared and mounted in 80% glycerol. Eight preparations were analyzed.
(6) Laser-scanning confocal microscopy. Digital images of GFP-expressing neurons and immunostained preparations were collected on a Nikon PCM 2000 laser-scanning confocal microscope equipped with green He/Ne (543 nm), red He/Ne (633 nm) and argon (488 nm) lasers. Scanning with an argon laser and using a band-pass filter at 510 nm detected GFP and FITC fluorescence. Cy5 and TR fluorescence were detected respectively with red and green He/Ne laser lines and using a band-pass filter at 650 nm (Cy5) or 565 nm (TR). Stacks of digitized images were merged by using Simple PCI (Compix In., Cranberry Township, PA) as image acquisition software. Corel Draw and Corel Photopaint (Corel Corp., Ottawa, Ontario, Canada) were the software used to enhance contrast and provide color when needed. Because GFP-fluorescence at the level of small, terminal processes is reduced by prolonged fixation, the color of the colocalized immunostaining occasionally prevails over the GFP-fluorescence when the two confocal images are merged. Prints were made by using a Tektronix Xerox Phaser 6200 printer.

3.3 RESULTS

Flies have a pulsating dorsal vessel that runs along the body dorsal midline (Fig. 3.1A). The dorsal vessel is composed of an abdominal multi-chambered heart and an unchambered thoraco-cephalic aorta (Rizki, 1978). Each cardiac chamber contains a pair of lateral incurrent ostia that act as valves to prevent the escape of blood during systole (reviewed by Chapman, 1969). The larval abdominal heart has three chambers, each with pairs of ostia. The first abdominal cardiac chamber of adult flies, termed the conical chamber (Ritzki, 1978), represents a new addition to the larval heart. This cardiac
chamber, located between the posterior end of the aorta and the anterior region of the heart (Fig. 3.1A-B), develops during the last day of the pupal stage. Another important aspect of the heart remodeling that occurs during metamorphosis is the formation of a layer of longitudinal muscle fibers along the ventral side of the abdominal heart (Curtis et al., 1999; Molina et al., 2001). This layer extends from the conical chamber along the length of the abdominal heart, but it is absent in the caudal chamber (Miller, 1950).

The alary muscles are segmental pairs of visceral muscles that suspend and support the heart (Fig. 3.1B). Their fibers come into contact with the inner layer of circular muscles of the heart (Rizki, 1978). Two rows of spherical pericardial cells flank the dorsal vessel (Fig. 3.1A-B). These are large, stationary macrophages comparable to the reticuloendothelial system of vertebrates (Rizki, 1978).

The adult peripheral nervous system that innervates the skeletal muscles and visceral organs of the abdomen is organized into segmental abdominal nerves that originate from the caudal portion of the thoracico-abdominal ganglion. The first two pairs of abdominal nerves, which serve the first and second abdominal segments, arise directly from the ganglion. The remaining abdominal nerves of the more posterior segments originate as branches from a ventral, midline, nerve trunk (Miller, 1950). Each abdominal nerve branches into two major nerves, the segmental nerve and the intersegmental nerve, both innervating muscles of abdominal segments (Gorczyca et al., 1994). One thin, peripheral nerve, the transverse nerve (TN), arises from the dorsal midline of the thoracico-abdominal ganglion and fasciculates with the abdominal nerve to innervate the abdominal segments (Gorczyca et al., 1994).
A class of embryonic peripheral neurons has been described by Bodmer and Jan (1987) as bipolar dendrite neurons. As their name indicated, they were described to have divergent bipolar dendrites. Lucifer Yellow dye fills have shown, however, that the ventral branch projects toward the CNS and that the dorsal branch fasciculates with the transverse nerve (Gorczyca et al., 1994). During embryonic development, both transverse nerves and bipolar dendrite neurons innervate the base of the alary muscles with a thick ending (Gorczyca et al., 1994). Because the dorsal branch represents the axon terminating in the alary muscles rather than a dendrite, we will refer to these neurons as bipolar neurons (BpNs). Based upon their cell body position and peptidergic character BpNs are apparent orthologues of L1 neurons described in M. sexta (Taghert et al., 1986; Davis et al., 1993; Davis et al., 2001).

The ectopic expression of GFP in neurons of GFP transgenic flies made it possible to visualize the complete innervation of the adult abdominal heart and alary muscles, and this innervation was found to rise from the transverse nerves of abdominal segments 1-5 (Fig. 3.2A, TN_{1-5}). Each transverse nerve has a characteristic branching pattern that is consistent among specimens and is serially homologous in the pregenital abdominal segment (Fig. 3.2B, S_{1-3}). The transverse nerve branches from the segmental nerve (Figs. 3.2B, arrowheads; 3.2G, TN_{3}, SN_{3}), extends obliquely forward beneath the tergum to the next preceding abdominal segment where it terminates on the cardiac chamber and the alary muscles of that chamber. The innervation of the right and left side of the cardiac chamber is symmetrical (Fig. 3.2D, TN_{SR-L}). The transverse nerve arborizations ramify extensively on the respective sides of cardiac chambers two through
six but usually do not meet. In contrast, the transverse nerve arborizations on each side of the first cardiac chamber (conical chamber) always converge with one another to form a transverse bridge (Figs. 3.2B, box; 3.2C, TNIR-L).

Numerous dissections of the abdominal heart of adult *D. melanogaster* failed to reveal any nerves comparable to the lateral cardiac nerves found in some other insects such as *Periplaneta orientalis* (Alexandrovič, 1926), *Locusta migratoria* (Roussel, 1972) and *Bombyx mori* (Kuwana, 1932). The lateral cardiac nerves represent longitudinal nerves on either side of the dorsal vessel containing axons from both the corpora cardiaca and segmental nerves from which nerve endings ramify in the heart wall and alary muscles (Chapman, 1969; Miller, 1997). Similarly, we could not locate these nerves on the abdominal heart of GFP transgenic flies, when examined under the fluorescence and confocal microscope, while, as noted above, we did observe the segmental innervation by the transverse nerves, as previously described in other dipterans, such as *Anopheles quadrimaculatus* (Jones, 1954) and *Musca domestica* (Fordan, 1971), and in the lepidopteran *Manduca Sexta* (Davis et al., 2001).

The transverse nerves serving the conical chamber (Fig. 3.2C, TNIR-L) form a distinctive synapse-like structure. Their fibers (thickness 1 \( \mu \)m) increase gradually in diameter as they reach the myocardium to give rise to a thick (8 \( \mu \)m) transverse bridge (Fig. 3.2C, arrow) across the chamber. Numerous longitudinal processes originate from it and most of them extend posteriorly toward the abdominal heart. In 50% of the specimens, independently of the sex of the fly, an alternative pattern of conical chamber innervation was observed (Fig. 3.2E). In these preparations, left and right transverse
nerves of segments 1-2 (Fig. 3.2E, TN1, TN2) join together to form a structure composed of two thick, transverse bridges interconnected by longitudinal processes extending both anteriorly and posteriorly. With either pattern, several of the longitudinal processes show bouton-like terminals (Figs. 3.2C; 3.2F, arrowheads). The conical chamber also receives innervation from the median nerve as visualized in GFP transgenic flies. The median nerve, which is the continuation of the recurrent nerve, extends ventrally along the entire aorta and terminates with a thick, unbranched, process in the most anterior portion of the conical chamber (data not shown).

Synaptotagmin, a synaptic vesicle protein, was used to label putative synaptic structures on the abdominal heart of adult flies. The antiserum to \textit{D. melanogaster} synaptotagmin has been used as a reliable label of presynaptic sites in \textit{D. melanogaster} skeletal muscle (Littleton et al., 1993). We have found abundant immunoreactivity on every chamber of the abdominal heart (Fig. 3.3A, DV). The cardiac nerve terminals make bouton-like terminals (2-3 \textmu m in diameter) on the myocardium after branching multiple times (Fig. 3.3B, DV). They are not organized in a row of boutons as is typical of the segmental skeletal muscles (Fig. 3.3B, DM). To demonstrate that the synaptotagmin-immunolabeled sites belong to branches of the transverse nerve, synaptotagmin localization was observed along with the GFP fluorescence of the peripheral nervous system of abdominal segments 1-6 of adult GFP transgenic flies (Fig. 3.4). Synaptotagmin was clearly localized at transverse nerve terminals of each chamber of the heart (Figs. 3.4A, TN1,4; 3.4C, TN3,5), as well as in regions of transverse nerve axons that run along the alary muscles (Fig. 3.4A, arrowheads). Synaptotagmin was not detected
where transverse nerves of two adjacent segments contact each other ipsilaterally running longitudinally along the lateral aspect of the dorsal vessel (Figs. 3.4C, box D; 3.4D, arrows), but was detected mostly where transverse nerve endings formed bouton-like structures (Fig. 3.4D, arrowheads). Anti-synaptotagmin staining also labeled the transverse bridge extending across the conical chamber (Fig. 3.4B, arrow).

Seven pairs of peripheral neurons, the BpNs, innervate the abdominal heart and associated alary muscles. The first five BpN pairs, which serve most of the dorsal vessel, have their somata located bilaterally in each abdominal segment near the row of spiracles (Figs. 3.5A, BpN1-3; 3.5C, BpN2-5), but the two caudal BpN pairs, which extend their processes on the terminal cardiac chamber (Figs. 3.2D, arrowhead; 3.4C, box E; 3.5C, BpN6), have their somata located close to the heart. Two thick processes extend from their four cell bodies to penetrate the myocardium (Fig. 3.6C, arrowheads). Extensive synaptotagmin immunostaining was associated with the BpN6 somata (Fig. 3.4E). Link nerves contact BpNs of adjacent abdominal segments (Figs. 3.5D, LN; 3.5B, LN) forming a complex intersegmental neuronal network (Fig. 3.5C, arrows). A similar arrangement of the abdominal peripheral neurons has been described in the fly *Glossina morsitans* by Anderson and Finlayson (1978).

Because CCAP increases the heart rate in adult *D. melanogaster* (Nichols et al., 1999a), a CCAP antiserum was used to determine if the extensive cardiac innervation observed in GFP transgenic flies is CCAP-IR. CCAP-immunostaining was detected in processes on the entire abdominal heart (Fig. 3.6A), and these processes have neurohemal-like endings that are organized as rows of thick (approximately 5 μm in
diameter) varicosities (Fig. 3.6B, arrowheads). All of the BpNs are CCAP-IR (Figs. 3.6D boxes E-H, BpN6; 3.6E-H, BpN2-5). Both ventral (Fig. 3.6F, arrowheads) and dorsal (Figs. 3.6G-H, arrowheads) BpN branches were immunolabeled. CCAP-IR fibers fasciculate with the transverse nerve soon after they arise from the BpN cell body (Figs. 3.6G-H, TN4-5) and terminate on the heart and alary muscles (Figs. 3.7A, AM; 3.7C, TN2). In all preparations, the BpNs6 showed the strongest CCAP immunoreactivity (Figs. 3.6C-D, BpN6). This caudal cluster contains the four largest BpNs (22-28 μm in diameter), as compared to BpN2-5 (6-10 μm in diameter). Their thick CCAP-IR branches (Fig. 3.6C, arrowheads) extend over the large pericardial cells boundaries to innervate the terminal cardiac chamber. This CCAP staining was not found in several other peripheral neurons (Figs. 3.6E and G, arrows) located within the same segmental clusters containing the BpNs.

To determine if the BpN CCAP-IR fibers account for the entire cardiac innervation, double-labeled whole mount preparations showing the CCAP-IR and GFP or 22C10-IR (see Methods) fluorescent terminals were analyzed. The CCAP-IR fibers (Fig. 3.7A) clearly represented only one component of the extensive innervation serving the abdominal heart (Fig. 3.7B). The CCAP-IR fibers start to branch at the tip of the alary muscles to run along the suspending strands of the cardiac chambers. Once they reached the myocardium, they rarely branch into secondary processes. More often, they follow the TN primary branch path and terminate with thick oblong varicosities (5-7 μm in diameter) along the way (Fig. 3.7C, TN2). Therefore, most of the highly branched processes extending across the dorsal vessel and their bouton-like terminals (Fig. 3.7D,
arrowheads) are not CCAP-IR (Fig. 3.7C, arrowheads).

Because the CCAP-IR processes do not overlap completely with the entire cardiac innervation, antibodies against classical neurotransmitters such as glutamate, GABA and octopamine were tested in adult, wild-type flies. GABA and octopamine immunostaining were not found in the abdominal heart of adult flies (data not shown). Glutamate immunostaining was detected in the abdominal heart (Fig. 3.8A, boxes B-C). In particular, the longitudinal processes extending from the transverse bridge of the conical chamber were immunolabeled. The glutamatergic projections ran along the surface of the myocardium, following the longitudinal cardiac muscles. They made moderately-branched rows of small (1-1.5 μm in diameter) endings intermingled with unbranched processes of large (3.5-4 μm in diameter), terminal varicosities (Fig. 3.8B, arrowheads). We classified these bouton-like structures as “type II” and “type I” endings, respectively, according to their size and distribution pattern, based on the classification used for terminals on skeletal muscles of D. melanogaster (Johansen et al., 1989). The glutamatergic processes that serve distal cardiac chambers had highly branched rows of type II endings (Fig. 3.8C) with occasional type I varicosities (Fig. 3.8C, arrowheads). The number of glutamatergic processes decreased toward the more distal cardiac chambers. No glutamate-positive terminals were observed on the terminal chamber (data not shown).

To determine the source of glutamatergic innervation, glutamate immunostaining was observed along with the GFP fluorescence of the peripheral nervous system of abdominal segments of adult GFP transgenic flies (Fig. 3.9). Glutamatergic fibers clearly
ran within the transverse nerves to terminate in the cardiac chambers (Figs. 3.9A-B, TNIR). The transverse bridge of the conical chamber appeared intensively immunostained (Figs. 3.9B, arrow; 3.9C). The thin longitudinal glutamate-IR processes (Fig. 3.9C, arrowheads), which run along the outer layer of longitudinal cardiac muscles, represent only part of the thick GFP-labeled structure (Fig. 3.9C, arrows). This glutamate-negative innervation includes the CCAP-IR processes, but might also include fibers and terminals (Fig. 3.9D, arrow) expressing another neurotransmitter or neuromodulator.

A number of orthograde backfills of the thoracic-abdominal ganglionic mass performed in wild type larvae showed the segmental innervation of the peripheral nervous system serving the dorsal musculature (data not shown) but failed to yield any clear evidence of processes extending to the aorta or abdominal heart. Moreover, thorough analysis of third-instar larvae of GFP transgenic flies gave no indication of cardiac innervation (Fig. 3.10A). In these preparations, we clearly observed fluorescent segmental nerves innervating the dorsal muscles (Fig. 3.10A, SN4.3, DM), as well as transverse nerves extending to the base of the alary muscles (Fig. 3.10A, arrows, TN4.5, AM). The distal tip of the transverse nerve terminal appears as a thick ending (Fig. 3.10E, arrow, TN3, AM) but does not extend onto the alary muscle strands suspending the myocardium (Fig. 3.10B, AM). These results confirmed what was previously described in D. melanogaster wild-type larvae by Gorczyca et al. (1994).

At this larval stage, BpNs6 are present and their somata are located bilaterally, close to the caudal cardiac chamber (Fig. 3.10A, BpN6, boxes C-D). Although, several thin branches originate from their somata (Figs. 3.10 C-D), they do not yet terminate in
the myocardium.

3.4 DISCUSSION

The first goal of this study was to determine whether the heart of adult *D. melanogaster* receives any neuronal input or whether its activity is independent from the nervous system. Our results demonstrated that the abdominal heart receives extensive segmental innervation (Fig. 3.1B). The transverse nerve fibers and BpN projections fasciculate with each other during embryonic development (Gorczyca et al., 1994) but it is only after metamorphosis that they innervate the abdominal heart.

The cardiac chambers located at each end of the abdominal heart showed distinctive variations in innervation. The pair of abdominal transverse nerves in the conical chamber always joins bilaterally to form the transverse bridge (Fig. 3.1B). Anterior to the transverse nerve bridge the median nerve, which extends longitudinally along the aorta, terminates with a thick process. This region of abundant neuronal terminals supports the hypothesis (Rizki, 1978) that the conical chamber represents the location of the retrograde pacemaker.

At the caudal end of the abdominal heart, the terminal chamber also showed a unique pattern of innervation. In addition to the bilateral transverse nerve innervation, which is present at every segment, the terminal chamber receives extra terminals originating from four large peripheral CCAP-IR neurons, the BpN56. Because the terminal chamber is where the anterograde heartbeat originates (Rizki, 1978) the cardioacceleratory effect of bath-applied CCAP (Nichols et al., 1999a) may mimic
release of CCAP by the BpNs to modulate the pacemaker properties of this cardiac region.

The organization of the peripheral nervous system in adult *D. melanogaster* shares some similarities with the segmental innervation of the adult fly *Glossina morsitans* (Anderson and Finlayson, 1978). Because of the larger size of *G. morsitans*, about twenty peripheral neurons distributed in a regular pattern in each abdominal segment could be studied both morphologically and electrophysiologically. In particular, the neurons innervating the alary muscles and the heart showed an organization similar to *D. melanogaster* cardiac innervation. Interestingly, in *G. morsitans* the neurons projecting to the alary muscles fired broad action potentials and contained many electron-dense granules. Because of these features, the authors suggested that these peripheral neurons might have a neurosecretory function. Our results support this hypothesis since the BpNs, innervating the alary muscles and the myocardium of *D. melanogaster*, are CCAP-IR.

The segmental organization of the cardiac innervation of adult *D. melanogaster* suggests a need to coordinate the neuronal activity of several abdominal segments. By extending between the BpNs and transverse nerves of adjacent abdominal segments, the link nerves (Fig. 3.5) could be a pathway through which the wave of contraction traveling to the series of cardiac chambers is coordinated. A study of the activity of these nerves would be an important step towards understanding whether BpN activity is segmentally coordinated by CNS projections or by peripheral interconnections.

The lack of lateral cardiac nerves observed in *D. melanogaster* has also been
described in other dipterans, such as *Anopheles quadrimaculatus* (Jones, 1954) and *Musca domestica* (Fordan, 1971). In contrast, lateral cardiac nerves were described in *G. morsitans* (Anderson and Finlayson, 1978). The significance of the absence of a direct descending input from the corpora cardiaca to the abdominal heart in *D. melanogaster* remains unknown.

During metamorphosis the dorsal vessel is remodeled. These morphological and neuroanatomical changes are likely related to the differences between adult and larval cardiac physiology. The larval heart beats at a rate of about 2.5-3.5 Hz (Robbins et al., 1999) and shows only an anterograde phase in which the wave of contraction is initiated from the caudal region and moves anteriorly (Rizki, 1978). The absence of a cardiac reversal, which is the cyclic alternation of the retrograde and anterograde phases of contraction, was also observed during the first day of the pupal stage (Curtis et al., 1999). Complete cardiac reversal develops sometime during metamorphosis (Rizki, 1978).

One of the most profound anatomical changes occurring during the last day of pupal life is the formation of a new conical cardiac chamber, which is added posterior to the aorta (Rizki, 1978; Curtis et al., 1999). This new region of the adult abdominal heart may to be the location of the retrograde pacemaker (Rizki, 1978). In addition, a new muscular layer is formed on the ventral region of the abdominal heart (Curtis et al., 1999; Molina et al., 2001). This functional and anatomical remodeling is accompanied by the simultaneous development of extensive cardiac innervation.

At least two distinct sources provide direct innervation to the abdominal heart of adult flies. Both glutamate and the CCAP immunoreactivity is detected at cardiac
synapse-like structures. The localization of the synaptic vesicle protein synaptotagmin in these structures throughout the abdominal myocardium (Fig. 3.3-3.4) suggests that some, if not all, of the cardiac innervation adopts a chemical mechanism of neurotransmission and modulation.

The demonstration of glutamate-IR at cardiac synapse-like processes is a novel finding in the circulatory system of insects. Octopamine-IR has been previously localized in identified heart-projecting neurons of other insect species, such as Periplaneta americana (Sinakevitch et al., 1996) and Locusta migratoria (Stevensen and Pfliiger, 1994), but we have not detected it associated with innervation of the adult D. melanogaster heart. Glutamate is known to be a neurotransmitter for cardiac ganglionic cells in crustaceans such as the isopod Bathynomus doederleini (Yazawa et al., 1998) and Ligia exotica (Sakurai et al., 1998). In the latter species, the heart beat changes from myogenic to neurogenic during juvenile development. Bath application of glutamate or excitatory junctional potentials depolarize the membrane of cardiac muscle cells in L. exotica (Sakurai et al., 1998) and the cardiac valves in B. doederleini (Tsukamoto and Kuwasawa, 2003).

The glutamate-IR fibers in D. melanogaster run through the transverse nerves and terminate on the myocardium of the adult cardiac chambers. Although we know the peripheral source of glutamate immunoreactivity, the location of the cell bodies of these neurons, which is probably within the CNS, remains unknown. Because glutamate-IR synaptic-like terminals seem to be concentrated on specific cardiac chambers and muscles, the glutamate-IR innervation might modulate or initiate the contraction of the
longitudinal muscle fibers located in the anterior portion of the abdominal heart.

Pharmacological studies (Zornik et al., 1999) on *D. melanogaster* demonstrated that glutamate is inactive in larva and pupa, but that it clearly affects the heart rate in adults. These results are consistent with our finding that the glutamate-IR innervation develops during the pupal stage.

A modest acceleratory effect of bath applied CCAP on heart rate was previously described in *D. melanogaster* larvae, pupae, and adults (Nichols et al., 1999 a) but no cardiac source of the peptide was identified. Our study demonstrates that the abdominal peripheral neurons, BpNs, show CCAP immunoreactivity and have processes that could release the peptide on the heart (Fig. 3.1A-B). The segmental distribution of the CCAP-IR varicosities throughout the abdominal heart suggests that this peptide could modulate the activity of every cardiac chamber as well as their alary muscles. Because a CCAP-IR cluster of four, large BpNs targets the terminal chamber, the contractile properties of this caudal pacemaker region are probably greatly affected.

Little is known about the physiology of BpNs in the adult stage. It would be of interest to know whether these peptidergic neurons receive any synaptic input or whether their neurosecretory function is modulated via hormonal signals. The detection of synaptotagmin on BpN₆ cell bodies might indicate presynaptic sites but newly synthesized presynaptic protein, which accumulates in the cytoplasm before being transported down to the terminals as well as sites of neurohemal release, could also account for the immunostaining.

The glutamate-IR and CCAP-IR processes do not account for the entire GFP-
fluorescent cardiac innervation. Fibers expressing some other neurotransmitter or modulatory substance are probably also innervating the myocardium of the conical chamber.

A number of FMRFamide-related peptides were demonstrated to inhibit the heart rate in early pupal stage when bath applied (Johnson et al., 2000), although bath application may not mimic the normal effects of neuropeptides that are released locally. In addition, the cardioinhibitory peptide SDNFMRFamide was isolated from adult *D. melanogaster* and immunolocalized to processes that project from subesophageal cells to the aorta in the pupa (Nichols et al., 1999b), but evidence was insufficient to determine whether FMRFamide-related peptide immunoreactivity is present in the abdominal heart. A homologous FMRFamide-positive innervation has been recently described in *Manduca sexta* (Davis et al., 2001). In the adult moth, the FMRFamide-IR neuroendocrine cells project from the subesophageal ganglion to the corpora cardiaca and aorta via the third cardiac nerve. Because other peptides such as CCAP are co-released on the corpora cardiaca and aorta, it has been hypothesized (Davis et al., 2001) that this neurohemal innervation might have a general systemic function in the release of cardioactive and non-cardioactive neuropeptides that are then transported by the circulatory system to the rest of the body.

The relatively simple vascular system of insects, found also in the most ancient living crustaceans and in many other arthropod groups (McMahon, 2001), has not only a circulatory function but also regulates the rate of hemolymph flow according to the animal’s needs. Cardiac reversal is a circulatory mechanism shared by most of the
Holometabolous insects to shunt the blood flow from the head-thoracic hemocoel to the abdominal hemocoel, and to create pressure changes that affect tracheal ventilation (Wasserthal, 1996).

The larval dorsal vessel in most insect species does not receive any direct innervation. At this stage, the myocardium produces a rhythm that is imposed by a pacemaker usually located at the caudal end of the abdominal heart. During metamorphosis, the heart develops new physiological properties and the cardiac reversal becomes a characteristic component of the adult cardiac cycle. Once the reversal is established, the cardiac impulse can also originate in a more anterior region of the dorsal vessel. The formation of an anterior cardiac pacemaker by itself would not be sufficient to explain how the alternating pattern of activity of the two pacemakers is produced. To cyclically trigger a reversal, the two cardiac pacemakers must be alternately dominant to each other. Interestingly, in addition to cyclic reversals, changes in the direction of cardiac contractions in insects can be initiated as a fast response to a variety of sensory stimuli (Kuwasa et al., 1999). Rapid regulation of the phase of cardiac contraction, and subsequently of the direction of the hemolymph flow, suggests neural control of heartbeat. Dulcis et al. (2001) demonstrated that the cardiac reversal in M. sexta is produced by the activity of an identified pair of motoneurons (MNi) in the eighth neuromere of the terminal abdominal ganglion. These CCAP-IR motoneurons determine when the posterior cardiac pacemaker becomes dominant so as to initiate the anterograde beat.

A neurogenic origin of the cardiac impulses in the anterior region of the dorsal
vessel represents an alternative mechanism that could produce a reversal. Rizki (1978) was the first to propose this hypothesis for adult D. melanogaster. Because the conical chamber has an independent development from the rest of the dorsal vessel and shows a faster rate of contraction as compared to the other cardiac chambers (Rizki, 1978), it was suggested that this region might represent the location of the retrograde pacemaker.

Previous attempts to reveal the possible influence of the nervous system on cardiac activity in D. melanogaster (Dowse et al., 1995; Gu and Singh, 1995; Johnson et al., 1997; 1998) led to the suggestion that the heart is completely myogenic, with neurohormones in normal background levels in the hemolymph playing a modulatory role. Because of the easier observation of heart activity, the animals analyzed in these studies were tested at the transition between the larval and pupal stage (Dowse et al., 1995; Johnson et al., 1997). Because cardiac reversal, the conical chamber, the longitudinal cardiac muscle layer, and the innervation of the abdominal heart all develop at the end of metamorphosis, the heart activity that is measured as pupariation begins is not representative of the adult cardiac physiology.

From the overall results described in our study and previously published data, the following model for the functional role of cardiac innervation in adult D. melanogaster is proposed. The caudal cardiac pacemaker, both in larvae and adults, initiates a myogenic impulse that travels along the dorsal vessel toward the aorta to produce the anterograde beat. In adults, the force of the heart contractions and the rate can be increased by CCAP release of the BpNs. These peptidergic neurons, which probably are interconnected via link nerves, may potentiate sequentially adjacent cardiac chambers to produce a
coordinated anterograde wave of contraction.

By contrast, the glutamatergic innervation may be involved in the activation of a newly developed anterior pacemaker in the conical chamber that may regulate the retrograde contraction. The activation of the processes from the TN bridge could cause an extensive glutamate release that would affect the newly formed ventral layer of longitudinal cardiac muscles, and the consequence could be a cardiac reversal. An alternative mechanism could be a retrograde impulse originated by a pacemaker located in the anterior end of the aorta. In this case, the conical chamber might act more as a booster of abdominal, retrograde heart contractions. The caudal cluster of the CCAP-IR neurons BpNs6 might contribute to the re-activation of the caudal pacemaker to complete the regular cardiac cycle of anterograde and retrograde beat alternation.

Further study needs to be done to reveal the role of the dual innervation of the *D. melanogaster* abdominal heart. In an electrophysiological study in *D. melanogaster* mutants, we are currently investigating the specific role of the glutamatergic and peptidergic innervation on adult cardiac physiology.
Figure 3.1: Diagram of cardiac innervation of adult *D. melanogaster*. **A**: ventral view of the dorsal half of the fly body (adapted from Miller, 1950). The location of the bipolar neuron cell body (BpN) is shown in segments I to VI with filled circles. The aorta, the conical chamber (CC), and the terminal cardiac chamber (TC) are also visible. **B**: ventral view of the conical chamber (conical ch.) and 2nd cardiac chamber (II cardiac ch.) showing the pattern of heart innervation. Transverse nerves (TN), bipolar neurons (BpN), the transverse bridge (TB), aorta, alary muscles (AM) and a representative pericardial cell (PC) are labeled.
Figure 3.2: PNS in the abdomen of adult flies. See next page for details.
**Figure 3.2: PNS in the abdomen of adult flies.** Laser-scanning confocal microscope images of whole-mount preparations of the peripheral nervous system in the abdomen of adult ELAV-GAL4/UAS-GFP transgenic flies. Abdominal sternites and visceral organs were removed to reveal the dorsal vessel and its innervation running along the ventral side of abdominal tergites. A: Ventral view of a fixed preparation of abdominal segments showing the dorsal vessel, surrounded by pericardial cells, along the midline (arrowheads) and transverse nerves (TN1-5) extending to the myocardium. B: Ventral view of fresh tissue of abdominal segments (S1-5) showing both motor fibers and sensory neurons of the peripheral nervous system. Segmental TNs, innervating the heart, originate from bifurcations (arrowheads) of the abdominal nerves (see also G). Peripheral neurons with relatively large cell bodies (arrow) innervate the terminal chamber of the abdominal heart. Box outlines region shown in C. C: Innervation of the same conical chamber shown in B (box). Left and right TNs join together to form a thick transverse bridge (arrow) extending across the chamber. Several longitudinal processes originate from it and extend posteriorly. D: Innervation of the terminal cardiac chamber. Fifth left and right TNs do not join together; instead they ramify before entering the heart wall to innervate the AMs. Processes of peripheral neurons (arrowhead) intermingle with TN terminals. Small cell bodies of sensory neurons (arrows) among the dorsal abdominal muscles are also visible. E: alternative pattern of conical chamber innervation observed in 50% of the analyzed specimens. Left and right TNs of segments 1-2 join together to form a structure composed of two thick transverse bridges interconnected by longitudinal processes extending both anteriorly and posteriorly. F: same synaptic formation shown in E (box). Several longitudinal processes, originating from the TN bridge (arrow) show bouton-like terminals (arrowheads). G: third abdominal nerve (AbN3) branching pattern showing how both segmental nerve (SN) and TN originate from it (see also in B, arrowhead). The TN fasciculates with the process of a peripheral neuron (arrow) which also innervates the abdominal heart. Scale bars = 100 μm in A and B, 50 μm in D and G, 20 μm in C, E and F.
Figure 3.3: Localization of synaptotagmin immunoreactivity. Synaptotagmin immunoreactivity in motoneuron terminals innervating the abdominal heart and dorsal musculature of adult ELAV-GAL4/UAS-GFP transgenic flies. Axonal branches and neural terminals were visualized by GFP fluorescence (white). Presynaptic vesicles were revealed with synaptotagmin antibodies, which were subsequently visualized with Cy5-conjugated secondary antibodies (red). Because GFP-fluorescence at the level of small, terminal processes is reduced by fixation, the color of the colocalized immunostaining occasionally prevails over the GFP-fluorescence when the two confocal images are merged. The abdominal fatbody was removed, as described in the methods, to reveal the dorsal musculature. A: ventral view of two abdominal segments showing the synaptic-like terminals on the dorsal vessel (DV), the large oblique dorsal muscle (ODM) and a number of longitudinal dorsal muscles (DM). B: same abdominal region shown in A (Box) at higher magnification. Cardiac nerve terminals make bouton-like synapses in the heart wall after branching multiple times. In contrast, DM synapses are typically organized in a row of boutons. The distinct sarcomere arrangement of the longitudinal cardiac muscles as compared to the segmental DM is also visible. Scale bars = 50 µm.
Figure 3.4: Synaptotagmin immunoreactivity in the PNS. See next page for details.
Figure 3.4: Synaptotagmin immunoreactivity in the PNS. Axonal branches and neuronal endings of TNs, peripheral and sensory neurons of adult ELAV-GAL4/UAS-GFP transgenic flies were visualized by GFP fluorescence (green). Presynaptic vesicles were detected by synaptotagmin antibodies, which were subsequently visualized with Cy5-conjugated secondary antibodies (red). Regions of colocalization of GFP and synaptotagmin appear yellow, but because GFP-fluorescence at the level of small, terminal processes is reduced by fixation, the color of the colocalized synaptotagmin-IR occasionally prevails over the GFP-fluorescence when the two confocal images are merged. A: ventral view of four abdominal segments showing localization of presynaptic vesicles at cardiac and skeletal muscle synapses. Synaptotagmin is localized in TN terminals but also in regions of TN axons that run along the AMs (arrowheads). B: higher magnification view of the same conical chamber shown in A (box). Anti-synaptotagmin staining labels both TN endings and the thick neural structure extending horizontally across the first cardiac chamber (arrow). Large poly-nucleated fat cells surrounding the cardiac chambers are also visible (arrowheads). C: ventral view of abdominal segments (3-6) showing localization of presynaptic vesicles at cardiac and musculature synapses. TNs of two adjacent segments often contact each other ipsilaterally running longitudinally along the lateral aspect of the DV (box D). Synaptotagmin immunoreactivity is colocalized with GFP at TN terminals; the remaining GFP-labeled structures, such as axons and sensory neurons, are not co-labeled. D: Same cardiac chamber shown in C (box D). The synaptotagmin immunostaining of the heart is detected mostly at the synaptic-like terminals where the TN endings form thick bouton-like structures (arrowheads). Very little synaptotagmin is localized along the ipsilateral connections (arrows) of adjacent TNs. E: same peripheral neurons shown in C (box E) innervating the caudal cardiac chamber. Abundant synaptotagmin immunoreactivity is detected at the level of the cell bodies. Scale Bars = 50 μm.
Figure 3.5: Peripheral bipolar neurons (BpNs). See next page for details.
**Figure 3.5: Peripheral bipolar neurons (BpNs).** Laser-scanning confocal microscope images of whole-mount preparations of the peripheral nervous system in the abdomen of adult ELAV-GAL4/UAS-GFP transgenic flies. The spiracle row in the abdominal tergites and the lateral pleural membrane were kept intact to reveal the peripheral bipolar neurons (BpNs) that innervate the dorsal vessel. **A:** ventral view of fresh tissue of the first three abdominal segments showing the cell body location of BpN1-3. **B:** same BpN3 shown in A (box) at higher magnification. Two neuronal branches originate from its cell body. The ventral projection contacts the third segmental abdominal nerve (AbN3). The dorsal projection bifurcates into two branches which fasciculate with the TN and link nerve (LN), respectively. **C:** ventral view of fixed abdominal tergites (2-5). The somata of BpN2-3 are located bilaterally, one per hemi-segment, and their fibers fasciculate with TN axons to innervate the abdominal cardiac chambers (arrowheads). In contrast, a median cluster of peripheral neurons (BpN6) serves the terminal cardiac chamber (see also 3C, E). Link nerves (arrows) interconnect abdominal nerves and TNs of adjacent abdominal segments. **D:** same BpN2-3 shown in C (box). The cell bodies of BpNs are often located at junctional positions among transverse, segmental, and link nerves. Scale bars = 100 μm in A and C, 20 μm in B and D.
Figure 3.6: Localization of CCAP immunoreactivity. See next page for details.
Figure 3.6: Localization of CCAP immunoreactivity. CCAP immunostaining in whole-mounts of the dorsal vessel and peripheral nervous system in the abdomen of adult wild type (A-C) and ELAV-GAL4/UAS-GFP transgenic (D-H) flies. The pericardial cell’s (PC) auto-fluorescence (green in A, brown in B-C) was detected by scanning with an argon laser line (488 excitation maximum) and using a band-pass filter at 510 nm. Because the GFP fluorescence greatly reduces after fixation, the peripheral nervous system in (D-H) was stained with 22C10 antibodies, which were subsequently visualized with FITC-conjugated secondary antibodies (green). In all images, CCAP antibodies were labeled with Cy5-conjugated secondary antibodies (red). A: the CCAP-IR varicosities are present throughout the whole dorsal vessel. The caudal cluster of BpN₆ is also CCAP-IR. White boxes indicate insets seen in B-C. B: CCAP terminals (arrowheads) in the abdominal heart form longitudinal rows of thick varicosities. Large pericardial cells (PC) surrounding the dorsal vessel are also visible. C: the caudal cluster of BpN₆ contains at least four cell bodies. Their CCAP-IR branches (arrowheads) run across the large pericardial cells (PC) boundaries to terminate in the cardiac terminal chamber. D: double staining of CCAP (red) and 22C10 (green) of the same whole-mount preparation shown in figure 5C. All segmental BpNs (boxes E-H) and the terminal BpN₆ are CCAP-IR. E: the BpN₂ has a large rounded cell body that is located at the junction where the second abdominal nerve originates the segmental, transverse, and link nerves. Another cell body (arrow), which is adjacent to BpN₂, does not show any CCAP immunoreactivity. F: Details of BpN₃ showing its triangular-shaped cell body that lies at the junction between transverse and link nerves. CCAP labeling can be detected also in the ventral projection (arrowheads) that contacts the third abdominal nerve. G, H: the somata of both BpN₄ and BpN₅ are included in a peripheral enlargement of the segmental abdominal nerves (SN₄₅). Their fibers, which contain large CCAP-IR varicosities (arrowheads), fasciculate with TN₄₅ toward the abdominal heart and AMs. A smaller neuron, adjacent to BpN₄ (arrow), does not show any CCAP immunoreactivity. Scale bars = 50 μm in A-D, 10 μm in E-H.
Figure 3.7: Localization of GFP and CCAP-Ir in the abdominal heart. Montage of confocal optical sections of a double-labeled whole-mount preparation showing the CCAP-IR (A) and GFP-fluorescent (B) terminals in the abdominal heart of adult ELAV-GAL4/UAS-GFP transgenic flies. White boxes in A-B indicate insets seen in C-D. A: the CCAP-IR endings originate from thin segmental fibers that fasciculate with the TN and terminate as varicosities in the AMs (AM) and the dorsal vessel. B: the GFP-fluorescent fibers of the segmental TNs ramify extensively to innervate both AM and cardiac wall. C, D: the CCAP-IR fibers that innervate the conical chamber (in C) do not branch before terminating as varicosities in the myocardium. In contrast, the TNs (in D) contain thick fibers that ramify horizontally across the same chamber and end with large bouton-like synapses (arrowheads). These structures are not CCAP-IR (arrowhead in C). Scale bars = 50 μm in A and B, 25 μm in C and D.
Figure 3.8: Localization of glutamate-Ir in the abdominal heart. Confocal photomicrographs of the dorsal vessel and its glutamate-IR innervation in adult wild-type flies. Glutamate antibodies were labeled with TR-conjugated secondary antibodies. A: montage of four confocal optical sections of the abdominal heart. The proximal conical chamber, as well as distal cardiac chambers, display extensive glutamate immunoreactivity. Pericardial cells (PC) are also visible. White boxes indicate insets seen in B-C. B: the glutamate-IR nerve endings of the conical chamber form longitudinal rows of bouton-like terminals. Some of the latter have a relatively large size (arrowheads). C: glutamate-IR innervation of a cardiac chamber showing how nerve terminals form symmetrical longitudinal rows of small varicosities, sometime ending with large bouton-like structures (arrowheads). Scale bars = 25 μm.
Figure 3.9: Glutamatergic innervation of the conical chamber. See next page for details.
Figure 3.9: Glutamatergic innervation of the conical chamber. Glutamate immunoreactivity in the conical chamber and abdominal muscles of adult ELAV-GAL4/UAS-GFP transgenic flies. Axonal branches and neuronal terminals were visualized by GFP fluorescence (white in A; green in C-D). Glutamate-IR processes were detected by glutamate antibodies, which were subsequently visualized with cy5-conjugated secondary antibodies (white in B; red in C-D). Regions of colocalization of GFP and glutamate appear yellow-orange (C-D), but because GFP-fluorescence at the level of small, terminal processes is reduced by prolonged fixation, the color of the colocalized glutamate immunostaining occasionally prevails over the GFP-fluorescence when the two confocal images are merged. A: ventral view of the conical chamber showing the GFP-fluorescent fibers of TNs₁ and the distinctive transverse bridge (arrow). B: confocal image of the same preparation shown in A collected by using a band-pass filter at 650 nm to visualize glutamate immunoreactivity. The glutamate-IR fibers ran within the TNs. The TN bridge (arrow) and its processes were glutamate-positive. C: merging of images shown in A and B. Glutamate immunoreactivity (red and yellow) was extensively localized at the level of the transverse bridge of the conical chamber. The glutamate-IR processes (arrowheads) represent only a portion of the GFP-fluorescent TN innervation. The extensive glutamate-negative green regions localized on the TN bridge and lateral longitudinal process (arrows) are accounted for in part by the CCAP-IR processes (see Fig. 7C), but might also include fibers expressing another neurotransmitter or neuropeptide. Glutamatergic terminals on the dorsal muscles (DM) are also visible. D: same terminals shown in C (box). The glutamate-IR bouton-like terminals (arrowheads, red-orange) are visible. The glutamate-negative (arrow, green) regions may represent terminals associated with additional cardiac innervation expressing another neurotransmitter or neuropeptide. Scale bars = 80 μm in A-B, 20 μm in C-D.
Figure 3.10: Lack of cardiac innervation in the larval stage. See next page for details.
Figure 3.10: Lack of cardiac innervation in then larval stage. Laser-scanning confocal microscope images of peripheral nervous system preparations in the abdomen of third-instar larvae of ELAV-GAL4/UAS-GFP transgenic flies. Visceral organs and dorsal tracheae were removed to reveal the dorsal vessel and abdominal musculature. A: Ventral view of abdominal segments (6-8) showing the last three chambers of the heart, which is surrounded by pericardial cells, running along the larval body midline. The segmental nerves (SN) give rise to several branches that innervate the dorsal muscles (DM). The TNS4,5, arising from the forth and fifth segmental nerves (SN4,5), contact the base of the AMs (arrows). The BpNs6 are not clustered along the caudal end fibers. Their somata (white boxes in A) are instead localized bilaterally at the base of the AMs of the caudal cardiac chamber. B: Same cardiac chamber shown in A (box). At this larval stage, there are no peripheral nervous system branches innervating the myocardium and the AMs. Synaptic terminals in the dorsal muscles (DM) are always visible. To visualize the non-innervated heart, the abdominal preparations were incubated with a coomassie brilliant blue solution, as described in the methods. This dye accumulates mostly in the pericardial cells (PC) as fluorescent intracellular granules. C, D: Higher magnification views of the cell bodies of BpNs6 (boxes in A) located bilaterally in the caudal cardiac chamber. Several thin, radial branches originate form their somata. E: TN3 terminal contacting the base of the AM. The distal tip of the TN appears as a thick, neuritic ending (arrow). Scale bars = 100 µm in A and E, 50 µm in B, 25 µm in C and D.
CHAPTER 4. GLUTAMATERGIC INNERVATION OF THE HEART INITIATES RETROGRADE CONTRACTIONS IN ADULT, DROSOPHILA MELANOGASTER

4.1 INTRODUCTION

Normal cardiac performance depends both on intrinsic excitability of cardiac pacemaker cells and on extrinsic neuronal activation or modulation of this specialized class of cardiomyocytes. The fine balance between cardiac pacemaker activity, conduction of electrical impulses to the working myocardium, and its regulation by classical neurotransmitters, neuropeptides and amines is, in many cases, still poorly understood (Beaulieu and Lambert, 1998). Here we investigate the role of glutamatergic innervation in the regular cardiac function of adult Drosophila. Octopamine and neuropeptides are expressed in cardiac neurons of a variety of insects (Stevenson and Pflueger, 1994; Sinakevitch et al., 1996; Duch et al., 1999; Davis et al., 2001), but the glutamate-Ir cardiac innervation reported recently in adult Drosophila (Dulcis and Levine, 2003) represents a novel finding in insect multi-chambered hearts. Glutamate-expressing axons grow onto the cardiac muscle in the first abdominal segment and fasciculate during metamorphosis to form a characteristic glutamate-Ir synaptic structure, the transverse bridge (Dulcis and Levine, 2003).

Glutamate is the major excitatory transmitter of the mammalian CNS (Collingridge and Lester, 1989; Monaghan et al., 1989), where it mediates not only normal synaptic transmission but also participates in functional plasticity during development and throughout life (Debanne et al., 2003; Kolleker et al., 2003; Leinekugel, 2003). The Drosophila neuromuscular junction is glutamatergic and has been broadly
used as a model, but the relatively large size of the novel cardiac synapses, together with the availability of genetic tools in Drosophila, provides a unique model system for investigating synaptic function and plasticity. Thus, the goals of this study were to investigate whether pre- and post-synaptic specializations accompany the glutamate-Ir cardiac innervation and to determine the role of these synapses in cardiac function.

Adult holometabolous insects display a cardiac cycle composed of two alternating pacemaker phases, the anterograde and the retrograde beats, which correlate with a reversal of hemolymph flow (Tenney, 1953; Queinnec and Campan, 1972, Wasserthal, 1976; Ichikawa and Ito, 1999; Smits et al., 2000; Dulcis et al., 2001). Previous studies in larval and pupal stages failed to describe a cardiac reversal in Drosophila (Dowse et al., 1995; Johnson et al., 1997). However, this may simply reflect the fact that in other species cardiac reversal develops only during metamorphosis and requires significant neuronal modification (Kuwasaawa and Matsushita, 1999; Davis et al., 2001; Dulcis et al., 2001; Dulcis and Levine, 2004).

In the present study, we employed immunocytochemical, electrophysiological, pharmacological, and optical approaches to investigate whether formation of the glutamatergic innervation correlates with changes in the regular cardiac function of the adult Drosophila. To this aim, we developed an optical technique based on the movement of GFP-labeled nerve terminals to monitor heartbeat in intact and semi-intact preparations. We found that cardiac reversal is indeed a feature of the adult heart function and that the excitatory effect of glutamatergic synapses on the myocardium provides the mechanism for originating the retrograde beat, and hence cardiac reversal, in adult flies.
4.2 MATERIALS AND METHODS

(1) *Drosophila strains and culture*. Flies were raised on medium consisting of instant food, agar, and oatmeal (Condie and Brower, 1989) supplemented with yeast. All stocks were maintained at 25°C under uncrowded conditions. Wild-type Oregon-R and *elav*-GAL4/UAS-GFP transgenic flies were used in this study. The data were collected from 2-3day old adults. The animals were anesthetized on ice for about 10 minutes then dissected in cold *D. melanogaster* saline (Stewart et al. 1994).

(2) *Immunocytochemistry*. *elav*-GAL4/UAS-GFP transgenic flies were used to visualize the peripheral fibers innervating the heart. In these animals, the expression of the green fluorescent protein (GFP) is controlled by the GAL4/UAS system with the GAL4 transcription factor driven by the pan-neuronal promoter *elav* (Estes et al., 2000). After removal of the ventral abdominal sternites and visceral organs the tissue was fixed with 4% paraformaldehyde for two minutes to keep the abdominal segments flat and to stop the heart from beating during confocal imaging. Because fixation greatly reduces GFP fluorescence, a GFP antiserum was applied as described below.

To examine the release sites at cardiac synapse in adult flies, a rabbit polyclonal antiserum to *Drosophila* DPAK (Harden at al., 1996; generously provided by Dr. N. Harden) and a mouse monoclonal antiserum (NC82; Hofbauer, 1991; generously provided by Dr. K. Zinsmaier), which recognizes an unidentified protein localized at active zones, were tested in skeletal muscle and heart preparations. The myocardium was fixed with Bouin solution for 1-2 minutes. After rinsing (3 times for 5 minutes each) in
1% PBST (pH 7.2), the preparations were incubated for 1 hour in the blocking solution comprising 2% bovine serum albumin (BSA, Sigma), 5% normal donkey serum (NDS, Sigma) in PBS with 1.5% Triton-x100 (T). The primary antisera (1:10 NC82 and 1:50 DPAK) made up in blocking solution were applied at 4°C overnight.

To determine whether DGluRIIA receptors localize at cardiac synapses, the myocardium of ELAV-GAL4/UAS-GFP transgenic adult flies, fixed and blocked as described above, was incubated for 2 hours at room temperature with a primary mouse monoclonal DGluRIIA (1:5) antibody ‘8B4D2’ developed by Schuster et al. (1991; obtained from the Developmental Studies Hybridoma Bank) and a rabbit polyclonal GFP (1:100) antiserum (Molecular Probes, Eugene, OR) made up in blocking solution. All of the preparations were subsequently washed with 1% PBST for a total of 15 minutes (3 times for 5 minutes each). A Cy5-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories, West Grove, PA) and the Cy2-conjugated donkey anti-rabbit IgG were used as secondary antibodies applied at a dilution of 1:25 for 1 hour at room temperature. The preparations then were washed several times in 1% PBST for 15 minutes, in PBS for 20 minutes and mounted in 80% glycerol solution. Rabbit polyclonal antiserum to *Drosophila* synaptotagmin (Littleton et al., 1993; generously provided by Dr. M. Ramaswami) was used to visualize cardiac terminals in wild type adult preparations. Synaptotagmin immunostaining was carried out as described in Dulcis and Levine (2003) and double labeled with DGluRIIAIr.

(3)Video microscopy and optical detection of heart activity. Recordings were made from
2-3 day old adults, as younger flies have too much fat tissue, thus making optical
detection through the cuticle difficult. The animals were anesthetized on ice for about 10
minutes then restrained dorsal side up by placing a bent pin around the neck of the adult
fly. Legs and wings were removed to keep the specimen immobile during optical
measurement. The cardiac chamber of interest was observed under the microscope with a
10x objective focused to distinguish heart movements either through the dorsal cuticle, in
intact preparations, or directly, for semi-intact preparations. To determine direction of the
heartbeat, two or more cardiac chambers were monitored simultaneously. Bright field
was used for detecting heartbeat of wild type flies; fluorescent light (fluorescein band
pass filter) was utilized for elav-GAL4/UAS-GFP transgenic flies. Simple PCI software
(Compix Inc., Cranberry Township, PA) was adapted to measure movement of the heart
during cardiac contractions. The software allowed collection of images at a speed of 19
frames/second. Although this is 3 times faster than the maximum adult Drosophila heart
rate (6 Hz) and should ensure detection of all cardiac contractions, there may have been
small errors in determining their precise onset and offset. Thus, the sequence of cardiac
contractions occurring in adjacent chambers could be determined accurately, but absolute
time difference was difficult to determine accurately.

(4) Cardiac volume measurements. Volume changes of the conical chamber were
detected optically. The area selected for light intensity measurement, performed with
Simple PCI software, was chosen to specifically detect diastolic and systolic movements
of the heart wall and distinguish them from higher rate mini-diastole/mini-systole cycles.
The lumen of the conical chamber was considered with approximation to a cylinder ($V = \pi r^2 h$), whose radius was measured at the level of the transverse bridge (Fig. 4.5A-B). Diastolic and systolic volumes expressed in $\mu m^3$ were converted in nanoliters by applying the following conversion ($10^9 \mu m^3 = 1,000$ nl).

(5) Intracellular recordings and local glutamate application. An axoclamp 2B amplifier (Axon Instruments, Union City, CA) was used for intracellular recordings from the myocardium of the conical chamber of both wildtype and elav-GAL4/UAS-GFP transgenic flies. Cardiac action potentials were recorded in bridge mode with thin-walled borosilicate electrodes (resistance, 25-30 MΩ) filled with 3M potassium chloride. Glutamate was applied with a Picospritzer II (General Valve, Fairfield, NJ) set at a pressure of 10 psi to deliver 10μl of Glutamate solution and reach a final concentration of $10^{-6}$M in the bath. The preparations were first perfused with a calcium-free saline to measure the glutamate-evoked depolarization in the absence of cardiac contractions, then the same stimulation protocol was performed in normal saline to monitor intracellular cardiac action potentials. Controls were made by injecting 10μl of normal saline solution. Intracellular signals were acquired with Clampex 9.0 (Axon Instruments). Clampfit 9.0 (Axon Instruments) was used for analysis.

(6) Pharmacology. For the pharmacological experiments we used the elav-GAL4/UAS-GFP transgenic fly line, since optical detection is optimal when nerve terminals are fluorescent. Glutamate (Sigma-Aldrich Corporation, St. Louis, MO) and CCAP (Backem,
Torrance, CA) were bath-applied in \textit{in vitro} preparations with a micropipette to reach final concentrations in the bath of $10^{-5}/10^{-6}\text{M}$ and $10^{-4}\text{M}$, respectively, while cardiac activity was being detected optically. The preparations were constantly perfused with normal saline (Stewart et al., 1994) to wash out the compound and to keep the heart well oxygenated.

\textit{(7) Electrical stimulation of transverse nerves}. Electrical stimulation of transverse nerves was performed in \textit{elav-GAL4/UAS-GFP} transgenic adult flies. Following removal of the abdominal viscera to expose the heart for optical detection, the first pair of abdominal TNs (1\(\mu\) in diameter) were visualized with fluorescence light (Fluorescein filter) and cut as far distally as possible from the conical chamber. The distal stump was drawn into the tip of a glass suction electrode connected to a S48 stimulator (GRASS Instruments, Quincy, MA), and a train of pulses (5s, 20 Hz, 1ms/pulse) was applied. The suction electrodes were fabricated from capillary tubing (Scientific Products, McGaw Park, IL). The tips were made with a pipette puller and then polished with a microforge to the correct size (about 1 micron) under a microscope. The nerve stumps were sucked into the tip of the pre-filled suction electrode by applying negative pressure with a syringe.

\textit{(8) Laser-scanning confocal microscopy}. Digital images of immunostained cardiac preparations were collected on a Nikon PCM 2000 laser-scanning confocal microscope equipped with green He/Ne (543 nm), red He/Ne (633 nm) and argon (488 nm) lasers. Cy2/GFP and Cy5 were detected respectively with argon and red He/Ne laser lines and
using band-pass filters at 510 nm (Cy2) and 650 nm (Cy5). Stacks of digitized images were merged by using Simple PCI as image acquisition software. Corel Draw and Corel Photopaint (Corel Corp., Ottawa, Ontario, Canada) software were used to enhance contrast and provide color when needed. Prints were made by using a Tektronix Xerox Phaser 6200 printer. Digital images of CCAP-immunoreactivity in the CNS were collected using a Leica DMR confocal microscope system.

4.3 RESULTS

The abdominal heart of adult *Drosophila* becomes extensively innervated during metamorphosis (Dulcis and Levine, 2003). Segmental abdominal transverse nerves (TNs) serve each of the cardiac chambers bilaterally. The conical chamber receives a characteristic innervation, the transverse bridge (Fig. 4.1A, arrow), which develops from fasciculation of right and left TNs (Fig. 4.1A, TN嵇R and TN嵇L). Some of the axons within the TNs are glutamatergic (Dulcis and Levine, 2003) and form longitudinal processes originating from the transverse bridge (TB) and extending posterior to it (Fig. 4.1A, arrowheads). The longitudinal processes terminate on the myocardium with glutamate-IR bouton-like endings (Dulcis and Levine, 2003; Fig. 4.1B, arrowheads).

To determine whether these cardiac terminals contain release sites for neurotransmitters, we used the NC82 antibody (Hofbauer, 1991) that recognizes an unknown protein localized presynaptically at the level of the active zones. In skeletal muscles, NC82Ir co-localizes but does not overlap with DPAKIr (Fig. 4.1C-E), a marker for active zones that recognizes a protein in the electron dense regions of the synaptic
cleft (Sone et al., 2000; Wan et al., 2000). Because of its localized punctate pattern (Fig. 4.1F, *arrowhead*), NC82 antibody may be a better marker than DPAK antiserum for quantification of active zones. The NC82-Ir putative release sites were localized extensively in the TB of the conical chamber (Fig. 4.1G-H, *arrows*) as well as in the cardiac bouton-like terminals (Fig. 4.1I, *arrowhead*).

We performed double labeling of the conical chamber with DGlurIIIA and synaptotagmin antisera to determine whether glutamate receptors were present and confined to the myocardium underneath innervation, or if their expression displayed a more diffuse pattern (Fig. 4.2A-D). Glutamate receptor subunit A formed rows of large clusters around the TB (Fig. 4.1A, *arrowheads*). In addition, GluRIIA-IR was detected on the longitudinal muscle fibers of the conical chamber in large clusters, which were always adjacent to neuronal terminals (Fig. 4.1D, *arrowheads*). To investigate the expression pattern of glutamate receptors in more caudal cardiac chambers we immunolabeled GFP and GluRIIA in elav-GAL4/UAS-GFP transgenic flies. As observed in the conical chamber, GluRIIA-IR in more caudal chambers was always localized where TNs contacted the myocardium (Fig. 4.2E, *arrowheads*).

Intracellular recordings from the ventral longitudinal muscle layer of the conical chamber (Fig. 4.3A) were performed to investigate the effect of glutamate on cardiac excitability. Local applications of $10^{-5}$M glutamate in Calcium-free saline caused long-lasting membrane potential depolarization ($5.8\pm2.4$ mV, mean±Std.Dev, n=5; Fig. 4.3B) that returned to the resting level following constant superfusion with saline. As expected, the heart did not beat in Ca-free saline since cardiac activity in *Drosophila* is mediated by
L-type calcium channels (Gu and Singh, 1995). Because resting membrane potentials of myocardial cells were always somewhat depolarized (-14±6 mV, Std.Dev, n=8), the presence of spontaneous MEJPs was used as an indication of successful intracellular recordings (Fig. 4.3D). The MEJPs had an amplitude of 0.22±0.11 mV (Std.Dev, n=53).

When glutamate was applied to hearts that were perfused with normal saline (HL-3), cardiac action potentials were initiated after the initial depolarization and continued to occur for several minutes afterwards, suggesting that regenerative pacemaker currents were activated (Fig. 4.3C). The cardiac action potential frequency, which was highest (1.7±0.16 Hz, mean±Std.Dev, n = 4) during the glutamate-evoked depolarization, decreased rapidly when the membrane potential repolarized (1.07±0.18 Hz, mean±Std.Dev, n = 4), and gradually diminished until the heart stopped beating (Fig. 4.3C). Cardiac action potentials that occurred after the membrane potential had repolarized displayed a slow depolarizing phase (Fig. 4.3E, arrowheads; Fig. 4.3F, arrow), typical of pacemaker potentials, that increased the duration, as measured at the base of the spikes, to 905±205 ms (mean±Std.Dev, n = 10). Cardiac action potentials occurring during the glutamate-evoked depolarization showed a faster rising phase (Fig. 4.3F, double arrow), narrower peak (Fig. 4.3F, arrowheads), and a significantly shorter duration (463±75 ms; mean±Std.Dev, n = 10; p < 0.0001 by Student’s t-test for unpaired data).

Optical detection of cardiac activity in intact adult flies allowed us to monitor two different aspects of heart wall displacement simultaneously. The conical chamber (CC) of adult Drosophila showed two distinct kinds of movement that occurred simultaneously.
High-frequency mini-systole/mini-diastole cycles were small individual contractions/relaxations of the chamber, whereas long lasting systole-diastole cycles were large changes of the conical chamber lumen. When these two kinds of movement were detected simultaneously and combined in one trace, the heart activity of resting flies was represented by a complex pattern of contractions with mini-systole/mini-diastole superimposed on the systole/diastole cycle (Figure 4.4A). The cardiac cycle was composed of two alternating phases with different rates of mini-systole/mini-diastole (heart rate), which were correlated with the systolic/diastolic phases of the CC (Fig. 4.4A). During systole, when the CC decreased its lumen, the heart rate increased to 4.9 ± 0.9 Hz (mean ± Std.Dev, n = 6). During diastole, when the CC increased its diameter, the frequency of mini-systole/mini-diastole decreased to 3.5 ± 1.6 Hz (mean ± Std.Dev, n = 6). The relative duration of systolic and diastolic phases showed high variability among specimens (13 ± 8 sec and 9 ± 7 sec (mean ± Std.Dev, n = 15) respectively. However, a long-lasting systole (7.3 ± 1.1 sec, mean ± Std.Dev, n = 10) in alternation with a shorter diastole (4 ± 0.4 sec, mean ± Std.Dev, n = 10) was observed in a large subset of our preparations. Total amount of circulating hemolymph and/or mechanical pressure applied to restrain the animals may represent the source of the observed variability in phase duration.

To determine the direction of heart contractions during the two cardiac phases, the imaging software was set to detect simultaneously mini-systole/mini-diastole cycles occurring in both the conical and second cardiac chambers (Fig. 4.4B-C). By analyzing the two superimposed traces at expanded time scale, it was determined that the higher
and lower rate phases (systole and diastole) corresponded to the anterograde and retrograde beats, respectively (Fig. 4.4 D). Cardiac reversals occurred cyclically in intact adult flies (Fig. 4.4C, arrows).

Selective optical detection of diastolic and systolic movements of the CC and measurement of its medial diameter during these two circulatory states allowed estimation of the volume of hemolymph that the chamber can exchange per cardiac cycle (Fig. 4.5). Video microscopy data revealed that the CC could reach its maximum diastolic volume \(1.35 \pm 0.1\) nl, mean \(\pm\) Std.Dev, \(n = 5\) by rapidly decreasing its muscle tone and starting from a systolic volume of \(0.45 \pm 0.05\) nl (mean \(\pm\) Std.Dev, \(n = 5\)), thus moving an average volume of approximately \(0.9\) nl of hemolymph per cardiac cycle.

To correlate the anterograde and retrograde beats with the volumetric changes occurring in the CC, mini-systole/mini-diastole (Fig. 4.6 A, upper trace) and systole/diastole (Fig. 4.6A, lower trace) cycles were recorded simultaneously, but were displayed in separate traces. The retrograde beat correlated with the conical chamber diastole and the anterograde beat with the systolic phase (Fig. 4.6B). Interestingly, the CC lumen rapidly increased to reach the maximum diastolic volume as soon as the retrograde beat started (Fig. 4.6B, left arrowhead). Similarly, as soon as the retrograde beat ceased the muscle tone of the CC rapidly increased to reach the systolic volume (Fig. 4.6B, right arrowhead).

Upon exposure to fluorescent light the abdominal hearts of semi-intact preparations of adult elav-GAL4/UAS-GFP flies usually stopped beating completely or showed only occasional anterograde contractions. Under these conditions, where the
majority of the segmental TNs were transected, bath application of glutamate to reach a final concentration of 30 μM had a chronotropic effect on cardiac activity in that mini-systole/mini-diastole were evoked (1.4 ± 0.5 Hz, mean ± Std.Dev, n = 6). These movements corresponded to the cardiac action potentials that were evoked by glutamate (Figure 4.3). There was a simultaneous reduction of the CC lumen (Fig. 4.7). As glutamate was washed out by constant superfusion with fresh oxygenated saline, the heart decreased its rate and eventually stopped (Fig. 4.7, upper trace). At the same time, CC volume gradually increased (Fig. 4.7, lower trace). A second glutamate application had the same effect (Fig. 4.7, arrows).

The direction of the glutamate-evoked cardiac contractions was retrograde (Fig. 4.8A). By simultaneous detection of mini-systole/mini-diastole cycles of the terminal and third cardiac chambers during the evoked retrograde beat, it was determined that when the third chamber completed a mini-diastole the terminal chamber was only at the beginning of the diastolic phase (Fig. 4.8B, dotted lines). In contrast, during the spontaneous anterograde beat, mini-diastoles in the two adjacent chambers were terminated synchronously. The delayed onset of the mini-systole of the third chamber determined the direction of the anterograde contraction waves (Fig. 4.8C-D). To determine whether the direction of the beat was influenced by glutamate or remained in the default anterograde direction of isolated hearts, we bath-applied glutamate to hearts that were spontaneously beating in the anterograde direction. Consistently (n = 5), bath-application of glutamate caused cardiac reversal (Fig. 4.8C).

CCAP-Ir neurons provide extensive innervation of the adult heart, particularly in
the caudal region (Dulcis and Levine, 2003). To test whether this cardio-active neuropeptide (Tublitz and Evans, 1986; Nichols et al., 1999; Dulcis et al., 2001) activated specifically the retrograde or the anterograde beat, CCAP was bath-applied to semi-intact preparations (Fig. 4.9A). By simultaneously monitoring heart contractions occurring in the conical, second and third cardiac chambers, it was demonstrated that application of $10^{-4}$ M CCAP potentiated the anterograde beat (Fig. 4.9B). As observed in spontaneous anterograde contractions (Fig. 4.9C), CCAP-induced contractions occurred first in the third chamber then sequentially invaded the second and conical chamber (Fig. 4.9D).

Pharmacological cardiac reversal was observed following $10^{-6}$ M glutamate bath-application to anterograde beating hearts that were pre-activated with CCAP (Fig. 4.9B-E). When CCAP and glutamate were both present in the bath, the conical and the third chamber contracted synchronously, both before the second cardiac chamber (Fig. 4.9E). As if the two cardio-active compounds were competing to produce their specific effect, an anterograde beat originated in the caudal portion of the heart and a retrograde beat originated in the CC occurred simultaneously (Fig. 4.9B-E).

To determine whether the effects of glutamate application reflected the function of the normal cardiac innervation, unilateral electrical stimulation of the first pair of glutamate-Ir transverse nerves (TNs) serving the conical chamber was performed as indicated in Figure 4.10A.

A train of electrical pulses (20Hz, 5s, 1ms/pulse) applied extracellularly with a suction electrode was sufficient to produce a chronotropic effect in the conical chamber (Fig. 4.10B). Following TN stimulation, the endogenous low-rate (0.5 ± 0.1, mean ± Std.Dev,
of anterograde beats that is characteristic of in vitro preparations (Fig. 4.10C) was replaced by a retrograde beat (2.1 ± 0.7, mean ± Std.Dev, n = 5) (Fig. 4.10D). The cardiac reversal was delayed with respect to the onset of the stimulus (Fig. 10 A). During the retrograde beat, whether it was initiated pharmacologically or evoked by nerve stimulation, the mini-systole/mini-diastole cycles of the terminal chamber always had a longer duration with respect to the contractions occurring in more anterior cardiac chambers, including the CC (Figs. 4.8A-B and 4.10B-C).

4.4 DISCUSSION

The adult *Drosophila* heart is innervated extensively by glutamate-IR neurons (Dulcis and Levine, 2003). A large glutamate-IR synaptic structure is formed during metamorphosis in the first cardiac chamber (the conical chamber), which has been suggested as the location of the retrograde pacemaker (Rizki, 1978; Dulcis and Levine, 2003). Pre- and post-synaptic specializations, including extensive synaptotagmin-Ir and clusters of GluRIIA-IR receptors, were present along the glutamatergic terminals. In addition, abundant NC82-Ir, which is a marker that co-localizes with DPAK at the level of active zones (Sone et al., 2000; Wan et al., 2000), revealed a number of putative release sites both in the transverse bridge and bouton-like terminals.

Local glutamate application in the conical chamber evoked a long-lasting depolarization of the membrane potential, which initiated pacemaker action potentials in normal saline. Both ionotropic (GluRs) and metabotropic (mGluRs) glutamate receptors have been described in the *Drosophila* CNS and at the NMJ (Shuster et al., 1991;
Parmentier et al., 1996; Petersen et al., 1997; DiAntonio et al., 1999; Ramaekers et al., 2001; Marrus et al., 2004). Although ionotropic glutamate receptors were localized at the cardiac synapses, the glutamate-evoked depolarization observed in myocardial cells might also be due in part to activation of mGluRs, which may cause an increase of postsynaptic excitability by, for example, blocking resting K\(^+\) currents or reducing voltage-gated and Ca\(^{2+}\)-activated K\(^+\) currents (Schrader and Tasker, 1997).

Ultrastructural, immunocytochemical and further electrophysiological analyses of these cardiac synapses must be undertaken to understand the mechanism of cardiac pacemaker cell activation in adult *Drosophila*.

To determine the influence of cardiac innervation on heart function, the first necessary step has been to produce a detailed description of the regular cardiac activity. The cardiac cycle of resting adult flies is composed of two alternating phases, the anterograde and retrograde beats, displaying different contraction rates. This phenomenon, known as cardiac reversal in other open circulatory systems, is associated with a change in the direction of blood circulation (Jones, 1977). Because cardiac contraction originates periodically at the two ends of the heart, two putative pacemakers must be alternately active in adult *Drosophila*. The caudal chamber, where the anterograde contractions originate, has been suggested as the location of the anterograde pacemaker (Rizki, 1978; Dowse et al., 1995; Johnson et al., 2002). By contrast, the retrograde pacemaker may reside in the conical chamber (Rizki, 1978; Dulcis and Levine, 2003). In addition to a constant beat, consisting of high-frequency cardiac contractions (mini-systole/mini-diastole cycles), the conical chamber also displays a superimposed
low frequency systole/diastole cycle that is characterized by a slow change in its
diameter, correlated with anterograde and retrograde beats, respectively. Unlike closed
circulatory systems in which each cardiac ventricular contraction/relaxation cycle
corresponds to a systole/diastole cycle, in open circulatory systems many anterograde
mini-systole/mini-diastole cycles must occur to complete a systolic phase. Similarly, it
takes several retrograde mini-systole/mini-diastole cycles before diastole is complete.
This ensures that in multi-chambered hearts blood moves backward (or forward) during
diastole (or systole) in order to achieve a complete filling (or emptying) of all four
cardiac chambers.

Larval cardiac activity is characterized by a constant anterograde beat that
originates in a pacemaker putatively located in the caudal chamber (Rizki, 1978; Dowse
et al., 1995; Johnson et al., 2002). During metamorphosis the adult conical chamber
forms between the existing abdominal heart and the thoracic aorta of the larva (Curtis et
al., 1999). Extensive glutamatergic innervation develops (Dulcis and Levine, 2003), and
cyclic cardiac reversal begins. The formation of a new retrograde cardiac pacemaker in
the conical chamber is not by itself sufficient to explain how the two adult cardiac
pacemakers are alternately active. Our hypothesis is that both intrinsic excitable
properties of the myocardium, and neuronal inputs participate in producing selective
activation/inhibition of the two pacemakers.

Both bath-application of exogenous glutamate and transverse nerve stimulation
had a chronotropic effect, involving an increase of the mini-systole/mini-diastole cycle
rate of conical chamber activity. The glutamate-evoked cardiac contractions originating
in the conical chamber were retrograde in direction and their rate correlated with the glutamate-evoked pacemaker potentials that were recorded intracellularly. Thus, cardiac reversal to the retrograde beat could be evoked in spontaneously anterograde-beating hearts. Similarly, retrograde contractions were initiated in the conical chamber by glutamate application to hearts that had been pre-incubated with CCAP, which by itself potentiated the anterograde beat.

One mechanism that is consistent with these results is that the muscle cells of the conical chamber may have faster intrinsic excitability and/or contractile properties (shorter cycle period) than the more posterior myocardial cells. The mini-systole/mini-diastole cycle was always shorter in the conical chamber with respect to more posterior chambers. This feature would allow the putative retrograde pacemaker in the conical chamber to impose its faster pace on the anterograde pacemaker of the caudal chamber. This mechanism alone, however, is not sufficient to explain cardiac reversal in the intact organism. Whereas bath-application of glutamate or TN stimulation evoked a retrograde beat that was always faster than the ongoing anterograde beat in semi-intact preparations, the retrograde beat that was recorded from intact animals always displayed a slower rate than the anterograde beat. This is analogous to what has been described in other holometabolous insects that show reversal (Dulcis et al. 2001; Dulcis and Levine, 2004). It is possible that in intact animals, where neuronal activity is functional and physiological conditions are preserved, the reciprocal alternation of pacemaker dominance is maintained by simultaneous inactivation of the anterograde pacemaker before or during activation of the retrograde pacemaker. In Manduca sexta, for example,
the motoneuron that serves the caudal chamber (MN-1; Davis et al., 2001) receives inhibitory synaptic input that stops its activation of the anterograde pacemaker and allows the slow-rate retrograde beat to begin (Dulcis and Levine, 2004). Innervation of the caudal chamber also develops during metamorphosis in Drosophila (Dulcis and Levine, 2003). The activity of these CCAP-IR neurons (BpNs) potentiates the anterograde beat (D. Dulcis, R. Levine and J. Ewer, unpublished observations) and might be inhibited by neuronal input during the retrograde beat of intact animals. As in Manduca, larval myogenic hearts do not need innervation to produce the anterograde beat, but once the reversal is established, and a new retrograde pacemaker develops, the alternation of the two adult pacemakers seems to require innervation to stop and/or reactivate the anterograde beat (Dulcis and Levine, 2004). This might represent a general mechanism in Manduca, Drosophila and other holometabolous insects. In Manduca the retrograde beat probably originates in the anterior aorta (Dulcis et al., 2001), which is heavily invested with CCAP- and FMRFamide-IR terminals (Davis et al., 2001). In Drosophila, by contrast, glutamatergic innervation of the conical chamber controls this function.

Another factor is that the adult heart is composed of two separate muscle layers, a circular layer which is present in the larval stage, and a ventral longitudinal layer which develops in the adult (Curtis et al, 1999; Molina et al., 2001). Electrical coupling between the two layers and their respective contractions could co-participate in producing the stereotyped cardiac pattern of activity. In addition, glutamatergic innervation and glutamate receptors were found only in the ventral longitudinal muscle layer. It is not known how glutamate-activated contraction of this layer influences the underlying
circular layer. This ventral muscle layer is well developed in the conical chamber but it is absent in the terminal chamber where the anterograde beat originates. The anterograde and the retrograde beats may travel along the two cardiac muscle layers independently if the two layers are not electrically coupled.

In summary, the retrograde glutamate-activated pacemaker could have faster intrinsic contraction properties that allow it to override ongoing, spontaneous or CCAP-evoked anterograde contractions in semi-intact preparations. However, this model would not be sufficient to explain cardiac function in vivo where the retrograde beat displays a slower rate than the anterograde beat. The regular cardiac cycle, as observed in intact preparations, may in fact result from a number of contributing factors.

Cardiac function in adult *Drosophila* needs to accommodate a variety of physiological conditions (for example, post-feeding versus dehydrated states) and behaviors, such as flight, locomotion, and ovoposition, which require specific variations of hemolymph circulation. Cardiac synapses may, therefore, undergo short-term and long-term synaptic plasticity that ultimately affects the activation of retrograde pacemaker cells. This system provides a unique model in which the effects of genetic manipulation on glutamatergic synaptic transmission can be analyzed not only at the molecular and cellular level, as with the skeletal muscle synapse, but also at the systems level.
Figure 4.1: Putative release sites at cardiac synapses. See next page for details.
Figure 4.1: Putative release sites at cardiac synapses. Laser-scanning confocal microscope images of the cardiac conical chamber (A-B, G-I) and skeletal muscles (C-F) in the abdomen of adult ELAV-GAL4/UAS-GFP transgenic flies. 

A, Ventral view of the conical chamber showing the GFP-labeled cardiac innervation (in yellow). Right and left abdominal transverse nerves (TN_{IR}, TN_{IL}) fasciculate bilaterally to form the transverse bridge (arrow) on the conical chamber. Longitudinal processes originating from it are visible (arrowheads).

B, GFP-expression pattern (orange/yellow) showing in detail the transverse bridge (TB), longitudinal processes, and their bouton-like terminals on the myocardium (arrowheads).

C, DPAK immunostaining of NMJs in adult skeletal muscles. D, NC82 immunostaining of same preparation shown in C. E, Merging of images shown in C and D. NC82 (pink) and DPAK (blue) immunoreactivities (-IR) co-localize at the level of adult NMJ. F, The same terminals shown in the box in E at higher magnification. NC82-IR and DPAK-IR show similar pattern of localization but are not completely overlapping. NC82-Ir putative active zones (arrowhead) are clearly visible in each bouton.

G, Double labeling of GFP (blue) and NC82-IR (pink) showing localization of putative release sites in the transverse bridge (arrow) and longitudinal processes of the conical chamber. The first pair of abdominal transverse nerves (TN) is also visible. H, Different confocal stack of the same preparation shown in G at higher magnification. I, The same bouton-like terminal shown in the box in H. Individual putative NC82-Ir active zone can be distinguished (arrowhead). Scale bars = 50 μm in A, 15 μm in B, G-H, 5 μm in C-E, 2 μm in F, I.
Figure 4.2: Localization of postsynaptic GluRIIA receptors. See next page for details.
**Figure 4.2: Localization of postsynaptic GluRIIA receptors.** Confocal micrographs showing the localization of postsynaptic GluRIIA at cardiac synapses. **A,** ventral view of the conical chamber longitudinal muscles showing GluRIIA-Ir clusters (arrowheads, green) localized along the transverse bridge (TB). The TB was visualized by synaptotagmin-IR (red). **B,** Synaptotagmin-Ir (red) innervation of the conical chamber. **C,** same preparation of the ventral layer of the conical chamber shown in B showing GluRIIA-IR (yellow). **D,** Merging of the same cardiac ventral layer preparations shown in boxes in B-C. Clusters of DGlurIIA-Ir receptors (yellow) along the synaptotagmin-Ir longitudinal processes extending posterior to the TB (red) are visible (arrowheads). **E,** ventral view of the third cardiac chamber. GFP-IR transverse nerves (TN3) and cardiac terminals are shown in red. GluRIIA-Ir clusters (green) are visible along both main neuronal branches and cardiac terminals (arrowheads). Regions of co-localization are visualized in yellow. Pericardial cells (PC) surrounding the chamber are also visible. The arrows indicate abdominal skeletal muscles. Scale bars = 15 μm in B-C, E, 10 μm in A, D.
Figure 4.3: Intracellular recordings from the myocardium of adult flies. See next page for details.
Figure 4.3: Intracellular recordings from the myocardium of adult flies. A, Epifluorescence micrograph of a representative preparation of the conical chamber showing the location of the intracellular electrode in the ventral longitudinal muscle layer served by the transverse bridge (TB, arrow). Scale bar = 50 μm. B, depolarization evoked by local application of 10^{-4}M glutamate and recorded in Ca-free saline. C, Glutamate-evoked depolarization and following cardiac potentials recorded in HL-3 saline. D, Portion of the recording shown in B at expanded time and voltage scales showing intracellular MEJPs in detail. E, Same recording shown in the box in C at expanded time scale. The slow depolarizing phase of cardiac action potentials is indicated (arrowheads). F, Difference between cardiac potentials occurring during (dotted line) and after (solid line) glutamate-evoked depolarization. Each trace represents a signal average of 10 cardiac potentials taken from a representative recording. Arrows and arrowheads indicate the rising phase and pick of the cardiac action potentials, respectively.
Figure 4.4: Optical detection of heart function in intact adult flies. A, Cardiac activity recorded from the conical chamber (CC) showing alternation of phases during which the heart beats at different rates, and superimposed diastolic/systolic cycles. Representative diastolic (filling) and systolic (emptying) movements of the CC are indicated by arrows. B, Schematic drawing of the anterior portion of the abdominal heart showing the regions of the CC (Conical ch., red dot) and second cardiac chamber (II cardiac ch., black dot) that were selected for simultaneous optical detection. Cardiac innervation and transverse bridge (TB) are also visible. C, Simultaneous recording of heart activity from the CC (red trace) and the second cardiac chamber (black trace). Spontaneous cardiac reversals are indicated (arrows). D, Superimposed traces at expanded time scale of the same recording shown in the box in C showing the cardiac reversal transition in detail (bracket at top). The direction of the heartbeat (anterograde versus retrograde) is determined by the relative delay between the CC (red trace) and the second cardiac chamber (black trace) contractions. The dotted lines mark representative CC movements.
Figure 4.5: Volume changes associated with diastole/systole cycle. A, Negative image of an epifluorescence micrograph of the conical chamber and drawing of a solid representing its volume. Cardiac innervation and transverse bridge (TB, arrow) are also visible (black). Scale bar = 50 μm. B, cartoon schematizing the two states of the conical chamber, whose volume was approximated to a cylinder ($V = \pi r^2 h$), during diastolic and systolic phases. The radius ($r$) was measured at mid-height (dotted line) approximately at the level of the TB. The arrows indicate the direction of hemolymph flow. C, Cyclic diastolic and systolic movements of the conical chamber detected optically. The Y axis was calibrated to volume (nl) changes.
Figure 4.6: Correlation of diastole/systole cycle with cardiac reversal. A, Simultaneous optical detection of heartbeat (upper trace) and cardiac volume changes (lower trace) recorded from the conical chamber. Anterograde and retrograde beats are easily distinguished by their different heart rates. B, superimposed traces at expanded time scale of the same recording shown in the box in A. Intersection of the cardiac volume trace with the heartbeat trace are indicated (arrowheads) to show the correlation of a rapid increase/decrease of the conical chamber volume with retrograde/anterograde beat, respectively. Maximum diastolic and systolic volumes are also indicated (arrows).
Figure 4.7: Effect of bath-applied glutamate on cardiac function. Simultaneous optical detection of heartbeat and cardiac volume recorded from the conical chamber. The arrows indicate the times of two bath-applications of glutamate, each reaching a final concentration in the bath of 30 μM.
Figure 4.8: Glutamate initiation of the retrograde beat in vitro. A, Simultaneous optical detection of the terminal (black trace) and third (red trace) cardiac chamber activity showing the effect on heartbeat following 30 μM Glutamate bath-application (arrow). B, Superimposed traces at expanded time scale of the same recording shown in the box in A showing the direction of glutamate-evoked heartbeat. Individual cardiac contractions and relaxation are labeled as mini-systole and mini-diastole cycles. The dotted lines indicate the end of the mini-diastole occurring in the third chamber (red trace) and project to the delayed mini-diastole of the terminal chamber (black trace). C, Simultaneous optical detection of the terminal (black trace) and third (red trace) cardiac chamber showing directionality of the beat, anterograde versus retrograde, before and after 30 μM Glutamate bath-application (arrow). D, Superimposed traces at expanded time scale of the same recording shown in the box in C. The dotted lines indicate synchronous end of the mini-diastole occurring in the third (red trace) and terminal chamber (black trace) during the spontaneous anterograde beat.
Figure 4.9: Pharmacologically-evoked cardiac reversal. A, Simultaneous optical detection of the conical (red trace), second (blue trace), and third (green trace) cardiac chamber activity showing the effect on heartbeat of $10^{-4}$M CCAP and $10^{-5}$M Glutamate bath-applications. The time of application is indicated (arrows). B, Superimposition of the same traces shown in A at expanded time scale. C-E, Same cardiac contractions shown in the boxes in B at expanded time scale showing heartbeat direction in the absence of CCAP and Glutamate (C), in the presence of CCAP only (D), and in the presence of both CCAP and Glutamate (E).
Figure 4.10: Effect of transverse nerve stimulation on heartbeat. A, Epifluorescence micrograph of a representative preparation of the conical chamber showing the location of the suction electrode on the transverse nerve (TN₁) terminating in the ventral longitudinal muscle layer with the transverse bridge (TB, arrow). Scale bar = 50 μm. B, Simultaneous optical detection of the conical (red trace) and terminal (black trace) cardiac chamber activity showing the effect of TN stimulation (5s, 20Hz, 1ms/pulse) on heartbeat. C-D, Superimposition of the same cardiac contractions shown in the boxes in B at expanded time scale showing heartbeat direction before (C) and during (D) TN electrical stimulation.
CHAPTER 5. ROLE OF NEUROPEPTIDE CCAP IN DROSOPHILA CARDIAC FUNCTION: INSIGHTS USING TARGETED ABLATION OF CCAP NEURONS

5.1 INTRODUCTION

Neuropeptides act as neurotransmitters, modulators, and hormones controlling important physiological functions and behaviors (Strand, 1999). The crustacean cardioactive peptide (CCAP) was first isolated in crustaceans (Stangier et al., 1987) but is involved in circulatory function, ecdysis behavior, and visceral organ activity in a variety of insect species and other invertebrates (Review: Dircksen, 1998). Here we investigate the role of CCAP in cardiac function in the adult Drosophila melanogaster. This widely used model organism allows CCAP function to be analyzed through genetic manipulations.

The peptide CCAP exerts its effects both on peripheral tissues and within the central nervous system. Its receptor has recently been cloned and characterized as member of the G protein-coupled receptor family (Park et al., 2002; Cazzamali et al., 2003). CCAP acts peripherally as a cardioacceleratory peptide in the moth Manduca sexta (Tublitz and Evans, 1986; Dulcis et al., 2001). In adults, its excitatory effect on the heart controls blood circulation during wing inflation (Tublitz and Truman, 1985) and flight (Tublitz, 1989). In addition, CCAP acts centrally to trigger ecdysis behavior at the larval-pupal molt in Manduca (Gammie and Truman, 1997) and is also released at the time of ecdysis in the crab, Carcinus maenas (Philippen et al., 2000).

Targeted ablation of CCAP-containing neurons demonstrated that CCAP is important for the normal execution and circadian timing of ecdysis behavior in
Drosophila (Park et al., 2003). CCAP KO animals that survived through metamorphosis also showed defects at adult eclosion which may result from compromised cardiac function. A cardioacceleratory effect of this peptide has been described for larval, pupal and adult Drosophila hearts (Nichols et al., 1999), although the authors found striking differences between the effect of this peptide on whole organism vs. that on isolated preparations.

The lack of a detailed neuroanatomical study describing cardiac innervation in adult Drosophila and an incomplete description of adult cardiac physiology, have led to the assumption that CCAP acts as a neurohormone to affect heart rate (Johnson et al., 1997). More recently, however, it has been found that the Drosophila adult heart is extensively innervated (Dulcis and Levine, 2003). Adult cardiac innervation develops during metamorphosis and is derived from at least two distinct sources. Glutamatergic innervation from the transverse nerves serves each abdominal segment bilaterally. In addition, peripheral bipolar CCAP-immunoreactive neurons (BpNs) terminate segmentally in each cardiac chamber (Dulcis and Levine, 2003).

The cardiac cycle of resting adult flies is composed of two alternating phases, the anterograde and the retrograde beats, which correlate with forward and backward hemolymph flow (Dulcis and Levine, 2004 b). This phenomenon, which is referred to as cardiac reversal, has been described in other holometabolous insects (Tenney, 1953; Queinnec and Campan, 1972; Wasserthal, 1976; Ichikawa and Ito, 1999; Smits et al., 2000, Dulcis et al., 2001). Glutamatergic innervation drives the retrograde beat in adult Drosophila (Dulcis and Levine, 2004 b). The goal of the present study was to determine
the role of cardiac innervation by CCAP expressing neurons.

Previous studies of CCAP and its role in insect cardiac function were performed by delivering exogenous neuropeptide (Tublitz and Evans, 1986; Nichols et al, 1999). When performed on isolated hearts *in vitro*, these studies do not take into account the contribution of cardiac innervation or blood pressure feedback loops that may occur in intact animals. Similarly, injections of exogenous peptide into intact preparations do not mimic the spatial and temporal conditions under which CCAP is released at specific cardiac chambers by the activity of peripheral BpNs terminals. In the present study we investigated the role of CCAP in cardiac activity using two different approaches, RNA interference and targeted cell ablation, to disable CCAP expression. We found that lack of CCAP innervation in intact preparations alters one of the cardiac phases, the anterograde beat, without affecting the cyclic cardiac reversal in adult *Drosophila*.

5.2 MATERIALS AND METHODS

(1) *Drosophila melanogaster strains and cultures*. Flies were either raised on medium consisting of instant food, agar, and oatmeal (Condie and Brower, 1989) supplemented with yeast or on standard agar/cornmeal/sugar media under a 12h:12h light:dark. All stocks were maintained at 25°C under uncrowded conditions. Wild type (Oregon-R) and *Df(3)23D1/TM3, Sb* flies, and actin-GAL4, and *elav-GAL4/UAS-green fluorescent protein* (GFP) transgenic flies were obtained from the Bloomington *Drosophila* stock center (University of Bloomington, Bloomington, Indiana).

UAS-CCAP RNAi transgenic flies were made by cloning a 666bp fragment of
CCAP cDNA (Park et al., 2003) (in normal 5’ to 3’ orientation) followed by an inverted, 3’-truncated, 574 bp fragment of CCAP cDNA into the pUAST P-element transformation vector (van Roessel and Brand, 2000). Thus, the resulting hairpin structure consisted of a 574 bp inverted repeat and a 92 bp spacer, the latter corresponding to the unpaired 3’ stretch of CCAP cDNA. Each fragment was made by PCR using primers containing appropriate restriction sites and a complete CCAP cDNA clone as template (Park et al., 2003). Each fragment was first cloned into the pGEM-T easy vector (Promega) and its sequence verified. Each fragment was then transferred sequentially into pUAST using Sure-2 competent cells (Stratagene). The resulting construct was verified by restriction digests and sequencing, and used for germline transformation using standard procedures. Two independent transgenic lines were obtained, with an insert on chromosome II and chromosome III, and are referred to here as UAS-CCAP RNAi (II) and UAS-CCAP RNAi (III), respectively. Flies homozygous for either and both inserts were made using standard techniques.

CCAP-GAL4 driver lines, bearing inserts on chromosome II and/or III, were used to drive gene expression in CCAP neurons (Park et al., 2003). CCAP-GAL4 flies were crossed to a UAS-GFP line to drive cytoplasmic expression of GFP in CCAP neurons. Targeted ablation of CCAP neurons was accomplished by driving expression of the apoptotic genes reaper (rpr) and head involution defective (hid) in the CCAP neurons by crossing flies bearing the CCAP-GAL4 (II) driver to either UAS-rpr or UAS-hid transgenic flies (Zhou et al, 1997). Flies expressing CCAP RNAi were obtained by crossing transgenic flies bearing CCAP-GAL4 and UAS-CCAP RNAi transgenes. Flies
bearing 1 or 2 copies of the CCAP-GAL4 driver and/or the UAS-CCAP RNAi transgene were produced.

(2) Optical detection of in vivo heartbeat. Recordings were made from 2-day old adults, as younger flies have too much fat tissue, thus making optical detection through the cuticle difficult. The animals were anesthetized on ice for about 10 minutes then restrained dorsal side up by placing a bent pin around the neck of the adult fly. Legs and wings were removed to keep the specimen immobile during optical measurement. The cardiac chamber of interest was observed under the microscope with a 10x objective focused to distinguish heart movements either through the dorsal cuticle, in intact preparations, or directly, for semi-intact preparations. Bright field was used for detecting heartbeat of wild type and CCAP transgenic flies; fluorescent light (fluorescein band pass filter) was utilized for elav-GAL4/UAS-GFP transgenic flies. Simple PCI software (Compix In., Cranberry Township, PA) was adapted to measure movement of the heart during cardiac contractions (Dulcis and Levine, 2004 b). Images were collected at a maximum speed of 19 frames/second. This is faster than the adult Drosophila heart rate, and ensures detection of all cardiac contractions. Following video acquisition, images were contrast-enhanced to reduce the blurring effect of the cuticle.

(3) CCAP bath application. The neuropeptide CCAP (Backem, Torrance, CA) was bath-applied to semi-intact preparations while cardiac activity was detected optically as described above. CCAP was applied with a micropipette to reach a final a concentration
in the bath of $10^{-4}$M. The preparation was continuously perfused with normal saline (Stewart et al., 1994) to wash out the compound and to keep the heart well oxygenated.

(4) Immunocytochemistry. Whole-mounted preparations of the abdomen of the adult progeny of a CCAP-GAL4xUAS-GFP cross were studied using immunofluorescence, as described in Davis (1993). Briefly, dissected tissues were fixed in 4% paraformaldehyde at 4° C overnight and washed 6x 1h in PBS + 0.5% TritonX-100 (0.5% PBST), followed by a final wash in 0.5% PBST at 4° C overnight. Tissues then were treated for about 24 hours at room temperature with rabbit anti-CCAP antiserum (generously provided by Dr. H. Agricola) diluted 1:1,000 in blocking solution made of 10% normal goat serum (NGS, Sigma) in 1% PBST. After 4 x 1h washes in 1% PBST tissues were blocked for 1 hour and incubated overnight at room temperature in a 1:400 dilution of a Cy5-conjugated donkey anti-rabbit IgG secondary antibody Jackson ImmunoResearch Laboratories, West Grove, PA). The preparations were then rinsed in 1% PBST and PBS and cleared and stored in 60% - 80% glycerol. Because the GFP-fluorescence expressed by the CCAP-immunoreactive neurons was reduced by prolonged fixation, the monoclonal mouse antibody anti-GFP (Molecular Probes, Eugene, OR) was used to double label with GFP. The GFP antiserum was added in a 1:100 dilution together with the other primary antibody and visualized with Cy2-conjugated donkey anti-mouse IgG (Jackson ImmunoResearch Laboratories).

The monoclonal mouse antibody 22C10, developed by Zipursky et al. (1984), and obtained from the Developmental Studies Hybridoma bank (University of Iowa), was
used to visualize cardiac innervation in CCAP KO animals. The 22C10 antiserum was used at a 1:100 dilution together with anti-CCAP and visualized with Cy5-conjugated donkey anti-mouse IgG.

Third instar larval CNSs were stained for CCAP immunoreactivity as described in (Park, 2003).

(5) Quantification of immunolabeling. Tissues to be quantitated were all processed and stained in parallel and under the same conditions. To quantify fluorescence in peripheral CCAP-immunoreactive BpNs, Z-series of confocal sections were collected at nonsaturated settings, then collapsed keeping the maximum intensity pixels. Images were then analyzed using Simple PCI software. Intensity of the CCAP immunoreactivity was measured in the BpN somata of 8 specimens for each transgenic line. Fluorescent CCAP immunostaining in the CNS was quantified as described in Park et al. (2003).

(6) Laser-scanning confocal microscopy. Digital images of immunostained cardiac preparations were collected on a Nikon PCM 2000 laser-scanning confocal microscope equipped with green He/Ne (543 nm), red He/Ne (633 nm) and argon (488 nm) lasers. Cy2 and Cy5 were detected respectively with argon and red He/Ne laser lines and using a band-pass filters at 510 nm (Cy2) and 650 nm (Cy5). Stacks of digitized images were merged by using Simple PCI as image acquisition software. Corel Draw and Corel Photopaint (Corel Corp., Ottawa, Ontario, Canada) were the software used to enhance contrast and provide color when needed. Prints were made by using a Tektronix Xerox
Phaser 6200 printer. Digital images of CCAP-immunoreactivity in the CNS were collected using a Leica DMR confocal microscope system.

(7) In situ hybridization. RNA in situ hybridization was carried out as described in (Park et al., 2003) using antisense and sense RNA probes derived from in vitro transcription of a cDNA clone.

5.3 RESULTS

The regular cardiac cycle of resting adult flies is composed of two phases in cyclic alternation, which are easily distinguishable based on their amplitude and duration (Fig. 5.1). Simultaneous optical detection of multiple cardiac chambers revealed the origin and propagation of contractions along the multi-chambered abdominal heart during the two phases (Dulcis and Levine, 2004 b). The long lasting cardiac phase (Fig. 5.1) represents the anterograde beat, during which contractions originating in the terminal chamber travel forward to the more anterior chambers (Dulcis and Levine, 2004 b). Conversely, the short-lasting phase (Fig. 5.1), which is referred to as the retrograde beat, originates in the conical chamber and travels backward towards the terminal chamber (Dulcis and Levine, 2004 b). This phenomenon, previously described as cardiac reversal in other holometabolous insects, correlates with a change in the direction of hemolymph flow within the dorsal vessel. Furthermore, the anterograde and retrograde beats correlate respectively with systole and diastole of this multi-chambered heart (Dulcis and Levine, 2004 b).
Bath-application of $10^{-4}$ M CCAP had a chronotropic effect on the cardiac activity of adult wild type and elav-GAL4/UAS-GFP transgenic flies (Fig. 5.2). When the heart was not beating at the moment of peptide application, as often occurred while GFP-expressing transgenic flies were briefly illuminated with fluorescent light, cardiac contractions always began within 40 seconds after CCAP application. This is in agreement with previous studies where, depending on the peptide concentration in the bath and on the developmental stage, the effect of CCAP took up to a minute to occur (Nichols et al., 1999). The CCAP-evoked cardiac contractions showed a gradual amplitude increase within the first 10 seconds until a maximum value was reached (Fig. 5.2, upward arrow). The CCAP effect washed out following constant perfusion with fresh oxygenated saline. The direction of CCAP-evoked cardiac contractions was always anterograde (data not shown), confirming that CCAP may potentiate the anterograde phase of the adult cardiac cycle in *Drosophila* (Dulcis and Levine, 2004 b).

Two approaches were used to investigate the role of CCAP in *Drosophila* cardiac function. First, we reduced CCAP expression by driving the expression of double stranded CCAP RNA (CCAP RNA interference, CCAP RNAi) in the CCAP neurons. Second, we caused the targeted ablation of CCAP neurons by expressing the apoptosis genes rpr and hid in these neurons.

RNAi has effectively been used to reduce the expression of numerous genes in a wide variety of organisms (review: Hannon, 2002), including *Drosophila* (e.g., Kim et al., 2004; Chapman et al., 2003; Kalidas and Smith, 2002; Fortier and Belote, 2000; Lam and Thummel, 2000). In order to test the ability of double stranded CCAP RNA to
reduce CCAP expression, we drove UAS-CCAP RNAi in the CCAP neurons using a CCAP-GAL4 driver (Park et al., 2003). Fig. 5.3B shows that expression of CCAP RNA was greatly diminished in the CCAP neurons of third instar larvae bearing 2 copies of the CCAP-GAL4 driver and 2 copies of the UAS CCAP RNAi transgene, as compared to the levels observed in the CNS of control larvae (bearing only 2 copies of the UAS CCAP RNAi transgene, Fig. 5.3A). Use of sense CCAP probes revealed that high levels of antisense CCAP RNA were present in RNAi expressing larvae (Fig. 5.3C), consistent with the hypothesis that the reduced levels of CCAP RNA found in these animals was due to interference of CCAP expression.

Although CCAP RNAi caused significant reduction in the levels of CCAP RNA (Fig. 5.3B), CCAP-IR was still clearly detectable in CCAP axons when either one (Fig. 5.3G) or two (Fig. 5.3H) copies of the UAS-CCAP RNAi transgene were driven using 2 copies of the CCAP-GAL4 driver. A quantitative analysis of CCAP-IR in the medial and lateral CCAP axons (cf. Fig. 5.3E) showed that this decrease was, nevertheless, highly statistically significant (Fig. 5.3I). It should be noted, however, that the levels of CCAP-IR in the lateral axons of CCAP RNAi-expressing larvae were similar to those of larvae hemizygous for CCAP (Fig. 5.3F), a condition that rarely leads to the expression of a mutant phenotype.

In order to determine the functional consequences of RNA interference of CCAP expression we examined the success of pupal ecdysis in CCAP RNAi-expressing animals. Targeted ablation of CCAP neurons has shown that CCAP neurons are essential for pupal ecdysis (Park et al., 2003), suggesting that the CCAP neuropeptide may be
critical for turning on ecdysis behavior at this stage. At pupal ecdysis the head is everted and the legs and wings are expanded to attain their final adult shape and length, Thus, the success of pupation can be determined indirectly by measuring the length of these appendages (Park et al., 2003). A quantitative analysis of the length of the prothoracic pair of legs and that of the wings of pharate adult animals revealed no statistically significant differences between their length in controls (progeny of w x UAS-RNAi [2 copies]) and in animals bearing one or two copies of the CCAP-GAL driver and one or 2 copies of the UAS-CCAP RNAi transgene (N=10 female flies per group [1 leg and 1 wing measured per animal]; p= 0.75 [wings] and p= 0.48 [legs] one-way ANOVA). Thus, CCAP RNAi, while sufficient to cause a measurable decrease in the levels of CCAP expression (Figs. 5.3B, G-I) was not effective in reducing CCAP function at pupal ecdysis. A similar result was obtained for cardiac function (see below).

We used the CCAP-GAL4 driver to cause CCAP RNAi expression in the peripheral neurons (BpNs) that segmentally innervate the heart (see Dulcis and Levine, 2003). In order to establish that this GAL4 transgene accurately reproduced the expression of the CCAP gene in the peripheral nervous system (PNS), it was first used to drive expression of GFP. The distribution of GFP was then compared to that of CCAP-IR. As shown in Fig. 5.4A the CCAP-GAL4 transgene caused the expression of GFP in BpNs that fasciculate with the abdominal transverse nerves (TNs) to serve the alary muscles and cardiac chambers bilaterally. By comparing GFP-immunoreactivity (GFP-IR) to CCAP-IR of abdominal segments we confirmed that the transgene expression pattern faithfully reproduced the number and location of CCAP-IR BpNs in adult
Drosophila (Fig. 5.4B-G).

To produce flies with reduced CCAP expression in the peripheral BpNs neurons, CCAP-GAL4 flies were crosses to flies carrying a UAS-CCAP RNAi transgene. In addition to CCAP-GAL4, an actin-GAL4 driver was also used.

To determine whether the RNAi method was effective in reducing CCAP expression in the peripheral CCAP-IR neurons, CCAP-IR was quantified in BpN6 somata of CCAP-GAL4 x UAS-CCAP RNAi and actin-GAL4 x UAS-CCAP RNAi transgenic flies and compared to the intensity measurement obtained for the controls (progeny of Wt x UAS-CCAP RNAi). The actin-GAL4 transgene, which drives CCAP RNAi expression ubiquitously, produced a decreased level of CCAP-IR in BpN6 that was significantly different (p<0.0001) from controls (Fig. 5.5). In contrast, the RNAi driven by CCAP-GAL4 transgene in CCAP neurons produced only a slight reduction of CCAP-IR, which was not significantly different from that of controls (Fig. 5.5).

The cardiac activity of intact adult CCAP RNAi transgenic flies, induced by both CCAP and actin promoters, was analyzed to identify abnormal cardiac phenotypes that might result from reduced levels of this cardioactive peptide. Cardiac reversal occurred regularly in adult CCAP RNAi transgenic flies. Other cardiac parameters, such as heart rate and duration of the anterograde and retrograde beats (Table 1), were also not significantly different from those of controls (P > 0.05 by Student’s t-test for unpaired data).

To produce flies lacking BpNs (CCAP KO animals), the expression of the apoptotic gene reaper (rpr) (White et al., 1994, 1996) was driven in the CCAP neurons.
by crossing UAS-rpr with the CCAP-GAL4 transgenic strain. As previously described (Park et al., 2003) CCAP is important for pupal ecdysis as well as adult eclosion and only 5% of adult CCAP KO flies emerged from their pupal cases. Recordings of the cardiac activity from 7 emerged CCAP KO adults were performed to investigate the consequences of loss of CCAP neurons on cardiac function. The cardiac cycle was profoundly altered in CCAP KO animals (Fig. 5.6). Although the anterograde and retrograde phases still occurred in alternation (Fig. 5.6, upper trace), the heart contractions were arrhythmic, the heart rate of both phases was decreased, and the anterograde phase had an abnormal pattern. Specifically, long-lasting retrograde contractions (Fig. 5.6, arrows), originating in the conical chamber of the first abdominal segment, often occurred during the anterograde phase (Fig. 5.6, second trace).

To confirm that the cardiac phenotype observed in CCAP KO animals was only due to the targeted ablation of CCAP neurons, a neuroanatomical investigation was performed to investigate whether the remainder of the cardiac innervation, as well as the heart itself, developed normally. The abdominal heart of emerged adult CCAP KO flies was fully developed. Each cardiac chamber was innervated normally by segmental TNs (Fig. 5.7A-B). The characteristic transverse bridge (TB, Fig. 5.7C), which carries the glutamatergic innervation, terminated bilaterally in the conical chamber and innervation of the more posterior chambers (Fig. 5.7D) was also as previously described in wild type animals (Dulcis and Levine, 2003). No CCAP-IR terminals were found on the myocardium of adult CCAP KO flies (Fig. 5.7E). Surprisingly, however, the cluster of peripheral neurons serving the terminal cardiac chamber (BpNs6) was present (Fig.
5.7B, arrow) and CCAP-immunoreactive (Fig. 5.8A, arrowheads) suggesting that these cells were resistant to the apoptotic effect of the rpr gene. Despite their survival, CCAP and 22C10 double-labeling showed that these BpNs6 did not send any CCAP-immunoreactive process into the terminal cardiac chamber (Fig. 5.8B, arrowheads) suggesting that their function may be compromised. The un-emerged CCAP KO animals, which represent fully developed pharate adults that were unable to emerge from the pupal case, also had the BpNs6 cluster (Fig. 5.8D, arrow) and their somata and processes were CCAP-IR (Fig. 5.8E-G).

To determine whether the ablation of the peripheral BpNs6 cluster failed because of its resistance to rpr-induced apoptosis, we drove, in CCAP neurons, the expression of hid, a gene that in some systems has been reported to have stronger apoptotic capabilities (e.g., McNabb et al., 1997). None of the CCAP-GAL4xUAS-hid transgenic flies were able to emerge from the pupal case, suggesting a more potent effect of hid expression on ecdysis behavior. Importantly, immunocytochemical analysis of adult pharate CCAP-GAL4xUAS-hid flies showed that the entire class of peripheral BpNs, including the caudal cluster BpNs6, was ablated (Fig. 5.8C). Unlike in wild type animals, the TNs of hid-induced CCAP KO flies were CCAP-negative (Fig. 5.8C, arrow) and there were no CCAP-IR terminals on the myocardium of the terminal chamber. Heart function of hid-induced CCAP KO flies was not examined because these pharate adults were unhealthy and did not survive.
5.4 DISCUSSION

Peripheral CCAP-IR neurons innervate the adult abdominal heart during metamorphosis (Dulcis and Levine, 2003). The goal of this study was to determine the role of the neuropeptide CCAP in cardiac function. Previous studies showed a cardioacceleratory effect of CCAP when applied ectopically to adult fly preparations (Tublitz and Evans, 1986; Nichols et al., 1999). However, it is difficult to predict from these data the role of CCAP on the function of this open circulatory system in the intact animal.

Circulatory systems, including multi-chambered insect hearts, are extremely sensitive to a variety of physiological changes that occur in the organism. The total amount of circulating hemolymph, mechanical pressure applied on the myocardium, activity of cardiac innervation, circulating hormones, and tracheal ventilation are all important parameters that are altered in dissected preparations. In the present study we employed two approaches, RNA interference (RNAi) and targeted cell ablation, to investigate the role of CCAP on heart function without affecting other components essential to normal cardiac physiology. Furthermore, a non-invasive optical detection of cardiac function allowed the study of cardiac phenotypes of intact adults in physiological experimental conditions.

In addition to the CCAP-expressing neurons localized in the CNS, CCAP is also expressed in segmental peripheral neurons, BpNs, that innervate the adult abdominal heart (Dulcis and Levine, 2003). Although the BpN pattern of activity and CCAP release modality have not been described, CCAP may influence a specific cardiac phase or
modulate heart rate depending on the site of release and concentration of the peptide.

Immunocytochemical analysis showed that the CCAP-GAL4 transgene drove GFP expression in all central and peripheral CCAP-IR neurons (Figs. 5.3-5.4; Park et al., 2003, and J.E., unpublished), accurately reproducing the expression of the CCAP gene. This finding confirmed that the CCAP-GAL4 was appropriate for selectively targeting the expression of CCAP RNAi and apoptosis genes to CCAP-expressing neurons.

Surprisingly, the RNAi approach did not succeed in reducing CCAP expression to levels that compromised normal pupal ecdysis behavior and cardiac function. Although we observed a reduction of the level of CCAP RNA in the cell body of central neurons (Fig. 5.3B), readily detectable levels of CCAP-IR were observed in their axons (Fig. 5.3G-I). Even when a stronger and ubiquitously-expressed driver, such as the actin-GAL4 transgene, was used to drive CCAP RNAi to significantly reduce, but not eliminate, CCAP-IR levels in the peripheral BpNs (Fig. 5.5), cardiac activity was not significantly different from that of controls (Table 1). Because a profound cardiac phenotype was observed when CCAP expression was eliminated completely by targeted cell ablation, we concluded that the RNAi is not an efficient approach to reduce peptidergic expression in this system. Peptides that are produced to circulate as neurohormones are usually synthesized at extremely high concentrations in the organism (Strand, 1999). Our CCAP RNAi experiments may have reduced CCAP concentration to a level that was still sufficient to produce normal ecdysis behavior and to control cardiac function. Alternatively, the neuropeptide CCAP may accumulate in intracellular stores or have a slow turnover.
The regular cardiac cycle of adult *Drosophila* is composed of two phases, the anterograde and the retrograde beats (Fig. 5.1). Our cell ablation experiments showed that only the anterograde beat was affected by lack of cardioactive peptide. Although regular cardiac reversals were observed in CCAP KO animals, retrograde contractions occurred frequently during the anterograde beat, suggesting that the reciprocal and cyclic dominance of retrograde/anterograde pacemakers, which is typical in wildtype animals (Dulcis and Levine, 2004 b), was altered in the absence of CCAP neurons. Because bath application of CCAP to the isolated abdominal heart initiates (Fig 5.2) and potentiates (Dulcis and Levine, 2004 b) the anterograde beat, our CCAP KO findings suggest that CCAP may control the level of activity of the anterograde pacemaker. A similar control mechanism of the anterograde beat has been described in *Manduca sexta* (Dulcis et al., 2001; Dulcis and Levine, 2004 a). Motoneuron MN-1 of the eighth neuromere, which is CCAP-immunoreactive in *Manduca* (Davis et al., 2001), drives the posterior cardiac pacemaker to initiate and maintain the anterograde beat (Dulcis and Levine, 2004 a).

From the results of the present study and previously published data, the following model for the functional role of CCAP in cardiac function in adult *Drosophila* is proposed. Release of CCAP at cardiac terminals might be temporally and segmentally coordinated by the activity of peripheral BpNs to synergistically re-activate and maintain the anterograde pacemaker activity during the anterograde phase. When central and peripheral CCAP-expressing neurons are eliminated by rpr expression, without affecting normal development of the remainder of cardiac innervation (Fig. 5.7), the anterograde pacemaker becomes weaker due to lack of CCAP activation. In this scenario, the
retrograde pacemaker takes over by producing retrograde contractions while the antagonist pacemaker is still active. A weakening of the anterograde pacemaker and consequent cardiac phenotype shown by CCAP KO animals (Fig. 5.6) could cause a reduction of the amount of hemolymph that is pumped from the abdominal heart into the aorta and the head per diastolic/systolic cycle. The consequent drop of hemolymph pressure in CCAP KO flies could represent the bases for the defects observed at adult eclosion (Park et al., 2003). During the larval and pupal stage, CCAP immunoreactivity occurs in numerous CCAP-expressing neurons in the CNS. After eclosion a number of these neurons undergo programmed cell death (Draizen et al., 1999). By periodically raising the level of circulating CCAP at different times during development, CCAP-IR central neurons might be specialized to produce systemic effects, such as activation of motor patterns involved in ecdysis behavior or even a general cardioacceleratory effect, that are distinct from those exerted by peripheral CCAP-IR BpNs in cardiac function. Future experiments are needed to determine whether central and peripheral CCAP-expressing neurons have a specific role in controlling cardiac function versus ecdysis behavior.
Figure 5.1: Optical measurement of cardiac activity in adult flies. A representative recording of consecutive cardiac cycles of resting flies is shown (upper trace). Each deflection corresponds to a cardiac movement. Two phases of heart contraction, which correlate with anterograde and retrograde beats, are visible (lower trace).
Figure 5.2: Chronotropic effect on cardiac activity. The time of peptide application is indicated (downward arrow). After a delay of about 40 sec, the initially non-beating heart begins to beat. A gradual increase in amplitude of the evoked cardiac contractions is visible (oblique arrow).
Figure 5.3: Consequences on CCAP expression of CCAP RNAi expression in CCAP neurons. See next page for details.
Figure 5.3: Consequences on CCAP expression of CCAP RNAi expression in CCAP neurons. *In situ* CCAP RNA expression in the CNS of third instar larval CNS of (A) control larvae (progeny of w x UAS-CCAP RNAi [2 copies of driver]) and in (B) progeny of CCAP-GAL4 x UAS-CCAP RNAi (2 copies of driver and UAS transgene). (C-D) *In situ* expression of antisense CCAP RNA in progeny of CCAP-GAL4 x UAS-CCAP RNAi (2 copies of driver and UAS transgene) (C) and in control animals (D). (E-F) CCAP-IR in control larvae (E), in animals hemizygous for CCAP (F), and in larval progeny of CCAP-GAL4 x UAS-CCAP RNAi bearing 2 copies of CCAP-GAL4 driver and either one (G) or two (H) copies of the UAS-CCAP RNAi transgene. (I) Quantitation of CCAP-IR in the medial (Medial) and lateral (Lateral) CCAP-immunoreactive axons (cf. Fig 3E). Values are averages ± SEM (N=8). Genotypes correspond to those of the relevant lettered panels. Lower case letters above the histograms indicate which groups of data were not statistically significantly different (P>0.05, ANOVA followed by Tukey’s HSD post hoc test). Scale bar: 80μm.
Figure 5.4: CCAP-ergic innervation in adult CCAP-GAL4xUAS-GFP flies. GFP and CCAP antibodies were labeled with Cy2- and Cy5-conjugated secondary antibodies, respectively. A: GFP immunostaining in whole-mounts of the dorsal vessel (heart) and peripheral nervous system in the abdomen of adult flies. Driven GFP expression was immunolocalized (in yellow) bilaterally in each abdominal segment at the level of transverse nerves (TN). The somata of peripheral bipolar neurons (BpN) are indicated with arrows. B-G: Double immunostaining showing GFP-IR (B-E, visualized in blue) and CCAP-IR (C-F, visualized in red) co-localized in the same somata of BpN6 (B-D) and BpN4 (E-G) shown in A. Regions of colocalization are visualized in purple in the merged images (D and G). Scale bars = 100 μm in A, 30 μm in B-G.
Figure 5.5: Immunoquantification of CCAP-Ir of bipolar neurons. CCAP-IR in the adult progeny of CCAP-GAL4 x UAS-CCAP RNAi and Actin-GAL4 x UAS-CCAP RNAi was compared to that of the progeny of Wt x UAS-CCAP RNAi flies (controls). The box plots represent the range of averaged values for each group and the bars are the standard errors; 8 preparations were quantified for each group. Double asterisks indicate the data that is significantly different (p<0.0001, t-student test) from controls. The horizontal line within each histogram reflects the median value.
Figure 5.6: Optical measurement of cardiac activity of CCAP knock-out flies. Four consecutive cardiac cycles, each composed of an anterograde and a retrograde phase, are shown (upper trace). Compare with Figure 5.1. A representative anterograde phase, in between retrograde phases, is shown at expanded time scale (lower trace). The arrows indicate occasional retrograde contractions occurring during the anterograde beat.
**Figure 5.7: PNS of adult CCAP knock-out flies.** Laser-scanning confocal microscope images of whole-mount preparations of the peripheral nervous system in the abdomen of adult progeny of CCAP-GAL4xUAS-rpr transgenic flies. 

**A:** Ventral view of the first four abdominal segments showing 22C10 immunostaining of the transverse nerves (TNs) innervating the heart. 

**B:** Ventral view of abdominal segments 3-5 showing the 22C10 immunoreactivity of TNs innervating the last three cardiac chambers. The arrow indicates the somata of the peripheral bipolar neurons (BpNs) serving the caudal cardiac chamber, which are still present, unlike more anterior BpNs. 

**C:** Same innervation of the conical chamber shown in the box in A. The transverse bridge (TB) is present in CCAP-rpr flies despite loss of BpNs. 

**D-E:** Double staining, 22C10-IR (D) and CCAP-IR (E), of the second cardiac chamber shown in the box in B. CCAP-rpr flies lack CCAP-IR innervation while the remainder of the cardiac innervation develops normally. Scale bars = 150 μm in A-B, 25 μm in C-E.
Figure 5.8: CCAP-Ir in the terminal chamber of CCAP knock-out flies. See next page for details.
Figure 5.8: CCAP-Ir in the terminal chamber of CCAP knock-out flies. CCAP immunoreactivity in the terminal chamber and caudal cluster of peripheral bipolar neurons (BpN₆) of adult (A-B) and pharate adult (D-G) progeny of CCAP-GAL4xUAS-rpr animals, and of pharate adult progeny of CCAP-GAL4xUAS-hid (C) transgenic flies. Axonal branches and neuronal endings were visualized by 22C10 immunostaining. CCAP and 22C10 antibodies were labeled with Cy5- and Cy2-conjugated secondary antibodies, respectively. A: ventral view of the caudal chamber visualized by autofluorescence (yellow/green) of the pericardial cells surrounding the heart in the progeny of CCAP-GAL4 xUAS-rpr. The CCAP immunoreactivity (CCAP-IR, red) of BpN₆ somata (arrowheads) is shown. B: Double labeling of CCAP-IR (red) and 22C10-IR (blue) of the peripheral nervous system serving the terminal chamber in the progeny of a CCAP-GAL4 xUAS-rpr. Regions of colocalization of the two antibodies are visualized in purple. Terminals of transverse nerves (TNs) serving abdominal segments 4-6 do not show any CCAP-IR, which is restricted to BpN₆ somata (arrowheads). Note the faint autofluorescence of the pericardial cells. C: Double labeling of CCAP-IR (green) and 22C10-IR (red) in the terminal chamber in the progeny of CCAP-GAL4 xUAS-hid. Unlike wild type animals, the TNs are CCAP-negative (arrow) and there are no CCAP-IR terminals on the heart. The peripheral cluster of BpN₆ is absent. D: ventral view of the terminal chamber and its 22C10-IR innervation in the pharate adult progeny of CCAP-GAL4 xUAS-rpr. BpN₆ somata are visible. E-G: same BpN₆ somata shown in the box in D plus a more caudal cell body. Colocalization of 22C10-IR (E, blue) and CCAP-IR (F, red) visualized in purple (G) showing that BpN₆ are resistant to the apoptotic rpr gene when driven by the CCAP promoter. Scale bars = 50 μm in A-D, 10 μm in E-G.
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<th>Anterograde phase</th>
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<td></td>
<td>Rate</td>
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<td>Wt x UAS-RNAi (controls)</td>
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<td>7.3 ± 1.1</td>
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<tr>
<td>CCAP-GAL4 x UAS-RNAi</td>
<td>4.1 ± 0.3</td>
<td>7.2 ± 2.2</td>
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**Table 5.1: Cardiac parameters.** Heart rate (Hz) and duration (sec) of the anterograde and retrograde phases were compared among the progeny of actin-GAL4 x UAS-CCAP RNAi, CCAP-GAL4 x UAS-CCAP RNAi, and Wt x UAS-CCAP RNAi (controls) transgenic flies. Values are mean ± standard deviation (n = 6).
SUMMARY AND CONCLUSIONS

This study allowed me to investigate and compare neuronal control of cardiac function in two representative examples of open circulatory systems. Manduca and Drosophila model systems were chosen for their valuable versatility and accessibility to electrophysiological recordings and genetic manipulation, respectively. Although the two systems share some similarities, the results showed profound differences both in the nature and function of cardiac innervation. Below, I summarized some novel similarities and differences emerged from this investigation.

**Similarities**

- Both species acquire the cardiac reversal pattern of blood circulation late during postembryonic development (adult stage);
- Cardiac innervation develops during the pupal stage of metamorphosis to control adult cardiac function;
- CCAP is the cardioactive peptide that is expressed both by central MNs-I₈ in Manduca and peripheral BpNs in Drosophila innervating the abdominal heart.
- Cardiac innervation initiates cardiac reversal in both species, although through a different mechanism.
- Cardiac cycles are composed of two phases. In both species, one of the phases is myogenic and the other seems to require neural activation, suggesting neurogenic properties.
Differences

- Cardiac innervation is segmental in Drosophila, with abdominal TNs serving each of the cardiac chambers, and restricted to one chamber in Manduca, with branches of DNs\(_{7,8}\) innervating the terminal cardiac chamber.

- The retrograde beat is the neurogenic phase in Drosophila; conversely, the anterograde beat in Manduca is the phase that requires neuronal activation.

- Duration of anterograde and retrograde phases and cardiac cycle frequency are extremely different in Manduca and Drosophila.

Despite the neuroanatomical differences of cardiac innervation between Manduca and Drosophila, the functional significance of cardiac reversal in the adult stage seems to be shared by these adult holometabolous insects. Why does cardiac reversal develop? There are few hypotheses that have been suggested in the literature. The most appealing are summarized below.

- Coordination of release of neuropeptides produced by neurosecretory cells in the brain and released in the CC/CA located nearby the aorta (Ichikawa and Okada, 2002). Their results support the idea that firing activity of neurosecretory cell system may be coordinated with circulation of hemolymph for rapid and pulsatile delivery of peptides released to target organs.

- Thermoregulation in an open circulatory system. Temperature increases in the thorax during flight. Inverting the hemolymph flow (anterograde to retrograde beat reversal) would allow heat transfer from the thorax, where it is produced, to compartments of the body (abdomen) that are better suited to loss heat via convection and evaporation being
less insulated and having a wider exchange surface (see chapter 31 in The Insects: structure and function, Chapman, 1969). The first attempt to correlate neuronal control of heartbeat with thermoregulation in Manduca was made by Heinrich (1971). In this study, when the thorax was artificially heated the pulsation rate of the abdominal heart increased, the amplitude of pulsation became larger, the beat was anterograde. This suggested mechanism implies that thermoreceptors are involved in detecting hemolymph temperature. Their experiments showed the effect of artificially elevated thoracic temperature but do not account for the possible intrinsic function of periodic reversals occurring repetitively during flight.

In closed circulatory systems, such as the mammalian system, the heart functions to meet similar needs. Thermoregulation, or in general changes of blood flow to different compartments of the body, take place at two separate levels: via heart rate changes and via modification of the vascular resistance in the periphery (vasoconstriction or vasodilatation). In an open circulatory system, the developed cardiac reversal makes the neurogenic heart both the pump and the vessel, being able to shunt and invert the flow where and when needed.

It is important to notice that cardiac reversal occurs in all holometabolous insects (Diptera, Coleoptera, Lepidoptera and Hymenoptera) but has never been reported in any hemimetabolous insects (Orthoptera, Blattoidea, Odonata, etc), although most of the species in both Holo- / Hemi-metabolous categories are able to fly. One fascinating explanation could be that cardiac reversal developed in those orders that produce more heat during flight. Wing beat frequencies of different insect species are plotted in the
graph below (modified from Chapman, 1969). Surprisingly, species that fly at higher wingbeat (Drosophila, Protophormia and Calliphora) show shorter cardiac cycles and higher reversal frequency than those one who have a slower wing stroke beat frequency (Manduca, Bombyx, etc). Furthermore, Hemimetabolous species, which have very slow wing beat frequency during flight such as Locusta, Periplaneta, Aeshna, Schistocerca, do not show any reversal.

In conclusion, the innervation of multi-chambered hearts seems to be evolved to make larval myogenic hearts able to accommodate the dynamic needs of adult open
circulatory systems. Cardiac reversal might represent the mean by which innervation influences circulation in open circulatory systems.

This study provided insights into how peptides and transmitters can activate or modulate the basal activity of cardiac pacemakers. For the first time it was shown that cardiac reversal, thus alternation of pacemaker dominance in insects, can be evoked and controlled pharmacologically. The approaches used in this investigation may represent general tools that would be applicable to the study of vertebrate hearts. The peptides that are expressed by the intrinsic ganglionated plexus neurons might have a parallel role in sculpting the excitability and dominance of the different cardiac pacemakers (sinoatrial and atrioventricular nodes) present in human myogenic hearts. It would be of interest to determine whether the neural activity or peptide expression of such neurons is altered in pathological conditions in which there is altered pacemaker activity. Similarly, the results presented in this dissertation prompt a careful comparison of pacemaker activity and its modulation in healthy and transplanted hearts.
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