Behavioral/Systems/Cognitive

Glutamatergic Innervation of the Heart Initiates Retrograde Contractions in Adult *Drosophila melanogaster*

Davide Dulcis and Richard B. Levine
Division of Neurobiology, University of Arizona, Tucson, Arizona 85721-0077

The adult abdominal heart of *Drosophila melanogaster* receives extensive innervation from glutamatergic neurons at specific cardiac regions during metamorphosis. Here, we show that the neurons form presynaptic specializations, as indicated by the localization of synaptotagmin and active zone markers, adjacent to postsynaptic sites that have aggregates of glutamate IIA receptors. To determine the role of this innervation in cardiac function, we developed an optical technique, based on the movement of green fluorescent protein-labeled nerve terminals, to monitor heart beat in intact and semi-intact preparations. Simultaneous monitoring of adjacent cardiac chambers revealed the direction of contractions and allowed correlation with volume changes. The cardiac cycle is composed of an anterograde beat in alternation with a retrograde beat, which correlate respectively with systole and diastole of this multichambered heart. The periodic change in hemolymph direction is referred to as cardiac reversal.

Intracellular recordings from muscles of the first abdominal cardiac chamber, the conical chamber, revealed pacemaker action potentials and the excitatory effect of local glutamate application, which initiated retrograde contractions in semi-intact preparations. Unilateral electrical stimulation of the transverse nerve containing the glutamatergic neuron that serves the conical chamber caused a chronotropic effect and initiation of retrograde contractions. This effect is distinct from that of peripheral crustacean cardioactive peptide (CCAP) neurons, which potentiate the anterograde beat. Cardiac reversal was evoked pharmacologically by sequentially applying CCAP and glutamate to the heart.

Key words: **cardiac pacemaker; DGluRIIA receptors; insect circulatory systems; crustacean cardioactive peptide; active zones; glutamate**

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 cardiomyocites. 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 melanogaster*. Octopamine and neuropeptides are expressed in cardiac neurons of a variety of insects (Stevenson and Pfluger, 1994; Sinakevitch et al., 1996; Duch et al., 1999; Davis et al., 2001), but the glutamatergic cardiac innervation reported recently in adult *Drosophila* represents a novel finding (Dulcis and Levine, 2003). Axons grow onto the cardiac muscle in the first abdominal segment and fasciculate during metamorphosis to form a characteristic glutamate-immunoreactive (IR) synaptic structure, the transverse bridge (TB) (Dulcis and Levine, 2003).

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Correspondence should be addressed to Davide Dulcis, Arizona Research Laboratories Division of Neurobiology, University of Arizona, P.O. Box 210077, Tucson, AZ 85721-0077. E-mail: dulcis@neurobio.arizona.edu.

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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 (NMJ) is glutamatergic and with the availability of powerful genetic tools has served as a valuable model system for investigating synaptic function and plasticity (Keshishian et al., 1996). The relatively large size of the novel cardiac synapses, however, may prove advantageous for many studies. Thus, the goals of this study were to investigate whether presynaptic and postsynaptic 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). In other species, cardiac reversal develops during metamorphosis and requires new neuronal input (Kuwasawa et al., 1999; Davis et al., 2001; Dulcis et al., 2001; Dulcis and Levine, 2004). *Drosophila* may follow a similar pattern, but this awaits confirmation (Rizki, 1978; Dowse et al., 1995; Johnson et al., 1997). During the larval stage in *Drosophila*, the heart does not receive innervation (Dulcis and Levine, 2003). The larval cardiac contractions are completely myogenic, originate in the caudal

chamber, and produce an anterograde heartbeat (Rizki, 1978). Profound anatomical changes occur during metamorphosis, including the formation of a new conical chamber, which is added posterior to the aorta, and an extensively innervated new muscular ventral layer (Curtis et al., 1999; Molina et al., 2001; Dulcis and Levine, 2003). Because the conical chamber has an independent development from the rest of the abdominal heart, Rizki (1978) hypothesized that this region might represent the location of the retrograde pacemaker whose neuronal activation could produce cardiac reversal in adult flies.

In the present study, we investigated whether formation of the glutamatergic innervation correlates with changes in the cardiac function of adult *Drosophila*. A novel optical technique, based on the movement of green fluorescent protein (GFP)-labeled nerve terminals, to monitor heartbeat in intact and semi-intact preparations, revealed that cardiac reversal is indeed a feature of adult heart function. The excitatory effect of glutamatergic synapses on the myocardium provides the mechanism for originating the retrograde beat and hence cardiac reversal.

Materials and Methods

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/upstream activation sequence (UAS)-GFP transgenic flies were used in this study. The data were collected from 2- to 3-d-old adults. For semi-intact preparations and immunocytochemistry, the animals were anesthetized on ice for ~10 min and then dissected in cold *D. melanogaster* hemolymph-like saline (HL-3) (Stewart et al., 1994).

Immunocytochemistry. elav-GAL4/UAS-GFP transgenic flies were used to visualize the peripheral fibers innervating the heart. In these animals, the expression of the 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 2 min 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* p21-activated kinase (DPAK; generously provided by Dr. N. Harden, Simon Fraser University, Burnaby, British Columbia, Canada) (Harden et al., 1996) and a mouse monoclonal antiserum (NC82; generously provided by Dr. K. Zinsmaier, University of Arizona, Tucson, AZ) (Hofbauer, 1991), 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 min. After rinsing (three times for 5 min each) in PBS containing 1.0% Triton X-100 (PBST), pH 7.2, the preparations were incubated for 1 hr in the blocking solution composed of 2% bovine serum albumin (Sigma, St. Louis, MO), 5% normal donkey serum (Sigma) in PBS with 1.5% Triton X-100. The primary antisera (1:10 NC82 and 1:50 DPAK) made up in blocking solution were applied at 4°C overnight.

To determine whether glutamate receptors (DGluRs) with the subunit DGluRIIA localize at cardiac synapses, the myocardium of *elav*-GAL4/UAS-GFP transgenic adult flies, fixed and blocked as described above, was incubated for 2 hr 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, University of Iowa, Iowa City, IA) 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 min (three times for 5 min each). A cyanine 5 (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 and were applied at a dilution of 1:25 for 1 hr at room temperature. The preparations then were washed several times in 1% PBST for 15 min and in PBS for 20 min and were mounted in 80% glycerol solution. Rabbit polyclonal antiserum to *Drosophila* synaptotagmin (generously provided by Dr. M. Ramaswami, University of Arizona, Tucson, AZ) (Littleton et al., 1993) was used to visualize cardiac terminals in wild-type adult preparations. Synaptotagmin immunostaining was performed as described by Dulcis and Levine (2003), and preparations were double labeled for DGluRIIA.

Video microscopy and optical detection of heart activity. Recordings were made from 2- to 3-d-old adults, because younger flies have abundant fat tissue, making optical detection through the cuticle difficult. The animals were anesthetized on ice for ~10 min and 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 10× 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 bandpass filter) was used for *elav*-GAL4/UAS-GFP transgenic flies. Simple PCI software (Compix, Cranberry Township, PA) was adapted to measure movement of the heart during cardiac contractions. Images were collected at a maximum speed of 19 frames/sec. This is faster than the adult *Drosophila* heart rate and ensures detection of all cardiac contractions. After video acquisition, images were contrast enhanced to reduce the blurring effect of the cuticle.

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 approximated as to a cylinder ($V = \pi \times r^2 \times h$), whose radius was measured at the level of the transverse bridge. Diastolic and systolic volumes expressed in cubic micrometers were converted to nanoliters by applying the following conversion: $10^9\ \mu m^3 = 1000$ nl.

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 wild-type 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 3 M potassium chloride. Glutamate was applied with a Picospritzer II (General Valve, Fairfield, NJ) set at a pressure of 10 ψ to deliver 10 μl of glutamate solution and to 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, and then the same stimulation protocol was performed in normal saline to monitor intracellular cardiac action potentials. Controls were made by injecting 10 μl of normal saline solution. Intracellular signals were acquired with Clampex 9.0 (Axon Instruments). Clampfit 9.0 (Axon Instruments) was used for analysis.

Pharmacology. For the pharmacological experiments, we used the *elav*-GAL4/UAS-GFP transgenic fly line, because optical detection is optimal when nerve terminals are fluorescent. Glutamate (Sigma-Aldrich, St. Louis, MO) and crustacean cardioactive peptide (CCAP) (Backem, Torrance, CA) were bath applied in *in vitro* preparations with a micropipette to reach final concentrations in the bath of 10^{-5}/10^{-6} M and 10^{-4}/10^{-6} M, respectively, while cardiac activity was being detected optically. At 10^{-5} M, CCAP increases adult *in vitro* and *in vivo* heart rate to 19 and 35%, respectively, over basal levels (Nichols et al., 1999). We examined the effect of lower levels (10^{-6} M) of the peptide CCAP *in vivo*. We also used a higher concentration (10^{-4} M) of the peptide, which induced a higher rate of anterograde beat, to test whether a subsequent glutamate application was able to cause cardiac reversal. In intact conditions, CCAP release is likely to be confined to cardiac terminals (Dulcis and Levine, 2003) rather than causing a general increase in the blood titer. The preparations were perfused constantly with normal saline (Stewart et al., 1994) to wash out the compound and to keep the heart well oxygenated.

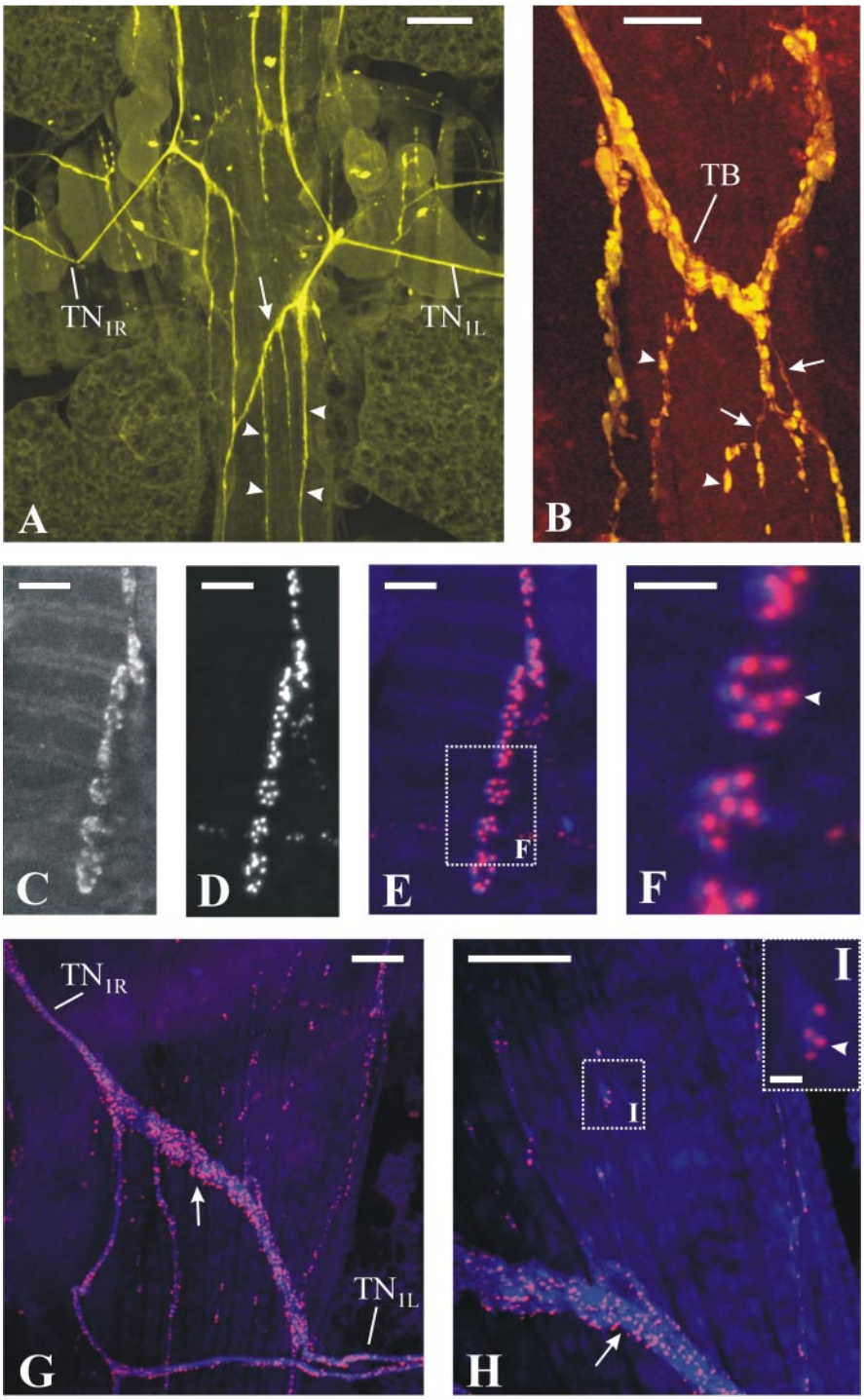

Figure 1. Laser-scanning confocal microscope images of the cardiac conical chamber (*A*, *B*, *G–I*) and skeletal muscles (*C–F*) in the abdomen of adult *elav*-GAL4/UAS-GFP transgenic flies. *A*, Ventral view of the conical chamber showing the GFP-labeled cardiac innervation (in yellow). TN_{1R} and TN_{1L} 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 TB, longitudinal processes, and their bouton-like terminals on the myocardium (arrowheads). Selective bundles of axons running within the TB and the thinner longitudinal processes (arrows) have been previously demonstrated to be glutamate-IR (Dulcis and Levine, 2003). *C*, DPAK immunostaining of NMJs in adult skeletal muscles. *D*, NC82 immunostaining of same preparation shown in *C*. *E*, Merged images shown in *C* and *D*. NC82 (pink) and DPAK (blue) immunoreactivities colocalize at the level of the adult skeletal muscle NMJ. *F*, The same terminals shown in the box in *E* at higher magnification. NC82 immunoreactivity and DPAK immunoreactivity show similar patterns 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 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: *A*, 50 μm; *B*, *G–H*, 15 μm; *C–E*, 5 μm; *F*, *I*, 2 μm.

Electrical stimulation of transverse nerves. Electrical stimulation of transverse nerves (TNs) was performed in *elav*-GAL4/UAS-GFP transgenic adult flies. After removal of the abdominal viscera to expose the heart for optical detection, the first pair of abdominal TNs (diameter, 1 μm) was visualized with fluorescence light (fluorescein filter) and was cut as far as possible from the conical chamber. The distal stump was drawn into the tip of a glass suction electrode connected to a S88 stimulator (Grass Instruments, Quincy, MA), and a train of pulses (5 sec; 20 Hz; 1 msec per 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 ~1 μm under a microscope. The nerve stumps were sucked into the tip of the prefilled suction electrode by applying negative pressure with a syringe.

Laser-scanning confocal microscopy. Digital images of immunostained cardiac preparations were collected on a Nikon (Tokyo, Japan) 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 bandpass 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, Ottawa, Ontario, Canada) software was used to enhance contrast and provide color when needed. Prints were made by using a Tektronix (Wilsonville, OR) Xerox Phaser 6200 printer.

Results

Release sites and receptors at glutamatergic cardiac terminals

The abdominal heart of adult *Drosophila* becomes extensively innervated during metamorphosis (Dulcis and Levine, 2003). Segmental abdominal TNs serve each of the cardiac chambers bilaterally. The most anterior chamber, the conical chamber, receives a characteristic innervation, the transverse bridge (Fig. 1*A*, arrow), which develops from fasciculation of right and left TNs (TN_{1R} and TN_{1L}) of the first abdominal segment (Fig. 1*A*). Some of the axons within the TNs are glutamate-IR (Dulcis and Levine, 2003) and form longitudinal processes originating from the TB and extending posterior to it (Fig. 1*A*, arrowheads). The longitudinal processes terminate on the myocardium with glutamate-IR bouton-like endings (Fig. 1*B*, arrowheads) (Dulcis and Levine, 2003). Double labeling for GFP (representing the entire innervation in *elav*-GAL4/UAS-GFP transgenic flies) and glutamate immunoreactivity showed that glutamatergic fibers clearly run within the TNs to terminate in the myocardium

(Dulcis and Levine, 2003). Glutamate-IR axons account for only part of the GFP-labeled structures, such as the transverse bridge and the longitudinal processes, which are shown in Figure 1, *A* and *B*.

To determine whether these cardiac terminals contain release sites for neurotransmitters, we used the NC82 antibody (Hofbauer, 1991), which recognizes an unknown protein localized presynaptically at the level of active zones. In skeletal muscles, NC82 immunoreactivity colocalizes but does not overlap completely with DPAK immunoreactivity (Fig. 1*C–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 staining pattern (Fig. 1*F*, arrowhead), the NC82 antibody may be a better marker than DPAK antiserum for localization of active zones. The NC82-IR putative release sites were localized extensively in the TB of the conical chamber (Fig. 1*G*,*H*, arrows) as well as in the cardiac bouton-like terminals (Fig. 1*I*, arrowhead).

We performed double labeling of the conical chamber with DGluRIIA and synaptotagmin antisera to determine whether glutamate receptors were present and confined to the myocardium underneath innervation, or whether their expression displayed a more diffuse pattern (Fig. 2*A*,*B*). DGluRIIA immunoreactivity was localized in rows of large clusters around the TB (Fig. 2*A*, arrowheads). In addition, DGluRIIA immunoreactivity was detected on the longitudinal muscle fibers of the conical chamber in large clusters, which were always adjacent to neuronal terminals (Fig. 2*B*, arrowheads). To investigate the expression pattern of glutamate receptors in more caudal cardiac chambers, we immunolabeled GFP and DGluRIIA in *elav*-GAL4/UAS-GFP transgenic flies. As observed in the conical chamber, DGluRIIA immunoreactivity in more caudal chambers was always localized where TNs contacted the myocardium (Fig. 2*C*, arrowheads).

Excitatory effect of glutamate on the myocardium

Intracellular recordings from the ventral longitudinal muscle layer of the conical chamber (Fig. 3*A*) were performed in semi-intact preparations to investigate the effect of glutamate on cardiac excitability. Local applications of 10^{-6} M glutamate in calcium-free saline caused long-lasting membrane potential depolarization (mean ± SD, 5.8 ± 2.4 mV; $n = 5$) (Fig. 3*B*) that returned to the resting level after constant superfusion with saline. As expected, the heart did not beat in Ca-free saline, because cardiac activity in *Drosophila* is mediated by L-type calcium channels (Gu and Singh, 1995). Although the resting membrane potentials of adult myocardial cells were always somewhat depolarized in our recordings (mean ± SD, −14 ± 6 mV; $n = 8$), the calculated reversal potential of glutamate receptor-mediated currents in the larval NMJ (+12 and 0 mV, respectively, for junctional and extra-junctional glutamate receptors) (Nishikawa and Kidokoro, 1995) suggests that the driving force could be sufficient to produce the observed glutamate-evoked membrane depolarization in the adult myocardium. The presence of spontaneous excitatory potentials provided an indication of successful impalement (Fig. 3*D*, arrows). These long-lasting excitatory potentials had an amplitude of 0.22 ± 0.11 mV (mean ± SD; $n = 53$) and a duration of 128 ± 49 msec (mean ± SD; $n = 53$). When glutamate was applied to hearts that were superfused with normal saline (HL-3), cardiac action potentials were initiated after the initial depolarization and continued to occur for several minutes afterward, suggesting that regenerative pacemaker currents were activated (Fig. 3*C*). The cardiac action potential frequency, which was highest right after and during glutamate application

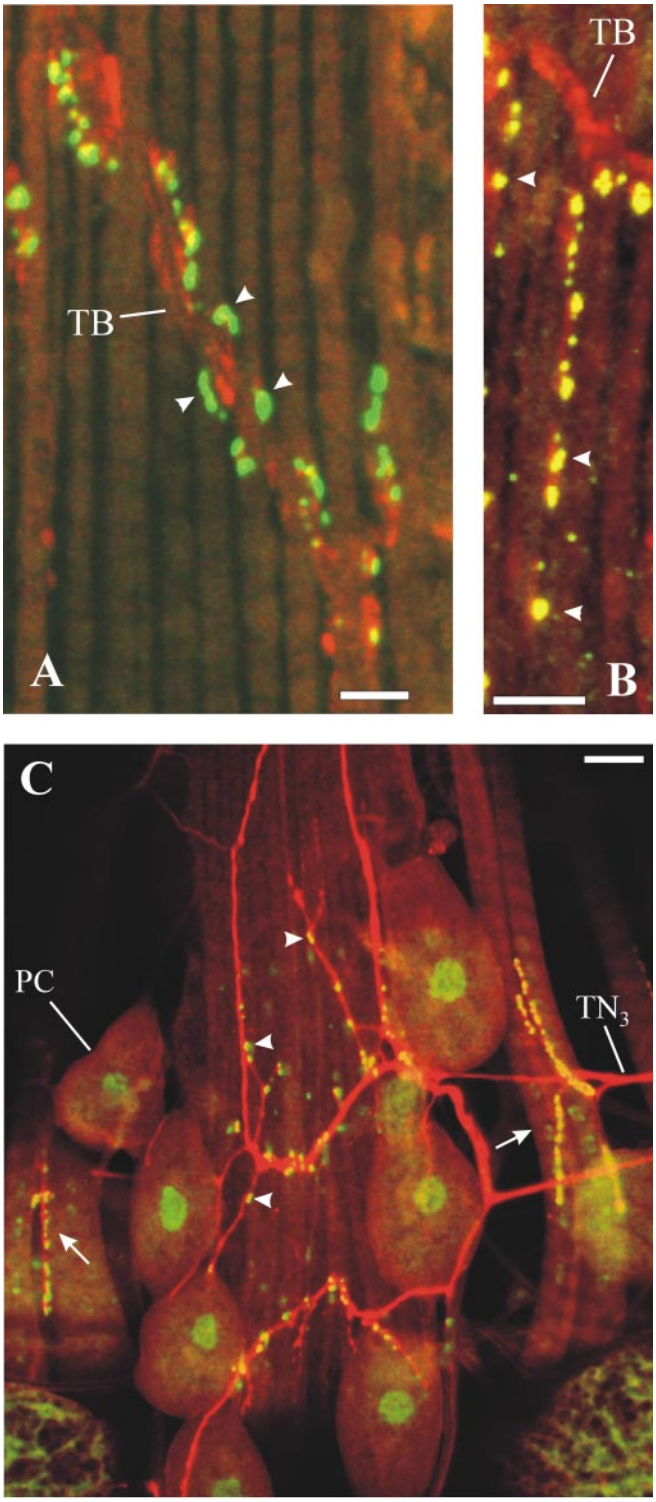

Figure 2. Confocal micrographs showing the localization of postsynaptic GluRIIA at cardiac synapses. *A*, Ventral view of the conical chamber longitudinal muscles showing GluRIIA-IR clusters (arrowheads; green) localized along the TB. The TB was visualized by synaptotagmin immunoreactivity (red). *B*, Synaptotagmin-IR (red) innervation of the conical chamber and GluRIIA immunoreactivity (yellow). Clusters of DGluRIIA-IR receptors (yellow) along the synaptotagmin-IR longitudinal processes extending posterior from the TB (red) are visible (arrowheads). *C*, Ventral view of the third cardiac chamber. GFP-IR transverse nerves (TN_3) and cardiac terminals are shown in red. GluRIIA-IR clusters (green) are visible along both main neuronal branches and cardiac terminals (arrowheads). Regions of colocalization are visualized in yellow. Pericardial cells (PC) with green fluorescent nuclei are also visible surrounding the chamber. The arrows indicate abdominal skeletal muscles. Scale bars: *A*, *B*, 10 μm; *C*, 15 μm.

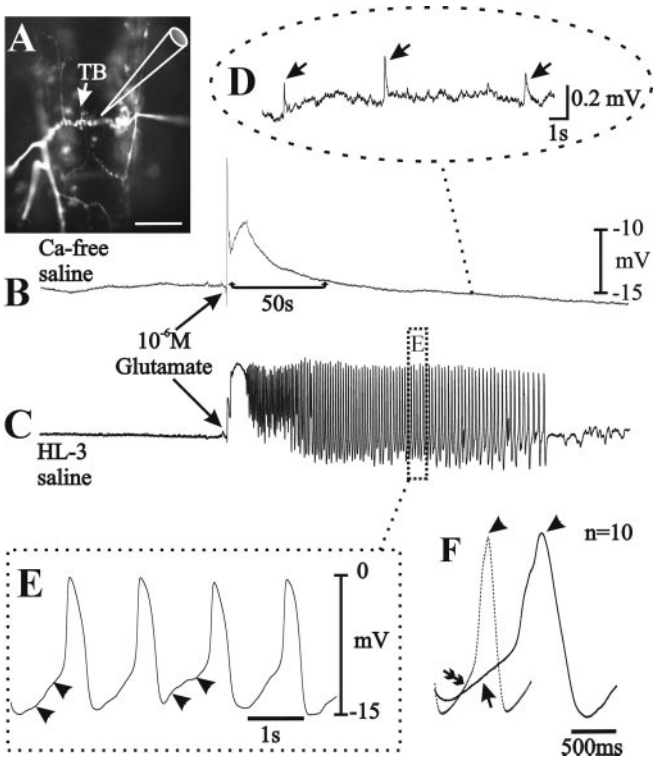

Figure 3. Intracellular recordings from the myocardium of adult flies. *A*, Epifluorescence micrograph of a representative preparation of the conical chamber showing the location of the intracellular electrode in the ventral longitudinal muscle layer served by the TB (arrow). Scale bar, 50 μm. *B*, Depolarization evoked by local application of 10^{-6} M glutamate and recorded in Ca-free saline. Spike-like artifact indicates time of glutamate puff. *C*, Glutamate-evoked depolarization and following cardiac action potentials recorded in HL-3 saline. *D*, Portion of the recording shown in *B* at expanded time and voltage scales showing spontaneous excitatory potentials (arrows) 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 peak of the cardiac action potentials, respectively.

(mean ± SD, 1.7 ± 0.16 per sec; $n = 4$), decreased rapidly by 50 sec after application (mean ± SD, 1.07 ± 0.18 per sec; $n = 4$) and gradually diminished until the heart stopped beating (Fig. 3*C*). This difference in frequency during and after glutamate application correlates in time with the glutamate-evoked depolarization and later repolarization of the membrane.

Cardiac action potentials that occurred after the membrane potential had repolarized displayed a slow depolarizing phase (Fig. 3*E*, arrowheads; *F*, arrow) and relatively long duration, as measured at the base (mean ± SD, 905 ± 205 msec; $n = 10$). Cardiac action potentials occurring during the glutamate-evoked depolarization showed a faster rising phase (Fig. 3*F*, double arrow), narrower peak (Fig. 3*F*, arrowheads), and a significantly shorter duration (mean ± SD, 463 ± 75 msec; $n = 10$; $p < 0.0001$ by Student's *t* test for unpaired data). The consistent changes in shape, rising phase, and duration of cardiac action potentials occurring during and after glutamate-evoked depolarization suggest that movement artifacts did not influence intracellular recordings.

Cardiac activity of adult *Drosophila*

Optical detection of cardiac activity through the dorsal cuticle of intact adult flies allowed simultaneous monitoring of two different aspects of heart wall displacement. The conical chamber 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 movement (Fig. 4*A*). Thus, there were two alternating phases of cardiac movement (systole–diastole) with different rates of mini-systole–mini-diastole (Fig. 4*A*, heart rate). During systole, when the conical chamber decreased its lumen, the heart rate increased to 4.9 ± 0.9 Hz (mean ± SD; $n = 6$). During diastole, when the conical chamber increased its diameter, the frequency of mini-systole–mini-diastole decreased to 3.5 ± 1.6 Hz (mean ± SD; $n = 6$). The relative duration of systolic and diastolic phases showed high variability among specimens (mean ± SD, 13 ± 8 and 9 ± 7 sec, respectively; $n = 15$). However, within a large subset of these preparations, a long-lasting systole (mean ± SD, 7.3 ± 1.1 sec; $n = 10$) was observed in alternation with a shorter diastole (mean ± SD, 4 ± 0.4 sec; $n = 10$). The 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*B*,*C*). By analyzing the two superimposed traces at an 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*D*). Cardiac reversals occurred cyclically in intact adult flies (Fig. 4*C*, arrows).

Selective optical detection of diastolic and systolic movements of the conical chamber 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. Video microscopy data revealed that the conical chamber could reach its maximum diastolic volume (mean ± SD, 1.35 ± 0.1 nl; $n = 5$) by rapidly decreasing its muscle tone starting from a systolic volume of 0.45 ± 0.05 nl (mean ± SD; $n = 5$), thus moving an average volume of ~0.9 nl of hemolymph per cardiac cycle.

To correlate the anterograde and retrograde beats with the volumetric changes occurring in the conical chamber, mini-systole–mini-diastole (Fig. 4*E*, top trace) and systole–diastole (Fig. 4*E*, bottom trace) cycles were recorded simultaneously but were displayed in separate traces. The retrograde beat correlated with the conical chamber diastole, and the anterograde beat correlated with the systolic phase (Fig. 4*F*). Interestingly, the conical chamber lumen rapidly increased to reach the maximum diastolic volume as soon as the retrograde beat started (Fig. 4*F*, left arrowhead). Similarly, as soon as the retrograde beat ceased, the muscle tone of the conical chamber rapidly increased to reach the systolic volume (Fig. 4*F*, right arrowhead).

Effect of glutamate on cardiac physiology

During exposure to fluorescent light (510 nm), the abdominal hearts of semi-intact preparations of adult *elav*-GAL4/UAS-GFP flies stopped beating completely or showed rare anterograde contractions. The cause of this reversible effect is unknown. Under

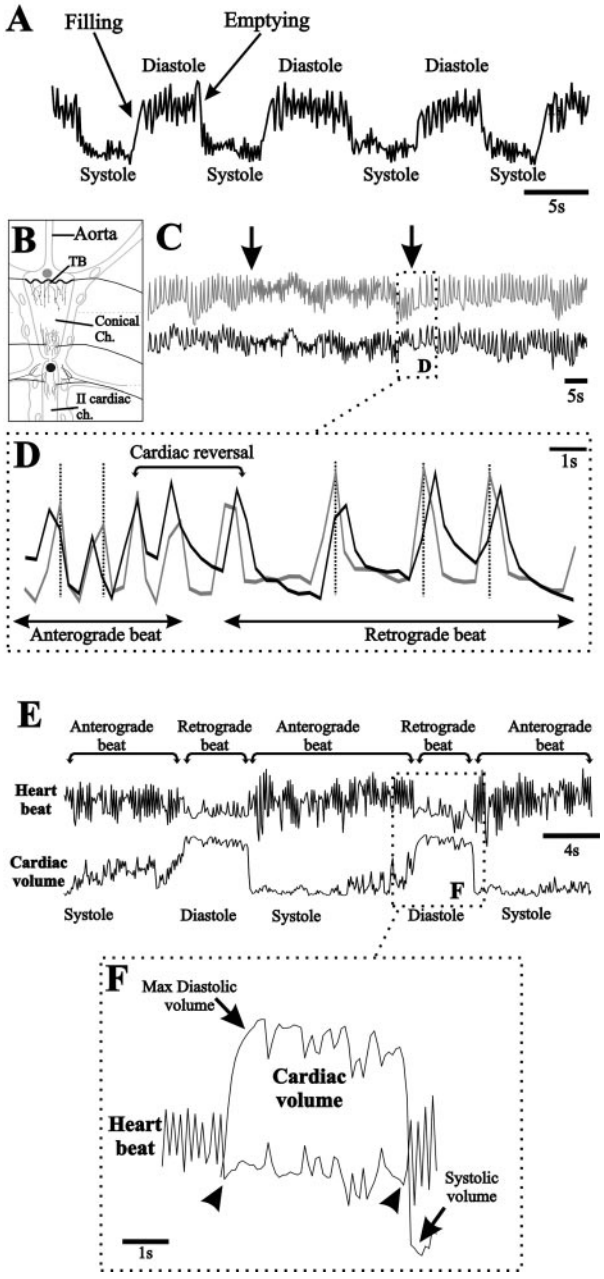

Figure 4. Optical detection of heart function in intact adult flies. *A*, Cardiac activity recorded from the conical chamber (CC) showing alternation of phases during which the heart beats at different rates and superimposed diastolic–systolic cycles. Representative diastolic (filling) and systolic (emptying) 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.; gray dot) and second cardiac chamber (II cardiac ch.; black dot) that were selected for simultaneous optical detection. Cardiac innervation and TB are also visible. *C*, Simultaneous recording of heart activity from the CC (gray 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 vs retrograde) is determined by the relative delay between the CC (gray trace) and the second cardiac chamber (black trace) contractions. The dotted lines mark the peaks of representative CC movements. *E*, Correlation of diastole–systole cycle with cardiac reversal in adult intact flies. Simultaneous optical detection of heartbeat (mini-systole–mini-diastole; top trace) and cardiac volume changes (systole– diastole; bottom trace) recorded from the conical chamber. Anterograde and retrograde beats are easily distinguished by their different heart rates. *F*, Superimposed traces at expanded time scale of the same recording shown in the box in *E*. 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).

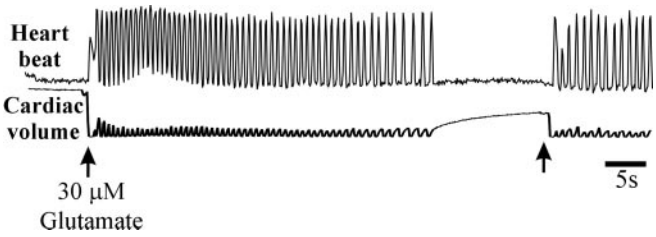

Figure 5. Effect of bath-applied glutamate on cardiac function of semi-intact adult heart preparations. Simultaneous optical detection of heartbeat and cardiac volume were recorded from the conical chamber. The arrows indicate the times of two bath applications of glutamate, each reaching a final concentration in the bath of 30 μM.

these conditions, in which 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 regular mini-systole–mini-diastole were evoked (mean ± SD, 1.4 ± 0.5 Hz; $n = 6$). These movements corresponded to the cardiac action potentials that were evoked by glutamate (Fig. 3). There was a simultaneous reduction of the conical chamber lumen (Fig. 5). As glutamate was washed out by constant superfusion with fresh oxygenated saline, the heart decreased its rate and eventually stopped (Fig. 5, top trace) as conical chamber volume gradually increased (Fig. 5, bottom trace). A second glutamate application had the same effect (Fig. 5, arrows).

The direction of the glutamate-evoked cardiac contractions was retrograde (Fig. 6*A*). This result was confirmed by video recordings of the entire abdominal heart which showed that, when glutamate was bath applied, contractions originated in the conical chamber, propagated backward to invade the more posterior chambers, and produced a continuous wave of retrograde contractions (data not shown). 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 mini-diastolic phase (Fig. 6*B*, 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. 6*C*,*D*). In five preparations, selected because there were spontaneously occurring anterograde contractions, bath application of glutamate consistently caused cardiac reversal (Fig. 6*C*).

CCAP-IR neurons provide extensive innervation of the adult heart, particularly in the caudal region (Dulcis and Levine, 2003). To test whether this cardioactive 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. 7*A*). 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. 7*B*). As observed in spontaneous anterograde contractions (Fig. 7*C*), CCAP-induced contractions occurred first in the third chamber and then sequentially invaded the second and conical chambers (Fig. 7*D*). Application of a lower CCAP concentration (10^{-6} M) also induced anterograde contractions, but the observed chronotropic effect (50% heart rate increase over basal cardiac activity; $n = 3$) was less robust (Fig. 7*F*). Pharmacological cardiac reversal was observed after 10^{-6} M glutamate bath application to anterograde beating hearts that were preactivated with

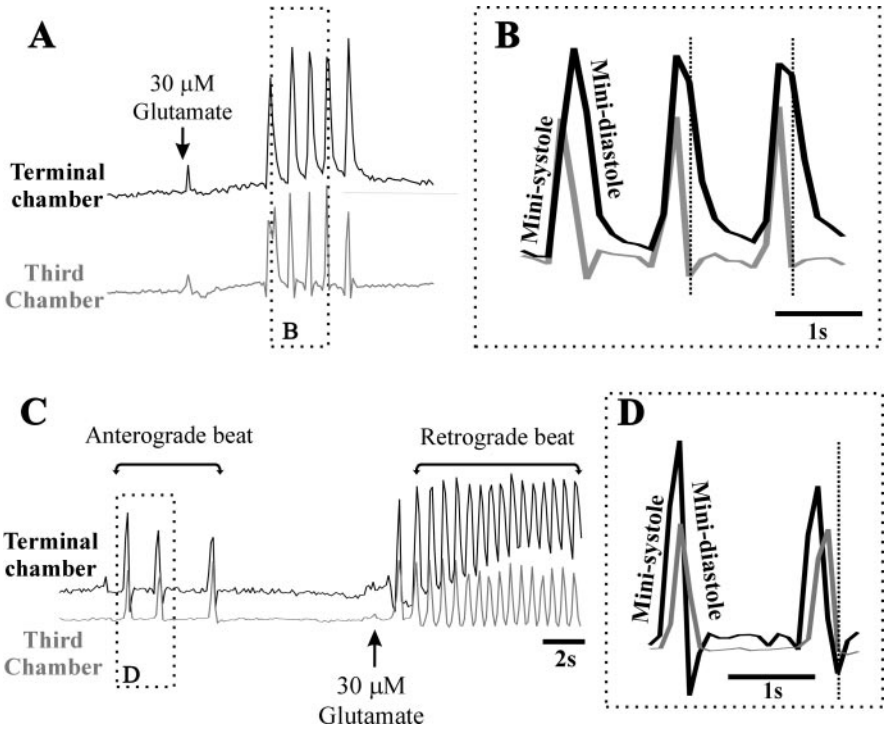

Figure 6. Glutamate initiation of the retrograde beat *in vitro*. *A*, Simultaneous optical detection of the terminal (black trace) and third (gray trace) cardiac chamber activity showing the effect on heartbeat after 30 μM glutamate bath application (arrow). *B*, Superimposed traces at expanded time 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 (gray 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 (gray 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 (gray trace) and terminal chamber (black trace) during the spontaneous anterograde beat.

CCAP (Fig. 7*B–E*). When CCAP and glutamate were both present in the bath, the conical and the third chambers contracted synchronously, both before the second cardiac chamber (Fig. 7*E*). As if the two cardioactive compounds were competing to produce their specific effect, an anterograde beat originating in the caudal portion of the heart and a retrograde beat originating in the conical chamber occurred simultaneously (Fig. 7*B–E*).

Transverse nerve stimulation initiates retrograde contractions

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 TNs serving the conical chamber was performed as indicated in Figure 8*A*. A train of electrical pulses (20 Hz; 5 sec; 1 msec per pulse) applied extracellularly with a suction electrode was sufficient to produce a chronotropic effect in the conical chamber (Fig. 8*B*). After TN stimulation, the endogenous low rate (mean ± SD, 0.5 ± 0.1; n = 5) of anterograde beats that is characteristic of semi-intact preparations (Fig. 8*C*) was replaced by a more rapid retrograde beat (mean ± SD, 2.1 ± 0.7; n = 5) (Fig. 8*D*). The cardiac reversal was delayed with respect to the onset of the stimulus (Fig. 8*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 conical chamber (Figs. 6*A*,*B*, 8*B*,*C*).

Discussion

Synaptic specializations at putative glutamatergic cardiac synapses

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). Presynaptic and postsynaptic specializations, including extensive synaptotagmin immunoreactivity and clusters of DGluRIIA immunoreactivity, were present along the glutamatergic terminals. In addition, abundant NC82 immunoreactivity, which is a marker that colocalizes 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 (Schuster 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 attributable 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 additional electrophysiological analyses of these cardiac synapses must be undertaken to understand the mechanism of cardiac pacemaker cell activation in adult *Drosophila*.

Cardiac pacemakers and systolic–diastolic phases of a multichambered heart

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 terminal 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). In

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 lower frequency systole–diastole cycle, which is characterized by a slow change in its diameter and 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 multichambered hearts, blood moves backward during diastole and forward during systole to achieve complete filling (or emptying) of all four cardiac chambers.

Functional role of glutamatergic innervation in adult cardiac physiology

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, however, is not by itself sufficient to explain cyclic alternation of the two adult cardiac pacemakers and other features of the heart beat in intact animals. Our hypothesis is that both intrinsic excitable properties of the myocardium and neuronal inputs participate in producing selective activation–inhibition of the two pacemakers.

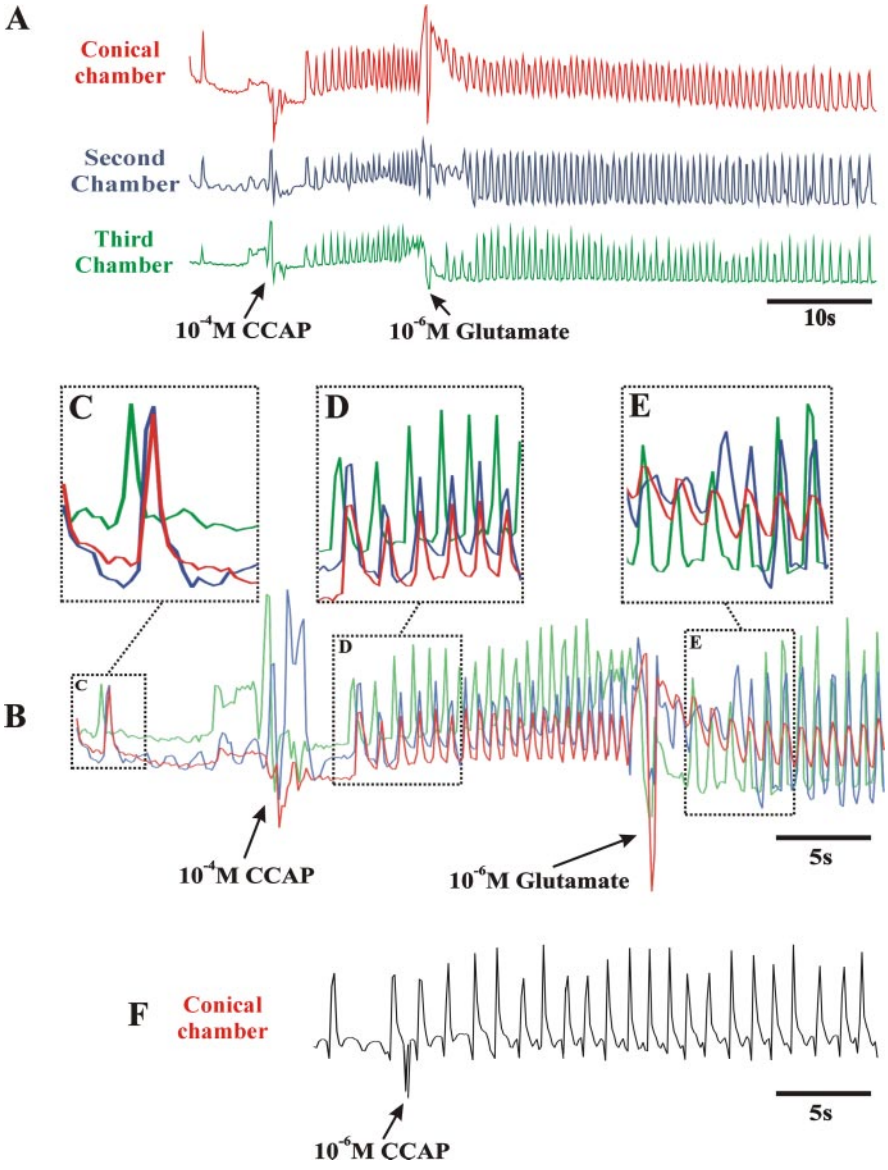

Figure 7. Pharmacologically evoked cardiac reversal in the isolated adult heart. *A*, Simultaneous optical detection of the conical (red trace), second (blue trace), and third (green trace) cardiac chamber activity showing the effect on heartbeat of 10^{-4} M CCAP and 10^{-6} M glutamate bath applications. 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*). Note, in particular, that CCAP accelerates the anterograde beat, whereas subsequent glutamate application advances the relative timing of the conical chamber beat. *F*, Optical detection of the conical chamber activity showing the effect on heartbeat of 10^{-6} M CCAP bath application (arrow).

Both bath application of exogenous glutamate and TN stimulation had a chronotropic effect in semi-intact preparations, involving an increase of the mini-systole–mini-diastole cycle rate of conical chamber activity. The glutamate-evoked cardiac contractions originated in the conical chamber and traveled in the retrograde direction. They were correlated with the glutamate-evoked pacemaker potentials recorded intracellularly from myocardial cells. Thus, cardiac reversal to the retrograde beat could be evoked in hearts that were spontaneously beating in the anterograde direction. Similarly, retrograde contractions were initiated in the conical chamber by glutamate application to hearts that had been preincubated 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 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. Although GluRIIA immunoreactivity and glutamatergic innervation are present at every cardiac chamber, a higher sensitivity of the glutamate receptors and/or faster properties of the putative pacemaker localized in the conical chamber may explain why retrograde contractions originate in the conical chamber when glutamate was applied to the entire abdominal heart.

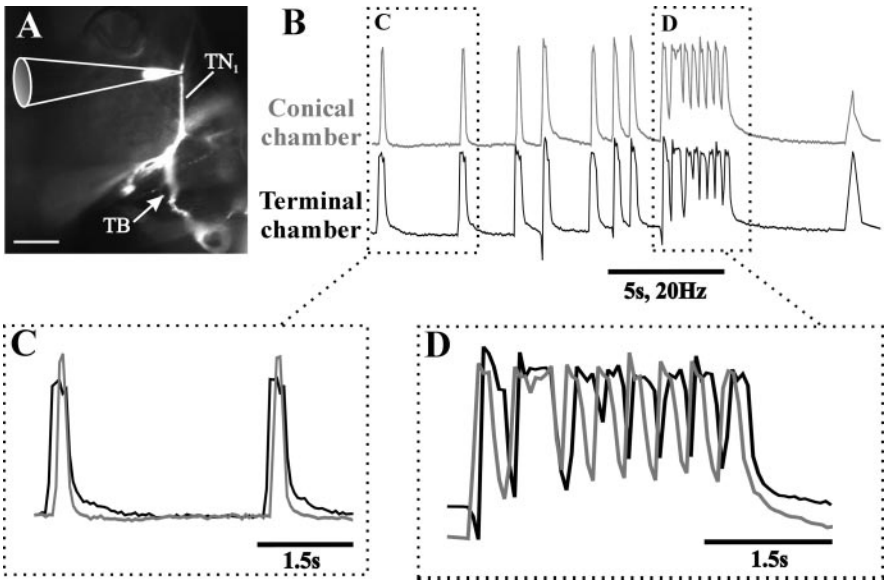

Figure 8. Effect of transverse nerve stimulation on heartbeat of *elav*-GAL4/UAS-GFP transgenic adult flies. *A*, Epifluorescence micrograph of a representative preparation of the conical chamber showing the location of the suction electrode on the transverse nerve (TN_1) terminating in the ventral longitudinal muscle layer with the TB (arrow). Scale bar, 50 μm. *B*, Simultaneous optical detection of the conical (gray trace) and terminal (black trace) cardiac chamber activity showing the effect of TN stimulation (5 sec; 20 Hz; 1 msec per 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. Note that TN stimulation advances the relative timing of the conical chamber beat.

There were, however, important differences between the results observed in semi-intact preparations and the heartbeat of the intact organism, suggesting that this mechanism alone is not sufficient to explain normal cardiac reversal. 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. This is analogous to what has been described in other holometabolous insects that show reversal (Dulcis et al., 2001; Dulcis and Levine, 2004). Perhaps in intact animals, in which neuronal activity 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 (Davis et al., 2001) receives inhibitory synaptic input that stops its activation of the anterograde pacemaker and allows the slower 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 potentiates the anterograde beat (D. Dulcis, R. Levine, and J. Ewer, unpublished observations). As in *Manduca*, the larval myogenic heart of *Drosophila* does 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 may require innervation to stop and/or reactivate the anterograde beat

Another factor is that the adult heart is composed of two separate muscle layers, a circular layer that is present in the larval stage and a ventral longitudinal layer that develops in the adult (Curtis et al., 1999; Molina et al., 2001). The ventral longitudinal muscle layer is well developed in the conical chamber but is absent in the caudal chamber, where the anterograde beat originates (Miller, 1950; Rizki, 1978). Glutamatergic innervation and glutamate receptors were found only in the ventral longitudinal muscle layer. The anterograde and the retrograde beats may travel along the two cardiac muscle layers independently if the two layers are not electrically coupled. It is not clear whether the relative activation of the two layers is altered in semi-intact preparations.

Finally, whereas the conical chamber is in diastole during the retrograde phase of cardiac activity in intact adults, bath-applied glutamate caused sustained contraction of the conical chamber while initiating the retrograde beat. This probably reflects differences between sustained bath application and the patterned glutamate release and more restricted access to targets that would occur during normal TN activity. In addition, although glutamate alone was sufficient for initiation of the retrograde beat, TN activity may cause the release of other neurotransmitters that have independent functions. The role of the glutamatergic and peptidergic (CCAP) innervation serving the second and third cardiac chamber is not known. One could hypothesize that each chamber requires innervation to potentiate and coordinate cardiac contractions occurring a different levels of the abdominal heart. To this aim, the pattern of activity of central (glutamatergic) and peripheral (peptidergic) segmental neurons, which is probably sculpted by sensory feedback loops, may be designed to sequentially activate adjacent cardiac chambers to produce a coordinated anterograde and retrograde wave of contraction.

Cardiac function in adult *Drosophila* needs to accommodate a variety of physiological conditions (for example, postfeeding vs 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.

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