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Short Communication

Direct innervation of the *Drosophila melanogaster* larval aorta

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ABSTRACT

The heart rate of larval *Drosophila* is modulated by various biogenic amines and peptides. The actions have always been assumed to be due to direct action on the heart since the larval heart was not known to be innervated. A recent study showed a difference in the sensitivity of the larval heart to serotonin when the CNS was ablated, thus suggesting a direct neural input. Here, we show that GFP tagged motor neurons and nerve terminals are present on the aortic region of the heart. Motor neuron cell bodies also exist outside the CNS. Transmission electron microscopy reveals the direct innervation in the aortic tissue. Thus, developmental and regulatory questions in this genetic model can now be addressed in relation to heart development and neural control.

The larval *Drosophila* heart tube is a model tissue for examining cardiac tissue development and differentiation as well as the biophysical nature of myogenic pacemaker properties (Ashton et al., 2001; He and Adler, 2001; Molina and Cripps, 2001; Ponzielli et al., 2002; Sláma and Farkaš, 2005; Wessells and Bodmer, 2004). The induction of selective gene transcription, some induced by hormones, is also of current interest in understanding the transformation from embryonic to larval to the adult heart (Bhalerao et al., 2005; Han and Olson, 2005). This developmental pattern change consists of degeneration in the caudal cardiac tissue in larvae and the development of predetermined aortic tissue for ostia formation which becomes cardiac in the adult (Molina and Cripps, 2001). As in mammals, cardiac function in adult flies is correlated with stress and age (Wessells and Bodmer, 2004); however, only a few studies have started to address this in larvae (Dasari and Cooper, 2005). The actions of biogenic amines (serotonin, octopamine) and catacholamines as well as peptides on the function of the heart have been extensively addressed in larvae, pupa and adult flies (Johnson et al., 1997, 2000, 2002; Nichols et al., 1999; Zornik et al., 1999). In the larvae, the actions of the hormones and applied substances have always been assumed to be direct on the heart tissue since the heart tube was not known to be neurally innervated.

A recent study sought to understand the physiological parameters on cardiac function with and without the intact CNS for neural regulation and documented that the action of serotonin on the heart rate is different without a CNS (Dasari and Cooper, in press). Thus, this current study was conducted to investigate whether neural processes are present on the aorta.

Since the *Drosophila* larval heart, also known as the dorsal vessel, is a continuous tube extending from the last abdominal segment to the cerebral hemispheres of the CNS, a logical location of a potential nerve track to the heart tube would occur close to the attachment of the vessel with the CNS. The heart is divided into an anterior aorta and a posterior heart (Rizki, 1978). There are a number of terminal ligaments of the aorta that are attached to the aorta close to the CNS by the pharyngeal region of the alimentary canal (Fig. 1). The neuronal processes of the GAL4/GFP line were visualized using a confocal "light" microscope. The nerves (or neuronal processes) innervating the aorta could not be detected without first labeling them or having GFP expressed. Thus, we made

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use of two *Drosophila* constructs that express GFP. One line expresses GFP in all motor, interneurons and sensory neurons (Bloomington, IN, stock 5146; P{w[+mW.hs]=GawB}elav[C155], P{w[+mC]=UAS-mCD8::GFP.L}LL4, P{ry[+t7.2]=hsFLP}1, w[*]), and the second line (Bloomington, IN, stock 6793; w[*]; P{w[+mC]=Cha-GAL4.7.4}19B P{w[+mC]=UAS-GFP.S65T}T2) expresses GFP in sensory neurons that produce acetylcholine as a neurotransmitter. Only in the line that expressed GFP in the motor neurons was a nerve observed from the CNS leading to the anterior of the aorta. Terminals were tracked to the first lymph node on the aorta (Fig. 1A). Five cell bodies, presumably motor neurons since they were not observed in the GFP line for acetylcholine containing neurons, were discovered in each of the tissue flanges from the aortic tube that attaches to the dorsal part of the cerebral lobes of the CNS. One cell body was seen in each preparation right in the middle, just ventral to, of the aortic tube at the same region where the flanges attach to the aorta. The nerve from the CNS branches both anterior and posterior when it reaches the aorta. Some of the terminals contain varicosities in the aorta as well as the terminal in the tissue flanges. These small swellings are analogous to varicosities observed at skeletal neuromuscular junctions. The terminals shown are for early 3rd instars. However, GFP-loaded terminals and cell bodies were observed in all three instars (1st, 2nd and early 3rds). In 1st and early 2nd instars, the cell bodies appeared to be in tighter clusters, making it difficult to distinguish individual stomata. The GFP was observed with a Leica confocal microscope with a 40× water dipping objective. All the GFP preparations were viewed in alive preparations with CO_2 gassed saline to stop contractions of the heart and body wall muscles. This procedure and the effects of CO_2 were previously described (Badre et al., 2005). The dissection time was 5 min, and the saline used to maintain the preparations was HL3 (Stewart et al., 1994).

In each preparation examined (n = 10), when ELAV-GFP expression was highly visible in the neural tissue, we observed the same expression for these neurons associated with the aorta. It should be noted that some preparations showed a very low level of GFP in all skeletal motor and sensory neurons. The reason for this is unknown, so we used only preparations which showed substantial GFP within the CNS or peripheral neurons. Larvae can be sorted readily by the strong GFP signal prior to dissection. The varied expression in larvae was only pertinent to the stock 5146, which expresses GFP pan neuronally. There are sensory neurons that do not produce ACh in *Drosophila* which then would not express GFP in the ACh-GFP line. As mentioned by Salvaterra and Kitamoto (2001), most if not all chemosensory, olfactory, chordotonal and auditory primary sensory neurons are cholinergic. The major types of primary sensory neurons which do not appear to be cholinergic are photoreceptor neurons in eyes and ocelli of

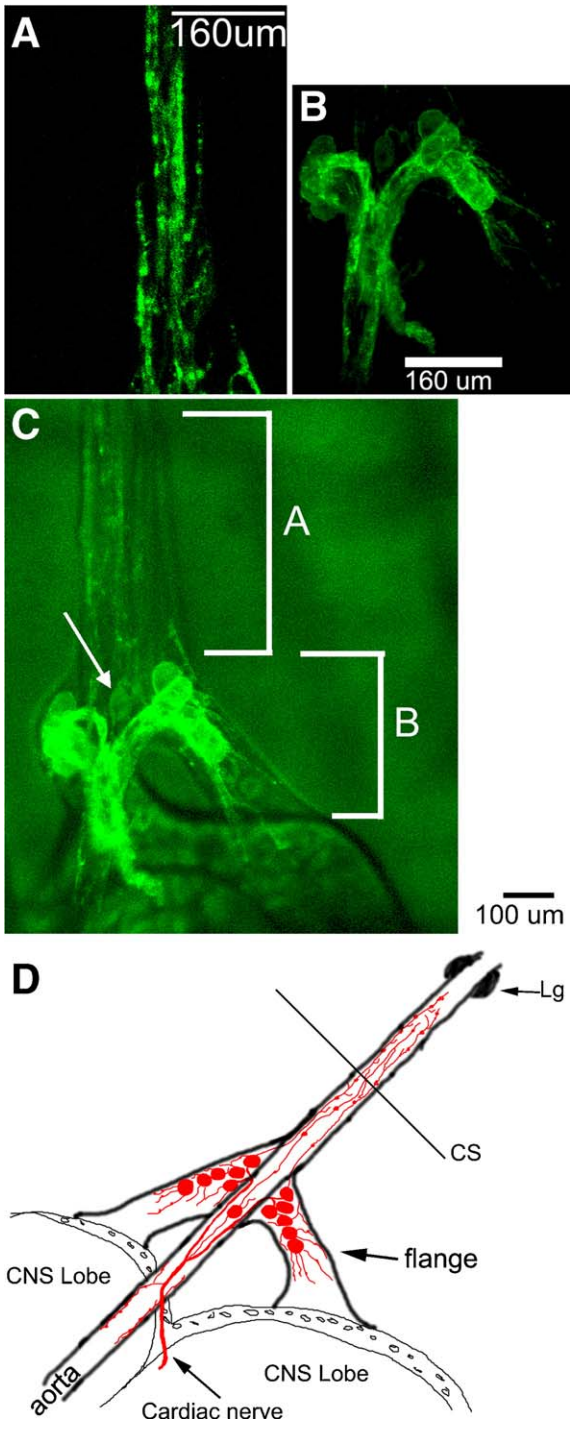

Fig. 1 – Innervation of the anterior end of the aorta. (A) Enlarged view of the GFP-loaded nerve terminals in the aorta caudal to the CNS. Note the multiple terminals on all sides of the aortic tube. (B) The 5 pairs of GFP-filled cell bodies are readily observed on one of the flanges that branches from the aorta to the connective tissue surrounding the cerebral lobes of the CNS. Note the nerve terminals within the tissue of the flanges. (C) Confocal image of the GFP-loaded neurons with the outline of the aorta and CNS. The lone cell body is seen just ventral to the aorta with terminals leading to and out of the cell (arrow is pointing to it). (D) Schematic of the aorta and innervation. The neural cells and terminals are in red. The terminals can be observed to proceed to the first pair of lymph glands (Lg). The location for the cross-section (CS) used for electron microscopy is approximated. The cardiac nerve leaves the CNS and once entering the aorta branches in both anterior and posterior directions.

adults. We examined also another GFP line. We used the Pickpocket (PPK)-GFP line. According to Adams et al. (1998), the PPK phenotype is associated with reduced function of sodium channels, and the protein is found in sensory dendrites of a subset of peripheral neurons in early larvae. The related sodium channel subunit is probably involved in mechanosensation. We did not see any GFP cell labeling in the same area related to the aorta as presented in our study. Nor did we observe any GFP labeling of the processes associated with the anterior part of the aorta where we observed putative motor nerve endings.

To determine if the nerve terminals are embedded within the aortic tissue and close to muscle, the tissue was processed for transmission electron microscopy by a standard procedure for invertebrate nerve terminals (Logsdon et al., 2005). The mid-region between the first lymph node and anterior end (Fig. 1D, at the line depicted as cross-section—CS) was sectioned on a Reichert Ultracut E microtome (~80 nm) and captured on slotted grids coated by 1% formvar, stained with uranyl acetate and lead citrate, then viewed on a Philips Tecnai 12 transmission electron microscope. A low magnification (1900×, Fig. 2A) of the entire cross-section of the aorta depicts a hollow tube with a thick endothelium (ranging from 4 to 10 μm). Histologically, higher magnification (18,500×) shows an area with many numerous terminals, with subsynaptic reticulum, directly innervating the *Drosophila* aorta (Fig. 2B), while even higher magnification reveals nerve terminals associated with vesicles (Fig. 2D, 98,000×). In addition to nerve terminal innervation, sarcomeres of striated muscle are visible at high magnification, with well-defined mitochondria to its periphery (Fig 2C, 18,500×).

To address the issue of whether synaptic varicosities similar to skeletal muscle appear, we used immunocytochemical staining. Anti-synaptotagmin staining revealed punctuate staining in the terminal regions, as observed for the terminals containing GFP (data not shown), which supports the electron microscopy results in the presence of synaptic vesicles (Fig. 2). The results imply that synaptic vesicle pools are present in these terminals. It is highly unlikely that these terminals are sensory. The procedures used for anti-synaptotagmin staining were as those previously described for crayfish and *Drosophila* skeletal NMJs (Cooper et al., 1995).

The origin of heartbeat in larvae is myogenic since even an excised heart and aorta continues to beat when removed from the animal (Dasari and Cooper, in press; Dowse et al., 1995; Johnson et al., 1997). The effect on heart rate in larvae by neurotransmitters and neuromodulators is of significant interest (Fowler et al., 1972; Gu and Singh, 1995; Johnson et al., 1997, 2000, 2002; Nichols et al., 1999; Zornik et al., 1999). Past studies were conducted primarily by injections into the hemolymph of intact larvae, which introduce agents to the heart as well as to the CNS. The larval CNS is very susceptible to actions of serotonin, octopamine and dopamine (Dasari and Cooper, 2004) and

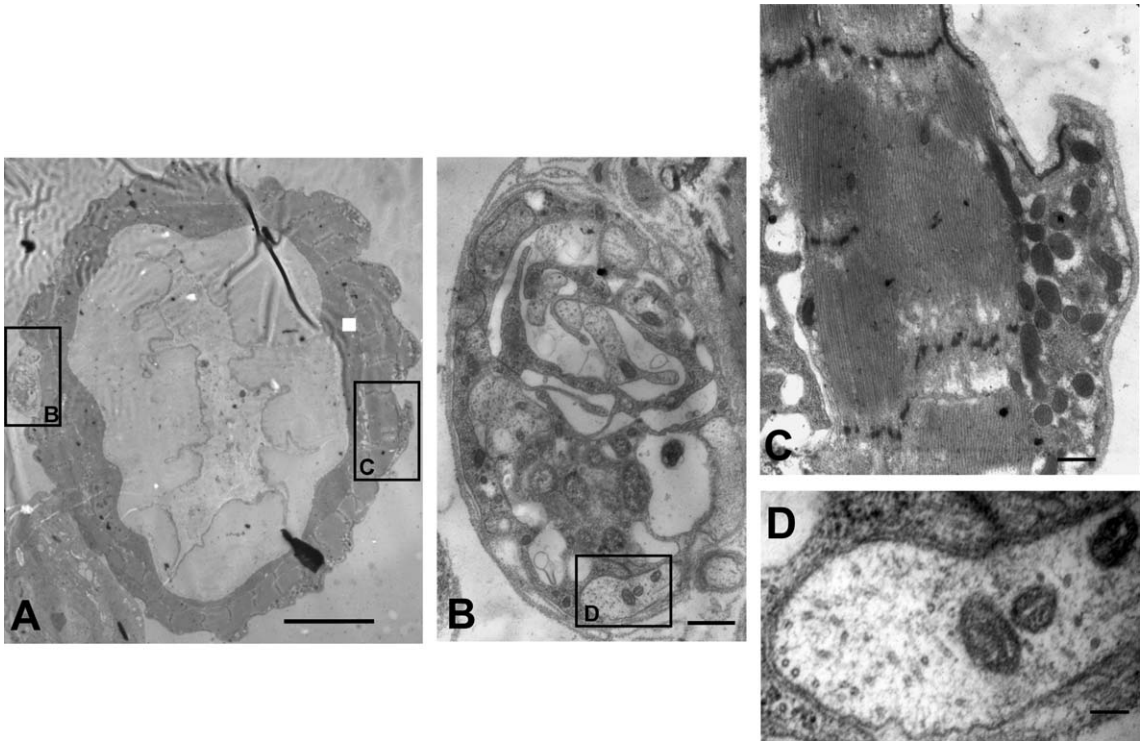

Fig. 2 – (A) Cross-section of the anterior end of the *Drosophila* aorta showing large layer of endothelium (scale bar = 10 μm, 1900× magnification). (B) Higher magnification of innervating nerve terminals (scale bar = 500 nm, 18,500× magnification). (C) High magnification of mitochondria within striated muscle (scale bar = 500 nm, 18,500× magnification). (D) High magnification of single nerve terminal from panel B, showing nerve terminal, mitochondria and synaptic vesicles (scale bar = 100 nm, 98,000× magnification).

thus could have a direct effect on the activity of the novel cardiac nerve we describe in this study. Since we have now shown neural innervation on the aorta and since a current study in which differences in the sensitivity to serotonin have been shown to occur with the nerve transected, it is plausible that the earlier compounds investigated need to be re-examined, with a focus of potential action on the neurally innervated heart vs. a deinnervated one, in attempts to determine direct actions on the heart. A recent study had noted also that a low pH of the bathing media or hemolymph can drastically increase heart rate (Badre et al., 2005) whereas dissolved carbon dioxide in the bathing media rapidly produces cardiac arrest with an intact or an ablated CNS (Badre et al., 2005). It would now be of interest to know if the CNS had a role in some of these actions.

The potential action of the neural innervation on the aorta can be wide ranging, from direct excitation for muscle contraction to altering pacemaker activity in the aorta. It is known that the caudal heart and the aorta can beat independently when transected at the valve region between the heart and aorta (Dasari and Cooper, in press). Furthermore, various studies have noted in intact preparations that the heart and aorta do not always beat in synchrony (Dasari and Cooper, in press). Thus, the innervation of the aorta could have an impact on aortic activity modulation by various neuromodulators. Since the electrical activity of the pacemaker and muscle along the length of the entire dorsal vessel may well be electrically coupled, the electrical activity in one region would have an impact on the overall electrical cable properties along the entire dorsal vessel. In adult *Drosophila*, retrograde contractions can be induced by neural activity of the anterior regions of the aorta (Dulcis and Levine, 2005). Even homodynamic differences in the vessel in the aortic region, from locally regulated contraction, could provide back-pressure on the heart and alter filling of the heart as in mammalian hearts. Possibly, contraction of the dorsal vessel anterior to the CNS as it proceeds to the head may regulate blood flow as needed to muscles associated with eating. This is not preposterous for invertebrates since crustaceans (Wachter and McMahon, 1996) as well as insects (Heinrich, 1976) are known to regulate blood flow to arterioles delivering hemolymph to various organs. Slama (2003) noted also that the heartbeat can reverse in the pupae of *Manduca sexta* possibly related to metabolic functions.

We feel that knowledge about the innervation of the aorta has opened new avenues of investigation into the regulation of the larval heart as well as into development of the aorta and the significance of neural input as currently addressed in the adult heart (Dulcis and Levine, 2003, 2005).

From this study, questions now arise: when does the aorta become functionally innervated-embryonic or later-instars? Is the larval innervation degraded at pupa stage or maintained for the neural-regulated adult aorta? What is the neurotransmitter in these terminals? Are there inhibitory as well as excitatory motor terminals? What are the 5 symmetrical cell bodies in the aortic flanges? If the cell bodies in the flanges are the motor neurons for the aorta, then to what does the cardiac nerve lead in the CNS?

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