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WHY THE OVOTESTIS OF *HELIX ASPERSA* IS INNERVATED*

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Although Schmalz described the innervation of the ovotestis in pulmonate snails as early as 1914, no functions have been attributed to it. In *H. aspersa,* the intestinal nerve branches profusely within the ovotestis and terminates in the walls of the acini and in the sheath surrounding the early portion of the hermaphroditic duct. We found both sensory and motor functions for this innervation. Significantly, there is a tonic sensory discharge generated by the mechanical pressure of growing oocytes, and the level of tonic afferent activity is strongly correlated with the number of ripe oocytes; this is probably a permissive signal that gates ovulation.

Tactile stimulation of the ovotestis causes a phasic sensory discharge and a pronounced cardio activation. Also, an efferent discharge is elicited in the ovotestis branch of the intestinal nerve. To study the motor consequences of efferent activity, the ovotestis branch was electrically stimulated. We found that such stimulation evokes peristaltic contractions of the initial portion of the hermaphroditic duct and increases beat frequencies of the cilia that line the interior of the duct. These effects could facilitate the transport of oocytes down the duct. Still other functions of afferent activity are implied by changes in the spontaneous activity of mesocerebral cells following nerve stimulation.

Putative sensory neurons and putative motoneurons have been identified in the visceral and right parietal ganglia.

Keywords: Ovotestis – ovulation – oviposition – oocytes – snails

INTRODUCTION

The gonad is not usually considered as either a source of sensory signals or as a target for motoneurons. Nevertheless, the ovary is innervated in most, if not all, species of mammals and in some species of reptiles. Although the function of innervation in these animals is far from clear, most of the evidence points to a motor function in releasing steroid hormones [1]. Studies of the ovary in bivalve molluscs have revealed that innervation may have a role in triggering ovulation, possibly through a serotonergic mechanism [8]. Other functions have also been proposed, including the modulation of oocyte maturation.

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The gonad of pulmonate snails is also innervated, at least in the genera *Lymnaea* [5] and *Helix* [9]. Although the gonad is hermaphroditic in these species, it is reasonable to expect that innervation serves only the female function, since sperm leave the ovotestis (OT) as soon as they mature. In any case, our studies have focused on the processing of oocytes. The motor control of ovulation is an obvious possible role for innervation, but our observations of egg laying suggested a sensory role as well. In the laboratory, as well as in nature, *Helix aspersa* lays eggs infrequently (about twice a year). Moreover, when snails from our laboratory colony were dissected and the oocytes in the ovotestes were examined, we found that very few animals were carrying enough mature oocytes to lay a normal clutch of fertilized eggs. These observations led us to hypothesize that one function of the nerve branch innervating the OT is to monitor the availability of ripe oocytes.

MATERIALS AND METHODS

Adult specimens (curled lips) of *Helix aspersa* were obtained from natural sites in California. The morphology of the innervation was examined after labeling the OT nerve branch with 8% Neurobiotin (Vector Labs) dissolved in 0.1 M tris buffer (pH 7.6); histochemistry was performed using the Vectastain ABC kit (Vector Labs).

Electrophysiological experiments were conducted *in vitro* using reduced preparations (for details, see ref. 2). In some cases, to identify serotonergic neurons, snails were treated with 5,7-dihydroxytryptamine (5,7-DHT) 30 days prior to use [11]. To distinguish afferent and efferent spikes in the OT nerve branch, two glass suction electrodes were placed side-by-side along the branch. The direction of spike conduction was then determined by measuring the timing of individual unit waveforms at the two electrode sites. To identify central neurons responsive to OT nerve branch electrical stimulation, a total of 68 cells was surveyed. Antidromic responses were distinguished from orthodromic responses by hyperpolarizing the soma and by recording simultaneously from the soma and the nerve. The cell numbering scheme is that of Kerkut et al. [6]. Cardiac activity was monitored using either a photocell placed over the auricle or a pressure probe placed inside the anterior aorta.

Observations of motor effects following nerve stimulation were conducted using isolated OT preparations. Muscle contractions were measured from digitized video images captured from a compound microscope. Measurements of ciliary beat frequencies were obtained from images displayed on a video monitor. A cadmium photocell, placed in front of the monitor, detected changes in light caused by the synchronized beating of cilia. The resulting waveforms were digitized and their periods were measured.

To count oocytes, the contents of the OT were removed to a shallow glass dish and viewed under a dissecting microscope at ×20 magnification. Only mature oocytes (>100 μm diameter) are reported here.

RESULTS

Morphology

From Figure 1 it can be seen that the OT lies embedded in the digestive gland. The oocytes develop while attached to the walls of the acini. After release from this attachment, they travel through small ductules and then exit the OT via the hermaphroditic duct (Figs 1A, 1B). We found that the OT branch of the intestinal nerve has two types of terminations within the OT. As shown in Fig. 1B, fine terminal branches are seen scattered among the acini and the ductules, and some of these fibers can be seen innervating the walls of the acini (Fig. 1C). We think that these are sensory endings. In addition, the wall of the hermaphroditic duct is richly innervated by a plexus of fine fibers at a site just proximal to its exit from the OT (not shown in Fig. 1); the fibers are studded with varicosities. From their appearance, we assume that these latter fibers are motor terminals.

When examined in cross-section with the electron microscope, the OT nerve branch was found to have a diameter of only 20 μm, but to contain 3025 axon profiles. The majority of these profiles (57%) are <0.2 μm in diameter, and none exceeds 2.1 μm in diameter.

Sensory functions

Centrally conducted action potentials in the OT nerve branch were either tonically (spontaneously) active, or phasic following tactile stimulation. The tonic activity was relatively minor in absolute quantity (≤ 80 spikes/min) but significant in that it was strongly correlated with the number of ripe oocytes present in the OT of the same animal (Fig. 2). Furthermore, when individual snails were compared, there was a striking increase in afferent activity in animals that possessed a sufficient number of mature oocytes to produce a clutch of normal size (mean, 87 eggs). Such snails tended to be individuals that were selected for study after they were observed excavating nests in the soil (designated 'diggers' in Fig. 2). We propose that the tonic sensory signal is permissive for egg laying in that it signals the availability of a minimum number of ripe oocytes.

As oocytes grow within the OT, they increase about 10-fold in diameter and about 1000-fold in volume. The diameter of the largest oocytes (250 μm) is greater than the diameter of a typical acinus (about 170 μm). To test whether the nerve branch innervating the OT might be sensitive to the mechanical consequences of oocyte growth, we artificially inflated the OT by the controlled injection of saline. To do this, we severed the hermaphroditic duct and inserted a cannula into the proximal stump. As shown in Figure 3, the afferent discharge increased as progressively larger volumes of saline were injected into the OT. It is therefore reasonable to conclude that the growing oocytes provide a continuous mechanical signal that is detected by sensory nerve endings.

Separate from the tonic afferent activity described above, we also noted a pronounced increase in afferent activity following tactile stimulation of the surface of the OT. Whereas brief, punctate stimuli were ineffective, a 3 s sweep of the OT surface with the blunt end of a wooden applicator stick elicited a strong discharge that continued for about 30 s after the cessation of the stimulus.

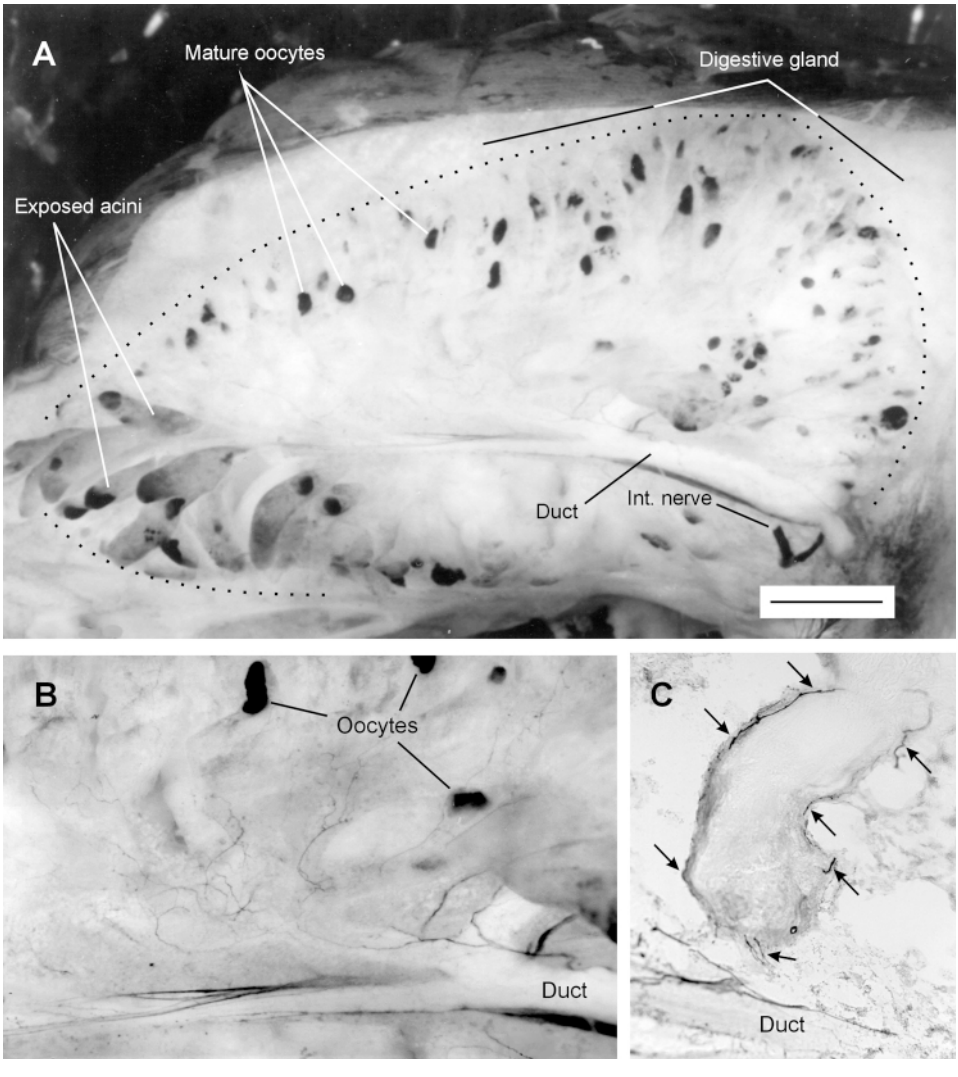

Fig. 1. Innervation of the ovotestis. A) Wholemount showing the ovotestis (delineated by the dotted line) embedded in the digestive gland. The nerve stump has been labeled with Neurobiotin, which also reacts nonspecifically with mature oocytes. B) Magnification of A showing fine terminal branching of the nerve in the region of the acini and small ductules. C) Frozen section (20 µm) from a different preparation showing innervation (arrows) of an acinus wall. Scale bar: A, 1 mm; B, 550 µm; C, 170 µm (Reproduced from [2])

Six putative sensory neurons were identified in the visceral ganglion (Fig. 4). These are all small cells (≤ 50 μm), and they all respond antidromically to electrical stimulation of the OT nerve branch.

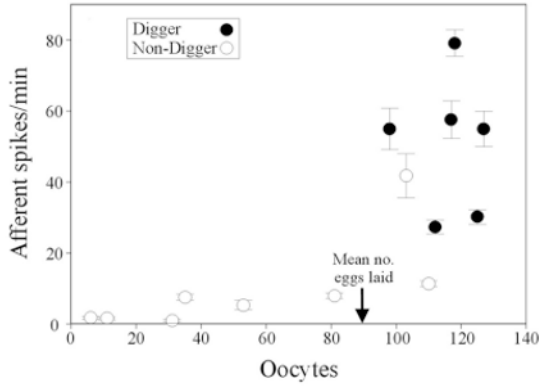

Fig. 2. Afferent activity in the ovotestis nerve branch correlates with the number of ripe oocytes in the ovotestis. Data points are means ± SEM for 5 randomly selected periods of 1 min duration. Diggers are snails that were observed excavating nests within 5 days prior to the recordings. Deposited eggs were counted in our laboratory (mean = 86.9 ± 2.7, N = 104) (Reproduced from [2])

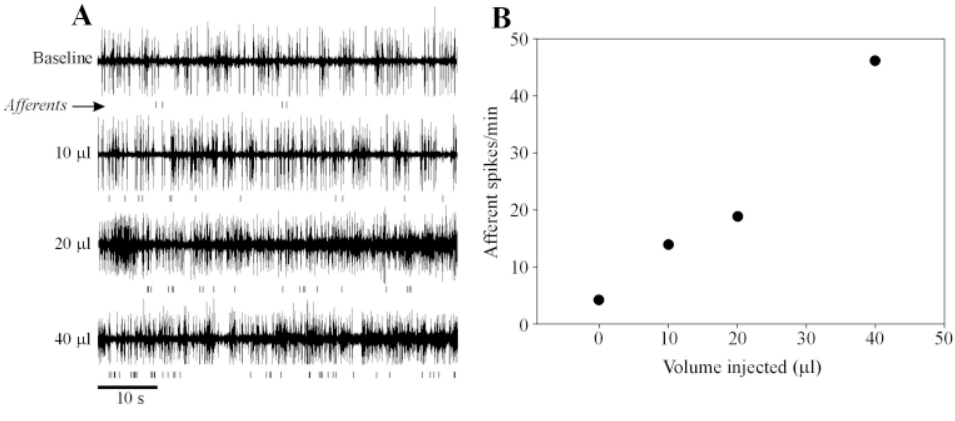

Fig. 3. Artificial expansion of the ovotestis by saline injection causes an increase in afferent activity. A) Traces show whole nerve activity 10 s after the indicated levels of ovotestis inflation. Afferent spikes are indicated in the raster plots. B) Quantification based on traces shown in A (Reproduced from [2])

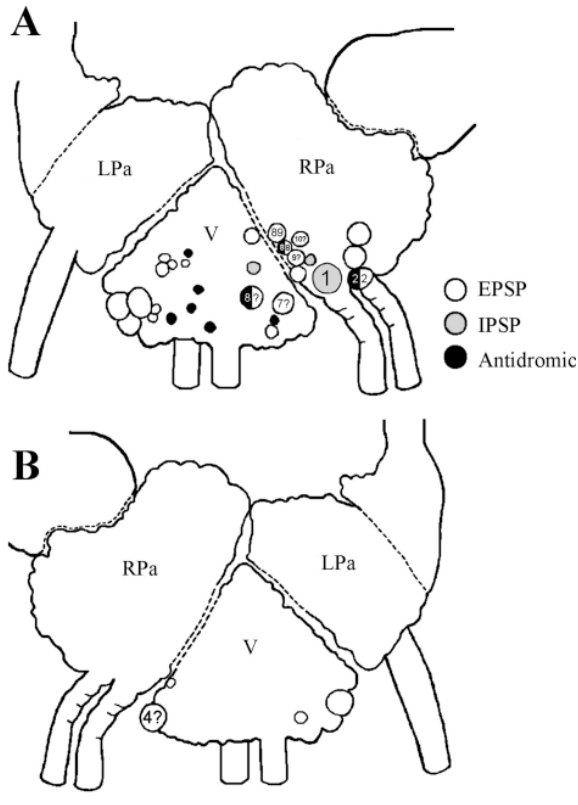

Fig. 4. Map of neurons responding to electrical stimulation of the ovotestis branch of the intestinal nerve. A) Dorsal surface. B) Ventral surface. Ganglion outlines and cell numbers are taken from [6]; question marks indicate uncertain identifications. Cells responding antidromically are either putative sensory neurons (small cells) or putative motoneurons (large cells)

Motor functions

When the OT nerve branch is electrically stimulated (2 ms pulses at 5 Hz for 5 s), two effects are observed in the hermaphroditic duct close to the site where the nerve branch provides a varicose innervation (see above). First to appear is a radial contraction of the duct wall at a location just proximal to the exit of the duct from the OT. The maximum reduction of the radial diameter is about 12%. By timing the contractions at two points separated by 150 μm along the length of the duct, it was determined that they consistently occurred approximately 1.5 s earlier at the proximal site than at the distal site, i.e. they are peristaltic contractions. Following these contractions, a second effect is observed, namely an increase in the beat frequency of the cilia that line the interior of the duct. Measurements of time-dependent beat frequencies revealed a robust increase from a mean of 6Hz before stimulation to a maximum

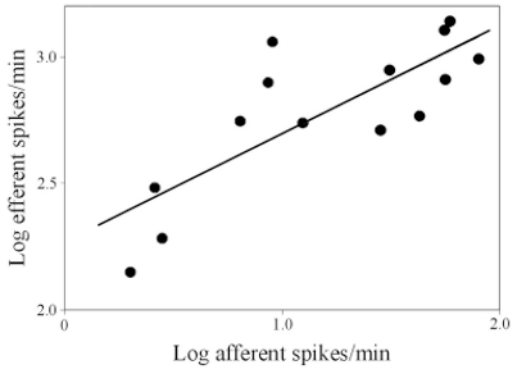

Fig. 5. Log transformed data showing the relationship between afferent spike rates and efferent spike rates in the ovotestis nerve branch ($r^2 = 0.812$, $P < 0.001$) (Reproduced from [2])

of 8.6Hz during stimulation, with a subsequent return to baseline. Both the peristaltic contractions of the duct wall and the increase in beat frequency of the interior cilia are consistent with the view that efferent activity in the OT branch of the intestinal nerve promotes the transport of oocytes out of the OT during ovulation.

Whereas the motor phenomena described above were transiently observed following brief periods of nerve stimulation, a correlation between the levels of tonic afferent activity and tonic efferent activity (Fig. 5) suggests a continuous regulation of OT processes. We suppose that, as the developing oocytes generate increasing levels of tonic afferent activity (Fig. 2), a centrally mediated reflex returns a tonic motor signal to the OT. This could activate the same mechanisms discussed in the previous paragraph, namely radial contractions of the hermaphroditic duct and ciliary excitation, or it could provide a chemical stimulus to promote oocyte maturation, as suggested for bivalves [8].

Three putative motoneurons were identified in the visceral and right parietal ganglia close to the septal junction between the ganglia (Fig. 4). Electrical stimulation of the OT nerve branch evokes combined orthodromic and antidromic responses in these cells. This response profile contrasts with that of the putative sensory neurons, which shows pure antidromic responses; also, the putative motoneurons are larger than the putative sensory neurons. It is noteworthy that two of the putative motoneurons (cell 8 in the visceral ganglion and cell 88 in the right parietal ganglion) stained positively for serotonin.

Afferent modulation of central nervous activity and cardiac function

Individual neurons were surveyed in the cerebral and sub-esophageal ganglia for responses to OT nerve branch stimulation. In the mesocerebrum, 15 neurons were tested. Of these, 8 showed no effect of stimulation, 5 were inhibited and 2 were excit-

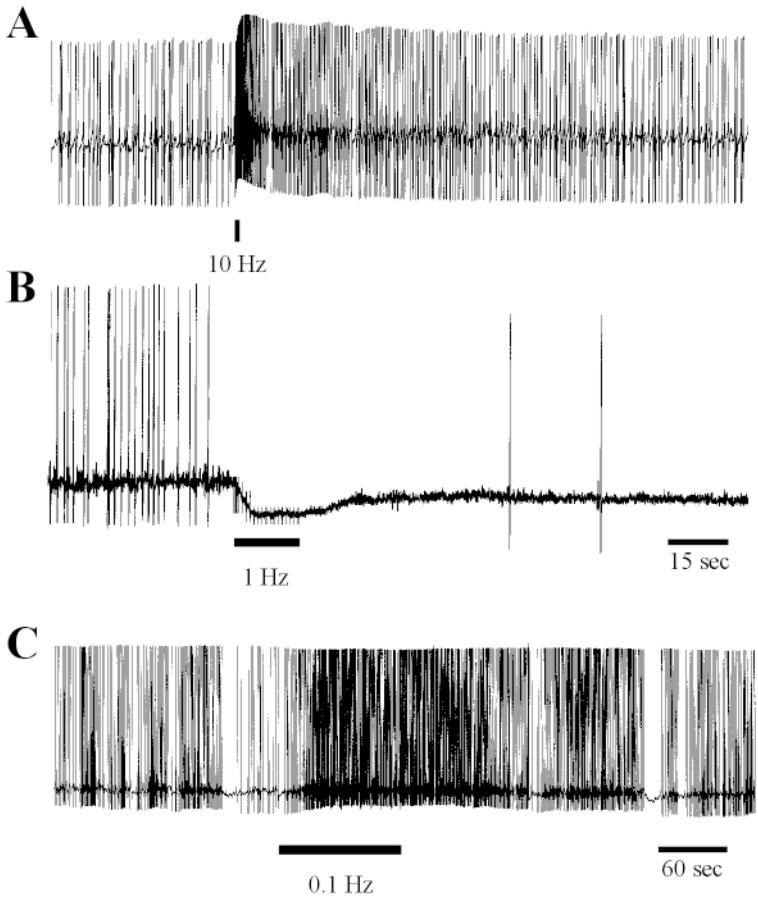

Fig. 6. Three examples (A, B, C) of mesocerebral cells responding to electrical stimulation of the ovotestis nerve branch. Stimulation rates and duration vary in each case. In total, 7 of 15 mesocerebral cells were affected by ovotestis nerve branch stimulation

ed. In no cases were discrete postsynaptic potentials observed in response to stimulation. Rather, as illustrated in Figure 6, responses were characterized by long-duration changes in spontaneous activity accompanied by shifts in the membrane potential. Since the mesocerebrum is an executive center for male mating behaviors [4] and possibly other reproductive functions [3], the present results suggest that mesocerebral commands are influenced by feedback or feedforward signals carrying information about the availability of gametes.

As already mentioned, tactile stimulation of the OT evokes a strong sensory response in the peripheral nerve branch. We have also observed that the same stimulus excites the heart (Fig. 7). The dominant effect of OT stimulation is an increase in beat amplitude, although small increases in beat frequency were also noted in some

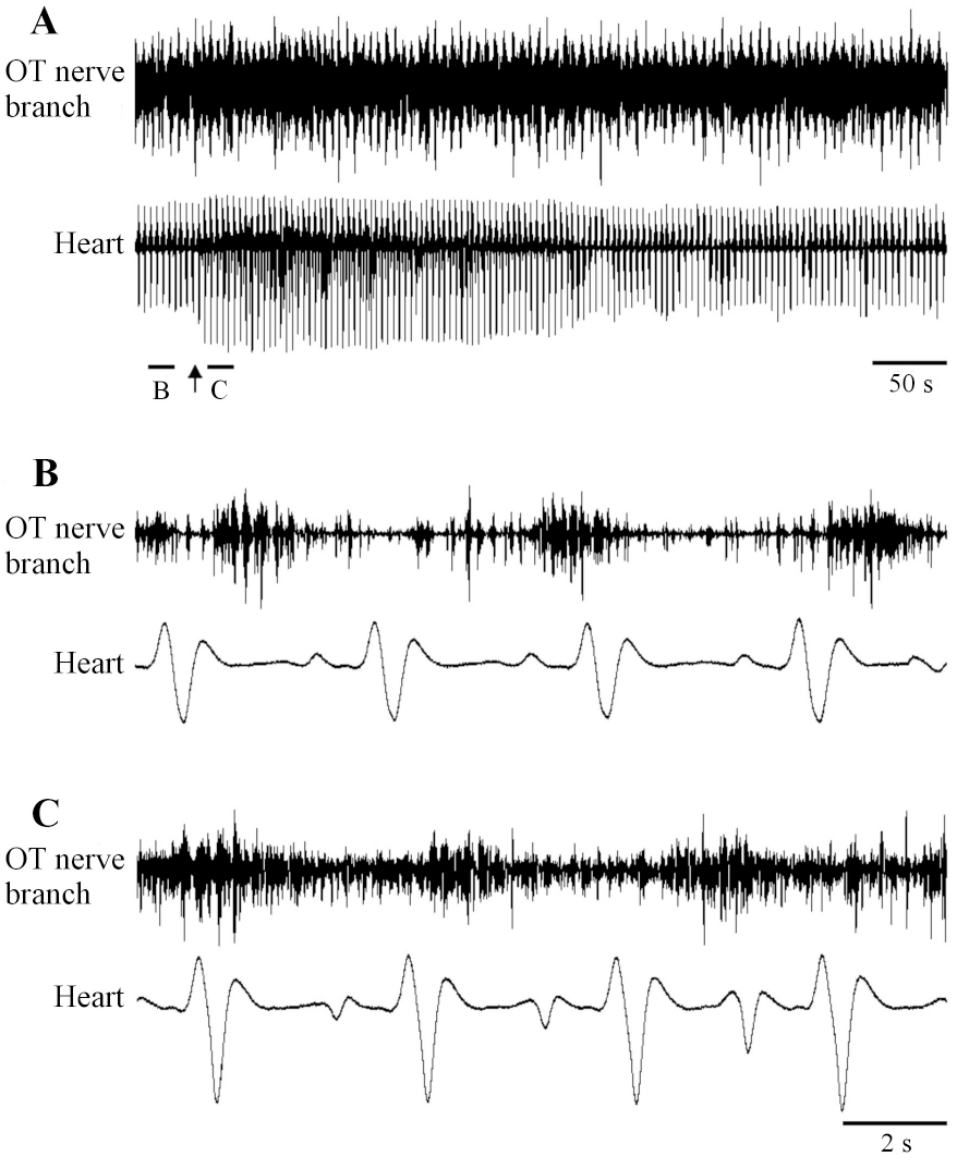

Fig. 7. Tactile stimulation of the ovotestis excites cardiac activity. A) Traces shown in a compressed time scale. Note that the extracellular nerve recording contains both afferent and efferent spikes. The arrow beneath the heart trace indicates the timing of a light brush stroke across the surface of the ovotestis. B) and C) Expanded views of recordings obtained immediately before and after tactile stimulation. Note that stimulation induced an increase in the number of small, presumably afferent, action potentials and a concomitant increase in heart beat amplitude

preparations. Correlated in time with the cardiac response, efferent activity increased significantly in the pericardial branch of the intestinal nerve upon tactile stimulation of the OT [2]. We identified 3 heart excitatory cells in the sub-esophageal ganglia, all of which are serotonergic based on 5,7-DHT staining. Two of these neurons, cell 89 in the right parietal ganglion and putative cell 7 in the visceral ganglion, are excited by OT nerve branch stimulation (Fig. 4). These data are consistent with the idea that ovulation triggers a sensory signal that travels centrally and excites the identified cardiac motoneurons. The increased firing of the motoneurons aids in meeting the metabolic demands of ovulation and oviposition.

DISCUSSION

Our results indicate a significant role for gonadal innervation in ovulation and oviposition. Additional roles relating to other reproductive functions are also suggested by the data. Notably, we have recorded both sensory and motor signals in the OT nerve branch. Some sensory responses are tonic whereas others are phasic. The tonic activity is particularly interesting because of its close association with the number of mature oocytes held in the OT. Egg laying is accomplished at a high metabolic cost, most of which is fixed regardless of the number of eggs deposited. This is because the snail works continuously for approximately 24 h digging its nest and expelling the eggs. Clearly, it would not be in the snail's interest to expend so much energy to deposit just a few eggs. We assume that snails balance the costs of egg laying against the reproductive benefits to arrive at an optimal clutch size. The tonic afferent activity in the nerve branch may inform the CNS when the minimum number of oocytes is available for fertilization. This would be one among many pieces of information necessary, but not alone sufficient, to initiate the final common command for ovulation and oviposition.

Efferent activity in the OT nerve branch is responsible for peristaltic contractions of the hermaphroditic duct and increases in ciliary beat frequencies, both of which should aid in the transport of oocytes from the OT to the fertilization pouch. In addition, we detected a high level of tonic efferent traffic in the nerve branch, the function of which is not yet apparent.

In addition to the direct afferent and efferent messages conducted in the OT nerve branch, the sensory signals affect central neural processes. We found numerous cells in the visceral and right parietal ganglia that are synaptically excited by OT nerve branch stimulation. Also, we documented a cardiac reflex and changes in the spontaneous electrical activity of mesocerebral neurons. In earlier publications, S.-Rózsa (ref. 10 and others cited therein) reported that tactile stimulation of the OT causes inhibition of the heart. We, however, consistently observed cardiac excitation. The difference in these results may be due to the methods used to record heart activity. We saw similar excitatory responses whether using a photocell or a pressure transducer, both of which are non-invasive devices. Other the other hand, S.-Rózsa used

a force transducer. It is possible that the mechanical hook-up of the force transducer caused the heart to behave in an abnormal manner after OT stimulation.

The putative sensory neurons and putative motoneurons that we identified should be studied more thoroughly. However, there may be more as yet unidentified central neurons innervating the OT because, remarkably, the nerve branch contains 3,025 axons. Even if many of these fibers belong to peripheral neurons or are branched from a smaller number of parent axons [7], the total number of central neurons may still be considerable.

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