1988

Studies of the Neural Basis of Evasive Flight Behavior in Response to Acoustic Stimulation in *Heliothis zea* (Lepidoptera: Noctuidae): Organization of the Tympanic Nerves

H. R. Agee
Insect Attractants, Behavior, and Basic Biology Research Laboratory, USDA-ARS, Gainesville, Florida 32604

E. Orona
Insect Attractants, Behavior, and Basic Biology Research Laboratory, USDA-ARS, Gainesville, Florida 32604

Studies of the Neural Basis of Evasive Flight Behavior in Response to Acoustic Stimulation in *Heliothis zea* (Lepidoptera: Noctuidae): Organization of the Tympanic Nerves

H. R. AGEE AND E. ORONA

Insect Attractants, Behavior, and Basic Biology Research Laboratory,
USDA–ARS, Gainesville, Florida 32604

Ann. Entomol. Soc. Am. 81(6): 977–985 (1988)

ABSTRACT The organization of the tympanic nerve within the thoracic ganglia of *Heliothis zea* (Boddie) was investigated. Cobalt chloride infiltration of cut axons was used to investigate the central terminations of the tympanic nerves. The axonal terminals of the A2 acoustic cell were confined to the meso-metathoracic ganglia, whereas the A1 acoustic and the nonacoustic B cell were found in the thoracic ganglia. The relevance of this organization for neural circuitry of evasive flight behavior to acoustic stimulation is discussed.

KEY WORDS Insecta, auditory processing, noctuid moths, neural circuits

THE AUDITORY SYSTEM of noctuid moths is especially amenable to experimental investigations because of its inherent simplicity and accessibility. Therefore, it serves well as a model system for understanding sensory processing in general, and particularly for studying the neural basis of evasive flight behaviors in insects. Each ear has only a single pair of acoustic cells, the A1 and A2 cells, as well as a nonacoustic B cell. The acoustic cells are especially sensitive to ultrasound (10–100 kHz) and have response spectra nearly identical, except that the A1 cell is about 20 dB more sensitive to sound pressure level at threshold (Agee 1967, Roeder 1975, Surlykke & Miller 1982). Our recent research at this laboratory has focused on the neural circuitry within the central nervous system (CNS) of the noctuid moth *Heliothis zea* (Boddie), known commonly as the corn earworm or bollworm moth (Agee 1985, Orona & Agee 1988).

The objective of this research on the auditory system was to understand better the axonal terminations and neural circuits of the acoustic nerves, so that areas within the ganglia of potential synaptic interaction with motoneurons could be identified. Understanding the neural coding within a simple sensory system should reveal general principles applicable to more complex systems. Our objective is to discover and understand the circuitry from the sensory inputs to the motoneurons that generate evasive flight behaviors, as has been attempted for the organization of auditory and motor circuits in locusts (Kien & Altman 1984, Boyan 1985, Boyan & Altman 1985, Reichert & Rowell 1986).

Materials and Methods

Adult male and female *H. zea* (3–4 d old) were reared in this laboratory. Tympanic nerves were infiltrated with a variation of the procedure of Tyrer & Altman (1974). The thoracic ganglia were exposed by dissecting from the venter. They were immersed in vivo in a drop of Carlson's saline until the ganglia and nerves were dissected free. Careful dissection allowed access to the penultimate branch of nerve IIIN1B (nomenclature of Eaton 1974), which includes the acoustic nerves and those bearing motoneurons to some of the dorsal longitudinal muscles. Nerve trunks to be filled were left at their maximal lengths, and all others were cut proximal to the ganglia.

Cobalt infiltration was performed in vitro on nerve IIIN1B. Occasionally, we could infiltrate either of the two branches (acoustic or motor) separately. The excised ganglia were placed in a drop of saline on a microscope slide, and the attached nerve was lifted into an adjacent drop of 1% cobalt chloride. The two drops were separated and surrounded by walls of petroleum jelly. The preparations were kept covered and refrigerated at 4°C for 18–24 h before reaction.

All specimens (n = 50), regardless of the infiltration method, were reacted in the following manner. The cobalt was precipitated in fresh ammonium sulfide (2 drops of 22% sulfide per ml of saline) for 10 min. The tissue was then fixed for at least 30 min in alcoholic Bouin's solution. The preparations were intensified using the modified Timm's

Mention of a commercial or proprietary product does not constitute an endorsement by the USDA.

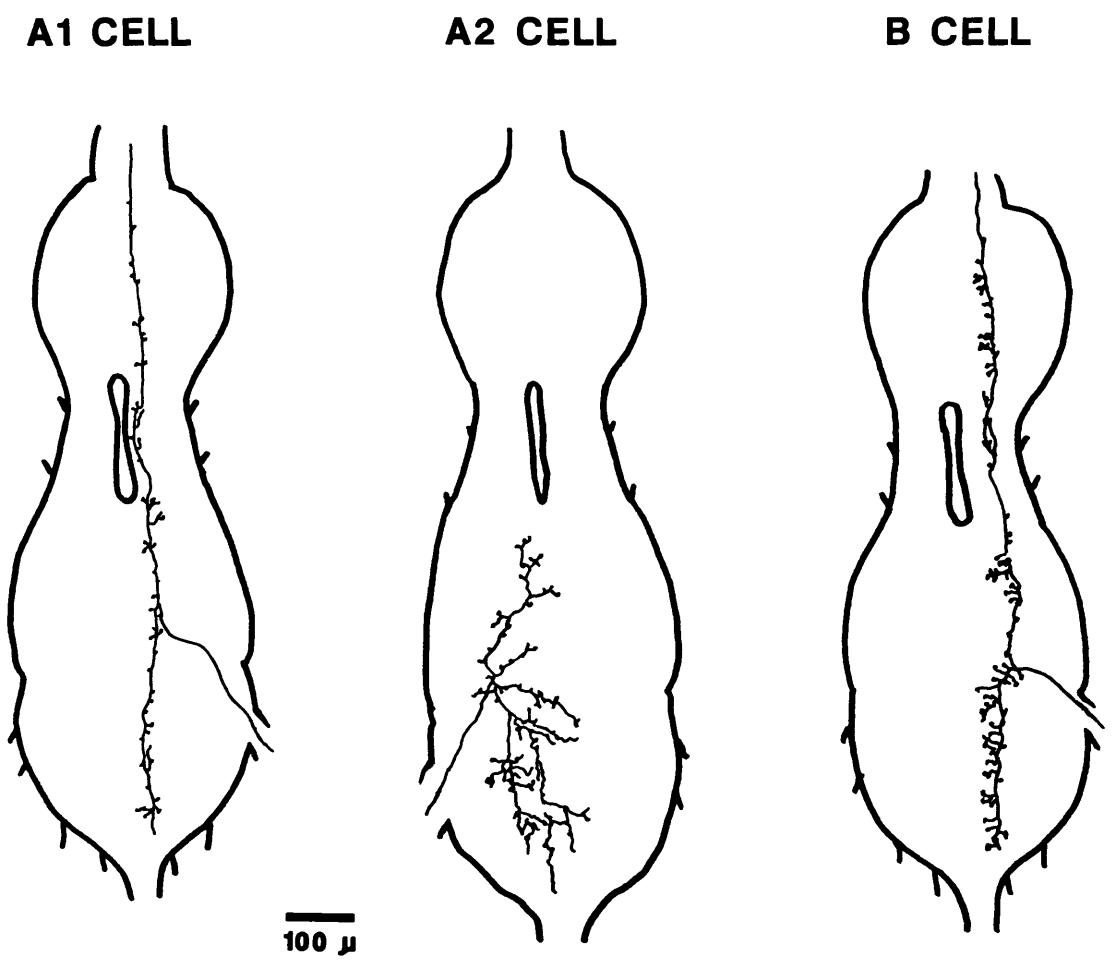


Fig. 1. Representative camera lucida reconstructions of the cells labeled following cobalt infiltration of the tympanic nerves. The A1 and B cells have synaptic terminals scattered throughout the thoracic ganglia. The axonal terminations of the A2 cell are confined to the meso-metathoracic ganglia.

method of Bacon & Altman (1977). Preincubation was usually at least 30 min, with the silvering step lasting between 15 and 45 min. Those specimens left as whole mounts were cleared in methyl salicylate, and then cover slipped on slides with Permount. Those specimens intended for paraffin embedding were cleared through a methyl benzoate–benzene sequence. Paraffin specimens were sectioned on a rotary microtome at a thickness of 10 or 15 mm cross and longitudinal sections.

The thoracic cavity and ganglia of some moths were stained with either methylene blue or hematoxylin-eosin stain so that branching of the different nerves and labeling of different tissues and structures could be studied with a light microscope as whole mounts and sectioned material.

Whole mounts and paraffin specimens of cobalt-infiltrated material were photographed with an Olympus OM-4 camera and microscope system, using Kodak Technical Pan 2415 film (at ASA 25) and Technidol developer. Camera lucida drawings were made at a 250 or 500× final magnification.

Voucher specimens will be deposited with the Department of Entomology and Nematology, University of Florida, Gainesville.

Results

The cobalt labeling allowed us to trace the morphology of the tympanic nerve specimens throughout the thoracic ganglia. In *H. zea*, the thoracic ganglia include an anteriorly located prothoracic ganglion and the more posterior pterygothoracic ganglia (Fig. 1). The tympanic nerve is a branch of nerve IIIN1B, which enters the anterior metathoracic ganglion dorsally. Each of the three cells (A1, A2, B) within the tympanic nerve appeared to have an anterior and posterior component within the pterygothoracic ganglia. The central branching patterns of the nerves were essentially similar, each remaining ipsilateral to the infiltrated side. Only in a few instances did any of the labeled processes appear to cross the midline.

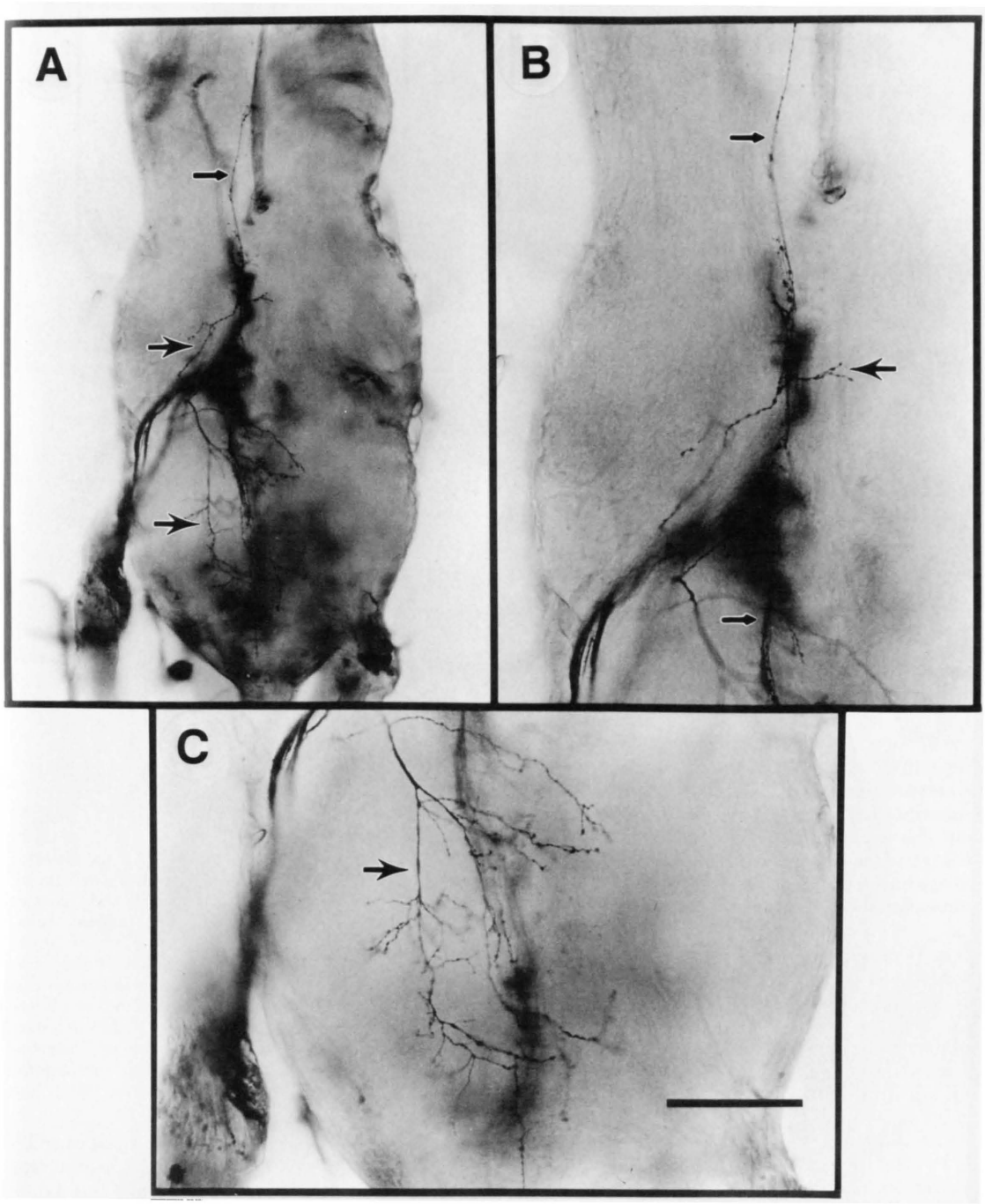
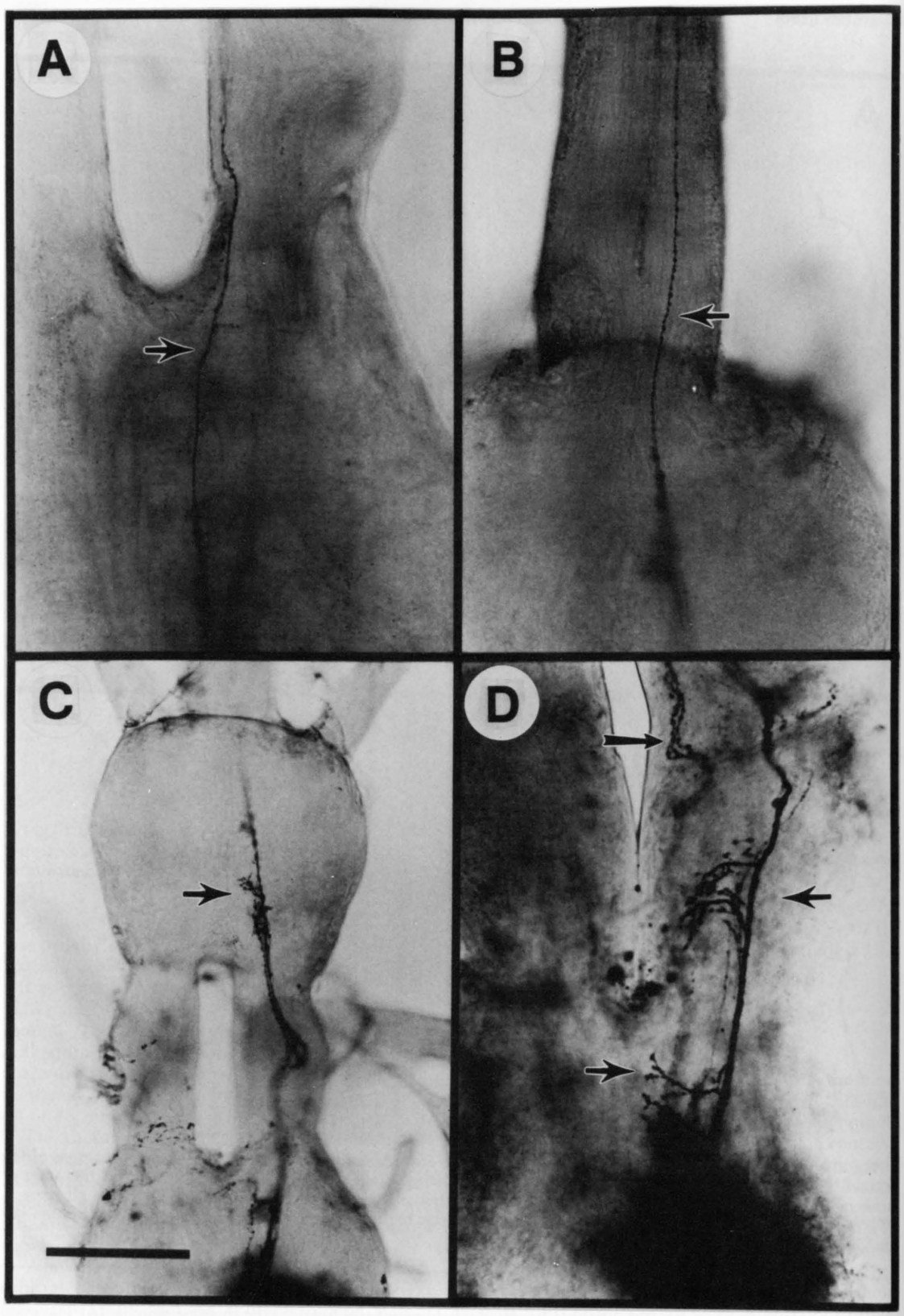


Fig. 2. Photomicrographs of the A2 and A1 cells from a wholemount preparation. (A) The arborizations of the A2 cell (large arrows) are confined to the meso-metathoracic ganglia. (B) Higher magnification of the anterior branches of the A2 cell within the mesothoracic ganglion, showing their close proximity to the A1 cell (small arrows). (C) View of the terminals of the A2 cell within the posterior region of the metathoracic ganglion. Calibration bar for (A) 200 μm; (B, C) 100 μm.

→

Fig. 3. Photomicrographs of the A1 and B cells in wholemount specimens of the thoracic ganglia. (A) The main axon of the A1 cell (arrow) occupies the most medial position within the ganglia compared with the more lateral position of the B cell in panel D. (B) The A1 cells have axons that leave the prothoracic ganglion, and ascend anteriorly into the cervical connective to the brain. (C) The B cell, like the A1 cell, has processes that could be



synaptic arborizations within the prothoracic ganglion. (D) Two sets of axonal terminals (short arrows) of the B cell are present in the anterior mesothoracic ganglia. Notice the more medial position (long arrow) of the A1 cells. Calibration bar for (A, B, D) 100 μm; (C) 200 μm.

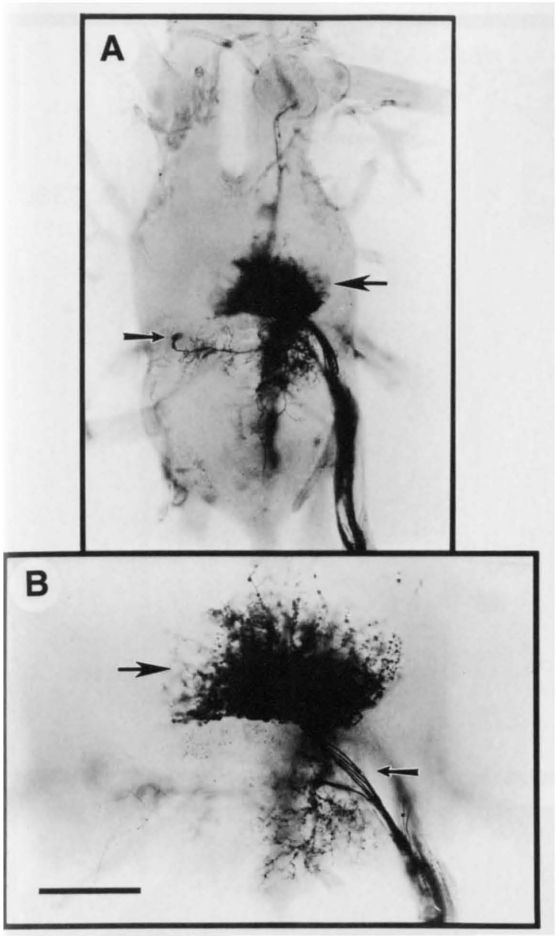


Fig. 4. Photomicrographs of the cobalt labeling of motoneurons following infiltration of nerve IIIN1B. (A) The motoneurons labeled (ipsilateral to the infiltrated nerve right side) have dendritic fields (large arrow) that occupy a considerable portion of the posterior region of the mesothoracic ganglion. A single contralateral cell body (small arrow) is found near the fusion of meso- with the metathoracic ganglion. (B) Higher magnification of the motoneuronal dendritic plexus (large arrow) located dorsally. The cell bodies (small arrow) are located ventrally (not shown) as in Fig. 5. The five axons to the motoneurons can be observed (arrow). Calibration bar for (A) 200 μm; (B) 100 μm.

The axonal terminations of the A2 cell were the most unusual; they differed greatly from those of the A1 and B cells (Fig. 1 and 2). All branching of the A2 cell was confined exclusively to the pterygothoracic ganglia. The A2 cell axon (about 3–4 μm in diameter) also had an anterior and a posterior component (Fig. 2B and C). The anterior branch was smaller and terminated in the central portion of the pterygothoracic ganglia. The posterior component branched more extensively and seemed to be confined to the metathoracic ganglion.

Within the pterygothoracic ganglia, the A2 terminations appeared to overlap somewhat with those of the A1 cell; the A2 cell seemed to have a more extensive arborization pattern (Fig. 2–4). In general, the A2 cell also had the most dorsal terminations within the pterygothoracic ganglia. These dorsal terminations were at about the same level as the dendritic fields of the motoneurons located ipsilaterally, as in a IIIN1B infiltration that labeled acoustic and nonacoustic fibers (Fig. 4 and 5). In many of these IIIN1B fills, much of the A2 branching was obscured; the branching could be traced only in high magnification reconstructions or in infiltrations involving the acoustic branch of the IIIN1B nerve. More ventrally, within the middle core of the pterygothoracic ganglia, the A2 terminations overlapped with those of the A1 and B cells.

The terminations of the A1 cell were similar to those of the B cell, whose branching extended from the posterior metathoracic to the prothoracic ganglion (Fig. 1 and 3). The A1 axons appeared the thinnest (1–2 μm) and remained more medial throughout the ganglia than those of the other cells. Terminations of A1 also were dispersed in several regions of the thoracic ganglia. A few axonal branches occurred in the pterygothoracic ganglia and an occasional process crossed the midline. Anteriorly, the axon was very fine, and it branched very little as it ascended into the cervical connective, probably to terminate in the brain.

The B cell was readily identifiable in the preparations (Fig. 1 and 3). Its axon appeared to have the largest diameter (4–5 μm) of the three. Its branching ranged from the most posterior part of the metathoracic ganglion, anteriorly to the prothoracic ganglion, and into the cervical connective to the brain. Within most of the pterygothoracic ganglia and the connective to the prothoracic ganglion, the B cell remained more lateral than the A1 cell. Axonal terminals were present in the central regions of the meta-, anterior meso-, and central regions of the prothoracic ganglion.

When cobalt infiltrations were performed on the entire IIIN1B nerve, in addition to the acoustic cells, some of the motoneurons innervating the indirect flight muscles also were labeled. The nonacoustic branch of IIIN1B innervated the dorso-longitudinal muscles. Six motoneurons were labeled; five were located ventrally within the ipsilateral side of the posterior mesothoracic ganglion; their neurites extended dorsally to their massive dendritic fields (Fig. 4 and 5). The cell body of the single contralateral motoneuron was dorsal and had a neurite extending across the midline to its dendritic field, which was located dorsally within the anterior region of the metathoracic ganglion (Fig. 5).

Thus, the acoustic A1 and A2 neurons, because of their widespread terminal fields within the thoracic ganglia, are in a good position to make synaptic contact with several interneurons and/or motoneurons (compare Boyan & Fullard 1986 and Madsen & Miller 1987). In the IIIN1B preparations, the high degree of overlap between the

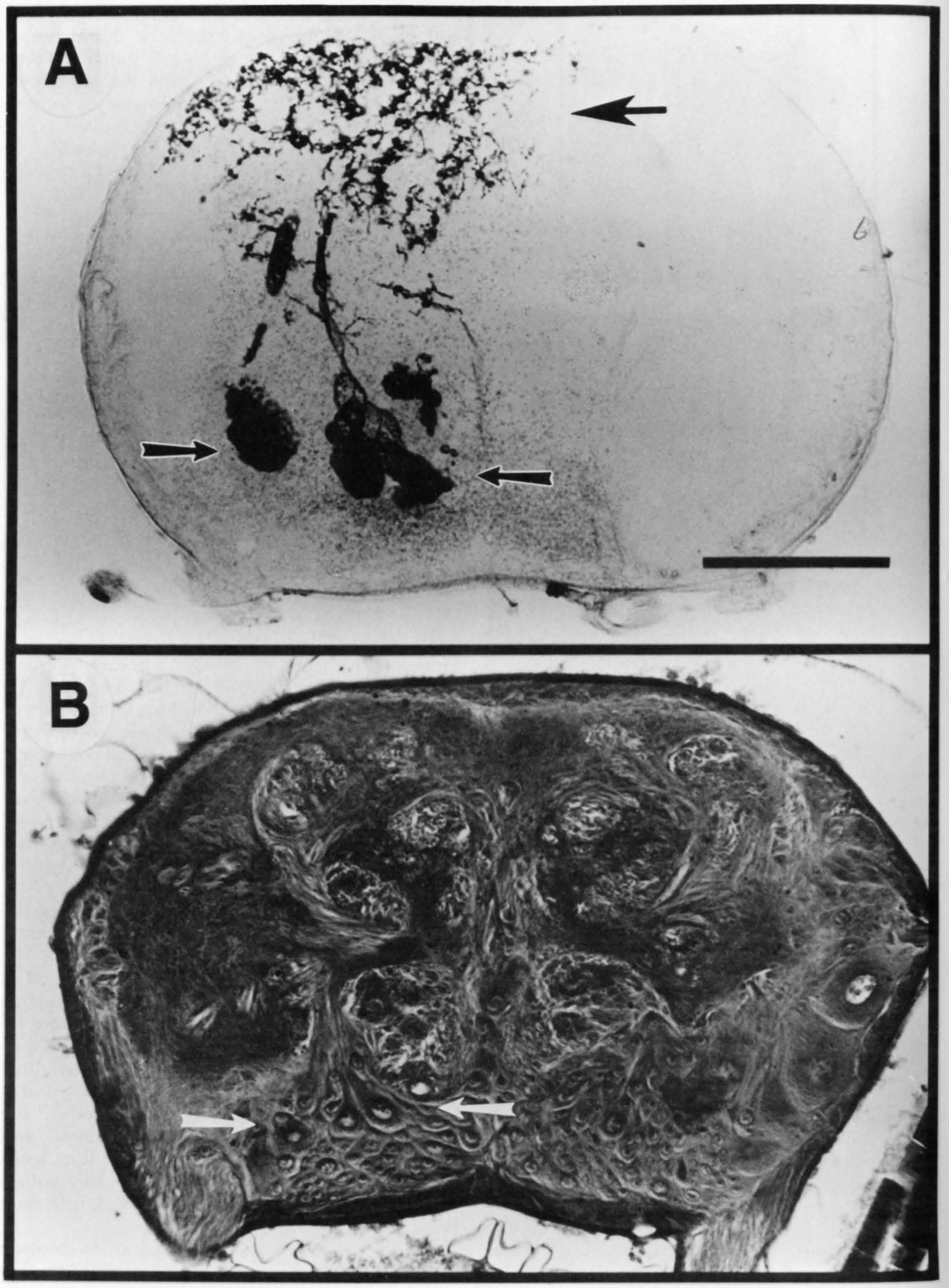


Fig. 5. Photomicrographs of motoneurons in cobalt (A) and noninfiltrated (B) specimens. (A) Cross-section of paraffin-embedded specimen showing the dorsally-located dendritic field (large arrow), neurites, and somata of the ipsilateral motoneurons (small arrows). (B) Cross-section of three of the motor cell nuclei and their neurites (small arrows). Calibration bar for (A, B) 100 μm.

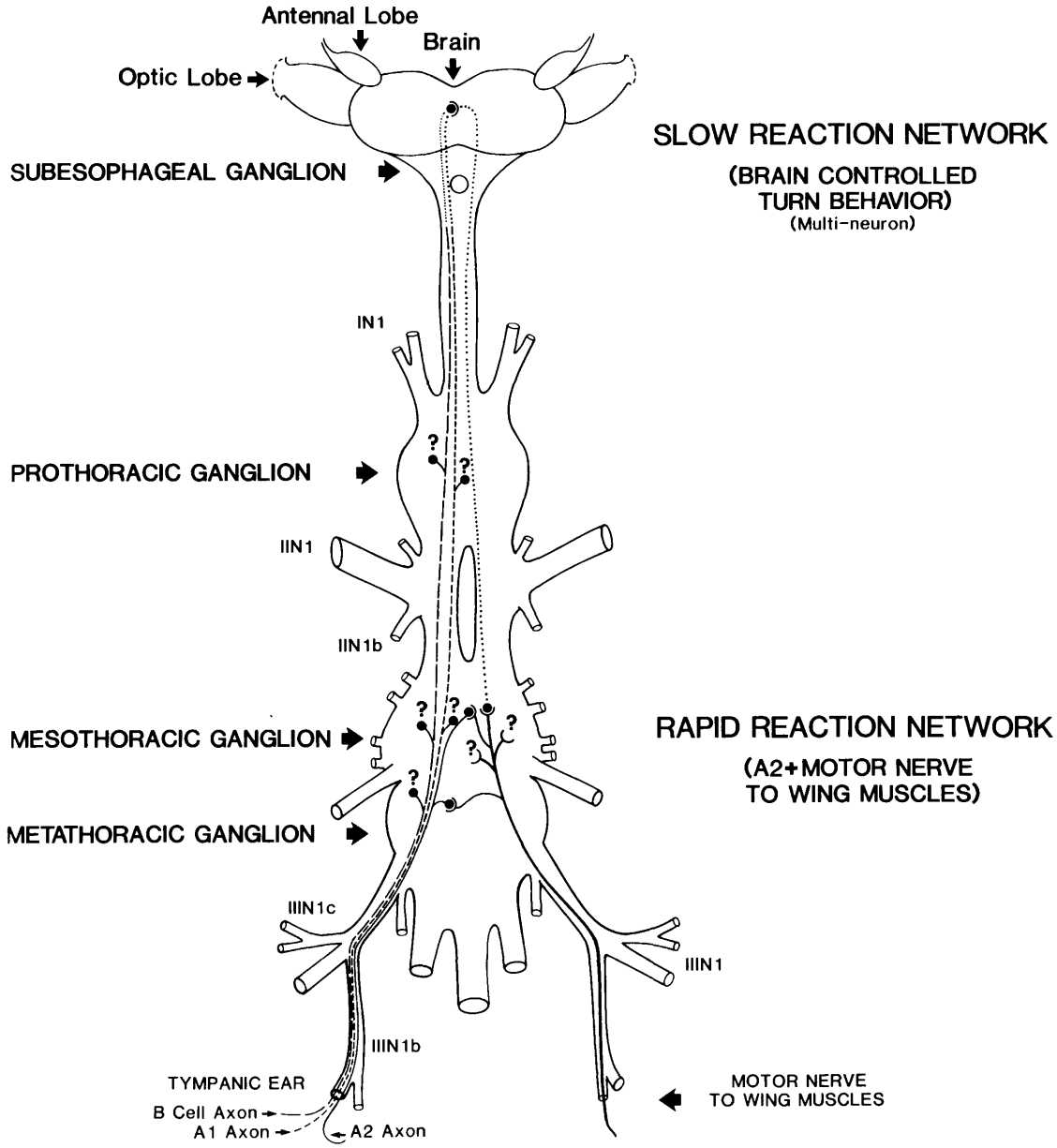


Fig. 6. Hypothesized schematic diagram of the tympanic neurons and the associated circuitry to the motoneurons involved in evasive flight behavior. Two behaviorally relevant systems appear to be present. The A2 acoustic cell has direct monosynaptic connections to the motoneurons within the meso-metathoracic ganglia, forming a rapid reaction network. On the other hand, the A1 and B cells appear to be additionally linked to interneurons and the brain in their involvement in a slower reaction network.

acoustic and motor processes made individual cellular reconstructions difficult. The axonal terminations of the tympanic nerves and dendritic fields of the motoneurons were largely coextensive (compare Fig. 2A with Fig. 4A). The proximity of the sensory to the motor cells itself is suggestive of possible monosynaptic connections. However, only transmission electron microscope studies of labeled cells would provide definitive anatomical evidence of the presumed synaptic contacts.

Discussion

Because of its simplicity, the auditory system of noctuid moths affords a unique opportunity for a system model for several research areas. Typically, most studies have stressed the importance of noctuid audition with regard to evasive flight behaviors. It is generally accepted that the ability of these nocturnal moths to detect ultrasound has evolved from the need to avoid their natural predators, bats

that produce ultrasonic signals (Roeder 1962, 1964; Agee 1969). However, ultrasonic communication in many insect species (including pyralids and noctuids) is very important in courtship behaviors, leading ultimately to mate attractance and reproduction (Spangler et al. 1984, Surlykke & Gogala 1986).

Field tests have been conducted to determine if *H. zea* can be chased from crops with sound generators that produce bat-mimicking ultrasounds (Roeder 1962, Agee 1969). These tests were only partially successful because the moths habituated behaviorally to the ultrasounds. Because economic control usually requires a 99% reduction in an insect population, more information is needed on the neural substrates of habituation (and dis-habituation) and on the neural processing of acoustic signals.

Our approach has investigated the processing of acoustic information within the thoracic ganglia and the circuits that control the evasive flight behaviors by using neuroanatomical, neurophysiological, and behavioral techniques (Agee 1985). These studies and those of others (Paul 1973, Surlykke & Miller 1982) on noctuid moths focused on the axonal terminations of the A1 and A2 cells within the thoracic ganglia. From these studies, we found that the A2 cell terminates exclusively within the meso-metathoracic ganglion, whereas the A1 cell terminates there and within the prothoracic ganglion, and possibly continues on to the brain. This possibility also is raised in the short latency of the neuronal responses (8–10 ms) to acoustic stimulation that has been recorded in the brain (Roeder 1969a,b).

Because they differ in terminations, these ipsilateral projections of A1 and A2 create the potential for the involvement of different sets of circuits in the second-order processing of the acoustic information (compare Roeder 1975). Although some earlier studies investigated thoracic interneurons (Roeder 1966, Paul 1974), they were conducted before the morphology of primary auditory or motoneurons was well known. We are currently mapping these circuits, by studying the physiological response properties of the acoustic interneurons and then filling them intracellularly with cobalt or fluorescent dyes (compare Boyan & Fullard 1986).

The motoneurons that innervate the wing flight muscles in moths are scattered throughout much of the pro- and meso-metathoracic ganglia (Kondoh & Obara 1982, Rind 1983, Kammer 1985). Typically, the cell bodies and their respective dendritic fields are confined to a small region of the neuropil, the axon leaving toward the motor nerve trunk at about the same level (Madsen & Miller 1987, unpublished data). We have observed that the axons of the motoneurons leave the smaller nerves of the ganglia, whereas the larger nerve trunks (such as IIN1 and IIIN1) convey sensory information primarily from the fore- and hindwing bases (Orona & Agee 1987).

The A2 cell axonal terminations, confined exclusively to the meso-metathoracic ganglia, are in excellent position to contact directly the dendritic fields of several of the motoneurons that innervate the wing muscles. In fact, physiological evidence indicates that the A2 cell contacts these motoneurons monosynaptically, forming a reflex arc: the A2 cell directly activates motor cells that control the wing flight muscles (Madsen & Miller 1987) (Fig. 6). Our behavioral data also support such a hypothesis. In field studies, high-intensity ultrasounds cause moths to engage in maneuvers distinctly evasive, such as quick turns or dives (Roeder 1962, 1967; Agee 1969). Because the A2 system is involved primarily with detection of sounds at these intensities, the behavioral system that the A2 cell initiates seems to be activated preferentially. Behavioral tests in which the cervical connectives to the brain have been cut indicate that many components of evasive movements are still intact and are not the result of descending influences (Treat 1955, Agee 1985). Thus, it appears that the auditory system directly activates motoneurons that are confined to the meso-metathoracic ganglia.

However, the A1 cell is associated with a set of interneurons (second order) located in the thoracic ganglia, the brain, or both, and hence the A1 cell does not contact directly the motoneurons for flight (Roeder 1975, Agee 1985). Such a neuronal system is more likely associated with slower or "decision-like" processes, such as steering away from distant sounds. Again, behavioral studies (Roeder 1962, 1967; Agee 1969) support a distinction between the A1 and A2 behavioral systems. There are more steering-away movements from the lower intensity ultrasounds, i.e., those sounds that only the A1 acoustic cells can detect (Fig. 6).

Finally, the importance of the other neuron, the B cell, in the moth ear (Treat & Roeder 1959, Lechtenberg 1971) should not be disregarded or underestimated. The B cell constitutes one-third of the tympanic output, has the largest cell body and axonal diameter, and has extensive projections throughout the thoracic ganglia and to the brain. Although apparently it is not involved in processing auditory information per se, the B cell may have important behavioral relevance, especially with evasive flight maneuvers. The B cell responds to auditory information in a unique manner: by a transient inhibition or cessation of activity, particularly at intense, highly repetitive ultrasounds (Lechtenberg 1971). Because of its response characteristics, the B cell appears more related to the A2 cell and its behavioral relevance. Considering the differences that have been reported in the acoustic and motor systems in flying compared with nonflying preparations (Madsen & Miller 1987), the B cell may be even more important in the evasive behaviors of intact flying moths. It is conceivable that the early arrival of B cell activity within the thoracic ganglia, at the onset of intense ultrasounds, is correlated with a clearing or dis-inhibition of

either auditory interneurons or motoneurons active in behavioral responses. Also, it is likely that the relative activity or inactivity of the B cell accounts for the behavioral evitability often observed in moth preparations (Roeder 1975).

In summary, available data indicate that there are two systems for processing acoustic information in this noctuid moth (Fig. 6). One system consists of the A2 cell and its direct connection to motoneurons that control the wing flight muscles. The A2 cells are activated preferentially by higher intensity sounds and can be considered part of a reflexive, rapid reaction network. The A1 cells, on the other hand, are more sensitive to lower intensities and presumably are connected to second-order interneurons and the brain. These cells are more likely to be involved with slower acting or longer range flight behaviors. Therefore, *H. zea* provides an excellent model system for studying sensory coding in general, and particularly for understanding the neuronal circuits that underlie behavioral habituation in insects.

Acknowledgment

The technical assistance of John C. Davis in the early phases of this research is appreciated.

References Cited

Agee, H. R. 1967. Response of acoustic sense cells of the bollworm and tobacco budworm to ultrasound. J. Econ. Entomol. 60: 366–369.

1969. Response of flying bollworm moths and other tympanate moths to pulsed ultrasound. Ann. Entomol. Soc. Am. 62: 801–807.

1985. Neurobiology of the bollworm moth: neural elements controlling behavioral responses to pulsed ultrasound. J. Agric. Entomol. 2: 345–350.

Bacon, J. P. & J. S. Altman. 1977. A silver intensification method for cobalt-filled neurons in wholemount preparations. Brain Res. 138: 77–86.

Boyan, G. S. 1985. Auditory input to the flight system of the locust. J. Comp. Physiol. (A)156: 79–91.

Boyan, G. S. & J. S. Altman. 1985. The suboesophageal ganglion: a "missing link" in the auditory pathway of the locust. J. Comp. Physiol. (A)156: 413–428.

Boyan, G. S. & J. H. Fullard. 1986. Interneurons responding to sound in the tobacco budworm moth *Heliothis virescens* (Noctuidae): morphological and physiological characteristics. J. Comp. Physiol. (A)158: 391–404.

Eaton, J. L. 1974. Nervous system of the head and thorax of the adult tobacco hornworm, *Manduca sexta*. Int. J. Insect Morphol. Embryol. 3: 47–66.

Kammer, A. E. 1985. Flying, pp. 391–552. *In* G. A. Kerkut & L. I. Gilbert [eds.], Comprehensive insect physiology, biochemistry, and pharmacology; vol. 5, Nervous system: structure and motor function. Pergamon, Oxford.

Kien, J. & J. S. Altman. 1984. Descending interneurons from the brain and suboesophageal ganglia and their role in the control of locust behavior. J. Insect Physiol. 30: 59–72.

Kondoh, Y. & Y. Obara. 1982. Anatomy of motoneurons innervating mesothoracic indirect flight muscles in the silkmoth, *Bombyx mori*. J. Exp. Biol. 98: 23–37.

Lechtenberg, R. 1971. Acoustic response of the B cell in noctuid moths. J. Insect Physiol. 17: 2395–2408.

Madsen, B. M. & L. A. Miller. 1987. Auditory input to motor neurons of the dorsal longitudinal flight muscles in a noctuid moth (*Barathra brassicae* L.). J. Comp. Physiol. 160: 23–31.

Orona, E. & H. R. Agee. 1987. Thoracic mechanoreceptors in the wing bases of *Heliothis zea* (Lepidoptera: Noctuidae) and their central projections. J. Insect Physiol. 33: 713–721.

1988. An insect model system for the analysis of sensory coding: auditory processing in the noctuid moth *Heliothis zea*. Proceedings, Symposium on "The Mind: Brain and Learning." Society for the Advancement of Chicanos and Native Americans in Science (SACNAS), September 1986, Pasadena, Calif.

Paul, D. H. 1973. Central projections of the tympanic fibres in noctuid moths. J. Insect Physiol. 19: 1785–1792.

1974. Responses to acoustic stimulation of thoracic interneurons in noctuid moths. J. Insect Physiol. 20: 2205–2218.

Reichert, H. & C. H. F. Rowell. 1986. Neuronal circuits controlling flight in the locust: how sensory information is processed for motor control. Trends Neurosci. 9: 281–283.

Rind, F. C. 1983. The organization of flight motoneurons in the moth, *Manduca sexta*. J. Exp. Biol. 102: 239–251.

Roeder, K. D. 1962. The behaviour of free-flying moths in the presence of artificial ultrasonic pulses. Anim. Behav. 10: 300–304.

1964. Aspects of the noctuid tympanic nerve response having significance in the avoidance of bats. J. Insect Physiol. 10: 529–546.

1966. Interneurons of the thoracic nerve cord activated by tympanic nerve fibers in noctuid moths. J. Insect Physiol. 12: 1227–1244.

1967. Turning tendency of moths exposed to ultrasound while in stationary flight. J. Insect Physiol. 13: 873–888.

1969a. Acoustic interneurons in the brain of noctuid moths. J. Insect Physiol. 15: 825–838.

1969b. Brain interneurons in noctuid moths: differential suppression by high sound intensities. J. Insect Physiol. 15: 1713–1718.

1975. Neural factors and evitability in insect behavior. J. Exp. Zool. 194: 75–88.

Spangler, H. G., M. D. Greenfield & A. Takessian. 1984. Ultrasonic mate calling in the lesser wax moth. Physiol. Entomol. 9: 87–95.

Surlykke, A. & M. Gogala. 1986. Stridulation and hearing in the noctuid moth *Thecophora fovea* (Tr.). J. Comp. Physiol. (A)159: 267–273.

Surlykke, A. & L. A. Miller. 1982. Central branchings of three sensory axons from a moth ear *Agrotis segetum*, Noctuidae. J. Insect Physiol. 28: 357–364.

Treat, A. 1955. The response to sound of certain Lepidoptera. Ann. Entomol. Soc. Am. 48: 272–284.

Treat, A. E. & K. D. Roeder. 1959. A nervous element of unknown function in the tympanic organ of moths. J. Insect Physiol. 3: 262–270.

Tyrer, N. M. & J. S. Altman. 1974. Motor and sensory flight neurons in a locust demonstrated using cobalt chloride. J. Comp. Neurol. 157: 117–138.

Received for publication 4 December 1987; accepted 23 May 1988.