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TA Christensen, H Itagaki, PEA Teal, RD Jasensky, JH Tumlinson, and JG Hildebrand

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Innervation and neural regulation of the sex pheromone gland in female *Heliothis* moths

(electrophysiology/octopamine/pheromone biosynthesis activating neuropeptide/ventral nerve cord/terminal abdominal ganglion)

THOMAS A. CHRISTENSEN*, HARUHIKO ITAGAKI*†, PETER E. A. TEAL‡, RONALD D. JASENSKY§, JAMES H. TUMLINSON‡, AND JOHN G. HILDEBRAND*

*Arizona Research Laboratories Division of Neurobiology, 611 Gould-Simpson Building and Center for Insect Science, University of Arizona, Tucson, AZ 85721; †Insect Attractants, Behavior, and Basic Biology Research Laboratory, Agricultural Research Service, U.S. Department of Agriculture, P.O. Box 14565, Gainesville, FL 32604; and ‡Arizona Research Laboratories Division of Biotechnology, University of Arizona, Tucson, AZ 85721

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ABSTRACT Female *Heliothis* moths normally produce their species-specific male attractant (sex pheromone blend) during scotophase, and this production is stimulated by pheromone biosynthesis activating neuropeptide (PBAN), presumably carried in the hemolymph. Several lines of evidence indicate that the central nervous system plays another critical role in this regulation. Pheromone biosynthesis was induced during photophase by electrical stimulation of the ventral nerve cord or the peripheral nerves projecting from the terminal abdominal ganglion to the pheromone gland in the tip of the abdomen. Electron microscopy further revealed that axonal branches innervate the gland tissue. Nerve branches associated with pheromone gland cells are enwrapped in glia and contain dense-core vesicles, suggesting that the innervation of the gland might be neurosecretory. Finally, the biogenic monoamine octopamine was nearly as effective as purified *Heliothis zea* PBAN in stimulating pheromone biosynthesis when injected into intact females during mid-photophase. Furthermore, both octopamine and PBAN stimulated significant increases in the pheromone content of the glands in isolated abdomens lacking a ventral nerve cord but only when abdomens were treated at the onset of scotophase. These data suggest that the regulation of sex pheromone production in *Heliothis* is more complex than previously thought. Activation of the gland appears to be governed by both neural and hormonal mechanisms, and these control mechanisms depend on photoperiodic cues.

The physiological mechanisms underlying the production of sex pheromone, its release from the pheromone gland (PG), and the associated "calling" behavior in female moths, have become the focus of much research and debate in recent years. Although it is clear that pheromone production is not controlled by the same mechanisms in all species, a major development was the demonstration that pheromone biosynthesis in certain species of moths can be stimulated by the injection of brain homogenates into intact females (1–19). Two structurally related pheromone biosynthesis activating neuropeptides (PBANs) were isolated and identified from homogenates of brains and subesophageal ganglia from the corn earworm moth *Helicoverpa zea* (formerly *Heliothis zea* and abbreviated *H. zea* in this report; see refs. 1 and 4) and the silkworm *Bombyx mori* (2, 3). These studies further revealed that PBAN is concentrated in the subesophageal ganglion (4–6).

Several theories about the mechanism of action of PBAN are currently being tested in numerous species. According to one hypothesis, PBAN is released into the hemolymph during the scotophase and circulates to the tip of the abdomen, where it directly stimulates the PG (4–6). In support of this

idea, recent evidence using isolated PGs from the redbanded leafroller moth suggests that synthetic PBAN can stimulate both the incorporation of radiolabeled acetate into pheromone and a small increase in the pheromone level (7). In *Heliothis armigera* PGs, PBAN also stimulates the incorporation of radiolabeled acetate into pheromone (20), but the amount of pheromone produced was not determined. Another study with *H. zea* and other moths proposed an alternative route for the action of PBAN (8). Teal *et al.* (8) found that injection of brain–subesophageal ganglion extracts into intact females during photophase resulted in a stimulation of pheromone biosynthesis, as has been reported by numerous investigators. This increase was not affected by transection of the ventral nerve cord (VNC) anterior to the terminal abdominal ganglion (TAG) before injection (7, 8). If the peripheral nerves posterior to the TAG were cut, however, pheromone biosynthesis could not be induced by injection of brain–subesophageal ganglion extract (8). These experiments suggest that PBAN stimulates one or more neurons in the TAG that provide a signal to the gland to produce pheromone and that this signal is blocked in the nerve transection experiments. Previous studies in the gypsy moth *Lymantria dispar* also had shown that pheromone production depends on an intact nervous system (21–23), but the neural mechanisms linking the nervous system to the PG remained unresolved.

In the present study, we have examined the connections between the TAG and the PG in *Heliothis virescens* and *H. zea*. The PG is innervated by neurosecretory-type nerve fibers, and electrical stimulation of the nerves arising from the TAG stimulates the production of sex pheromone. Furthermore, the actions of synthetic and purified *H. zea* PBAN on pheromone biosynthesis are paralleled by those of the biogenic monoamine octopamine.

MATERIALS AND METHODS

Insects. Female *H. virescens* and *H. zea* were reared according to published procedures (8). Pupae were shipped from Gainesville to Tucson and were maintained under both natural and reversed 14-hr light/10-hr dark photoperiodic cycles at 27°C and 70–80% relative humidity until adult eclosion. All experiments were performed during the 2nd or 3rd day after eclosion.

Gland Morphology. Abdomens were processed for light-microscopic paraffin histology according to standard proce-

Abbreviations: CNS, central nervous system; EAG, electroantennogram; PBAN, pheromone biosynthesis activating neuropeptide; PG, pheromone gland; TAG, terminal abdominal ganglion; VNC, ventral nerve cord.

†Present address: Department of Biology, Kenyon College, Gambier, OH 43022.

dures (24). For tracing the nerve fibers to the PG, the abdomen was transected at the fourth segment and pinned with its dorsal side up. The cuticle was cut along the dorsal midline toward the posterior segments, and the two flaps of cuticle were splayed open. The gut was displaced to one side to reveal the VNC and TAG underneath. The nerves arising from the TAG and innervating the terminal abdominal segments were cut and placed into a cobalt/lysine solution for 24–48 hr (25). The eighth and ninth segments were then removed from the abdomen and everted to expose the hemocoelom side of the PG, which is a monolayer of cells lining the intersegmental membrane between the eighth and ninth abdominal segments (26, 27). This manipulation gives various histological reagents rapid access to the PG tissue. The eighth and ninth segments were then fixed in alcoholic Bouin's solution, followed by standard treatment with H₂S and intensification with silver (28). Silver-intensified preparations were either examined in whole mount or embedded in Epon, cut in 6- μ m-thick sections, and stained with toluidine blue. For electron microscopy, the PG was excised, fixed in cold Karnovsky's fixative solution, osmicated, dehydrated, embedded in Epon, sectioned with a diamond knife (29), and then examined in a JEOL 1200EX transmission electron microscope.

Electrical Stimulation. The isolated abdomen preparations (transected at the fourth segment as described above) were flooded with a physiological saline solution [150 mM NaCl/3 mM CaCl₂/3 mM KCl/10 mM *N*-tris(hydroxymethyl)methyl-2-aminoethanesulfonic acid buffer, adjusted to pH 6.9] without allowing the external surface of the intersegmental cuticle overlying the PG to become wet. All peripheral nerves except those arising from the TAG were cut. After treatment with 3% collagenase/dispase (Boehringer Mannheim) for 1 min, part of the dorsal sheath of the TAG was removed with fine forceps to facilitate penetration of test solutions.

The peripheral nerves posterior, or the connectives anterior, to the TAG were stimulated with a pair of fine bipolar electrodes made from silver wire (100- μ m diameter). Every 2 sec, a 500-msec train of 0.1-msec pulses at 20 Hz was delivered. These trains were repeated for 5 min, 15 min, or 1 hr. Stimulation voltage was adjusted until slight contractions of abdominal intersegmental muscles could be seen. In this reduced preparation, movements of the distal abdomen typical of normal calling behavior were not observed. To isolate the TAG from the rest of the preparation, a petroleum jelly well was constructed around the TAG on a Parafilm substrate that had been pinned in place beneath the TAG. To block Ca²⁺-mediated transmitter release in the TAG without affecting the rest of the preparation, 20 mM MgCl₂ was added to the saline solution in the well (30).

Pharmacological Treatments. Intact females were injected with synthetic and purified *H. zea* PBAN (see below) or DL-octopamine hydrochloride in the physiological saline solution described above with 20 mM Mg²⁺ added to block endogenous transmitter release. Isolated abdomens, transected posterior to the sixth segment (thus, with the nerves between the TAG and the PG severed), were also incubated in the solutions. Each isolated abdomen was placed with the PG facing upward on a 10- μ l drop containing a test solution. Intact females and isolated abdomens were treated for 1 hr at ambient temperature (\approx 25°C). Control insects were treated in the same fashion with the saline solution plus 20 mM Mg²⁺.

Gland Extracts and Electroantennograms (EAGs). After electrical or pharmacological stimulation, the PGs were removed, and the pheromone was extracted in 50–60 μ l of gas chromatographic (GC) grade *n*-hexane for 1 min. The extracts were then used as olfactory stimuli for EAG measurements or they were sealed in glass ampoules and shipped by overnight courier from Tucson to Gainesville for capillary GC analysis by standard methods (8). To conceal the method of

treatment of samples that were to be analyzed by GC, an internal standard (1 ng/ μ l each of octadecane and nonadecane) was added, and each sample was coded before shipment. EAGs were recorded from antennae of mature *H. virescens* males (2–3 days after eclosion) (31). Statistical analysis of the data was carried out with SAS software (SAS Institute, Cary, NJ).

PBAN Synthesis. *H. zea* PBAN (4) was synthesized at the University of Arizona Macromolecular Structure Facility by standard automated solid-phase techniques (32) in an ABI 431A synthesizer using *N*-methylpyrrolidone/*N*-hydroxybenzotriazole/dicyclohexylcarbodiimide activation and fluorenylmethoxycarbonyl/*t*-butyl protection. The peptide was cleaved from the support by trifluoroacetic acid/phenol/ethanedithiol/thioanisole/water (82.5:5:2.5:5:5) (33) and purified by reverse-phase HPLC on an octadecylsilane AQ column (4.6 \times 250 mm; YMC, Morris Plains, NJ). Samples were eluted with a mixture of water/acetonitrile containing 0.1% trifluoroacetic acid. The water/acetonitrile ratio was programmed from 78/22 at 3 min after injection to 75/25 at 20 min. PBAN eluted from this column at 18.5 min under these conditions. The peptide was then analyzed for purity on the same reverse-phase HPLC column and by capillary zone electrophoresis (34). For structural confirmation, we performed amino acid analysis, fast atom bombardment mass spectrometry, and automated N-terminal Edman degradation (ABI model 477A). We used purified *H. zea* PBAN at a concentration of 5.0×10^{-7} M, which evokes about the same amount of pheromone production as a brain-subesophageal ganglion extract from one *H. virescens* female (8).

RESULTS AND DISCUSSION

The neural connections from the TAG to the PG of adult female *H. zea* were traced in serial paraffin sections of abdominal tips. Several nerve branches run in close proximity to the PG, which lines the soft intersegmental cuticle between the eighth and ninth abdominal segments (one nerve branch is shown in Fig. 1A). Anterograde filling (with cobalt/lysine) of the nerves arising from the TAG in *H. zea* and *H. virescens* revealed fine arborizations that had small, distinct swellings adjacent to the PG (Fig. 1B). Electron microscopy confirmed that these nerve fibers penetrated the basement membrane of the PG (Fig. 1C). The axonal branches were enwrapped in glia and contained dense-core vesicles, typical of neurosecretory nerve endings. These elements did not resemble terminals of motor neurons, nor were they associated with peripheral cell bodies typical of sensory neurons.

Ultrastructural studies in a wide variety of insects have demonstrated neurosecretory innervation of other epidermal glands (35–37), but previous studies of lepidopteran PGs failed to reveal such innervation (38–45). We have shown in *H. zea* that retrograde labeling from the PG through the terminal nerve with cobalt/lysine stains neuronal somata in the posterior portion of the TAG (46, 47). The number, sizes, and positions of these cells are similar to those revealed by back-filling the branch of the terminal nerve that innervates the common oviduct in the sphinx moth *Manduca sexta* (48). The possibility that this particular nerve branch also innervates the PG needs to be tested in a more detailed anatomical study.

The PGs in many female moths, including those of *Heliothis* sp., contain little pheromone during the photophase (49, 50), but electrical stimulation of the connectives anterior to the TAG (Table 1) or the nerves arising from the TAG (Fig. 2) induces production of pheromone in photophase females. Pheromone production was measured by two independent assays. In one series of experiments, *n*-hexane extracts of PGs from treated female *H. virescens* were tested in male EAG bioassays (31) to estimate the amount of pheromone

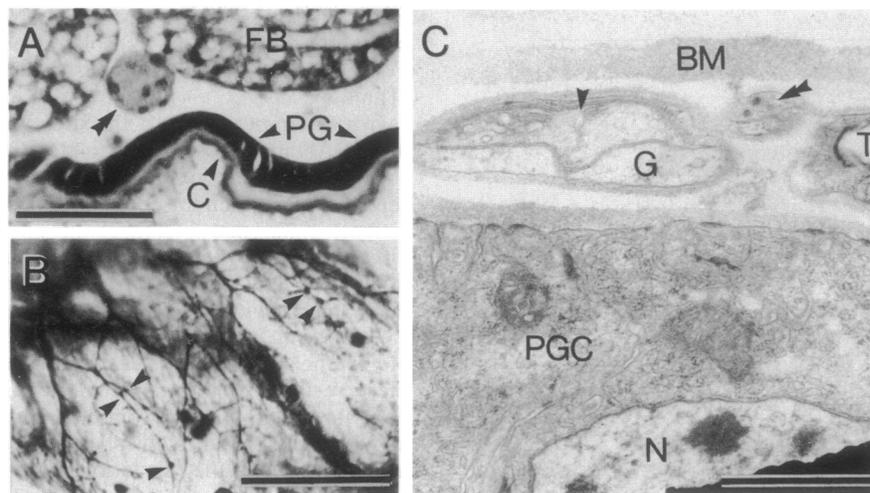


FIG. 1. Light- and electron-microscopic evidence for innervation of the PG. The oviduct is not visible in any of these preparations. (A) Paraffin cross-section (10 μ m) of the PG in *H. zea*. Double arrowhead points to a nerve cross-section in close proximity to the PG cell layer. C, cuticle; FB, fat body. (B) Anterograde cobalt impregnation of the nerves to the PG, located in the intersegmental membrane between the eighth and ninth abdominal segments. The cobalt staining reveals innervation by finely branching nerve fiber(s) with varicosities along the finer branches (arrowheads). (C) Electron micrograph of a PG cell in *H. virescens* showing innervation (single and double arrowheads) that penetrates the basement membrane of the PG. One of the axonal profiles exhibits dense-core vesicles (double arrowhead). BM, basement membrane; G, glia; T, tracheole; PGC, PG cell; N, nucleus of the PGC. (A and B, bars = 100 μ m; C, bar = 1 μ m.)

extracted from the PG. Tests of extracts were repeated on three males to account for antennal variability (Fig. 2). Stimulation of TAG nerves for as little as 5 min yielded PG extracts that elicited EAG responses that were always significantly greater than those elicited by extracts of unstimulated control glands. This finding suggests that stimulation of efferent nerves to the PG increases production of pheromone. Stimulation of TAG nerves for longer periods (up to 1 hr) did not result in greater EAG responses. In such experiments, it is possible that over the longer periods of stimulation, the pheromone produced is released into the air or degraded. To measure more precisely the pheromone levels produced by these treatments, the major pheromone component (*Z*)-11-hexadecenal in PG extracts was quantified directly by capillary GC procedures (8). When the VNC anterior to the TAG

was stimulated for 15 min, the amount of pheromone extracted from the PG was almost 4 times that of the control level ($P < 0.05$) (Table 1). When the TAG (but not the PG) was treated with saline solution containing 20 mM $MgCl_2$ for 10 min prior to stimulation of the VNC, pheromone levels were not significantly different from background levels (Table 1). This result suggests that the elevated Mg^{2+} concentration inhibited pheromone production by blocking the descending neural input to the TAG. The Mg^{2+} treatment of the TAG did not affect the contraction of intersegmental muscles driven by stimulation of the VNC, indicating that this blockade resulted from interruption of Ca^{2+} -mediated synaptic transmission (30) rather than elevation of axonal threshold. In *M. sexta*, motor neurons that arise in the abdominal ganglion immediately anterior to the TAG send axons through the

Table 1. Treatments used to stimulate pheromone biosynthesis in *H. virescens* females

	Pheromone level, ng	% control	n
Electrical stimulation of the ventral nerve cord during photophase			
Control	6.2 \pm 2.1 (A)	100	7
Stimulated 15 min	22.3 \pm 4.0 (B)	360	8
Mg^{2+} block of TAG, then stimulated	7.4 \pm 1.4 (A)	119	5
Pharmacological stimulation of pheromone biosynthesis			
Intact females during photophase			
Control	4.0 \pm 1.6 (A)	100	14
30 μ l, 0.5 μ M PBAN	45.1 \pm 6.8 (B)	1128	14
30 μ l, 0.1 μ M octopamine	29.3 \pm 7.4 (B)	733	14
Isolated abdomens transected posterior to the TAG			
Treated during mid-photophase			
Control	5.0 \pm 1.5 (A)	100	8
10 μ l, 0.5 μ M PBAN	4.8 \pm 1.3 (A)	96	6
10 μ l, 10 μ M octopamine	10.1 \pm 3.6 (A)	202	8
Treated at onset of scotophase			
Control	3.0 \pm 0.7 (A)	100	15
10 μ l, 0.5 μ M PBAN	17.7 \pm 2.2 (B)	590	10
10 μ l, 1.0 μ M octopamine	19.3 \pm 4.8 (B)	643	14

Each pheromone value is the amount (ng) of (*Z*)-11-hexadecenal, the major pheromone component in *H. virescens* glands, as measured by GC analysis (8). All values are means \pm SEM. Means followed by the same letter are not significantly different (SAS *t* test; $P < 0.05$).

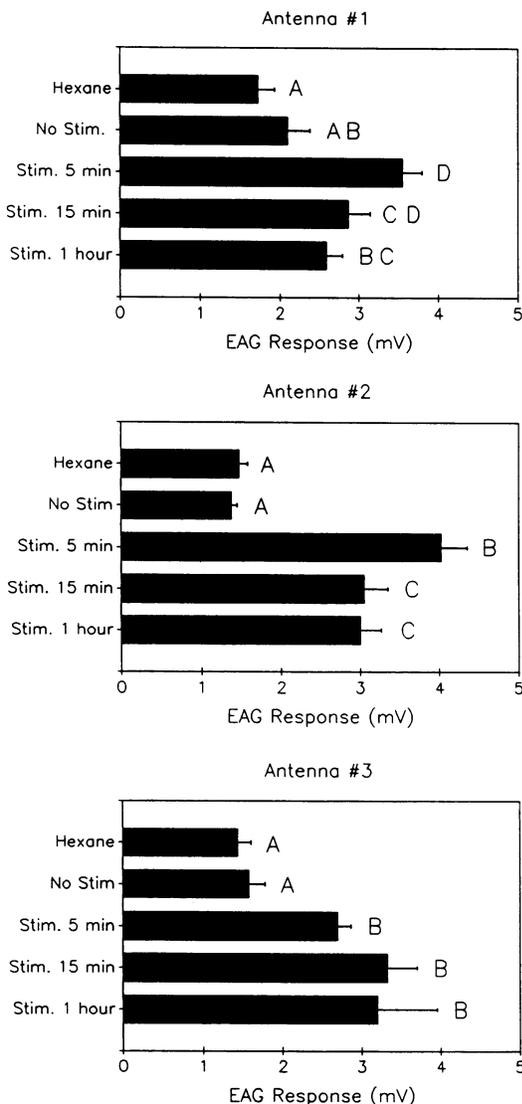


FIG. 2. Effects of electrical stimulation of TAG nerves on pheromone biosynthesis in *H. virescens* females during photophase as measured by EAG assays of PG extracts. Eight females were used for each treatment and extracts of their PGs were made. Extracts were tested on three males to account for antennal variability. Each value represents the mean \pm SEM for the eight extracts (seven extracts for antenna 3). Means with the same letter are not significantly different ($P < 0.05$) as determined by Duncan's multiple range test. For comparison, eight glands taken from untreated females 4 hr into scotophase yield a mean EAG response of 4.5 ± 0.2 mV (from three antennae).

TAG without synapsing (ref. 51; R. B. Levine, personal communication), and we believe that corresponding motor neurons in *H. virescens* were not affected by the Mg^{2+} treatment.

To begin to identify candidates for the role of neurotransmitter(s) regulating pheromone biosynthesis, we tested the effects of octopamine, which is known to function in a variety of insect neuroeffector systems (52–61). Octopamine was injected into intact *H. virescens* females or incubated with isolated abdomens lacking the TAG (Table 1). The PGs from intact females treated with octopamine for 1 hr yielded >7 times the amount of pheromone extracted from untreated control glands. Similarly, PGs in isolated abdomens treated with octopamine produced significant amounts of pheromone, but the effect clearly depended on photoperiod (Table 1). Only abdomens treated at the onset of scotophase showed a significant increase in pheromone levels ($P < 0.05$). Simi-

larly, purified synthetic *H. zea* PBAN elicited an increase in pheromone production in isolated abdomens (without the TAG) at the onset of scotophase but not during photophase (Table 1).

In an attempt to identify the site(s) of action of octopamine and PBAN in the abdomen, isolated (but not denervated; see Fig. 1) PGs were treated with octopamine or PBAN. In repeated trials, neither octopamine nor PBAN induced consistent, significant increases in pheromone levels in *H. virescens* or *H. zea* females under our experimental conditions. This may be due to the absence of a tissue such as fat body that provides pheromone precursors to the PG (18).

At present, our data suggest that multiple factors contribute to the regulation of sex pheromone biosynthesis, and these results point to some interesting avenues for future experiments. It is clear from our morphological and physiological results that the central nervous system (CNS) plays an important role in the regulation of pheromone production in *H. virescens*, but whether this applies generally to other species of moths remains unknown. Previous studies in *H. zea* concluded that PBAN acts on neurons in the TAG, because production of pheromone was induced by injection of brain-subesophageal ganglion extract only when the nerves between the TAG and the PG were intact (8). These treatments, however, were applied only to photophase females. Our results from experiments testing purified PBAN on intact *H. virescens* females and isolated abdomens during photophase are consistent with these findings. Furthermore, electrical stimulation of the VNC anterior to the TAG results in the production of pheromone, and our data suggest that this production depends on the Ca^{2+} -mediated release of a neurotransmitter or modulator from the terminals of descending fibers entering the TAG.

Three lines of evidence suggest that these descending fibers contain and release PBAN in the TAG. First, pheromone activity is associated with extracts of the VNC taken during the night when females actively produce pheromone (8). Second, using paraldehyde/fuchsin to stain putatively neurosecretory cells, we found a pair of axons, one in each connective, that appear to arise in the subesophageal ganglion, project through the VNC, and have varicose terminals in the TAG (46). Recently, similar patterns of PBAN-like immunoreactivity were observed in the VNC of *H. zea* (62), and varicose collaterals of the immunoreactive fibers were found in the TAG (N. T. Davis, personal communication). Axons that are immunoreactive for other peptides (e.g., eclosion hormone) are known to descend through the VNC in *M. sexta* (63) and in *H. zea* (N. T. Davis and H.I., unpublished observations). Finally, in *M. sexta*, some dorsal midline TAG neurons with axons in the terminal nerve are stimulated by application of PBAN (47). Further experiments are necessary to reveal the termination sites of these TAG neurons. Some of these neurons may innervate the PG or muscles that regulate female calling behavior, which also exhibits circadian rhythmicity (49, 64). Thus, PBAN might trigger several neuroeffector systems in the insect CNS.

Our results do not preclude the possibility that PBAN may also function as a circulating neurohormone, as has been widely proposed (1–7, 9–19, 49, 50), but the peptide has not yet been isolated from the hemolymph. The presence of "PBAN-like activity" in the hemolymph also is not sufficient proof of blood-borne PBAN, because many bioactive substances circulate in the hemolymph. Nevertheless, our data show that purified PBAN can stimulate pheromone production through two routes in *H. virescens*. Intact females injected with PBAN during mid-photophase produced pheromone, but PGs from isolated abdomens treated at the same time of day did not (Table 1). This result suggests that one target of PBAN is in the CNS (probably in the TAG as discussed above), and this activation mechanism does not

depend on photoperiod (8). In the absence of the CNS, PGs from isolated abdomens treated with PBAN at the onset of scotophase also produced pheromone. This suggests that some other factor associated with photoperiod was required for this second effect.

It is not surprising to find that PBAN may have multiple sites of action, as it is also found in males (1) and is active in larvae of the common cutworm *Spodoptera litura* (3). Perhaps a more surprising finding is that the actions of PBAN are paralleled by those of octopamine. These findings add a new dimension to the complexity of sex pheromone biosynthesis in these insects. While much work remains to be done, our initial observations indicate that octopamine could play several roles in regulating the production or release of female sex pheromone. Octopamine induces the release of hyperlipemic hormones from the glandular cells of the corpora cardiaca (65, 66). Recently, PBAN-like immunoreactivity has been localized in the corpora cardiaca (among other areas of the CNS; ref. 62), and it is therefore possible that injection of octopamine into intact females stimulates pheromone production by inducing the release of PBAN into the hemolymph. Octopamine also exerts an action on pheromone production in the absence of the CNS (Table 1), possibly by stimulating lipid (pheromone precursor) release from fat body (67). At present, therefore, the sites of action of octopamine and PBAN in these preparations remain unknown. Future work will need to examine further the roles of octopamine and PBAN in regulating pheromone production and should seek to define the role of photoperiod in this regulation. Anatomical studies will need to focus on characterizing the neurons that innervate the gland tissue.

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