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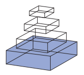

Regulatory role of PBAN in sex pheromone biosynthesis of heliothine moths

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Both males and females of heliothine moths utilize sex-pheromones during the mating process. Females produce and release a sex pheromone for the long–range attraction of males for mating. Production of sex pheromone in females is controlled by the peptide hormone (pheromone biosynthesis activating neuropeptide, PBAN). This review will highlight what is known about the role PBAN plays in controlling pheromone production in female moths. Male moths produce compounds associated with a hairpencil structure associated with the aedaegus that are used as short-range aphrodisiacs during the mating process. We will discuss the role that PBAN plays in regulating male production of hairpencil pheromones.

Keywords: pheromone, PBAN, *Heliothis*, *Helicoverpa*, moth

BACKGROUND ON PBAN

The hormonal regulation of pheromone biosynthesis in moths was first demonstrated in the heliothine *Helicoverpa zea* (Raina et al., 1987) and a peptide isolated shortly thereafter (Raina et al., 1989). Pheromone biosynthesis activating neuropeptide (PBAN) was identified as a 33 amino acid *C*-terminal amidated peptide from the brain–subesophageal ganglion complex of adult female moths. Immunohistochemical procedures traced the neuronal production of PBAN to three groups of neurons in the subesophageal ganglion (Blackburn et al., 1992; Kingan et al., 1992; Rafaeli and Jurenka, 2003). The gene encoding for PBAN was subsequently identified (Davis et al., 1992; Ma et al., 1994, 1998). In addition to encoding for PBAN four other neuropeptides could be produced. One of these had been identified as the diapause hormone that regulates embryonic diapause in the silkworm moth (Imai et al., 1991). The analysis of mRNA from a number of moth species now indicates that five peptides could be produced by the PBAN gene (Choi et al., 2004). Evidence indicates that these peptides could be processed and released into circulation as active neuropeptides (Ma et al., 1996).

Sequence similarities to the pyrokinins became evident after it was determined that the minimum activity required was the last five *C*-terminal amino acids (Raina and Kempe, 1990, 1992). Leukopyrokinin was first identified in the cockroach *Leucophaea maderae* due to the stimulation of hindgut muscle contraction (Holman et al., 1986). A variety of PBAN/pyrokinin peptides have been found in all insects to date based on gene sequence and peptide isolation (Jurenka and Nusawardani, 2011). These peptides are found in neurons localized to the brain–subesophageal ganglion and ganglia of the ventral nerve cord. Most insects possess another peptide produced by the *capa* gene. This gene, first identified in *Drosophila melanogaster* (Kean et al., 2002), can produce three peptides. Two of the peptides are periviscerokinins that usually have an FPRVamide *C*-terminal ending. Periviscerokinins are involved in a variety of functions including stimulating heart rate and affecting Malpighian tubule functions. The third peptide, that can be produced by the *capa* gene, is related to the diapause hormone because it has a WFGPRLamide *C*-terminal ending.

As indicated above the first functions described for the PBAN/pyrokinin family of peptides was stimulation of pheromone biosynthesis in female moths and stimulation of hindgut muscle contraction in cockroaches. Other functions were soon described for peptides in the family including induction of embryonic diapause in females and cuticle melanization in larvae of the silkworm moth, *Bombyx mori* (Matsumoto et al., 1990; Imai et al., 1991). Once the peptides were identified it became apparent that these peptides belong to the same family with cross-reactivity of function. Subsequently other functions were identified including acceleration of puparium formation in higher flies (Zdarek et al., 1997) and pupal diapause development in heliothine moths (Sun et al., 2003; Zhang et al., 2004). This list of functions indicates the pleiotropic nature of the PBAN/pyrokinin family of peptides across the Insecta. Other functions could potentially be found for the family because these peptides are found in all insects (Jurenka and Nusawardani, 2011).

The target tissue for the action of PBAN in adult female moths is the pheromone gland, which is found as intersegmental tissues located between the eighth and ninth abdominal segments of the ovipositor in heliothines (Rafaeli and Jurenka, 2003). Pheromone biosynthesis can be stimulated by either injecting peptides into intact female moths or peptides can be incubated with an isolated pheromone gland on saline. The *in vitro* studies demonstrate that the PBAN-receptors are located in the epidermal cells of the

pheromone gland. A biologically active biotinylated-PBAN analog was used to demonstrate specific binding to a protein from isolated pheromone glands (Rafaeli et al., 2003). Cloning and functional expression of a PBAN-receptor was first reported in *H. zea*, the same moth in which PBAN was first identified (Choi et al., 2003).

PBAN-RECEPTOR

The PBAN-receptor was identified based on sequence similarities with a group of three receptors from *D. melanogaster*. After the *D. melanogaster* genome was sequenced and annotated peptide G-protein coupled receptors (GPCR) were identified based on sequence alignment with known vertebrate peptide GPCRs (Hewes and Taghert, 2001). One group of three receptors (CG8784, CG8795, CG9918) had sequence similarities with the neuromedin U (NmU) receptor from mammals. The ligand, NmU, has a *C*-terminal ending of FRPRNamide, which is similar to the *C*-terminal ending of PBAN, FSPRLamide. Choi et al. (2003) demonstrated that the vertebrate NmU peptide stimulated pheromone biosynthesis in female moths indicating cross-reactivity and receptor activation. The *D. melanogaster* sequences were used in a PCR based sequencing approach to obtain the full-length sequence from pheromone glands of *H. zea*. The three receptors from *D. melanogaster* were also characterized as being activated by pyrokinins (Park et al., 2002) indicating that all of these receptors belong to a similar family.

The functional expression of the *H. zea* PBAN-receptor indicated that PBAN activated the receptor at a half-maximum effective concentration of 25 nM (Choi et al., 2003). Several other peptides produced by the PBAN-gene were also active at similar concentrations. Although, concentrations required to stimulate pheromone biosynthesis *in vitro* in *Helicoverpa armigera* showed that PBAN was active at 0.5 nM and the other peptides at significantly higher concentrations (Stern et al., 2007). PBAN-receptors have been characterized from several moths including *B. mori* (Hull et al., 2004), *Heliothis virescens* (Kim et al., 2008), and *Plutella xylostella* (Lee et al., 2011). These studies indicate that PBAN will activate receptors at concentrations in the low nanomolar range. Several other PBAN-receptors have been identified based on sequence homology from other moths including *H. armigera* (Rafaeli et al., 2007), *Spodoptera exigua* (Cheng et al., 2010), and *Spodoptera littoralis* (Zheng et al., 2007).

Only one PBAN-receptor sequence was identified from pheromone glands of *H. zea* (Choi et al., 2003). However, three PBAN-receptor sequences were identified from cDNA obtained from the central nervous system of *H. virescens* fourth instar larvae (Kim et al., 2008). All three receptor sequences were identical except for *C*-terminal extensions. The N-terminal and seven-transmembrane domain regions of the *H. virescens* PBAN-receptors are about 98.5% identical with the *H. zea* and *H. armigera* receptors. The *H. virescens* PBAN-receptor subtype C, was identified in pheromone glands of adult female *H. virescens*. The other two were identified from the larval central nervous system and did not have activity when tested in a calcium mobilization assay using a heterologous expression system (Kim et al., 2008). The *C*-terminal extension of *H. virescens* PBAN-receptor subtype C is similar to the *C*-terminal of the *B. mori* PBAN-receptor (Hull et al., 2004). The *C*-terminal extension in the *B. mori* PBAN-receptor is involved in efficient internalization of the receptor after activation (Hull et al., 2004). It is interesting to note that the PBAN-receptors identified from pheromone glands of other moths have a shortened *C*-terminal ending similar to that of the *H. zea* PBAN-receptor. Functional significance of a short *C*-terminal ending indicates that the receptor could remain active in the pheromone gland cell membrane for a longer period of time. Time course studies on induction of pheromone biosynthesis in isolated pheromone glands indicated that the PBAN-receptor does remain active for a period of time after stimulation by PBAN (Choi et al., 2004).

The diapause hormone-receptor (DH-receptor) has high sequence homology to the PBAN-receptor especially in the transmembrane domains (Jurenka and Nusawardani, 2011). In Lepidoptera the DH-receptor has only been characterized from *B. mori* (Homma et al., 2006). Other insects have a pyrokinin 1-receptor (PK1-receptor) that is similar to the DH-receptor in sequence and are activated by DH-like peptides with an FGPRLamide *C*-terminal ending. Other insects also have PK2-receptors that are similar to the PBAN-receptor. A third GPCR is the perivicerokinin-receptor (PVK-receptor) that is activated by PVKs but not by other PBAN/pyrokinin-like peptides (Iversen et al., 2002; Park et al., 2002; Olsen et al., 2007). Phylogenetic relationships of these receptors from insects indicate three groups of receptors that follow a typical evolutionary origin for orders of insects (**Figure 1**).

G-protein coupled receptors have a seven-transmembrane domain motif that appears to be structurally conserved. With the X-ray crystal structure of rhodopsin, β_1 and β_2 adrenergic receptors, and A_{2A} adenosine receptors published, it is possible to construct homology models based on these solved three-dimensional structures. A detailed model of the *H. zea* PBAN-receptor was built using the crystal structure of rhodopsin as a template and *in silico* binding studies indicated possible interactions with PBAN as a ligand (Stern et al., 2007). A putative ligand binding pocket was indicated in a study utilizing an evolutionary trace approach in comparing the insect PBAN-receptors (Jurenka and Nusawardani, 2011). The conserved nature of the transmembrane domains and structural features of the ligand binding pocket for GPCRs allows the prediction of ligand interactions in a binding pocket of the PBAN-receptor. A model illustrating a putative *H. zea* PBAN-receptor binding pocket is shown in **Figure 2**. This model will need to be verified with mutation studies to determine if the specified amino acids are involved in ligand binding.

Several mutation studies have been conducted to determine which domains of the PBAN-receptor are involved in ligand recognition and activation. One study utilized chimeras where the extracellular domains were exchanged sequentially between the *H. zea* PBAN-receptor and the *D. melanogaster* PK1-receptor (Choi et al., 2007). All extracellular domain chimeras reduced activity of the chimera receptor when challenged with PBAN. However the *H. zea* PBAN-receptor chimera with the third extracellular loop exchanged had increased activity when challenged with the diapause hormone. The *D. melanogaster* PK1-receptor chimera with the third extracellular loop exchanged had increased activity when challenged with PBAN. These results indicate that the third extracellular loop is important for peptide recognition and

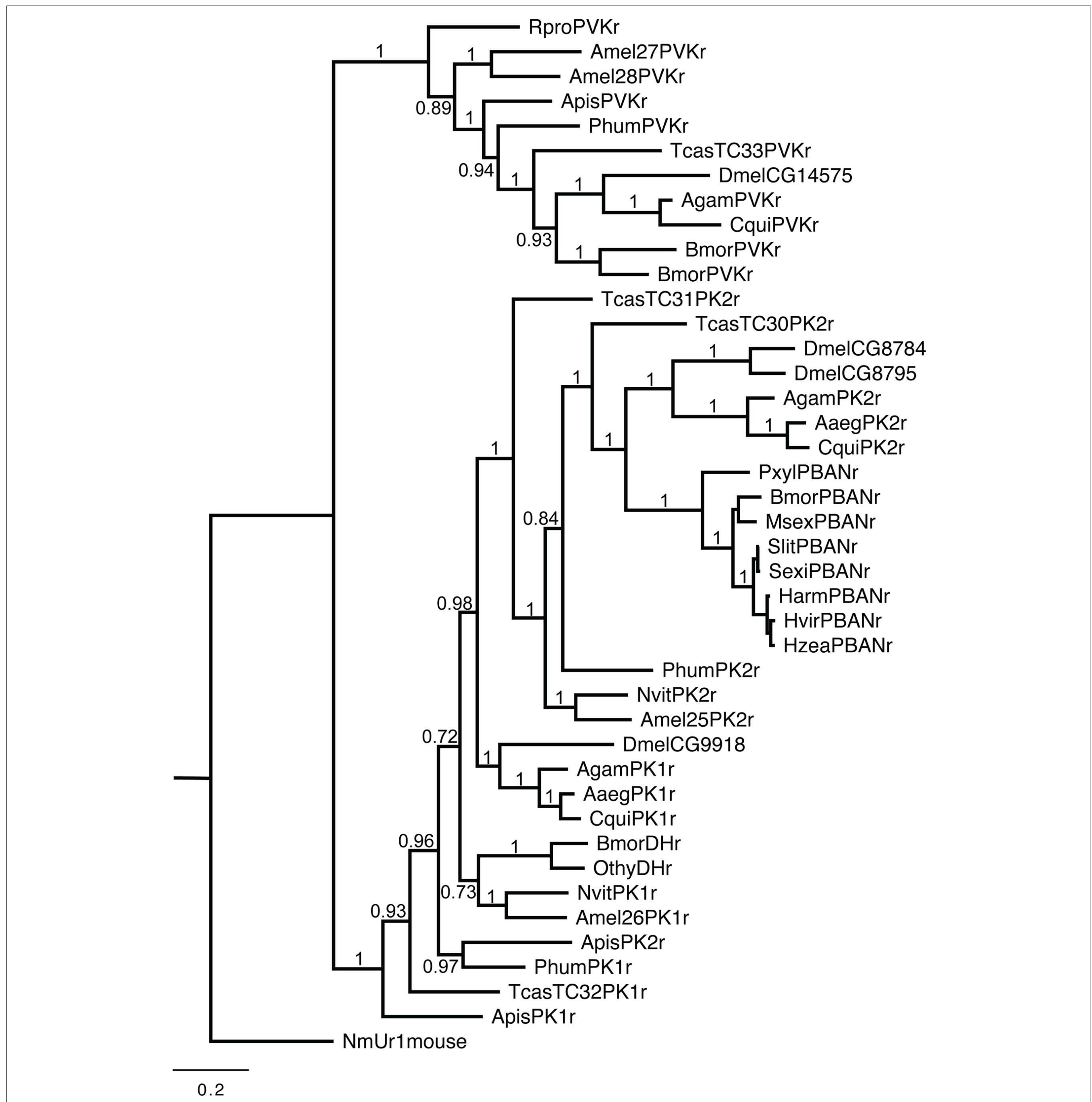

FIGURE 1 | Phylogenetic relationships of the PBAN/PK2-receptor, DH/PK1-receptor, and PVK-receptor. Species abbreviations: *Aaeg, Aedes aegypti*; *Agam, Anopholes gambiae*; *Agos, Aphis gossypii*; *Amel, Apis mellifera*; *Apis, Acyrthosiphon pisum*; *Bmor, Bombyx mori*; *Cfor, Coptotermes formosanus*; *Cqui, Culex quinquefasciatus*; *Dvir, Diabrotica virgifera virgifera*; *Dmel, Drosophila melanogaster*; *Harm, Helicoverpa armigera*; *Hzea, Helicoverpa zea*; *Hvir, Heliothis virescens*; *Msex, Manduca sexta*; *Nvit, Nasonia vitripennis*; *Othy, Orgyia thyellina*; *Phum, Pediculus humanus*; *Pxyl, Plutella xylostella*; *Rpro, Rhodnius prolixus*; *Sinv, Solenopsis invictus*; *Sexi, Spodoptera exigua*; *Slit, Spodoptera littoralis*; *Tcas, Tribolium castaneum* (Jurenka and Nusawardani, 2011).

could be involved in accepting the correct peptide for binding to a receptor activation site which is the ligand binding pocket located in the transmembrane domain area of the receptor. Alanine substitution mutations made in the third extracellular loop indicate that specific amino acids could be involved in peptide recognition (Choi and Jurenka, 2010). Further studies will be required to validate these models and establish how this family of receptors recognize specific peptides and activate the receptor.

PBAN MODE OF ACTION

Pheromone biosynthesis activating neuropeptide activation of the receptor induces the influx of extracellular calcium and the subsequent increase in cytosolic calcium (Jurenka et al., 1991; Jurenka, 1996). In heliothine moths, as in all moth species examined to date, the presence of calcium in the extracellular medium is a prerequisite for PBAN action (Rafaeli, 1994; Choi et al., 2004; Choi and Jurenka, 2006) suggesting that the opening of cation channels and the concomitant influx of calcium are most likely conserved features of PBAN activation. In the absence of calcium or the presence of calcium–calmodulin inhibitors such as W7, pheromonotropic activity is abolished (Rafaeli and Gileadi, 1996a) and, conversely, the pheromonotropic effects of PBAN can be duplicated with calcium ionophores such as ionomycin, thapsigargin, and A23187 (Rafaeli and Gileadi, 1996a; Rafaeli and Jurenka, 2003).

However, unlike the signal transduction pathway determined for the silkworm *B. mori*, in the heliothine moths there is evidence that the increase in cystosolic calcium activates a calcium–calmodulin sensitive adenylate cyclase which in turn promotes the production of cyclic-AMP (Rafaeli and Soroker, 1989; Soroker and Rafaeli, 1995; Rafaeli and Gileadi, 1996a). Furthermore, pharmacological compounds that affect cyclic-AMP levels such as cyclic-AMP analogs, isobutyl methyl xanthine (a phosphodiesterase inhibitor), and forskolin (an adenylate cyclase activator) have been shown to stimulate downstream events and thereby pheromone biosynthesis in *H. armigera* (Rafaeli and Soroker, 1989; Rafaeli, 1994) and *H. zea* (Jurenka et al., 1991). In addition, in *H. armigera* sodium fluoride, a G-protein activator can induce intracellular cyclic-AMP levels and subsequent downstream events leading to pheromone production, independent of the ligand PBAN (Rafaeli and Gileadi, 1996b). As an outcome of the activation of the second messengers, kinase, and/or phosphatase is activated, which, in their turn, stimulate fatty acid biosynthesis in the pheromone biosynthetic pathway (**Figure 3**).

What enzyme in the biosynthetic pathway is affected by the signal cascade brought about through PBAN binding to its receptor? Demonstration of the enzymatic key regulatory step in the biosynthesis of sex-pheromones primarily relies on following labeled precursors and intermediates into pheromone molecules in the absence and presence of PBAN. Thus, if production of a labeled pheromone component from incorporation of labeled precursor occurs in the absence of PBAN to the same extent as in its presence the labeled precursor must be acting downstream of the regulatory enzyme and therefore regulation must occur upstream. The effect of PBAN on the different steps in the biosynthetic pathway has been investigated in several Lepidopteran species including *B. mori* (Arima et al., 1991; Ozawa et al., 1993), *Thaumetopoea pityocampa* (Arsequell et al., 1990), *S. littoralis* (Martinez et al., 1990; Fabrias et al., 1994), *Manduca sexta* (Fang et al., 1995; Tumlinson et al., 1997), *H. zea* (Jurenka et al., 1991), *H. armigera* (Tsfadia et al., 2008), and *Plodia interpunctella* (Tsfadia et al., 2008). Studies

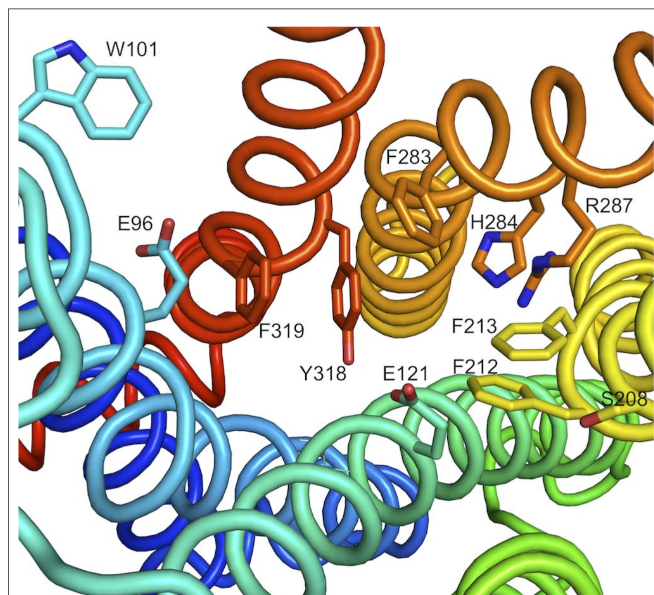

FIGURE 2 | A model of the *Helicoverpa zea* PBAN-receptor illustrating amino acids that could be involved in binding PBAN. Reproduced with permission from Jurenka and Nusawardani (2011).

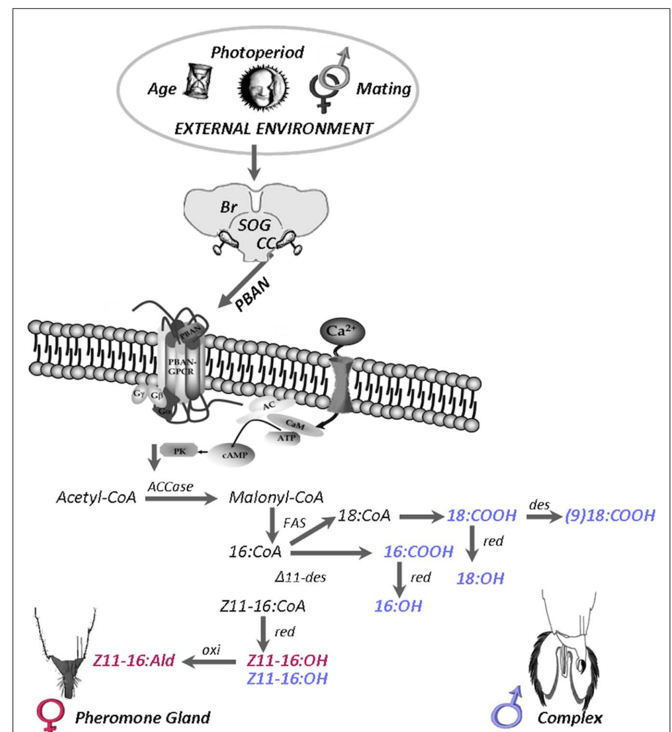

FIGURE 3 | A diagrammatic representation of sex pheromone biosynthesis resulting from PBAN release into the hemolymph and its interaction with the PBAN GPCR in female pheromone glands (PG) and male hairpencil–aedaegus-complexes of *Helicoverpa armigera.* Up-regulation of female components in red and male components in blue. (Male-complex illustration taken from Bober and Rafaeli, 2010 with permission). Abbreviations: AC, adenylate cyclase; ACCase, acetyl-CoA carboxylase; Br, brain; CaM, calcium–calmodulin; cAMP, cyclic-AMP; CC, corpora cardiaca; Δ11-des, Δ11 desaturase; FAS, fatty acid synthetase; GPCR, G-protein coupled receptor; oxi, oxidase; PBAN, pheromone biosynthesis activating neuropeptide; PK, protein kinase A; red, reductase; SOG, subesophageal ganglion.

of this nature have so far indicated that PBAN does not influence desaturase activity.

Using both stable isotopes and specific enzyme inhibitors the rate limiting step of PBAN pheromone biosynthesis regulation in *H. armigera* was determined (Tsfadia et al., 2008). These studies showed that only incorporation of labeled acetate is affected by PBAN and that this incorporation can be inhibited by the acetyl coenzyme A carboxylase (ACCase) inhibitor, tralkoxydim. Levels of incorporation of labeled malonyl CoA or palmitic acid (downstream of acetate) were unaffected by the presence or absence of PBAN (Tsfadia et al., 2008). Thus, in *H. armigera*, the rate limiting step for PBAN control is regulation of the ACCase which catalyzes the rate limiting enzyme of fatty acid biosynthesis, prior to the action of fatty acid synthetase (**Figure 3**). PBAN is also thought to influence ACCase activity in *Argyrotaenia velutinana* (Tang et al., 1989), *P. interpunctella* (Tsfadia et al., 2008), and *H. zea* (Jurenka et al., 1991). In contrast, in *B. mori*, *T. pityocampa*, *S. littoralis*, and *M. sexta* PBAN stimulates a reductase that converts an acyl-CoA to an alcohol precursor (Arsequell et al., 1990; Martinez et al., 1990; Ozawa et al., 1993; Fang et al., 1995). In *H. virescens* a two-step regulatory role for PBAN was demonstrated (Eltahlawy et al., 2007). This two-step theory entailed a push (ACCase) and a pull (acyl-CoA) for the action of PBAN and may explain the controversial hypotheses suggested for identifying the rate limiting steps controlled by PBAN in the different moth species.

An interesting feature revealed by studies on the mode of PBAN action is that in those species in which PBAN has been shown to regulate reductase activity, cyclic-AMP has proven to be ineffective as a pheromonotropic agent whereas in those species in which PBAN regulates ACCase activity, cyclic-AMP appears to be involved as a second messenger. Moreover, it is interesting that different receptor subtypes maybe correlated with the different downstream intracellular signal cascades that are induced. It is apparent that at least two subtypes of PBAN-receptors could be located in pheromone glands: one with calcium signaling cascade including cyclic-AMP and a shorter *C*-terminal tail (*H. zea*, *H. armigera*); the other dependent only on calcium having a longer *C*-terminal tail (*B. mori*). Allocating these characteristics may be too premature until more evidence becomes available as to the involvement of cyclic-AMP in the signal transduction of other PBAN-receptor subtypes. For example, PBAN induces calcium elevations by the *P. xyllostella* PBAN-receptor, which also has a short *C*-terminal tail; however cyclic-AMP levels were not analyzed in the *P. xyllostella* study (Lee et al., 2011).

PBAN's INFLUENCE ON MALE PHEROMONAL COMPONENTS

Male pheromone production has been studied in several insect species. Male insects often possess scent-releasing organs in the form of hairpencils, coremata, or androconial scales (Birch et al., 1990). In the Lepidoptera, studies have identified hairpencil secretions produced by several species (Chow et al., 1986; Phelan et al., 1986; Teal and Tumlinson, 1989; Heath et al., 1992; Thibout et al., 1994; Huang et al., 1996). The behavioral role of these secretions is not well understood, but most often these odors have been deemed important in courtship behavior. In general male pheromones can be considered to have many possible functions: they can act as aphrodisiacs to stimulate female receptivity during courtship (Birch, 1974); they have been reported to induce female calling (Szentesi et al., 1975), females become immobile allowing copulation; and they have been reported to function as male-to-male inhibitory compounds, thereby minimizing male-competition (Hirai et al., 1978; Teal et al., 1984; Teal and Tumlinson, 1989; Wu et al., 1991; Huang et al., 1996). Odors released by male hairpencils are important in male acceptance by the female and may play a role in mate choice and species isolation (Hillier and Vickers, 2004; Lassance and Löfstedt, 2009). In the European corn borer, *Ostrinia nubilalis*, scents released during courtship by males provide critical information for female acceptance (Lassance and Löfstedt, 2009). Close-range chemical cues have also been proposed as a trait used by females to assess male quality (Eisner and Meinwald, 1995).

In species of Lepidoptera belonging to the Arctiidae and Danaidae, scent gland composition is related to host plant consumption and sequestration of compounds during the larval and adult stages (Birch and Hefetz, 1987). However, in other moth species, as in the heliothine moths, these male odors are derived from the fatty acid biosynthetic pathway, and as such are similar to the female sex-pheromone blends (Teal and Tumlinson, 1989; Huang et al., 1996). In *O. nubilalis* the male chemical signal is also analogous to the female signal in that structurally similar compounds are being used by both sexes and are governed by

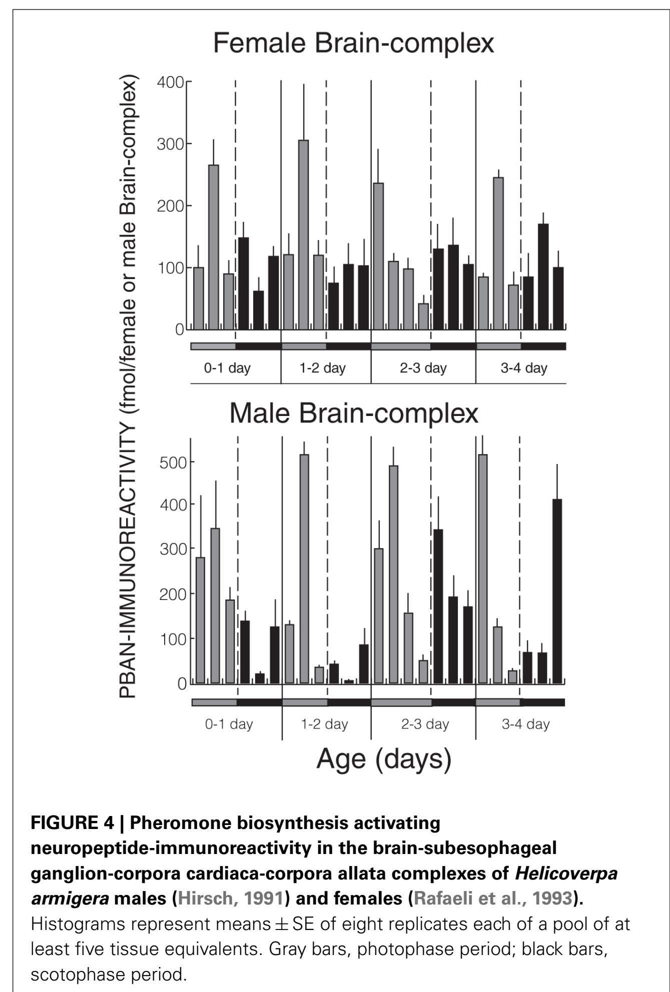

FIGURE 4 | Pheromone biosynthesis activating neuropeptide-immunoreactivity in the brain-subesophageal ganglion-corpora cardiaca-corpora allata complexes of *Helicoverpa armigera* males (Hirsch, 1991) and females (Rafaeli et al., 1993). Histograms represent means ± SE of eight replicates each of a pool of at least five tissue equivalents. Gray bars, photophase period; black bars, scotophase period.

the same genes encoding biosynthetic enzymes (Lassance and Löfstedt, 2009).

Pheromone biosynthesis activating neuropeptide was originally isolated from female *H. zea* suboesophageal ganglia and its distribution in the female nervous system was studied using ELISA (Kingan et al., 1992) however the gene transcript was shown to be present in both the male and female central nervous system (Ma et al., 1998). Using an immunoassay, PBAN was also found in both female and male *H. armigera* central nervous systems and throughout the photoperiod (**Figure 4**; Hirsch, 1991; Rafaeli et al., 1993).

The function of PBAN in the males, however, has been a mystery since its discovery but its presence has been speculated to have other functional significances particularly in light of the pleiotropic action of PBAN and PBAN-like peptides in other insects. However, a recent temporal differential expression study of the PBAN-R revealed the presence of gene transcripts in both the male-complexes (hairpencil–aedaegus complex) and female pheromone glands of two moth species, *H. armigera* and *B. mori* (Bober et al., 2010) with levels dependent on the age of the adults and up-regulated on or just before eclosion. Whilst the presence of PBAN in males can be understood in terms of the ubiquitous characteristic of this peptide family, the presence, as well as the transcriptional regulation of its receptor in the male-complex called for a re-examination of a possible function for PBAN and its receptor in this tissue.

As discussed above, and, since fatty acid-derived pheromonal compounds were identified in male-complexes of several moth species including heliothine moths, we aimed to determine whether PBAN plays a role in the regulation of the biosynthesis of these compounds in male *H. armigera*. We utilized both physiological bioassays and RNA interference (RNAi) technology to identify several male pheromone components that are responsive to PBAN stimulation and to photoperiod, and are also significantly affected by silencing of the PBAN-R (Bober and Rafaeli, 2010). We hypothesized that these components are key in the chemical communication between females and males during copulation (**Figure 4**). It remains to be demonstrated that those PBAN up-regulated male produced compounds are indeed responsible for a successful mating encounter by either arresting females for copulation, increasing their receptivity, or deterring co-specific males from competing.

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