Chapter 6
A Sexy Moth Model – The Molecular Basis of Sex Pheromone Biosynthesis in the Silkmoth *Bombyx mori*

J. Joe Hull and Adrien Fónagy

Abstract The reproductive behaviors of many insects are coordinated by the synthesis and release of species-specific volatiles that communicate the location of potential mates. Given their biological importance, structural elucidation of these compounds (i.e., sex pheromones) and molecular determination of the underlying biosynthetic pathways have been the focus of numerous studies. Among the various model species that have been examined, the silkmoth (*Bombyx mori*) has had an outsized impact on the research field. Indeed, it was Adolf Butenandt's pioneering publication in 1959 on chemical characterization of the silkmoth sex pheromone (E,Z)-10,12-hexadecadien-1-ol (i.e., bombykol) that ushered in a new era of chemical ecology. Since then, *B. mori* has been at the forefront of each new advancement in our understanding of the pre- and postadult eclosion processes that culminate in pheromone production – from demonstration of hormonal regulation by a neuropeptide to identification of the cognate receptors and characterization of the genes comprising the biosynthetic and regulatory pathways. In honor of the 60th anniversary of bombykol's elucidation, we provide a perspective on the spectrum of studies that have made Butenandt's "sexy" moth one of the principal models for sex pheromone biosynthesis.

Keywords *Bombyx mori* · Bombykol · Sex pheromone biosynthesis

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6.1 Introduction

The reproductive behaviors of many insects are coordinated by the synchronous synthesis and release of species-specific chemical mixtures that communicate the location of potential conspecific mates. Typically, these compounds (referred to as sex pheromones) are produced as blends of volatiles with differing chemistries and varying component ratios. Over the years, sex pheromones have been the focus of numerous studies from chemical identification to elucidation of the biosynthetic pathways, olfactory detection components, and neural processes that culminate in a behavioral response. Initially viewed through the prism of pest management, pheromone-based studies have broadened to provide critical insights into the evolutionary underpinnings of insect chemical communication and speciation. Lepidopteran-based studies have arguably dominated the research field since chemical elucidation of the first sex pheromone from 5 x 10^5 silkmoth (*Bombyx mori*) females in 1959 (Butenandt et al. 1959). Today, the pheromone database encompasses 67 lepidopteran families representing >1500 species (El-Sayed 2018).

Elucidation of lepidopteran sex pheromone chemistries has revealed conserved pathways for most species that utilize relatively simple straight-chain C_{10}–C_{18} aliphatic compounds containing varying degrees of unsaturation and an oxygenated functional group (i.e., type I); however, straight-chain hydrocarbons and epoxide hydrocarbons (i.e., type II) have also been identified, albeit less frequently (Ando et al. 2004). In parallel with chemistry-based structure determination, research into the biochemical and molecular basis of sex pheromone biosynthetic pathways has established that fatty acid metabolism intermediates (e.g., palmitic acid/hexadecanoic acid) serve as substrates for the downstream modifications (selective β-oxidation reactions, desaturations, and diverse reductive modifications) that generate the species-specific pheromone blends (Bjostad et al. 1987). These pathways along with their regulation, evolution, and product detection have been the topic of numerous excellent reviews (Sakurai et al. 2014; Zhang et al. 2015; Groot et al. 2015; Yew and Chung 2015; Allison and Cardé 2016; Jurenka 2017). Here, however, in honor of the 60-year anniversary of the structural determination of (*E*,*Z*)-10,12-hexadecadien-1-ol (i.e., bombykol) as the principal silkmoth sex pheromone (Butenandt et al. 1959), we provide a perspective on the spectrum of studies that have made Butenandt's "sexy" moth the foremost model for understanding the pre- and posteclosion processes that culminate in moth sex pheromone production (i.e., pheromonogenesis).

6.2 Regulation of Pheromone Production

6.2.1 Circadian-Based Control

For most moths, pheromone production and release typically exhibit circadian oscillations that coincide with specific points of the day:night cycle (Groot 2014). The basis for this circadian output was the focus of early biochemical studies.

Observations that decapitation/neck ligature inhibited normal scotophase pheromone production in *Helicoverpa zea* and *Chilo suppressalis* (Raina and Klun 1984; Ohguchi et al. 1985) suggested involvement of a brain-derived hormonal factor. A subsequent study showing female brain homogenates could restore pheromone production in decapitated *H. zea* females confirmed the cephalic nature of the factor (Raina and Klun 1984). That same study provided a potential mechanism for the circadian signal as pheromonogenic activity was most pronounced in the hemolymph of scotophase female *H. zea* rather than photophase. In their model, Raina and Klun (1984) suggested that the hormonal factor accumulated during photophase and was subsequently released into the hemolymph to act on the target tissue during scotophase. Similar to other moths, *B. mori* also exhibits circadian oscillations in pheromone production, albeit with bombykol titers lowest at early photophase, peaking at midphotophase, and declining again at late photophase (Ichikawa 1998; Fujii et al. 2018). Building on the early findings of *H. zea*, Ando and coworkers (1988a) examined the relevance of the hormonal mechanism described in *H. zea* for species that exhibit photophase-based pheromone production. They found that head extracts from photophase *B. mori* females were able to stimulate pheromone production in decapitated females, suggesting that bombykol production was likewise mediated by a circadian-released neuroendocrine factor (Ando et al. 1988a). Subsequent studies performed a decade later provided further support for humoral-based circadian control of pheromone production in *B. mori* with evidence that pheromonogenic neurosecretory cells also exhibit diurnal firing activity (Ichikawa 1998; Tawata and Ichikawa 2001).

6.2.2 Pheromone Gland as the Neuroendocrine Target Tissue

The site of sex pheromone production and release for most female moths is typically a bulbous, extrudable gland (i.e., pheromone gland, PG) located at the intersegmental membrane of the eighth and ninth segments (Bjostad et al. 1987). Early histological and ultrastructural analyses in a number of species, including pioneering work with *B. mori* PGs (Hayashi and Ito 1933; Steinbrecht 1964; Waku and Sumimoto 1969), revealed the glands are well suited for hydrocarbon-based pheromone biosynthesis and secretion. PGs are composed of hypertrophied secretory cells and modified epidermal cells with an apical brush border, a well-developed smooth ER network, and varying numbers of electron-lucent lipid vesicles (Ma and Ramaswamy 2003). In *B. mori*, the PG is a symmetrical pair of ventrolateral sacs termed *sacculi laterales* (Percy-Cunningham and MacDonald 1987; Fónagy et al. 2001) composed of two distinct layers – an outer cuticular layer and an inner layer consisting of 9000–10,000 homogenous epidermal cells arranged in a monolayer (Fónagy et al. 2000). This inner layer is characterized by the presence of cytoplasmic lipid droplets that accumulate 1–2 days prior to adult eclosion and which fluctuate in size and number in accordance with bombykol production and photoperiod (Fónagy et al. 2001). In contrast, the cuticle overlaying the gland consists of a lamellate endocuticle, protein epicuticle, a thin electron-dense cuticulin layer, and

an outer epicuticle (Steinbrecht 1964; Waku and Sumimoto 1969). Using a microsome-based bioassay consisting of fractions generated from each of the *B. mori* PG layers, Fónagy and coworkers (2000) confirmed that bombykol production was restricted to the inner epidermal cell layer.

Although the role of the PG in sex pheromone production and release had been well established, it was less clear if it was also the target of the brain-derived pheromonogenic factor as suggested for some species (Soroker and Rafaeli 1989; Rafaeli et al. 1990). Or, as suggested by other studies, the brain-derived factor acted on a non-PG target tissue(s) that either released a secondary stimulus to trigger pheromone production or provided pheromone precursor substrates (Tang et al. 1989; Teal et al. 1989). Multiple in vitro studies using isolated *B. mori* PGs (Arima et al. 1991; Fónagy et al. 1992b, c; Ozawa et al. 1993; Matsumoto et al. 1995a, Ozawa et al. 1995; Fónagy et al. 2001), however, revealed the glands to be the principal target of neuroendocrine control and confirmed that all of the cellular machinery necessary for bombykol biosynthesis and release are specific to the PG.

6.2.3 HPLC-Based Purification of the Neuroendocrine Factor

Neuroendocrine-based regulation of sex pheromone synthesis was initially proposed by Riddiford and Williams based on *corpora cardiaca* excision studies using saturniid moths (Riddiford and Williams 1971). Later observations that decapitation/neck ligature likewise inhibited sex pheromone production (Raina and Klun 1984; Ohguchi et al. 1985; Ando et al. 1988a) and that multiple moth species had a brain-derived pheromonotropic factor (Raina and Klun 1984; Raina et al. 1987; Ando et al. 1988a) provided further support for neuroendocrine regulation. Using high-performance size exclusion chromatography, a *B. mori* factor was shown to be both susceptible to protease treatment and peptidergic in nature (Ando et al. 1988a). Using a 10-step isolation procedure, a pheromonotropic peptide was purified to homogeneity from 75,000 adult male *B. mori* heads (Nagasawa et al. 1988). N-terminal sequencing of the purified peptide yielded a 10-amino acid (aa) sequence (Leu-Ser-Glu-Asp-Met-Pro-Ala-Thr-Pro-Ala-) with 100% identity to a previously isolated neuropeptide, melanization and reddish coloration hormone (MRCH)-I (Matsumoto et al. 1986). Sufficient quantities of the pheromonotropic peptide were subsequently purified from 6 x 10^5 adult male *B. mori* heads for complete sequence determination of the factor as a 33-aa, C-terminal amidated peptide termed pheromone biosynthesis activating neuropeptide (PBAN) (Kitamura et al. 1989). A second, later eluting pheromonotropic fraction yielded *B. mori* PBAN-II, which is differentiated from the first *B. mori* PBAN by the inclusion of a single N-terminal Arg residue (Kitamura et al. 1990). A similar 33-aa, C-terminal amidated PBAN was independently purified from 5000 adult *H. zea* male/female brain-suboesophageal ganglion (SOG) complexes (Raina et al. 1989). High sequence identity (79%) between the *B. mori* and *H. zea* PBANs and pheromonotropic cross-reactivity in species with differing sex pheromone chemistries confirmed that

neuroendocrine regulation of pheromone production was evolutionarily conserved (Raina et al. 1989; Fónagy et al. 1992b).

6.2.3.1 Structure–Function Analysis of PBAN

Initial structure–function analyses of *B. mori* PBAN (BmPBAN or BommoPBAN) highlighted the importance of the C terminus, rather than the N terminus, in mediating pheromonotropic activity. Loss of the C-terminal Leu33 abolished activity, whereas deletion of residues 1–23 had no effect on activity (Kitamura et al. 1989; Kitamura et al. 1990). Comparison of amidated, hydroxylated, and methyl ester versions of the peptide underscored the critical importance of the C-terminal amide (Kitamura et al. 1989; Kitamura et al. 1990; Kuniyoshi et al. 1991a; Nagasawa et al. 1994). Further studies determined that the minimal sequence necessary for activity (albeit reduced) resided in the pentapeptide (Phe-Ser-Pro-Arg-Leu-NH_2); higher activity, however, was retained in the hexapeptide (Tyr-Phe-Ser-Pro-Arg-Leu-NH_2) (Kuniyoshi et al. 1991a). Replacing the first three residues (Tyr, Phe, and Ser) of the hexapeptide had inconsequential effects on pheromonotropic activity, whereas substitution of Pro, Arg, and Leu had severe effects (Kuniyoshi et al. 1991a). A more recent substitution study revealed that the Arg functional group is essential for receptor binding (Kawai et al. 2012).

Kitamura and coworkers (1989) reported that purified BommoPBAN co-eluted with the oxidized version of the synthetic peptide, suggesting that the natural peptide was in the fully oxidized state. Furthermore, the oxidized peptide was reported to have significantly higher activity (Kitamura et al. 1989; Kitamura et al. 1990). To explore the basis for this modification, Nagasawa and coworkers generated a series of oxidation products examining the importance of each of the three Met residues (Met5, Met14, and Met22). Oxidation of single residues enhanced activity over the nonoxidized form, but the most pronounced effects were only apparent when all three residues were in their oxidized state (Nagasawa et al. 1994). Additional peptide modifications designed to generate highly active analogs of the penta- and hexapeptides were also examined for potential use in developing PBAN mimetics. Addition of acetyl, benzoyl, ethyl, benzyl, pyroglutamyl, or D-alanyl groups to the N-terminal position enhanced activity relative to nonmodified peptides. The observed increase in activity though was the result of enhanced hemolymph stability rather than receptor activation (Kuniyoshi et al. 1991b; Kuniyoshi et al. 1992b). Attempts at peptide cyclization, which restricts the available conformational space of a linear peptide, via derivatization of the Arg and Leu sites had limited activity, whereas cyclization outside of the core sequence [e.g., cyclo(-Asn-Thr-Ser-Phe-Thr-Pro-Arg-Leu)] yielded an analog of equal potency as the linear hexapeptide alone (Nagasawa et al. 1994). More recent efforts to develop pheromonotropic agonists/antagonists based on the BommoPBAN decapeptide sequence (i.e., residues 24–33) determined that a linear synthetic derivative with a Tyr-Asn substitution (Ser-Lys-Thr-Arg-Asn-Phe-Ser-Pro-Leu-NH_2) of the carbon 6 aa upstream of the terminal residue (i.e., C6 position) was a partial agonist (Kawai et al. 2010). Cyclic

peptides [cyclo(Thr-Cys-Asn-Phe-Ser-Pro-Arg-Leu) and cyclo(Thr-Cys-Tyr-Phe-Ser-Pro-Arg-Leu)] based on this substitution likewise yielded partial agonists and demonstrated that the C6 position and the restricted conformational space were important for receptor recognition (Kawai et al. 2011). These findings provided a biological context for an earlier NMR study (Okada et al. 2009) that showed the bioactive decapeptide (Ser-Arg-Thr-Arg-Tyr-Phe-Ser-Pro-Arg-Leu-NH_2) and the inactive free acid form assume different conformations with the amide exhibiting a type I β-turn similar to that reported for the *H. zea* peptide (Nachman et al. 1993). Given their potential for next-generation pest management tactics, PBAN peptidomimetics, many based on the initial *B. mori* structure–function studies, have been the focus of multiple research groups with developments the topic of numerous reviews (Altstein et al. 2007; Nachman 2014).

6.2.4 Molecular Cloning of the BommoPBAN cDNA

Determination of the BommoPBAN aa sequence facilitated molecular elucidation of the encoding mRNA transcript and provided a means for localizing PBAN expression. Using dissimilar degenerate methods with cDNAs generated from adult female brain–SOG complexes or pooled SOG from day 0 pupae, two groups with differing aims separately identified a transcript encoding a 192-aa peptide precursor that included the PBAN sequence (Kawano et al. 1992; Sato et al. 1993). Although alternative splicing is a common mechanism for enhancing neuropeptide diversity, subsequent molecular studies failed to find any evidence for alternative splice variants of the *B. mori* transcript (Sato et al. 1993; Sato et al. 1994). Post-translational proteolytic processing of the prepropeptide was predicted to yield the PBAN sequence along with four additional amidated peptides including diapause hormone (DH), a previously purified peptide critical for induction of embryonic diapause (Imai et al. 1991) that was the target of the pupae-based study. The DH active core (Phe-Gly-Pro-Arg-Leu-NH_2) is similar to the critical PBAN pentapeptide motif as is the C terminus of the three other peptides (Phe-Thr-Pro-Arg-Leu-NH_2; Phe-Ile-Pro-Arg-Leu-NH_2; Phe-Ser-Pro-Arg-Leu-NH_2) termed suboesophageal ganglion neuropeptides (SGNPs) α, β, and γ (Kawano et al. 1992; Sato et al. 1993). Structurally, the prepropeptide consists of the initial signal peptide (23 aa) followed by the DH sequence (residues 24–47), then α-SGNP (residues 97–103), β-SGNP (residues 106–122), PBAN (residues 125–158), and γ-SGNP (residues 161–168) with each of the peptides flanked by mono- or dibasic cleavage sites and amidating Gly signals. Processing of the PBAN peptide via the dibasic Arg125-Arg126 site yields PBAN-I, whereas alternative processing at Arg125 alone, which follows the "rule" for single Arg processing sites with a basic aa (Arg122) in the −4 position (Veenstra 2000), would generate PBAN-II (Kitamura et al. 1990). HPLC-based purification of *B. mori* SOG extractions confirmed that the respective SGNPs were generated following proteolytic processing of the PBAN prepropeptide (Sato et al. 1993). Consistent with the early structure–function analyses that highlighted the

importance of the PBAN C-terminal pentapeptide (Kuniyoshi et al. 1991a), both DH and the SGNPs are pheromonotropic and activate heterologously expressed PBAN receptor at low to submicromolar concentrations (Sato et al. 1993; Watanabe et al. 2007). PBAN encoding transcripts with similar multipeptide processing sites have since been cloned from a number of moths (see Jurenka and Nusawardani 2011; Jurenka 2015).

6.2.4.1 Expression Profiling of the PBAN Transcript

Given the two main products of the DH-PBAN transcript regulate different physiological processes (i.e., pheromone production and diapause induction), the timing and location of DH-PBAN expression can have profound biological effects. Initial expression profiling of the PBAN transcript (commonly referred to as DH-PBAN) based on Northern blots showed a strong hybridization signal in the SOG of day 0 pupae as well as brain–SOG complexes of day 4 pharate adult females (Sato et al. 1993; Sato et al. 1994). A somewhat reduced signal was also apparent in brain–SOG complexes of day 4 pharate adult males. More extensive profiling revealed robust hybridization signals in day 8 eggs as well as SOGs from day 5 fifth instar larvae, day 3 pupae, and day 6 pharate adults with a weaker signal in day 3 fourth instar larvae and newly eclosed adults (Xu et al. 1995a). PCR-based analyses using primers designed to anneal to the 5′-ends of the DH (sense primer) and PBAN (antisense primer) coding sequences revealed that DH-PBAN expression was limited to the late pupal stage of nondiapausing silkworms, whereas peaks of expression were observed throughout embryonic, larval, and pupal development of diapause silkworms (Xu et al. 1995a). The observed expression in late-stage pharate adults is most consistent with the role of PBAN in pheromone production, whereas expression across developmental stages is more reflective of the role DH has in diapause induction. Similarly, given the critical role environmental conditions have on the induction of embryonic diapause in *B. mori* (Watanabe 1924), reports of temperature-linked DH-PBAN expression (Xu et al. 1995a) would be associated with the DH-driven activity of the transcript rather than pheromone production. Dopamine-induced expression of the DH-PBAN transcript is also associated with DH activity as dopamine and/or DOPA exposure switches the diapause programming state (Noguchi and Hayakawa 2001).

6.2.4.2 Localization of the PBAN Transcript/Peptide

In situ hybridization using a probe designed to the DH coding sequence localized the *B. mori* DH-PBAN transcript to 12 neurosecretory cells grouped into three clusters along the ventral surface of the mandibular, maxillary, and labial neuromeres of the SOG (Sato et al. 1994). Similar cell groupings corresponding to four mandibular cells (also termed anterior or SMd cells), six maxillary cells (medial or SMx cells), and two labial cells (posterior or SLb cells) were likewise identified immunohistochemically

using antibodies against DH, PBAN, and/or peptides with the shared FXPRLamide (Phe-xxx-Pro-Arg-Leu-NH_2, where x = any aa) pentapeptide C terminus (Ichikawa et al. 1995; Ichikawa et al. 1996; Sato et al. 1998; Morita et al. 2003; Shiomi et al. 2007; Hagino et al. 2010). Similar sets of PBAN-immunoreactive neurosecretory cells have been identified in other Lepidoptera, including *H. zea*, *Mamestra brassicae*, *Ostrinia nubilalis, Manduca sexta,* and *Pseudaletia separata* (Kingan et al. 1992; Tips et al. 1993; Ma and Roelofs 1995a; Davis et al. 1996; Raina et al. 2003). Based on the SOG immunoreactivity, translation of the DH-PBAN transcript occurs throughout *B. mori* larval, pupal, and adult development as well as embryonically with earliest detection at the embryonic blastokinesis stage (Morita et al. 2003). Surprisingly, targeted ablation of the differing immunoreactive cells in *B. mori* resulted in cell-specific phenotypes with SLb ablation affecting ovariole accumulation of 3-hydroxykynurenine (a marker of diapause-destined eggs) and SMd/SMx removal resulting in reduced sex pheromone production (Ichikawa et al. 1996). This apparent functional differentiation of the DH-PBAN immunoreactive neurosecretory cells is supported by reports of diapause-linked differences in the immunoreactive staining and firing activity patterns of SLb cells during pupal-adult development (Sato et al. 1998; Ichikawa and Suenobu 2003; Hagino et al. 2010). Similar differences were not observed in the firing of SMd/SMx cells, and their activity during a diapause sensitive state had no effect on diapause induction (Ichikawa and Kamimoto 2003), further supporting functional differentiation. Reports of weak PBAN-specific immunoreactivity observed in SLb cells relative to SMd/SMx cells coupled with the ablation studies support anatomical and functional differentiation of these cells and suggest that they are specialized for diapause determination (Hagino et al. 2010). The staining profile of neurite projections from DH-PBAN immunoreactive cells suggests that axonal transport from the SMd/SMx and SLb cells also differs (Ichikawa et al. 1995). SMd/SMx-derived axons that terminate in the *corpus cardiacum* (a neurohemal organ that functions in neuropeptide storage for hemolymph release) pass through the maxillary nerve and *nervus corporis cardiaci-ventralis* (also referred to as NCC-V), whereas those from SLb cells utilize circumesophageal connectives and NCC-3. Intriguingly, the respective DH-PBAN peptidergic activities also exhibit differences in neurosecretory control with SLb-derived DH activity controlled by the brain (Matsutani and Sonobe 1987) and likely involve γ-aminobutyric acid (Shimizu et al. 1989; Hasegawa and Shimizu 1990; Ichikawa et al. 1997). In contrast, PBAN is regulated by a circadian pacemaker (Ando et al. 1988c; Ichikawa 1998; Tawata and Ichikawa 2001).

6.2.5 Identification of the PBAN Gene

By using radiolabeled probes corresponding to the DH and PBAN coding regions to screen a genomic library, Xu and coworkers (1995b) determined that the *B. mori* DH-PBAN gene was organized across 5 introns and 6 exons. The exons are arranged such that the signal peptide and the first four aa of DH are on exon 1, the remaining

20 DH aa on exon 2, an uncharacterized peptidergic sequence on exon 3, complete sequences for the α and β SGNPs and first 15 aa of PBAN on exon 4, the remaining 19 PBAN aa and γ SGNP on exon 5, and the stop codon on exon 6. Splicing of the five introns follows the "GT-AG" rule (Mount 1982) and consists of a 0, 2, 1, 2, 1 phase pattern (0 = codon that does not include sites from 3′ donor, 1 = codon that has two sites from 3′ donor, and 2 = codon that has one site from 3′ donor). The genomic organization of the DH-PBAN gene appears to be evolutionarily conserved in Lepidoptera with identical exon coding regions reported in other moth species (Zhang et al. 2005; Jing et al. 2007; Chang and Ramasamy 2014; Fodor et al. 2017; Senthilkumar and Srinivasan 2019); however, the size of the intronic regions varies with larger introns typically occurring in *B. mori*.

6.2.5.1 PBAN Promoter Region

An initial scan of the *B. mori* DH-PBAN gene revealed a number of potential promoter sites, including a canonical TATA box (position −46) and a potentially modified CAAT box (position −90) (Xu et al. 1995b). Subsequent in vitro analyses, however, demonstrated transcriptional activity of a proximal promoter (position −75 to −65) upstream of the TATA box corresponding to a POU-binding domain, and expression of the corresponding transcription factor (POU-M1/M2) coincided with DH-PBAN expression (Zhang et al. 2004). That study also identified an enhancer element 3.5–5 kb upstream of the DH-PBAN transcription site. In vivo studies also identified a *cis*-acting element upstream of the DH-PBAN start site (Shiomi et al. 2003), refinement of which localized the regulatory element to a Pitx-binding core sequence that resides in a different region (position −1117 to −1088) of the DH-PBAN gene than the POU motif (Shiomi et al. 2007). As with POU-M1/M2, the expression of the BmPitx transcription factor paralleled that of the DH-PBAN transcript and co-localized in the 12 DH-PBAN-producing neurosecretory cells of the SOG. Viral-based overexpression of the transcription factor enhanced DH-PBAN expression in pupal SOGs, whereas knockdown reduced expression (Shiomi et al. 2007). The identification of different DH-PBAN promoter regions may reflect limitations of the disparate assays used (POU-M1/M2 – in vitro; Pitx – in vivo) and/or *B. mori* strain-specific sequence variations (POU-M1/M2 – Jingsong x Haoyue; Pitx – Tokai x Asahi). However, reports of transcriptional activation involving physical cooperativity between Pitx homologs and POU domain factors (Tremblay and Drouin 1999; Quentien et al. 2002) may indicate that DH-PBAN transcription is similarly regulated. This proposed mechanism does not appear to be evolutionarily conserved across Lepidoptera as the POU-M1/M2 transcription factor was unable to drive expression from a conserved region of the *Helicoverpa armigera* DH-PBAN gene (Zhang et al. 2005). In that species, an E-box element (CAGCTG) rather than the POU domain is critical for transcriptional activation (Hong et al. 2006). Two ecdysone responsive element-like regions (position −753 to −743 and position −709 to −609) have also been identified in the 5′-region of the *B. mori* DH-PBAN gene (Xu et al. 1995b). Given the role ecdyster-

oids have in lepidopteran reproduction, the response elements may link PBAN transcription with reproductive competence; however, their role in pheromone production remains to be ascertained.

6.2.6 PBAN-Related Family of Peptides

The C-terminal FXPRLamide pentapeptide motif that characterizes BommoPBAN is critical for pheromone production (Nagasawa et al. 1988; Kitamura et al. 1989) as well as different physiological processes in the silkworm, including induction of embryonic diapause (Imai et al. 1991) and ecdysteroidogenesis in larval prothoracic glands (Watanabe et al. 2007). The peptide was also shown to exhibit pheromonotropic activity in other species (Matsumoto et al. 1990; Fónagy et al. 1992b; Fónagy et al. 1992d) in addition to having myotropic (Kuniyoshi et al. 1992a; Fónagy et al. 1992d) and cuticular melanization (Matsumoto et al. 1990) activities. Reciprocal cross-species pheromonotropic activity in *B. mori* has also been reported for FXPRLamide peptides from other species (Matsumoto et al. 1992a; Kuniyoshi et al. 1992a; Fónagy et al. 1992a). In the years since the initial purification and identification of BommoPBAN, the C-terminal pentapeptide has become a defining feature of the FXPRLamide family of pleiotropic neuropeptides (i.e., pyrokinins, PBANs, myotropins, DH, and SGNPs), which are expressed throughout the Insecta via the *capa* and *DH-PBAN/hugin* genes (Jurenka 2015; Yaginuma and Niimi 2015; Schoofs et al. 2017). In addition to pheromonotropic activity in moths, these peptides also regulate an astounding array of functions, including the induction of cuticular melanization in moth larvae (Matsumoto et al. 1992b; Altstein et al. 1996), the termination of pupal diapause in heliothine moths (Xu and Denlinger 2003; Zhang and Denlinger 2012), the induction of embryonic diapause and seasonal polyphenism in moths (Imai et al. 1991; Uehara et al. 2011), prothoracic gland ecdysteroidogenesis (Watanabe et al. 2007), visceral muscle contraction in cockroaches (Holman et al. 1986; Schoofs et al. 1993), acceleration of puparium formation in flies (Žďárek et al. 1998; Žďárek et al. 2004), pheromone synthesis in male *H. armigera* (Bober and Rafaeli 2010; Zhao et al. 2018), and trail pheromone biosynthesis in *Solenopsis invicta* (Choi and Vander Meer 2012).

6.3 The PBAN Receptor

6.3.1 Molecular Cloning of the PBAN Receptor

Although the molecular identities of BommoPBAN and its encoding gene had both been well elucidated by the mid 1990s, similar identification of the corresponding receptor proved more elusive. Based on the hypothesis that receptor/ligand coevolu-

tion would yield closely aligned receptor families, *Drosophila melanogaster* receptors phylogenetically related to the mammalian neuromedin U receptor (NmUR), which is activated by a peptide with a C-terminal FRPRNamide sequence, were assayed for activation by peptides with similar C-terminal cores (Park et al. 2002). Among the receptors assayed in that study, the Drosophila receptors CG8795 (AF522190) and CG8784 (AF522189) were activated by FXPRLamides. A homology-based cloning approach based on the two Drosophila receptors facilitated amplification of a *B. mori* PG-derived transcript encoding a 413-aa protein with significant sequence similarity to class A rhodopsin-like G protein-coupled receptors (GPCRs) and which was dose-dependently activated by *B. mori* PBAN (Hull et al. 2004). Based on the transcript expression profile, ligand activation, and similarity with the NmUR family, the *B. mori* protein was identified as a PBAN receptor (PBANR). Subsequent RNA interference (RNAi)-mediated knockdown confirmed the role of the receptor in bombykol production (Ohnishi et al. 2006). Similar cloning strategies enabled amplification of receptors from *H. zea* PGs (Choi et al. 2003) and *B. mori* ovaries (Homma et al. 2006) that were activated by synthetic PBAN and DH, respectively. Surprisingly, sequence identity between the *B. mori* PBANR and DH receptor (41%) is less than between the two PBANRs (83%), suggesting differences in efficacy between DH and PBAN (Sato et al. 1993; Homma et al. 2006; Watanabe et al. 2007) may be linked to receptor-based ligand discrimination. PBANRs have since been amplified and/or identified from a number of moth species (Zheng et al. 2007; Rafaeli et al. 2007; Kim et al. 2008; Cheng et al. 2010; Lee et al. 2012a; Nusawardani et al. 2013; Wu et al. 2015; Ding and Löfstedt 2015; Fodor et al. 2018; Jiang et al. 2018; Cha et al. 2018).

Initial comparative analyses of PBANRs were confounded by a 67-aa C-terminal extension critical for ligand-induced receptor internalization in BommoPBANR (Hull et al. 2004; Hull et al. 2005). The absence of this C-terminal extension suggested that the "shorter" PBANRs, such as the *H. zea* and *H. armigera* PBANRs, utilized a different regulatory mechanism and lead to speculation that the varied C-terminal lengths reflected species-specific differences in the cellular signaling pathways activated. Equally perplexing were results from previous photoaffinity-based PBAN-binding studies that had identified a ~ 50-kDa membrane protein in isolated *H. armigera* intersegmental membrane preparations (Rafaeli and Gileadi 1997; Rafaeli et al. 2003) that was closer in size to BommoPBANR (45.9 kDa) than either of the cloned heliothine PBANRs at that time (*H. armigera* PBANR – 38.7 kDa or *H. zea* PBANR – 38.6 kDa). Subsequent modification of PBANR amplification conditions resolved the controversy as transcripts encoding multiple variants (PBANR-As, −A, -B, and -C) that differed only in the length of their respective C-terminal ends were amplified from PG cDNAs in *B. mori* and a number of other species (Kim et al. 2008; Lee et al. 2012a; Nusawardani et al. 2013; Fodor et al. 2018). Although the BommoPBANR-C variant was the first identified, the nomenclature was amended to better reflect that used in other moths. BommoPBANR-As is a 306-aa receptor with an incomplete seventh transmembrane (TM) domain that does not traffic to the cell surface and is instead retained in the endoplasmic reticulum and/or Golgi complex. BommoPBANR-A, which more

closely resembles the initial *H. zea* PBANR, is a 345-aa receptor with a 20-aa C-terminal truncation relative to the C variant. BommoPBANR-B is the largest (475-aa) of the *B. mori* receptors and, like the C variant, undergoes ligand-induced internalization (Lee et al. 2012a). Overall, transcripts for the "shorter" A variants, which correspond to the initial *H. zea* and *H. armigera* PBANRs, were found to be less abundant, had lower PBAN efficacies, and exhibited different internalization kinetics (Lee et al. 2012a; Lee et al. 2012b). Preferential amplification of the shorter variants reported by the earlier studies was likely methodological based given that the high GC content (55–80%) of the extended C-terminal ends can adversely affect PCR amplification efficiencies (McDowell et al. 1998).

6.3.2 Identification of the PBANR Gene

The modular nature of the cloned *PBANR* variants (i.e., differences restricted to the C terminus) is consistent with known GPCR alternative splicing events (Markovic and Challiss 2009; Maggio et al. 2016). When aligned to the *B. mori* genome (Shimomura et al. 2009), the *BommoPBANR* gene localized to a > 50 kb segment of chromosome 12 on scaffold Bm_scaf84 (Lee et al. 2012a). The gene is composed of six exons and five introns with exon 1 consisting of the 5′-untranslated region, exons 2–4 encoding the N terminus through TM7, and exons 5–6 encoding the C terminus and stop codon. Based on sequence analyses, BommoPBANR-As and BommoPBANR-A appear to be generated from the introduction of premature stop codons following retention of introns 3 or 4, respectively. BommoPBANR-C results from a five-nucleotide (nt) insertion at the 3′-end of exon 5 that shifts the codon usage for residues 404–413 and introduces a stop codon that generates a C-terminal tail truncated 62-aa compared to BommoPBANR-B, which is generated from conventional splicing of exons 2–6 (Lee et al. 2012a).

6.3.3 Expression of Multiple PBANR Variants

Although the biological significance underlying the concomitant expression of multiple PBANR variants in PGs remains to be fully elucidated, similar variants in other systems have been reported to impact ligand specificity/potency, receptor trafficking, endocytotic regulation, and spatial/temporal regulated expression (Markovic and Challiss 2009; Maggio et al. 2016; Donaldson and Beazley-Long 2016). We have speculated previously that the multiplicity of transcripts may provide a mechanism for fine-tuning cellular responsiveness to the PBAN signal. In one theoretical model, expression of the shorter, less active PBANR-A at the cell surface could function as a ligand sink that competes with PBANR-C for ligand binding. The net result would be a reduction in peptide available to activate the GPCR-mediated cellular response, thus reducing overall sensitivity to the extracellular signal. Alternatively, heterodi-

merization of the longer variants (PBANR-B and PBANR-C) with the shorter variants (PBANR-As and PBANR-A) could impact normal cell surface trafficking, ligand specificity, and/or cellular signaling. In support of this, "short" (i.e., truncated) GPCRs exert dominant negative effects on their full-length isoforms when the two are co-expressed (Seck et al. 2005; Zmijewski and Slominski 2009; Chow et al. 2012). Rhodopsin-like GPCR dimerization, however, remains a controversial topic with support both for (Fotiadis et al. 2006; Ferré et al. 2014) and against (Chabre and le Maire 2005; Whorton et al. 2008) the event. In addition to altered ligand binding/receptor activation, receptor variants have also been reported to exhibit distinct spatial and temporal expression profiles (Markovic and Challiss 2009). Consequently, multiple PBANR transcripts may reflect a spatiotemporal dependence of functionality. This hypothesis is especially attractive given the pleiotropic complexity of the *DH-PBAN* gene and evidence for BommoPBANR activation by the various FXPRLamide peptides (Watanabe et al. 2007).

6.3.4 PBANR Structure–Function Relationships

GPCRs function at the cell surface to turn an extracellular signal into a cellular response. Mechanistically, ligand (e.g., PBAN) binding triggers conformational changes in the receptor that promote the activation of a complex signaling network that culminate in the associated cellular response and feedback regulation (i.e., desensitization and internalization) of the receptor (Cong et al. 2017; Wang et al. 2018). Although elucidation of the specific GPCR structural motifs that mediate these processes is an area of active research among vertebrate receptors, it is not as well developed for insect GPCRs. Of the insect GPCRs that have been characterized, structure–function studies of BommoPBANR are among the most extensive and have provided insights into PBAN functionality in terms of ligand-mediated internalization, which rapidly proceeds via a clathrin-mediated pathway that requires C-terminal interactions and protein kinase C phosphorylation (Hull et al. 2004; Hull et al. 2005; Hull et al. 2011).

6.3.4.1 G-Protein Coupling

Typically, propagation and termination of a peptide ligand signal requires GPCR coupling and activation of specific classes of heterotrimeric (α, β, and γ) G proteins. Interactions between these proteins and the GPCR promote release of G protein-bound GDP (guanosine 5′-diphosphate), which results in recruitment of GTP and subsequent dissociation of the α and β/γ subunits from the receptor, freeing them to activate downstream effector proteins. Hydrolysis of GTP to GDP leads to reassembly of the heterotrimeric complex, effectively resetting the system (Duc et al. 2015). Receptor-G protein coupling has been reported to involve ionic interactions between basic residues in intracellular loop 3 (ICL3) and anionic residues in the C terminus

of the G protein (Yang et al. 2002; Kleinau et al. 2010). A dibasic site (R263 and R264) near TM6 of BommoPBANR that is highly conserved among other PBANRs is critical for feedback regulation of the receptor post-activation. Site-directed mutagenesis of the double Arg site with either neutral (Ala) or anionic (Glu) residues significantly reduced internalization of the ligand-bound receptor (Hull et al. 2011). The disruption in internalization is consistent with perturbed PBANR signaling and provided the first evidence for this region in PBANR-G protein coupling.

6.3.4.2 C-Terminal Motifs Critical to Ligand-Induced Internalization

A number of C-terminal motifs critical for vertebrate GPCR desensitization and endocytosis have been identified (Ferguson 2001; Kristiansen 2004; Pandey 2009; Calebiro and Godbole 2018), two of which are present in the longer BommoPBANRs: NPxxY (residues 325–329) and YxxΦ (residues 360–363). The NPxxY sequence (N = Asn, P = Pro, x = any aa, Y = Tyr) has been reported to function in the internalization of some (Barak et al. 1995; Gripentrog et al. 2000; He et al. 2001; Bouley et al. 2003) but not all vertebrate GPCRs (Slice et al. 1994). The YxxΦ internalization motif (Y = Tyr, x = any aa, and Φ = aa with a bulky hydrophobic side chain) typically located 10–40 aa downstream of TM7 has been likewise implicated in receptor internalization (Collawn et al. 1990; Paing et al. 2004; Pandey 2009). In this tetrapeptide sequence, the Tyr residue appears to be the most critical signal (Pandey 2009). C-terminal truncations of BommoPBANR-C localized the internalization motif to a 10-aa region spanning residues 357–367 that encompass the YxxΦ motif (Hull et al. 2005). Impaired internalization following Ala-substitution of the Y and Φ residues (Tyr383 and Leu 386) confirmed the importance of the signal. The YxxΦ motif, YSAL, is highly conserved in lepidopteran PBANRs and a number of related FXPRLamide receptors (i.e., pyrokinin 2 receptor) but has diverged somewhat in DHRs (YTAM/V) and is not readily apparent in pyrokinin 1 receptors. This variance suggests that regulation of those receptors either utilizes a different internalization signal or proceeds via a nonendocytotic pathway. Whether or not this sequence is sufficient in and of itself to promote internalization has yet to be experimentally determined.

6.3.4.3 Phosphorylation-Dependent Internalization of BommoPBANR

Ligand-induced receptor internalization is typically triggered following phosphorylation of sites in the ICLs or C terminus. For BommoPBANR, protein kinase C (PKC) appears to fulfill this function as endocytosis was blocked by the general kinase inhibitor staurosporine (Hull et al. 2005), mutations to consensus PKC sites (S333 and S366) in the C terminus of BommoPBANR-C (Hull et al. 2011), and RNAi knockdown of endogenous PKC in cultured insect cells expressing the receptor (Hull et al. 2011). The consensus PKC sites are highly conserved in other

PBANRs, which may indicate that feedback regulation of this class of receptors is evolutionarily conserved.

6.3.4.4 Effects of the N Terminus

For some GPCRs, structural determinants in the N terminus, such as N-linked glycosylation, can impact efficient cell surface trafficking and functional activation (Duvernay et al. 2005; Dong et al. 2007). Glutamine substitution of two consensus N-glycosylation sites (N19 and N22) in the *H. zea* PBANR N terminus significantly reduced receptor activation (Choi et al. 2007). Deletion of the first 27 residues from the BommoPBANR-C N terminus, which contains the corresponding N-glycosylation sites (N18 and N21), had no noticeable effects on receptor trafficking or ligand-induced internalization (Hull et al. 2011). While the variation in responses between the studies may simply be methodological based, it might also reflect functional differentiation of the two receptors given the low (37%) sequence identity across the two N termini.

6.3.4.5 Mapping the PBANR Ligand-Binding Pocket

To provide insights into the structural determinants underlying PBAN–PBANR interactions, an in silico model of BommoPBANR-C was constructed using spatial coordinates derived from crystal structures for the human β2 and A_{2A} adrenergic receptors, both of which, like PBANR, are class A GPCRs (Kawai et al. 2014). Based on alignment with known ligand recognition sites in the two human receptors and conserved residues across FXPRLamide receptors, 27 aa interspersed across the TM domains and extracellular loops (ECLs) were predicted to comprise a portion of the PBAN-binding pocket. The functional importance of the individual residues in terms of cell surface trafficking, ligand binding, and receptor activation was evaluated via sequential Ala-substations (Kawai et al. 2014). Substitution of four residues (Ser207, Phe211, Phe212, and His284) affected cell surface expression and, given their interhelical localization, are predicted to contribute to receptor stabilization. Eleven residues (Glu95, Glu120, Asn124, Val195, Phe276, Trp280, Phe283, Arg287, Tyr307, Thr311, and Phe319) influenced ligand-binding and receptor activation, three residues (Phe209, Phe303, GLy315) influenced ligand-binding alone, and a single residue (Tyr318) influenced receptor activation alone. This latter substitution was the only change that resulted in a receptor with normal ligand binding but impaired signaling abilities, which suggests that the Tyr side chain may play a significant role in the PBAN-induced conformational change in PBANR that results in receptor activation. The effects of Ala-substitutions on TM residues Phe212, Phe276, Trp280, Phe283, and Phe319, which are highly conserved in class A GPCRs, are likewise consistent with a proposed receptor conformational switch (Holst et al. 2010; Trzaskowski et al. 2012; Hulme 2013; Tehan et al. 2014).

Extension of the proposed in silico BommoPBANR model to include molecular docking simulations of a pentapeptide FXPRLamide analog identified a number of the same TM bundle localized receptor–ligand interaction sites (Kawai et al. 2014). Many of these sites are evolutionarily conserved across insect FXPRLamide receptors and human NmURs with Glu residues in TM2 and TM3 (i.e., E95 and E120) predicted to play a role in FXPRLamide binding as conservation of those sites in other class A GPCRs is more limited. Incorporation of a 10-aa PBAN analog into the molecular docking analyses identified two additional residues (V195 in ECL2 and F303 in ECL3) as contact points that were not predicted for the smaller 5-aa analog (Kawai et al. 2014). Interactions between these ECL-localized contact points and noncritical portions of the peptide could potentially stabilize ligand binding and/or serve as a selectivity filter for differentiating PBAN and DH, as suggested by the reported differences in peptide efficacies (Sato et al. 1993; Homma et al. 2006; Watanabe et al. 2007). Indeed, among the functionally relevant ligand contact points in BommoPBANR, only V195 (Glu in DHR) and F303 (Pro in DHR) are not conserved in *B. mori* DHR.

A similar approach that incorporated spatial coordinates from the bovine rhodopsin crystal structure to map potential ligand-binding sites in the *H. zea* PBANR predicted an inner-binding pocket surface of 20 contact points (Stern et al. 2007). Of those potential ligand-binding points, only three were also identified in the *B. mori* study (Ile113, Lys196, and Tyr307) and only Ala-substitution of Tyr307 perturbed receptor functionality via altered ligand binding and receptor activation (Kawai et al. 2014). The discrepancy in potential contact sites likely reflects methodological variances, which include the use of an early GPCR structural template (i.e., rhodopsin) that is now recognized to be less optimal for modeling peptide receptors (Sabio et al. 2008; Mobarec et al. 2009; Congreve et al. 2011). In contrast, all of the residues identified based on evolutionary trace analysis of PBANR-related sequences (Jurenka and Nusawardani 2011), which sought to identify a conserved TM-bounded pocket, are represented in the *B. mori* model.

Elucidation of the structure–function relationships underlying BommoPBANR functionality has expanded our understanding of the PBAN mode of action and provided insights into the molecular determinants that discriminate diverging peptidergic signals (i.e., PBAN vs DH). These insights into the endocrinological/peptidergic control of critical insect physiological functions can be used to further develop peptidomimetic agonists and/or antagonists of various insect GPCRs identified as potential targets for next-generation pest management strategies (Scherkenbeck and Zdobinsky 2009; Van Hiel et al. 2010; Bai and Palli 2013; Audsley and Down 2015). Indeed, PBAN antagonists have been the focus of much research over the years (Altstein 2001; Altstein et al. 2007; Altstein and Hariton 2009; Nachman 2009; Hariton et al. 2010; Nachman 2014).

6.4 PBAN Signal Transduction

6.4.1 Molecular Events Pre-Ca^{2+} Influx

6.4.1.1 Activation of the Secondary Messenger Cascade

A driving focus for numerous studies over the years was elucidating the signal transduction cascade activated in response to PBAN binding. The initial steps in most GPCR-linked cascades require receptor-mediated dissociation of an associated G protein complex with subsequent activation of the downstream effector proteins involved in generating the secondary messenger molecules that drive the signal transduction cascades. The predominant Gα subunits involved in receptor signaling include the following: Gαs – stimulate cAMP production, Gαi/o – inhibit cAMP production, and Gαq – stimulate Ca^{2+} influx. Although four Gα subunits (2 – Gαs, 1 – Gαo, and 1 – Gαq) have been identified to date in *B. mori* PGs, RNAi-mediated knockdown demonstrated that only the Gαq subunit functions in PBAN signal transduction (Hull et al. 2007a; Hull et al. 2010).

Determination that Gαq plays a role in PBAN signaling provided the initial molecular basis for early studies that reported pharmacological manipulation of intracellular Ca^{2+} levels via chelators (EGTA), ionophores (ionomycin, A23187, thapsigargin), or Ca^{2+} channel blockers (lanthanum, SKF-96365, 2-APB) could affect pheromone production (Fónagy et al. 1992b; Fónagy et al. 1992c; Matsumoto et al. 1995a). Fluorescent Ca^{2+} imaging techniques provided more direct evidence for PBAN-mediated Ca^{2+} influx in *B. mori* PGs (Hull et al. 2007a). The role of Ca^{2+} in PBAN signal transduction appears to be invariant as the pheromonotropic effects of the second messenger have been reported in a number of species (Jurenka et al. 1991a; Fónagy et al. 1992b; Rafaeli 1994; Jurenka et al. 1994; Soroker and Rafaeli 1995; Ma and Roelofs 1995b; Matsumoto et al. 1995b; Zhao et al. 2002; Choi and Jurenka 2004; Choi and Jurenka 2006). In contrast, the utilization of cAMP (cyclic adenosine-3′, 5′-monophosphate) as a co-messenger is species dependent. Pharmacological compounds (cAMP analogs, IBMX, and forskolin) that affect intracellular cAMP levels are pheromonotropic in a number of species (Rafaeli and Soroker 1989; Jurenka et al. 1991a; Jurenka et al. 1994; Soroker and Rafaeli 1995; Jurenka 1996), and cAMP levels are elevated following PBAN stimulation (Rafaeli and Soroker 1989; Rafaeli 1994; Soroker and Rafaeli 1995; Rafaeli and Gileadi 1996). In *B. mori*, however, the compounds do not exhibit pheromonotropic effects (Fónagy et al. 1992c; Hull et al. 2007a), and there is no PBAN-mediated increase in cAMP levels (Hull et al. 2007a). In species that utilize the second messenger, PBAN regulates the activity of acetyl-CoA carboxylase (ACC), an enzyme in fatty acid biosynthesis (Tang et al. 1989; Jurenka et al. 1991b; Tsfadia et al. 2008; Du et al. 2017a). Conversely, in species that utilize cAMP-independent pathways, PBAN

regulates later steps in biosynthesis, which in *B. mori* includes both lipase (release of pheromone precursors from storage droplets) and fatty acyl reductase (FAR) activities, the final step in bombykol biosynthesis (Ozawa A et al. 1993; Ozawa and Matsumoto 1996; Fónagy et al. 2000; Ohnishi et al. 2011a; Du et al. 2012a).

6.4.1.2 Role of Phospholipase C

The influx of extracellular Ca^{2+} is tightly regulated by various cation channels including receptor-activated Ca^{2+} channels, which open in response to receptor-mediated phospholipase C (PLC) activation (Berridge et al. 2000). PLC hydrolysis of PIP_2 (phosphatidylinositol-4,5-bisphosphate) yields two products, the phosphoinositide IP_3 (inositol 1, 4, 5-triphosphate) and diacylglycerol (DAG), which act on two different types of Ca^{2+} channels. IP_3 works through endoplasmic reticulum Ca^{2+} stores to trigger an influx of extracellular Ca^{2+} via store-operated channels (SOC), whereas DAG and/or its metabolites act directly on a different subset of channels (Shuttleworth et al. 2004; Parekh 2006; Hardie 2007). Pharmacological manipulation of the two channel types implicated the involvement of SOC activity in *B. mori* pheromone production (Hull et al. 2007b). Building on that study, a PBAN-mediated increase in *B. mori* PG phosphoinositides coupled with reduced pheromonotropic effects of PBAN in the presence of PLC inhibitors confirmed a role for the enzyme downstream of PBANR activation (Hull et al. 2010). To date, three PLC transcripts (PLCβ1, PLCβ4, and PLCγ) and an IP_3 receptor (IP_3R) have been amplified from *B. mori* PGs with RNAi-mediated knockdown revealing PBAN-linked functionalities for the IP_3R as well as PLCβ1 and PLCγ (Hull et al. 2010). While the specific mechanistic roles the two PLCs have in propagating the PBAN signal remain to be fully elucidated, findings from other systems suggest that PLCβ1 likely functions in PIP_2 hydrolysis and that specific domains in PLCγ stabilize protein–protein interactions essential to formation of the SOC complex (Patterson et al. 2005).

6.4.1.3 Role of Ca^{2+} Channels

SOC complexes typically consist of stromal interaction molecule 1 (STIM1) functioning as the sensor of stored Ca^{2+} levels and Orai1 as the pore-forming unit at the plasma membrane (Derler et al. 2016; Nwokonko et al. 2017). Depletion of ER Ca^{2+} levels in response to an extracellular signal triggers redistribution of STIM1 to areas near the cell surface where interactions with Orai1 promote conformational changes in the pore sufficient for Ca^{2+} influx. Consistent with a role in propagating the PBAN signal, RNAi-mediated knockdown of the two *B. mori* homologs (BmSTIM1 and BmOrai1) impacted pheromone production without affecting non-pheromonotropic enzyme activities (Hull et al. 2009). In vitro analyses conducted in parallel showed that BmSTIM1 and BmOrai1 interactions were stoichiometrically dependent and required a cluster of basic residues in BmSTIM1. Expression of only the shorter of

two BmOrai1 splice variants in the PG, in contrast to the expression of both variants, suggests that its role in the PBAN signaling cascade may be mechanistically distinct than in other tissues (Derler et al. 2016).

6.4.2 Post-Ca^{2+} Influx Signaling

6.4.2.1 Role of Calmodulin

Intracellular Ca^{2+} functions as a highly versatile signaling molecule that controls the regulation of diverse cellular processes (Berridge et al. 2003). As discussed above, PBAN-mediated Ca^{2+} influx is critical to not only pheromone biosynthesis in *B. mori* but also all other moth species. The predominant post-influx mediator of Ca^{2+} signaling is calmodulin, a multifunctional Ca^{2+}-binding protein that regulates the activity of a host of downstream processes (Sharma and Parameswaran 2018; Urrutia et al. 2019). Although transcriptional and pharmacological evidence support a role for calmodulin in the *B. mori* PBAN-signaling pathway, in vivo functionality remains to be demonstrated. Calmodulin has been purified from *B. mori* PGs (Iwanaga et al. 1998), and digital gene expression analyses of the tissue revealed significant upregulation of the encoding transcript within 72 hr of adult eclosion (Du et al. 2012a). Furthermore, pharmacological agents (W-7 and trifluoperazine) that inhibit calmodulin activity blocked in vitro pheromone production (Matsumoto et al. 1995a). Similar pheromonostatic effects of calmodulin inhibition have been reported in other species (Matsumoto et al. 1995b; Rafaeli and Gileadi 1996); however, contradictory results with other inhibitory compounds (Soroker and Rafaeli 1995) suggest that inferences regarding the presumed role of calmodulin will require more direct in vivo demonstration of functionality.

6.4.2.2 Phosphorylation Cascade

GPCR-mediated signaling pathways typically proceed via a phosphorylation cascade involving diverse kinase (phosphorylation) and phosphatase (dephosphorylation) steps, many of which are regulated by Ca^{2+}-bound calmodulin (Sharma and Parameswaran 2018). Although early studies assessing the pharmacological effects of kinase inhibition (H-89, PKA; staurosporine, PKC; KN-62, calmodulin-dependent kinase II, CaMKII) or activation (phorbol 12-myrstate 13-acetate, PKC) found no effect on in vitro pheromone production in *B. mori* (Matsumoto et al. 1995a; Ozawa et al. 1995), subsequent demonstration of PKC function in PBANR feedback regulation (Hull et al. 2011) implicated a PBAN-linked phosphorylation cascade. PG-derived immunoblots probed with anti-phosphoamino acid antibodies provided direct demonstration of kinase activity with multiple proteins shown to undergo rapid PBAN-mediated phosphorylation (Ohnishi et al. 2011a). RNAi-targeted knockdown of three kinases (PKA, PKC, and CaMKII), two of which are expressed in the PG (PKC and CaMKII), revealed CaMKII functions in *B. mori* sex

pheromone production by regulating the lipolytic release of stored pheromone precursors (Ohnishi et al. 2011a). Phosphoproteomic analysis of *H. armigera* PGs revealed similar PBAN-induced phosphorylation of multiple proteins (Du et al. 2017a); however, in that species, PKA activity is critical for pheromone biosynthesis in both males and females (Du et al. 2017b; Du et al. 2017a). Unlike *B. mori*, the pheromone biosynthetic point regulated by PBAN in heliothines is ACC, a fatty acid biosynthesis enzyme that catalyzes carboxylation of acetyl-CoA to generate the malonyl-CoA used in fatty acid chain elongation. Based on their phosphoproteomic analyses and in vivo knockdown, Du and coworkers suggest that PKA indirectly regulates PBAN-mediated ACC activity by inhibiting the kinase (AMP-activated protein kinase) that maintains ACC in the inactive state.

In contrast to the kinase studies, pharmacological inhibition of phosphatase activity had pronounced in vitro pheromonostatic effects in *B. mori* (Matsumoto et al. 1995a; Ozawa and Matsumoto 1996; Fónagy et al. 1999). Inhibitor profiling implicated calcineurin (also called protein phosphatase 2b), a serine/threonine phosphatase activated by Ca^{2+}-bound calmodulin, in the PBAN-signaling cascade (Fónagy et al. 1999). In support of this role, a calcineurin homolog with 85% sequence identity to the *D. melanogaster* protein was amplified from a *B. mori* PG-specific cDNA library (Yoshiga et al. 2002). Although the encoding transcript is expressed in multiple tissues, it undergoes significant upregulation around the time of adult eclosion in concert with other transcripts in the *B. mori* sex pheromone biosynthetic pathway (Yoshiga et al. 2002). Determination of the rate-limiting steps in *B. mori* suggests that calcineurin or calcineurin-like phosphatase activity regulates the terminal fatty acyl reduction (Ozawa et al. 1993, 1995; Ozawa and Matsumoto 1996). Direct demonstration of calcineurin on FAR activity in *B. mori*, however, remains to be experimentally demonstrated. Knockdown studies have recently demonstrated the importance of calcineurin for sex pheromone biosynthesis in both male and female *H. armigera* (Du et al. 2017a; Zhao et al. 2018). In females, the phosphatase appears to directly regulate ACC activity, which is inactive when phosphorylated (Du et al. 2017a). In males, the control point remains to be elucidated; however, a FAR has been reported to be critical for the biosynthesis of some of the pheromonal components (Du et al. 2017b). It is intriguing to speculate that PBAN-mediated activation of calcineurin as the principal control point for FAR activity may be evolutionarily conserved.

6.5 Pheromone Gland Lipid Droplet

6.5.1 Lipid Droplet Dynamics

Unlike many other moth species, bioactive sex pheromone (i.e., bombykol) can be extracted from the PGs of newly eclosed *B. mori* females (Ando et al. 1988c). The pheromone-producing cells that comprise the inner layer of the PG are morphologi-

cally distinguished by the presence of large cytosolic lipid droplets that begin to accumulate 2 days prior to adult eclosion (Fónagy et al. 2000; Fónagy et al. 2001; Yokoyama et al. 2003). The size and number of lipid droplets fluctuate in relation to the circadian clock as well as pheromonotropic stimuli (Fónagy et al. 2000; Fónagy et al. 2001). Under pheromonostatic conditions, the lipid droplets are large and limited in number; conversely, smaller lipid droplets predominate in response to a pheromonotropic stimulus. Structural and chemical characterization revealed that the lipid droplets are largely composed of triacylglyerols (TAGs) that sequester five long-chain fatty acyls: two unsaturated C16 fatty acyl bombykol precursors (i.e., Δ10,12-hexadecadienoate and Δ11-hexadecenoate) at the *sn*-1/*sn*-3 positions and some combination of conventional diet-derived oleate, linoleate, and linolenate C18 fatty acyls (Matsumoto et al. 2002). Knockdown of the *B. mori* PBANR confirmed that lipolytic release of the stored precursors for entry into the pheromone biosynthetic pathway is regulated by the PBAN signal (Ohnishi et al. 2006). The utilization of lipid droplets for storage of pheromone precursor presumably predates silkworm domestication (Fujii et al. 2018) and may be a conserved storage mechanism for rapid pheromone production/release in some moth species (Fang et al. 1996; Foster 2001; Foster 2005).

6.5.2 Lipid Droplet Formation

Although our understanding of the mechanisms driving lipid droplet biogenesis in moth PGs remains to be as fully developed as that of vertebrate systems (see Guo et al. 2009; Pol et al. 2014; Gao and Goodman 2015), significant advances in elucidating aspects of the molecular framework have been made. Using an initial *B. mori* PG expressed-sequence tag database (Yoshiga et al. 2000) as well as digital gene expression (Du et al. 2012a) and iTRAQ-based quantitative proteomic (Du et al. 2015) analyses, a number of transcripts were found to be specifically/predominantly PG expressed and significantly upregulated at the time of adult eclosion, suggesting potential roles in pheromone biosynthesis. Among the transcripts identified to date are a number of genes critical for TAG synthesis, and by extension, cytoplasmic lipid droplet formation.

6.5.2.1 Fatty Acid Transport Protein

Fatty acid transport proteins and/or lipid transport proteins typically facilitate extracellular import of long-chain fatty acids and diacylglycerols (DAGs) across the plasma membrane (Shapiro et al. 1988) with subsequent ATP-dependent esterification to the corresponding acyl-CoA derivatives (Stahl 2004). Although the *B. mori* sex pheromone is de novo synthesized from acetyl-CoA via fatty acid biosynthesis, targeted knockdown of the *B. mori* FATP (BmFATP) affected pheromone production via impaired lipid droplet accumulation (Ohnishi et al. 2009). Chemical analy-

sis revealed disrupted stoichiometric integration of fatty acyl components. Typically, a subset of the TAGs that comprise the PG lipid droplets are composed of two unsaturated C18 fatty acyls (i.e., oleate, linoleate, linolenate) and one of the C16 bombykol precursors – Δ10,12-hexadecadienoate or Δ11-hexadecenoate (Matsumoto et al. 2002). In BmFATP knockdown PGs, these TAGs were significantly reduced. The disrupted availability of C18 fatty acyls for incorporation into these TAGs impeded normal lipid droplet formation and, as a result, less pheromone precursor was available for flux into the pheromone biosynthetic pathway. Thus, BmFATP functions in pheromone production by importing the extracellular diet-derived C18 fatty acids critical for synthesizing a subset of lipid droplet TAGs that store sex pheromone precursors.

6.5.2.2 Triacylglyceride Synthesis Enzymes

Two major pathways contribute to TAG biosynthesis: the glycerol phosphate pathway, which proceeds from acylation of glycerol-3-phosphate, and the monoacylglycerol pathway, which utilizes diet-derived monoacylglycerol to generate TAGs (Takeuchi and Reue 2009; Hussain 2014). The two pathways converge at the final reaction point that converts DAG into the final TAG molecule via diacylglycerol acyltransferase (DGAT). The rate-limiting steps in the two pathways are the acylation of glycerol 3-phosphate by glycerol-3-phosphate acyltransferase (GPAT) and conversion of monoacylglycerol to diacylglycerol by acyl-CoA:monoacylglycerol acyltransferase (MGAT). Analysis of *B. mori* PG transcripts at various points in pupal-adult development revealed that the expression of GPAT and DGAT is consistent with a role in pheromone biosynthesis (Du et al. 2012a). Further analysis of the PG DGAT indicated that it was most similar to DGAT2 family members, which are characterized by two TM domains rather than the nine TMs of DGAT1. Although both DGAT1 and DGAT2 catalyze similar reactions, there was no evidence in the digital gene expression analyses for *B. mori* DGAT1 upregulation in the post-eclosion PG (Du et al. 2012a). RNAi-mediated knockdown of DGAT2 and GPAT both affected sex pheromone production with GPAT also reported to impact lipid droplet formation (Du et al. 2012b; Du et al. 2015). Similar to DGAT1, there is currently no transcriptional support for MGAT functionality in generating the pheromone-associated TAGs (Du et al. 2012a), suggesting TAG formation in the PG is largely dependent on the glycerol phosphate pathway.

6.5.2.3 Acyl-CoA-Binding Proteins

Similar disruption of *B. mori* TAG synthesis and PG lipid droplet formation was reported following RNAi-mediated knockdown of two transcripts encoding acyl-CoA-binding proteins (ACBPs) – mgACBP and pgACBP (Ohnishi et al. 2006). Although ACBPs typically bind and protect long-chain (C14-C22) acyl-CoA esters from hydrolysis (Gossett et al. 1996), mgACBP and pgACBP are thought to impact

TAG-dependent lipid droplet synthesis, and by extension downstream pheromone production, differently (Ohnishi et al. 2006). Unlike pgACBP, which is predominantly expressed in the PG, mgACBP is also expressed in the larval midgut, suggesting a potential dietary role (Matsumoto et al. 2001). Given the different expression patterns of the two transcripts, the current model for PG TAG synthesis suggests that mgACBP donates acyl-CoAs from the C18 fatty acyls imported by BmFATP, whereas pgACBP provides the de novo synthesized pheromone precursor fatty acyl-CoAs.

6.5.2.4 Acyl Carrier Protein

Since bombykol is de novo synthesized from acetyl-CoA through palmitate (16: acyl) (Yamaoka et al. 1984; Ando et al. 1988b; Arima et al. 1991), it is not surprising that disruption of steps in the fatty acid biosynthetic pathway also affects lipid droplet formation in the PG. An acyl carrier protein (ACP) homolog was among a group of transcripts identified in a PG EST database (Yoshiga et al. 2000) with an expression profile consistent with a pheromonogenic role (Ohnishi et al. 2011b). ACPs function early on in fatty acid biosynthesis by binding and presenting acyl chain intermediates to other enzymes in the pathway (Byers and Gong 2007; Chan and Vogel 2010). Targeted knockdown of the ACP transcript impaired lipid droplet production and specifically affected the synthesis of TAGs storing the C16 pheromone precursor products Δ10,12-hexadecadienoate and Δ11-hexadecenoate (Ohnishi et al. 2011b).

6.5.3 Lipolysis of the Lipid Droplet

6.5.3.1 Lipid Storage Droplet Protein

Studies in other organisms have revealed a complex network of protein interactions and phosphorylation events underlying lipid droplet dynamics (Barbosa et al. 2015; D'Andrea 2016; Sztalryd and Brasaemle 2017). For *B. mori*, the role of PBAN on lipid droplet dynamics (i.e., changes in size and number in response to pheromonotropic stimuli) has been well documented (Fónagy et al. 2000; Fónagy et al. 2001; Matsumoto et al. 2002; Yokoyama et al. 2003; Fónagy et al. 2005; Ohnishi et al. 2006) as has the importance of PBAN-mediated phosphorylation (Ohnishi et al. 2011a). Among the group of proteins phosphorylated in response to PBAN stimulation is a member of the perilipin family of lipid droplet proteins, *B. mori* lipid storage droplet protein-1 (BmLsd1). BmLsd1 has high sequence identity with a similar protein in *Manduca sexta* implicated in adipokinetic hormone-mediated lipolysis of fat body lipid droplets (Patel et al. 2005). Similar to that protein, BmLsd1 localizes to lipid droplets and is critical for lipolytic release of pheromone precursors stored in the PG lipid droplets (Ohnishi et al. 2011a). BmLsd1, however, does not appear

to be the lipolytic enzyme. Rather, it is thought that the protein functions analogously to mammalian perilipins with PBAN-activated CaMKII phosphorylation of Ser/Thr residues in BmLds1, promoting a conformational change that either exposes the lipid droplet surface to associated lipases or allows for lipase binding in conjunction with other sequestered co-activators such as the CGI-58 protein that has been described in adipocytes (Sztalryd and Brasaemle 2017). Although homologs of CGI-58 are present in the *B. mori* genome (accession #s XP_004927228.1, XP_004927229.1, XP_012546725.1), there is currently no transcriptional or biochemical evidence for their role in PBAN-mediated lipolysis.

6.5.3.2 Lipases

Expression analyses by differing groups have identified seven lipase-like genes that are upregulated in the PG within 72 hr of adult eclosion (Ohnishi et al. 2011b; Du et al. 2012a; Zhang et al. 2013), a time period that is consistent with a role in pheromone production. RNAi-mediated knockdown, however, revealed that only four of the lipases have a role in pheromone production. Among the lipases identified include homologs of triacylglycerol lipase (NRPG0023/BGIBMGA005695), *D. melanogaster* lipase 3 (NRPG1187), *Aedes aegypti* lipase (NRPG1885), and a pancreatic lipase-like gene (BmPLLG/BGIBMGA011864). Although stimulated lipolysis typically proceeds through a phosphorylation cascade the culminates in lipase activation (D'Andrea 2016), the role of PBAN-mediated phosphorylation on lipase activity remains to be determined.

6.6 Bombykol Biosynthetic Pathway Enzymes

Unlike other Type I pheromones, the bombykol biosynthetic pathway is relatively simple in that the palmitic acid backbone does not undergo chain-shortening reactions or further modification of the terminal hydroxyl group. Rather, the bioactive pheromone is generated by stepwise conversion of fatty acid biosynthesis-derived palmitate via two desaturation steps and a terminal fatty-acyl reduction step (Ando et al. 1988b; Arima et al. 1991). The first desaturation step is a general Z11 desaturase reaction common in the pheromone biosynthetic pathways of numerous moth species (Roelofs et al. 2002). In contrast, the second desaturation step, which generates a conjugated diene system through 1,4-elimination of two allylic hydrogens at the double bond in the Z11-monoene C16 intermediate, is less common. Neither of the desaturation steps, however, is controlled by PBAN; rather, the peptide regulates the terminal fatty acyl reduction reaction (Ozawa A et al. 1993; Ozawa et al. 1995; Ozawa and Matsumoto 1996). As with other components of the pheromone pathway, transcripts encoding the respective enzymes are predominantly expressed in the PG and are upregulated at adult eclosion. Molecular characterization of the enzymes via a yeast expression system revealed the desaturase (Desat1 also referred

to as Bmpgdesat1) catalyzes both desaturation steps and that the reductase (pgFAR) exhibits strong substrate specificity for the immediate precursor, Δ10,12-hexadecadienoate (Moto et al. 2003; Moto et al. 2004). RNAi-mediated knockdown of the respective transcripts confirmed the role of the two enzymes in bombykol biosynthesis (Ohnishi et al. 2006). Since the initial identification of the enzymes, additional multifunctional desaturases critical for moth pheromone production have been reported (Serra et al. 2006b; Serra et al. 2006a; Matoušková et al. 2007; Serra et al. 2007) as have a number of FARs selective for pheromone precursors (Antony et al. 2009; Liénard et al. 2010; Hagström et al. 2012; Lassance et al. 2013).

6.7 Summary

Based on the aggregate of the studies highlighted above, a model for the cellular and molecular processes that govern bombykol biosynthesis has emerged (Fig. 6.1). Prior to adult eclosion, DH-PBAN expression is upregulated in a subset of neurosecretory cells and post-translationally processed PBAN is transported along axons that pass through the maxillary nerve to the *corpus cardiacum*. Concomitantly in the PG, an array of pheromonogenic genes are upregulated and active TAG biosynthesis leads to the accumulation and enlargement of cytoplasmic lipid droplets (upper panel, Fig. 6.1). Soon after eclosion, stimulation of a central circadian pacemaker triggers release of stored PBAN into the hemolymph with subsequent binding of PBAN to cell surface localized PBANRs in the PG. The ensuing PBANR conformational change results in dissociation of the heterotrimeric G protein complex with subsequent Gαq activation of PLCβ1-mediated hydrolysis of PIP2 into DAG and IP_3. The soluble IP_3 diffuses through the cytosol to activate IP_3 receptors in the endoplasmic reticulum membrane resulting in the subsequent release of stored Ca^{2+}. The drop in luminal Ca^{2+} levels promotes translocation of STIM1 to the plasma membrane where it triggers an influx of extracellular Ca^{2+} through Orai1 channels, presumably via interactions with a scaffolding complex that includes PLCγ (lower left panel, Fig. 6.1). The concomitant rise in intracellular Ca^{2+} allows for the formation of Ca^{2+}-calmodulin complexes, at which point the PBAN pathway exhibits species-dependent divergence. In *B. mori,* and presumably species in which PBAN regulates a step late in pheromonogenesis, the Ca^{2+}-calmodulin complexes activate both calcineurin and CamKII. Calcineurin, in turn, activates the FAR that catalyzes the terminal step in pheromone biosynthesis (i.e., reduction of Δ10,12-hexadecadienoate to 10,12-hexadecadien-1-ol), while CamKII-dependent phosphorylation of BmLdsp-1 promotes lipolytic release of stored pheromone precursors from the cytoplasmic lipid droplets (lower right panel, Fig. 6.1).

Building on the *B. mori* framework, other groups have shown in species that utilize cAMP as a secondary messenger, and calcineurin-mediated dephosphorylation promotes ACC (i.e., the rate-limiting step in fatty acid biosynthesis) activity. In concert with this action, elevation of cAMP levels in response to Ca^{2+}-calmodulin activation of an adenylate cyclase leads to a PKA-initiated cascade that inhibits the

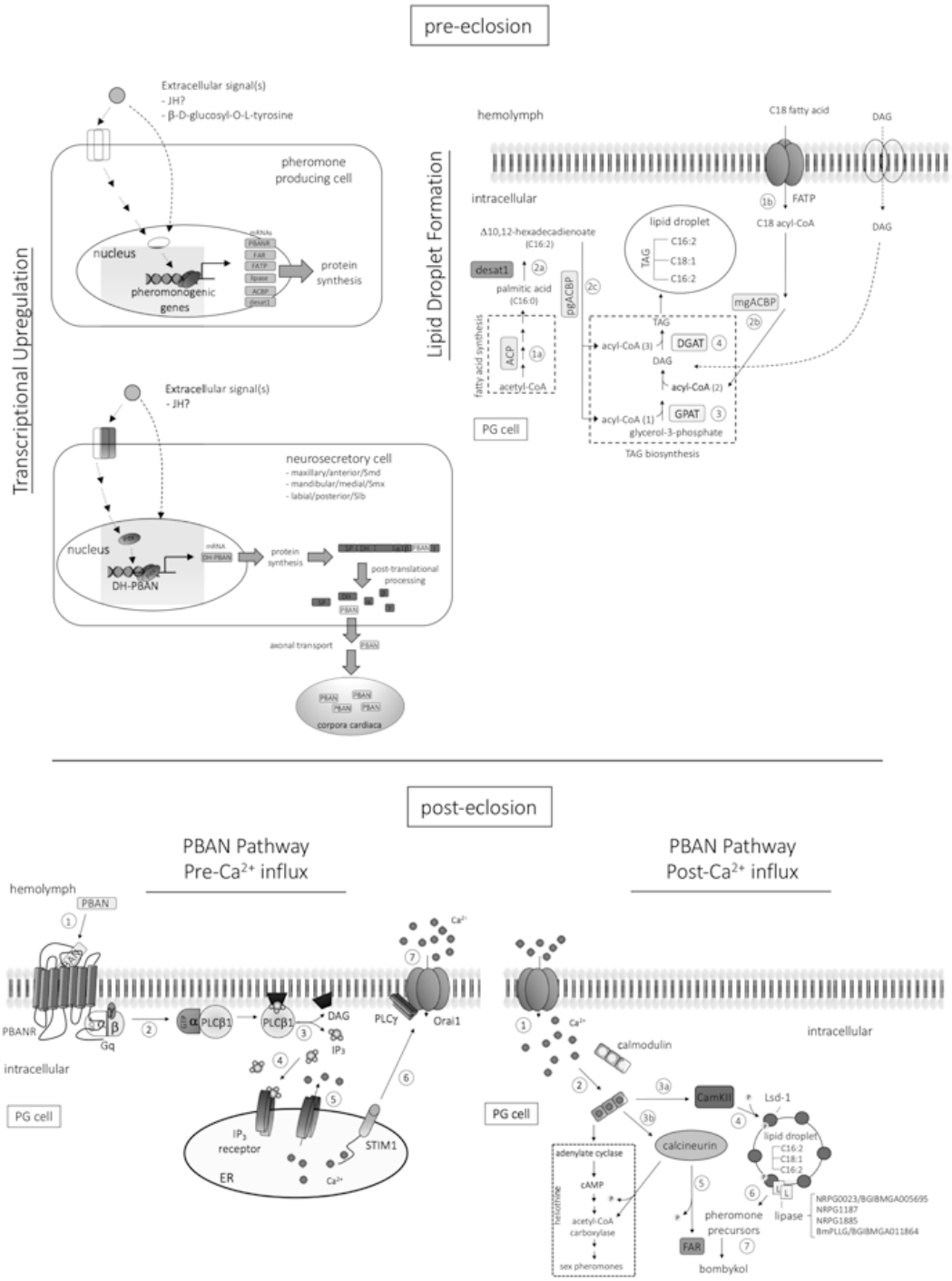

Fig. 6.1 Pre- and posteclosion cellular and molecular events driving bombykol production. Upper panel: Pre-eclosion events. (Left) Transcriptional upregulation of pheromonogenic genes in the developing PG and upregulation of DH-PBAN transcription in a subset of neurosecretory cells with axonal transport of processed PBAN peptide to the *corpora cardiaca* for subsequent circadian-controlled release into the hemolymph. (Right) Lipid droplet formation in the cytoplasm of developing PG cells. Fatty acid synthesis-derived palmitic acid is converted to pheromone precursor and incorporated into TAG via the glycerol-3-phosphate pathway along with diet-derived C18 acyl-CoAs and imported DAGs. Lower panel: Post-eclosion events. (Left) Signal transduction steps downstream of PBAN/PBANR binding prior to the influx of extracellular Ca^{2+}. (Right) PBAN pathway steps post-Ca^{2+} influx involve a calmodulin-dependent cascade that culminates in lipolytic release of pheromone precursors and their subsequent FAR-dependent modification to the final bioactive product – bombykol

phosphorylation event that maintains ACC in an inactive state (see Du et al. 2017a; Jurenka 2017).

6.8 Conclusion

The six decades of research since Butenandt's pioneering study have witnessed an explosion in both identification of pheromonal compounds and molecular elucidation of the associated biosynthetic pathways. Among the Lepidoptera, *B. mori* has been at the forefront of each new advancement in our understanding of the processes underlying moth pheromonogenesis – from purification of the regulatory peptide (PBAN) to identification of the cognate receptor to characterization of the genes comprising the biosynthetic and regulatory pathways. Going forward, advances in gene editing and transgenesis techniques hold great promise for *B. mori* to continue providing critical discoveries in relation to pheromonogenesis (see Moto and Matsumoto 2012; Shiomi et al. 2015).

To date, each new insight into the processes that comprise pheromonogenesis has highlighted the complexity of the system and provided new puzzles for us to unravel. Some of the questions raised by the current paradigm of pheromonogenesis that we find the most intriguing, and for which *B. mori* is well positioned to address, include:

1. What is the pre-eclosion signal that initiates transcription of pheromonogenic genes? Early studies linked β-D-glucosyl-*O*-L-tyrosine with transcription of pgACBP (Ohnishi et al. 2005), whereas more recent studies suggested a role for juvenile hormone signaling in priming the PG for pheromonogenesis (Zhang et al. 2014). Additional studies will be needed to determine if a lone signal drives transcription or if multiple signals are involved.
2. What is the molecular basis for regulation of the pleiotropic FxPRLamide peptide/receptor system?
3. What mechanism drives SLb-specific enrichment of DH relative to PBAN?
4. How is ligand selectivity of PBANRs/DHRs achieved?
5. What biological role do the concomitantly expressed PBANR variants play in PBAN signaling?
6. How is alternative splicing of PBANRs regulated?
7. What is the evolutionary significance of the different PBAN-mediated control points (fatty acid biosynthesis vs terminal fatty acyl reduction), and how did this divergence arise?

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commercial products in this article is solely for the purpose of providing specific information and does not imply recommendation or endorsement by the United States Department of Agriculture (USDA). USDA is an equal opportunity provider and employer.

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