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Bursicon-expressing neurons undergo apoptosis after adult ecdysis in the mosquito *Anopheles gambiae*

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ABSTRACT

Neuropeptides are important regulators of diverse processes during development. The insect neuropeptide bursicon, a 30 kDa heterodimer, controls the hardening of the new cuticle after the shedding of the old one (ecdysis) and the inflation and maturation of adult wings. Given this specific functional role, its expression should only be required transiently because adult insects no longer undergo ecdysis. Here we report the transient expression of bursicon in the mosquito, *Anopheles gambiae*. Quantitative RT-PCR revealed that transcription of the bursicon monomers, *burs* and *pburs*, steadily increases through the larval stages, peaks in the black pupa stage, and decreases to below detectable levels by 8 h after adult ecdysis (eclosion). Immunohistochemistry on the adult nervous system showed that bursicon is co-expressed with crustacean cardioactive peptide (CCAP) in specific neurons of the abdominal ganglia, but that labeling intensity wanes by 14 h post-eclosion. Finally, detection of disintegrating DNA by TUNEL labeling demonstrated that the bursicon expressing neurons successively undergo apoptosis following eclosion. Taken altogether, these data describe *A. gambiae* as another holometabolous insect in which bursicon ceases to be produced in adults, and in which the bursicon expressing neurons are removed from the ventral nerve cord.

1. Introduction

Insect bodies are supported by a chitinous exoskeleton (cuticle), which, once hardened, prevents growth. In order to grow or morph into a different life stage, insects must shed their old cuticle (ecdysis) and replace it with a new one. The new cuticle allows for a short phase of growth, but it must harden quickly (sclerotize) to confer protection from predators, infectious agents and desiccation, and to provide rigid attachment sites for muscles. The sclerotization of the new cuticle is regulated by the neuropeptide bursicon, which is released after the completion of ecdysis at all developmental stages (Honegger et al., 2008). Recent data have also shown that bursicon regulates the inflation and maturation of adult wings (Dewey et al., 2004; Honegger et al., 2008; Luan et al., 2006; Peabody et al., 2008). Thus, bursicon is of critical importance for insect survival because without a hard cuticle and functional wings insects would lack their protective armor, and their ability to walk, crawl, and fly.

Bursicon is co-expressed with crustacean cardioactive peptide (CCAP) in large bilateral neurosecretory cells present in most ganglia of the insect ventral nerve cord (Honegger et al., 2008). These cells, first named Cells 27 in the abdominal ganglia of *Manduca sexta* by Taghert and Truman (1982), release bursicon and CCAP into the hemolymph during ecdysis (Honegger et al., 2008; Luan et al., 2006; Peabody et al., 2008). The axons of these cells project to the alary muscles of the heart (Davis et al., 1993), to type III boutons on larval muscles 12 and 13 in *Drosophila melanogaster* (Loveall and Deitcher, 2010), and also possess many en passant varicosities (Davis et al., 1993; Honegger et al., 2008; Luan et al., 2006; Peabody et al., 2008). CCAP is known to increase heart activity (Davis et al., 2001; Dulcis et al., 2005; Lehman et al., 1993; Loi et al., 2001; Tublitz and Truman, 1985), and thus, likely enhances the delivery of bursicon to its target sites. A second neuron, interneuron IN 704, is located adjacent to Cell 27 and is only CCAP-immunoreactive (IR) in some insects (*Periplaneta americana*, *M. sexta*) but co-expresses bursicon and CCAP in *D. melanogaster* larvae (Luan et al., 2006). INs 704 most likely drive the ecdysis motor pattern through the action of CCAP (Truman, 2005), and in *D. melanogaster* might co-activate Cells 27 to release bursicon and CCAP into the hemolymph (Luan et al., 2006).

Development into the adult insect represents the final ecdysis (termed eclosion). Consequently, given that bursicon is only known to function in the sclerotization of the exoskeleton and wing maturation, and that adult insects do not undergo these processes after eclosion has been completed, bursicon should no longer be required during this life stage. Interestingly, in *M. sexta*

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and *D. melanogaster*, two holometabolous insects, the bursicon expressing Cells 27 undergo apoptosis soon after eclosion (Ewer et al., 1998; Peabody et al., 2008) while in the hemimetabolous insects *P. americana* and *Teleogryllus commodus* these cells survive through late adulthood and remain bursicon-IR (Honegger et al., 2008). We show here that the mosquito, *Anopheles gambiae*, is another holometabolous insect in which bursicon expressing cells undergo apoptosis following eclosion, suggesting that there is a divide between hemimetabolous and holometabolous insects with respect to bursicon function in adults.

2. Methods

2.1. Mosquito rearing and specimen collection

A. gambiae (G3 strain) were reared and maintained in an environmental chamber at 27 °C and 75% relative humidity, with 12 h light and 12 h dark photoperiods as described (Andereck et al., 2010). For gene expression analyses, mosquitoes were collected as eggs (with developing 1st instar larvae), 2nd through 4th instar larvae, callow pupae, black pupae, and adults. Under our rearing conditions, the onset of adult eclosion occurs approximately 2 h into the dark photoperiod, and emerging adults initially rest on the surface of the water until their wings have expanded and sclerotized sufficiently to sustain the stress of flight. To establish distinct age groups, pupae were transferred to hatching cages and freshly eclosed adults were collected at 0.5 h intervals. Adult mosquitoes were then either processed immediately (0 h adults) or kept in containers for various time periods before processing. For immunohistochemistry, mosquitoes were processed and imaged individually. For gene expression analyses, cDNA was synthesized from pools of individuals (approximately 200 eggs, 50 2nd instar larvae, 40 3rd instar larvae, 30 4th instar larvae, 20 pupae, and 15 adults).

2.2. RNA collection, cDNA synthesis and PCR

Gene expression analyses were done as described (Hillyer and Estevez-Lao, 2010). Mosquitoes were triturated in TRIzol reagent (Invitrogen, Carlsbad, CA) and total RNA was purified as per manufacturer's instructions. To remove contaminating DNA, RNA was re-purified using the PureLink™ Micro-to-Midi™ Total RNA Purification System (Invitrogen) and subsequently treated with RQ1 RNase-Free DNAse (Promega, Madison, WI). Purified RNA was then quantified, and cDNA was synthesized from up to 5 μg of total RNA using an Oligo(dT)$_{20}$ primer and the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen).

Gene expression was initially assayed by conventional PCR using gene specific primers and Choice-Taq™ DNA Polymerase (Denville Scientific Inc., Metuchen, NJ) on a BioRad DNA Engine® Thermal Cycler, using amplification of *rpS7* as a loading reference. To quantify relative gene expression (quantitative Real-Time PCR; qPCR), cDNA was amplified using gene specific primers and Power SYBR® Green PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI 7300 Real-Time PCR System. Amplification of *rpS7* was used as a loading reference, and relative quantification was done using the $2^{-\Delta\Delta C_T}$ method (Livak and Schmittgen, 2001). Quantitative PCR data are presented as the average fold-change relative to eggs or black pupae, depending on the experiment, and bars represent the standard deviation. As a means of evaluating the detectability of *burs* and *pburs* mRNA, the cycle thresholds (CT) and ΔCTs are also reported. This latter measurement represents *burs* or *pburs* CT minus *rps7* CT, which compensates for variability in template loading. Primers used to amplify *burs* (Genbank ID AY735443), *pburs* (Genbank ID AY823259), and *rpS7* (Genbank ID XM_314557) are listed in Table 1, and were designed such that the cDNA amplicon spans at least one intron. Amplicon size (conventional PCR) and melting curve analyses (quantitative RT-PCR) confirmed that only cDNA, and not genomic DNA, was amplified.

2.3. Immunohistochemistry

Male and female mosquitoes were cold-anesthetized and their wings and legs were removed. Subsequently they were intrathoracically injected with approximately 0.3 μl of Bouin's fixative using a microcapillary glass needle, immersed in a drop of phosphate buffer (pH 7.4), and dissected along a coronal plane. The dorsal cuticle and viscera were removed, and the ventral half of the abdomen, containing the ventral nerve cord, was flattened and processed for immunohistochemistry. Because of the considerable amount of time required for each individual dissection, age variation within each time point can vary up to 2 h (e.g., 0 h collection represents mosquitoes 0–2 h post-eclosion).

Whole-mount immunolabeling procedures were carried out as described previously (Dai et al., 2008). Following fixation in aqueous Bouin's for 2–6 h, tissues were washed using phosphate buffered saline (PBS) and treated with 0.02% collagenase in PBS for 10 min at room temperature. Tissues were then rinsed in PBS containing 0.3% Triton X-100 (PBST), blocked in 10% normal goat serum in PBST for 2 h, and incubated in a PBST solution containing mouse anti-PBURS ((Luo et al., 2005); dilution 1:1000) and rabbit anti-CCAP (provided by H. Agricola, U. of Jena, Germany; dilution 1:5000) antibodies for 2–4 days. After primary antibody incubation, tissues were washed in PBST, followed by incubation in a PBST solution containing 1:500 dilutions of goat anti-mouse and goat anti-rabbit IgGs coupled to Cy2 and Cy3, respectively (Jackson Immunoresearch, West Grove, PA). Tissues were then washed several times in PBS, mounted on glass slides using Aqua Poly/Mount (Polysciences Co., Warrington, PA), and observed using a ZEISS LSM 510 META laser scanning confocal microscope. Images were displayed as Z-stack projections, saved in the Zeiss LSM 5

Table 1
Sequences of primers used in this study.

Gene	Genbank ID	Primer	Nucleotide sequence (5′–3′)	Transcript amplicon	Genomic amplicon	Application
burs	AY735443	Forward	AGTTGGCGTTCTTCCTTCTG	206 bp	NA	PCR; qPCR
		Reverse	GATCTTGCTGCCGGACAC			
pburs	AY823259	Forward	CCGTCGGAAATTCATCTCAT	298 bp	390 bp	PCR; qPCR
		Reverse	TGAAGCACTTGCAATCAACC			
rps7	XM_314557	Forward	CGTGAGGTCGAGTTCAACAA	480 bp	711 bp	PCR
		Reverse	GCTGCAAACTTCGGCTATTC			
rps7	XM_314557	Forward	GACGGATCCCAGCTGATAAA	132 bp	281 bp	qPCR
		Reverse	GTTCTCTGGGAATTCGAACG			

NA, no amplification. *A. gambiae burs* is *Trans*-spliced: exons 1, 2 and 4 are located in chromosome arm 2L while exon 3 is located in chromosome arm 2R (Robertson et al., 2007). Because the sequences of the forward and reverse primers used to amplify *burs* reside on chromosomes 2L and 2R, respectively, this primer combination does not amplify genomic DNA.