Description of a Brain Neuropeptidome of the Malaria Vector Nyssorhynchus Albimanus and Neuropeptide Differential Expression During Infection with Plasmodium Berghei

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Research

Keywords: Neuropeptide, adipokinetic hormone, corazonin, pyrokinin, Nyssorhynchus albimanus, ookinete

Posted Date: May 8th, 2020

DOI: https://doi.org/10.21203/rs.3.rs-25368/v1

Abstract

Background Insect neuropeptides, mainly synthesized in the brain, play a central role in the control of many physiological processes. A neuropeptidome of the mosquito *Nyssorhynchus albimanus* was described based in a comparative analysis of the mosquito genome complemented with high-throughput sequencing of brain transcriptomes. In addition, neuropeptides differentially expressed during *Plasmodium* infection were identified.

Results We identified 3,811 transcripts associated to translation, oxidation-reduction process, protein binding, ATP binding, integral components of membrane and ribosome, among others. We identified 29 neuropeptide transcripts that predicted at least 60 biopeptides, including pyrokinin, glycoprotein hormone alpha (GPA2), prothoracicotropic hormone, neuropeptide-like precursor 1 (NPLP1), allatostatin C, orcokinin, corazonin, adipokinetic hormone I, SIFamide, pyrokinin capa-like, pigment-dispersing factor, adipokinetic hormone/corazonin-related peptide (ACP), tachykinin-related peptide, trissin, neuropeptide F, short neuropeptide F (sNPF), diuretic hormone 31, bursicon, crustacean cardioactive peptide (CCAP), allatotropin, allatostatin 1, partner of bursicon (PBURS), ecdysis triggering hormone (ETH), diuretic hormone 44 (Dh44), insulin-like peptides 5, 1, 3, 7, 2 (ILPs) and eclosion hormone (EH) and seven neuropeptide receptors. Transcript mapping to the *Ny. albimanus* genome provided evidence for the re-annotation of the myosuppressin gene. A quantitative analysis documented increased expression of adipokinetic hormone/corazonin-related peptide, pyrokinin and corazonin in the mosquito brain after *Plasmodium berghei* infection.

Conclusion This work represents an initial effort to characterize the neuropeptide repertoire of *Ny. albimanus* and provides new information for understanding neuroregulation of the mosquito response during *Plasmodium* infection .

1. Background

The Insect central nervous system (CNS), formed by the brain, thoracic and ventral segmental ganglia, control feeding behavior, muscle activity, and other relevant functions, that in mosquitoes are implicated in vectorial competence [1]. Neuropeptides are synthesized by the CNS neurons as pre-pro-neuropeptide. These include transmitter peptides and peptide hormones involved in neuron communication, neuroendocrine regulation, and many physiological and behavioral processes [2–4], such as reproduction, metamorphosis, growth and metabolism [5]. Pre-pro-peptides consist of an N-terminal signal peptide, one to several potentially active biopeptides flanked by cleavage sites in basic residues like lysine-arginine and a conserved C-terminal amidated motif [6]. C-terminal alpha-amidation promotes peptide stability and bioactivity [7–9]. Some neuropeptides are secreted into the hemolymph and regulate several functions in various organs of the insect [10–13].

Insect neuropeptides are functionally and structurally heterogeneous [3, 4, 12, 14–16]. Adipokinetic hormones induce the mobilization of lipids from the fat body to the hemolymph, increase trehalose levels, stimulating the insect heartbeat frequency [14, 15, 17] and enhancing the activation of the prophenoloxidase (PPO) cascade [18]. Myosuppressins inhibit food intake [19, 20], and the visceral and cardiac muscle contractions, while increasing the skeletal muscle activity [21] and midgut enzyme secretion [22]. Tachykinin-related peptides possess stimulatory effects on visceral muscle [23], regulate the release of adipokinetic hormones (AKH) [24], olfactory processes [25] and the insect aggressiveness [26]. Pirokinins modulate visceral muscle contraction [27], activate pheromone biosynthesis [28], regulate melanin synthesis [29], the embryonic diapause [30], and accelerate pupation [31]. Corazonin regulates the heart contraction rate [32][33], ecdysis [34], and stress responses [35]. In ants, corazonin is also involved in the control of social behavior [36], while in *Drosophila*, SIFamides promotes sleep and modulates sexual behavior [37, 38].

Anopheline mosquitoes transmit malaria. Although the genome sequence of more than 20 anopheline species are available [39], only the neuropeptidome of *Anopheles gambiae* has been described [40]. This neuropeptidome comprises at least 35 genes coding for tachykinin-related peptides, pyrokinins (Pk/PBAN, CAPA), myosuppresins, adipokinetic hormones, insulin-like peptides (ILP), sulfakinins, among others [40]. This information contributed to the understanding of the functional role of neuropeptides in diverse physiological and developmental processes of this mosquito [32, 41–43]. In addition, the role of ILP in mosquito growth and reproduction has been documented in *An. stephensi*, [40]. Various factors, including *Plasmodium* infection, alter the expression of these neuropeptides, indicating their participation in other mosquito functions [44]. However, the information about neuropeptides in other anophelines remains limited.

The subgenus *Anopheles Nyssorhynchus* was recently raised to the genus status *Nyssorhynchus* [45, 46]. *Nyssorhynchus albimanus* is an important malaria vector in México, Central America, and northern South America [47]. In this work, we refer our results to *Nyssorhynchus albimanus*, but keep *Anopheles albimanus*, as registered, when referring to genome and proteome data sets. Some neuropeptides of *Ny. albimanus* have been described in the brain, thoracic, and abdominal tissues [48]; however, a comprehensive description of its neuropeptidome and the effect of *Plasmodium* infection is still lacking.

Novel approaches, such as genome/transcriptome mining, have been employed for discovery and characterization of diverse neuropeptides, and several peptides have been predicted for a variety of species [49, 50]. Knowledge of neuropeptides and their role in physiological processes involved in malaria transmission could contribute to the development of novel control strategies [40]. We present herein the description of a neuropeptidome of *Ny. albimanus* constructed through a brain transcriptome and available genomic data analysis. As well as the analyses of the expression of various *Ny. albimanus* neuropeptides during ookinete *Plasmodium betghei* infection. Our results provide insights into the interactions between neuroregulation and immune response in this mosquito, and open new ways to characterize the regulatory network underlying these interactions.

2. Material And Methods

insect rearing

White stripe strain *Ny. albimanus* females [51] were obtained from the insectary of the National Institute of Public Health (INSP) in Cuernavaca, Mexico. Mosquitoes were bred under a 12:12 photoperiod at 28 °C and 70–80% relative humidity and 8% sucrose in cotton pads *ad libitum*. During the 72 h before infection with *P. berghei*, mosquitoes were provided with PSN 1 × (5,000 U of Penicillin, Streptomycin at 5 mg/ml, and Neomycin at 10 mg/ml) and gentamicin (50 µg/ml) (Thermo Fisher Scientific, Waltham, Massachusetts, USA). Cotton pads were changed daily. This antibiotic treatment eliminates almost all bacteria in mosquitoes' midguts [52].

Plasmodium berghei infection

Four days post-emergence mosquitoes were fed with ookinetes of *P. berghei* ANKA strain expressing the Green Fluorescent Protein (GFP) [53] (kindly donated by Robert E. Sinden, Imperial College, UK). Ookinetes were produced by culturing gametocyte-infected mouse blood as described previously [54]. Groups of 300 female mosquitoes were fed for 1 h using artificial membrane feeders with: (i) mouse blood + approximately 800 per µl GFP *P. berghei* ookinetes (infected group), or (ii) uninfected mouse blood (control group). Unfed mosquitoes were removed, and the engorged ones were incubated at 21 °C to allow for parasite invasion and interaction with the mosquito midgut. At 24 h post-blood feeding, mosquito midguts were analyzed under a 40 X fluorescence microscope (Leica DM1000) to confirm the presence (*P. berghei*-infected group) or absence (control group) of parasites. We observed ookinetes and retorts forms. Only midguts containing more than 300 parasites were included in the infected group. Only brains from infected mosquitoes with confirmed infection from the infected group and all brains from the control group were collected.

Neurotranscriptome preparation and sequencing

Mosquitoes were cold anesthetized for 10 min at 4 °C and maintained on ice. Three-hundred brains were obtained from each mosquito group (N = 300). Dissected tissues were kept in 200 µl of Trizol Reagent (Invitrogen Waltham, Massachusetts, USA) and stored at -70 °C until processing.

Total RNA from pooled *P. berghei*-infected and control mosquito brains was obtained using Trizol Reagent following the manufacturer's instructions (Thermo Scientific). The RNA clean up kit (Zymoclean, Irvine, CA, USA) was used to eliminate the possible contamination with mosquito eye pigment. Total RNA concentration, integrity, and yield were determined using Agilent's 2100 Bioanalyzer with the RNA 6000 Pico kit, according to the manufacturer's instructions (Agilent Technologies, Santa Clara, CA, USA).

Full-length cDNA libraries were synthesized using the Mint-2 cDNA synthesis kit (Evrogen, Moscow, Russia), according to the manufacturer's instructions. Briefly, 1 µg of RNA from each sample group (previously digested with DNase I (Invitrogen) to remove contaminating DNA) was used for first-strand cDNA synthesis with a dT oligo (CDS-*Gsu*I : 5'-AAGCAGTGGTATCAACGCAGAGTACTGGAG(T)20VN-3') and a Plug oligo adapter (5'-AAGCAGTGGTATCAACGCAGAGTGGCCATTACGGCCGGGGG-3') (Evrogen). The first-strand cDNA was used for second-strand cDNA synthesis by PCR amplification with the M1primer (5'-AAGCAGTGGTATCAACGCAGAGT-3') (Evrogen). Later, 3 µg of each double-strand cDNA was digested with *Gsu*I (15U) for 6 h at 30 °C. The cDNA libraries were prepared using the GS FLX Titanium Rapid Library Preparation kit according to the manufacturer's instructions (Roche). The cDNA libraries were sequenced in a full Pico titer plate using the Genome Sequencer FLX Titanium platform (454-Roche).

Data filtering, trimming, and mapping

The output raw sequences were filtered according to length (> 100 bp), sequence complexity, and quality. Primer adaptors were trimmed using the SeqClean software [55]. The filtered reads were mapped to the *Anopheles albimanus* transcriptome [56] using GS Reference Mapper software v.2.5.3, with default parameters, and genome (Strain: STECLA, version Gene set: AalbS2.6.), using Exonerate v.2.2 (Slater GS and Birney E, 2005) with the EST2 genome mode, and a threshold score of 300, and a maximum intron length of 20,000 bp. Output bam files were used to verify mappings to the *An. albimanus* genome to identify putative transcribed genes that were not annotated. The count of reads by gene was done with the HTseq count v0.11.1 [57] software. The dataset used in this work is available at [58].

Transcriptome brain analysis

To characterize the *Ny. albimanus* brain transcripts that we obtained, we retrieve gene ontology (GO) annotations and performed comparisons between identified GOs using BLAST2GO v4.0.2. Additionally, we conducted a large-scale data mining with our *Ny. albimanus* brain transcripts as queries to retrieve a set of 1:1 orthologs in the *An. gambiae*, *Aedes aegypti* and *Drosophila melanogaster* genomes with the web interface BioMart in VectorBase [59] and the *D. melanogaster* genome in Flybase [60]. The *Ny. albimanus* ortholog set was used to identified genes with recognized expression in the brain of the aforementioned insects [60–62]. A GO type enrichment analysis for biological processes, molecular functions and cellular components was performed using the R TopGO library [63]. Transcripts were translated into six frames and analyzed using InterProScan against the InterPro database for both mosquito groups. Transcripts were translated into six frames and analyzed using InterProScan against the InterPro database [64]. Transcripts containing neuropeptides and or hormone domains were selected and validated with tBLASTx and BLASTp using a cut-off e-value of 1.0e-5. BLAST outputs were retrieved, listed and compiled by descending sequence identity percentage and score, and ascending e-value. Each of them was used to verify mappings to our *Ny. albimanus* brain transcriptome.

Neuropeptide identification

To identify putative neuropeptides in both *Ny. albimanus* groups (*P. berghei*-infected and control), we compiled a dataset (*reference-dataset*) of FASTA sequences from the Database for Insect Neuropeptide Research (DINeR) [65], as well as previously published neuropeptides and neuropeptide receptors of *An. gambiae* [61] and *Ae. aegypti* [62], and neuropeptide and brain expressed genes of *D. melanogaster* [60]. The *reference-dataset* was used to perform multiple BLAST searches (BLASTn, tBLASTx and BLASTp) against the genome of *An. albimanus* using a cut-off e-value of 1.0e-5. BLAST outputs were retrieved, listed and compiled in the order of descending sequence identity percentage and score, and ascending e-value. The putative neuropeptides and neuropeptide receptors of *Ny. albimanus* were identified through BLAST [66], pfam [67], prosite [68], superfamily [69], smart [70], panther [71], gene3D [72], Conserved Domain Database (CDD) [73], prints [74] and ProDom [75]. Recognized domain signatures were visually inspected and compared against the genes of the *reference-dataset* to corroborate their architecture similarities. Also, we retrieve from VectorBase 1:1 ortholog of *An. albimanus*, *An. gambiae*, *Ae. aegypti* and *D. melanogaster* to investigate their neuropeptide orthologue relationships. The set of neuropeptides and neuropeptide receptors was annotated with BLAST2GO v4.0.2 [76]. The signal peptide prediction of putative neuropeptides was conducted with SignalP v5.0 [77]. The presence of neuropeptide precursors were detected with the software NeuroPID [78]; the prediction of cleavage sites and neuropeptides were analyzed using the NeuroPred programm [79] and to predict transmembrane helices of neuropeptide receptors we used TMHMM Server v.2.0 [80].

Expression of neuropeptides in Ny. albimanus mosquitoes

Based on the available genomic data, we generated specific oligonucleotides for eighteen *Ny. albimanus* identified neuropeptides (Additional file 1: Table S1) using Oligo Analyzer v3.1 [81]. We conducted real-time PCR assays and amplification of each potential biopeptide region by triplicate using an Applied Biosystems(ABI) Step One Plus Real-Time PCR System. The qRT-PCR program was 95 °C for 10 min, 95 °C for 15 seconds and 64 °C for 1 min, repeated for 40 cycles; then 95 °C for 15 sec, 64 °C for 15 sec, and 95 °C for 15 sec, for one cycle. The specificity of the SYBR green PCR signal was confirmed by melting curve analysis and 1.5% agarose gel electrophoresis.

Differential expression of neuropeptides in P. berghei-infected and uninfected mosquitoes

The expression of adipokinetic hormone/corazonin-related peptide, tachykinin related peptide, SIFamide, myosuppressin, pk/PBAN, corazonin, ILP-2, ILP-3, and IL-5 were investigated in the brain of *P. berghei*-infected and control mosquitoes through real-time PCR assays. For every neuropeptide gene, three biological replicates were conducted for both conditions. As an internal control, a fragment of actin was amplified using primers RTActU R: (CGA TCC ACT TGC AGA GCC AGT) and RTAct3.2 F: (5´-TAC GCC AAC ATT GTC ATG TCC – 3´) [82]. The real-time PCR program was conducted as described above. Generated qRT-PCR Ct values were analyzed using the 2-ΔΔCt method [83] and tested with one-way ANOVA, followed by a Kruskal-Wallis post-test ($\alpha = 0.05$).

3. Results

Brain transcriptome analysis:

Sequencing yielded 101,520 raw reads from control group and 109,383 from the *P. berghei*-infected group. The average read lengths in both groups were of 145 bp. Almost half of the total reads of infected and control group mapped to the *An. albimanus* genome (45.1%, and 45.9%, respectively). The reference mapping identified 3,811 transcripts, 30.03% of the total transcripts set currently registered in the transcriptome of *An. albimanus* (12,687 transcripts) (AalbS2.6), 947 transcripts were located in control, 1,220 transcripts in infected, and 1,633 were shared by both groups (Fig. 1). Of these, 2,131 had been previously annotated and 1,671 lack of available metadata (Additional file 2: TableS2). Most transcripts were associated to translation, oxidation-reduction process, transmembrane transport and proteolysis (biological process), protein binding, ATP binding, structural constituent of ribosomes, nucleic acid binding (molecular function); integral component of membrane, ribosome, nucleus, cytoplasm and intracellular (cellular component). Molecular functions were the most representative sequences identified in the GO analysis. These included sequences associated to ATP synthesis coupled to proton transport (GO:0015986); SRP-dependent co-translational protein targeting to membranes (GO:0006614); retrograde vesicle-mediated transport, Golgi to endoplasmic reticulum biological processes (GO:0006890); cytoplasm (GO:0005737); mediator complex (GO:0016592); integrator complex (GO:0032039), cellular component; NADH dehydrogenase (ubiquinone) activity (GO:0008137); transcription co-regulator activity (GO:0003712) and ATP binding (GO:0005524). (Additional file 3: Table S3).

The InterPro analysis identified more than 300 domains; among these, Na + channel auxiliary subunit TipE (IPR031578), choline/carnitine acyltransferase domain (IPR039551), Wnt (IPR005817), mitoguardin (IPR019392), ribosomal RNA small subunit methyltransferase H (IPR002903) and actin-related protein 2/3 complex subunit 4 (IPR008384). (Additional file 4: Table S4). Also, we identified transcripts that codified proteins related to the central nervous system (Additional file 2: TableS2).

Identification of neuropeptide transcripts

We identified 368 reads that mapped with 19 transcripts associated to neuropeptides in the *An. albimanus* genome: SIFamide, pigment dispersing hormone, insulin-like peptide 1,3,7, diuretic hormone 31, myosuppressin, trissin, prothoracicotropic hormone, pk/PBAN, pirokinin/CAPA, corazonin, allatostatin-C, orcokinin, bursicon alpha, tachykinin related peptide, adipokinetic hormone/corazonin-related peptide, adipokinetic hormone I, short neuropeptide F, glycoprotein GPA2 and neuropeptide F (Fig. 2).

The comparative *in silico* analysis using neuropeptide transcripts sequences of *An. gambiae*, *Ae. aegypti* and *D, melanogaster* yielded ten neuropeptides in addition to the above mentioned: partner of bursicon, insulin-like peptide-2, insulin-like peptide-5, ecdysis triggering hormone, eclosion hormone, crustacean

cardioactive peptide, allatotropin, allatostatin A, allatostatin C and diuretic hormone 44. To confirm these identities, eighteen nucleotide sequences corresponding to these pre-pro-peptides were matched against *An. gambiae* genome in Vectorbase. Matching sequences were used to design oligonucleotides for amplification by RT-PCR of *P. berghei*-infected and uninfected mosquito brain samples (Additional file 5. Figure S1).

Of the 29 neuropeptides identified in mosquito brains and the *An. albimanus* database, 12 peptide sequences had at least one domain of the respective putative peptide (ILP-1,3,7, ILP-2, ILP-5, pk/PBAN, allatostatin-A, allatostatin-C, corazonin, CCAP, ACP, AKH-I, PDH and EH). Three sequences had unrelated putative domains (allatotropin and glycoprotein GPA2 and prothoracicotropic hormone). While no domains were identified in tachykinin related peptide, trissin, SIFamide, orcokinin, bursicon, partner of bursicon, neuropeptide F, short neuropeptide F, myosuppressin, ETH, Dh31, Dh44, NPLP1 and pyrokinin/CAPA (Table 1).

Table 1

Neuropeptides and receptors identified in *An. albimanus* brain and annoted transcriptome.

Name	VectorBase ID (*An. gambiae, Ae. Aegypti*)[1]	SignalP[2]	TMH[3]	NPPs	VB AALB ID[4]	NPPs	InterPro shared domains	Group
Pyrokinin/PBAN	AGAP002292	Yes	-	5	AALB008609	5	Pyrokinin, conserved site (IPR001484)	Share
Glycoprotein GPA2*****	AGAP008301	Yes	-	2	AALB001547	1	DAN (IPR004133)	Share
Prothoracicotropic hormone*	AGAP000859	Yes	-	0	AALB007627	1	Cystine-knot cytokine (IPR029034)	Contr
Neuropeptide-like precursor 1 (NPLP1)*	AGAP010366	Yes	-	0	AALB001239	1	None predicted IPR	*Plasn*
Allatostatin C	AGAP010157	No	-	3	AALB007903	1	Allatostatin, insect (IPR020161)	Share
Orcokinin	AGAP012220	No	-	6	AALB009966	5	None predicted IPR	Share
Corazonin	AGAP003675	Yes	-	3	AALB010867	1	Procorazonin (IPR020190)	Share
Adipokinetic Hormone 1	AGAP008834	Yes	-	1	AALB015568	1	Adipokinetic hormone/red pigment-concentrating hormone (IPR010475)	*Plasn*
SIFamide	AGAP007056	Yes	-	2	AALB005004	1	None predicted IPR	Share
Pyrokinin capa-like	AGAP000347	Yes	-	4	AALB006405	2	None predicted IPR	Share
Pigment-dispersing hormone	AGAP005776	Yes	-	1	AALB006094	1	Pigment-dispersing hormone (IPR009396)	Share
Adipokinetic hormone/corazonin-related peptide	AGAP002430	Yes	-	1	AALB008450	1	Adipokinetic hormone, conserved site (IPR002047)	Share
Tachykinin-related peptide*	AGAP010014	No	-	2	AALB000888	4	None predicted IPR	Share
Trissin*	AGAP012496	Yes	-	2	AALB015467	1	None predicted IPR	*Plasn*
Neuropeptide F	AGAP004642	No	-	1	AALB002883	1	None predicted IPR	Contr
short neuropeptide F (sNPF)*	AAEL012542	No	-	3	AALB007863	3	None predicted IPR	Share
Diuretic hormone 31	AAEL008070	No	-	2	AALB009260	1	Peptide hormone DH31-like (IPR034439)	Share
Myosuppressin***, %**	AGAP001474	Yes	-	1	AALB009255	1	C2 domain superfamily (IPR035892)	Share
Bursicon alpha*	AGAP002537	No	-	1	AALB009577	1	Cystine-knot cytokine (IPR029034)	Share
Crustacean cardioactive peptide (CCAP)**	AGAP009729	Yes	-	1	AALB000113	1	Crustacean cardioactive peptide (IPR024276)	Share
Allatotropin**	AGAP012130	Yes	-	1	AALB004051	1	EF-Hand 1, calcium-binding site (IPR018247)	Share
Allatostatin A**	AGAP003712	Yes	-	4	AALB015793	7	Allatostatin (IPR010276)	Share
Partner of Bursicon (PBURS)**	AGAP004506	Yes	-	1	AALB003562	1	Bursicon subunit beta (IPR034441)	Share
Ecdysis triggering hormone (ETH)**	AGAP007062	Yes	-	1	AALB004998	2	None predicted IPR	Share
Diuretic hormone 44 (Dh44)**	AGAP003269	Yes	-	1	AALB009504	1	Corticotropin releasing factor (IPR000187)	Share
Insulin Like Peptide 5 (ILP-5)**	AGAP003927	Yes	-	2	AALB008758	3	Insulin like superfamily (IPR036438)	Share
Insulin Like Peptide 1, 3, 7 (ILP-1, 3, 7)*	AGAP010604, AGAP010603	No	-	2	AALB010411	7	Insulin like superfamily (IPR036438)	Share
Insulin Like Peptide 2 (ILP-2)**	AGAP010600	Yes	-	1	AALB010410	2	Insulin like superfamily (IPR036438)	Share
Eclosion hormone (EH)*****, **, "	AGAP010437	Yes	-	1	AALB007602	1	Eclosion hormone (IPR006825)	*Plasn*
Dopamine/ecdysteroid receptor*	AAEL000266	No	Yes	-	AALB004495	-	G protein-coupled receptor, rhodopsin-	Share

							like (IPR000276)	
Leucokinin receptor*	AAEL006636	No	Yes	-	AALB010632	-	G protein-coupled receptor, rhodopsin-like (IPR000276)	Contr
Myosuppressin receptor*	AAEL006283	No	Yes	-	AALB004350	-	G protein-coupled receptor, rhodopsin-like (IPR000276)	*Plasn*
short neuropeptide F (sNPF) receptor*	AAEL007924	No	Yes	-	AALB003789	-	G protein-coupled receptor, rhodopsin-like (IPR000276)	*Plasn*
Eclosion hormone receptor*	AAEL008387	Yes	Yes	-	AALB000935	-	Receptor, ligand binding region (IPR001828)	*Plasn*
Capa receptor*	AAEL017335	No	Yes	-	AALB009517	-	Neuromedin U receptor (IPR005390)	Contr
Tachykinin-like receptor (TkR99D)*	AAEL006947	No	Yes	-	AALB002458	-	G protein-coupled receptor, rhodopsin-like (IPR000276)	*Plasn*
* No metadata available for this ID transcript in AalbS2.6								
** No found in putative *An. albimanus* brain transcriptome but identified in AalbS2.5 data base								
" With Eclosion hormone domain (Pfam)								
All transcripts have startt and stop codon, except allatostatin and diuretic hormone 31 transcripts without start codon								
% Identified at intronic sequense of AALB009255								
[1, 4] https://www.vectorbase.org/								
[2] http://www.cbs.dtu.dk/services/SignalP/								
[3] http://www.cbs.dtu.dk/services/TMHMM/								

[1]ID´s of transcripts correspond to *An. gambiae* and *Ae. aegypti* of https://www.vectorbase.org

[2]Signal peptide was predicted by SignalP-5.0 Server (http://www.cbs.dtu.dk/services/SignalP/)

[3] Prediction of transmembrane helices in proteins was predicted by

TMHMM Server v. 2.0 (http://www.cbs.dtu.dk/services/TMHMM/)

[4] ID´s of transcripts corresponds to *Ny. albimanus* of https://www.vectorbase.org

Page 2. Neuropeptides orthologes genes.

[1]https://www.vectorbase.org/

[2]https://orthomcl.org/orthomcl/

[3]ID Ortholog Group by orthomcl database

N/A (No Assigned Ortholog)

The identified transcripts encode for at least 60 putative biopeptides. Their sequences are shown in Table 2. We also identified five neuropeptide receptor transcripts (Table 1) with G protein-coupled rhodopsin-like domains (AALB004495-RA, AALB010632-RA, AALB004350-RA, AALB003789-RA, and AALB002458-RA), that belong to dopamine, leucokinin, myosuppressin, short neuropeptide F and tachykinin orthologues receptors, respectively; these contain rhodopsin-like domains. Transcript AALB000935-RA, contains a binding ligand-receptor and transcript AALB009517-RA, a neuromedin U receptor domain.

For brevity, we describe only neuropeptides with increased expression in the brains of the infected group compared to those of the control group. We also describe a myosuppressin, identified within the sequence of transcript AALB009255-RA. Details of the sequences of the other transcripts are presented in the supplementary material (Additional file 6 Figure S2).

Structural characterization of the myosuppressin gene:

The polypeptide AALB009255-PA is a predicted polypeptide of 1,166 amino acid residues long (Fig. 3A) and is coded by fourteen exons and thirteen introns. It is predicted to contain C2 domains (calcium-dependent membrane-targeting module involved in signal transduction or membrane trafficking), which are absent in myosuppressins [84]. However, Tblastn searches, mapping, and insect transcriptome data [85], previously published myosuppressins sequences [86, 87] and a close sequence analysis, identified an open reading frame between the first two exons and the second intron of AALB009255-RA (Fig. 3B). This codes for an identical peptide of *An. gambiae, D. melanogaster, M. domestica* and *Ae. aegypti* myosuppressins (Fig. 3F).

Adipokinetic hormone/corazonin-related peptide (ACP)

One transcript-encoding a precursor of ACP was identified (AALB008450-RA). This transcript encodes for a 106-residue product with a predicted signal peptide of 31 amino acid residues in the N-terminus, followed by a predicted neuropeptide-coding sequence for 11 amino acid residues (QVTFSRDWNA**G**). It has a G residue and KR cleavage sites in the C-terminus (Fig. 4A). ACP is identical to the ACP of *D. melanogaster* [88] and *An. darlingi*, *An. gambiae*, *Ae. aegypti* and *C. pipiens* [89](Fig. 4B).

Table 2

Alphabetical list of peptides and their best BLAST hit on NCBI and Vector Base. Except tachykinin related peptide (APSFGLGMRa) that aligned with pre-pro-tachykinin-B of Panulirus interruptus, best BLAST hits of all peptides were with other insect peptides. In red are shown the cleavage sites for arginine (R) and lysine (K). In blue are shown the conserved motifs in each bio-peptide. *Sequences of these peptides are shown in supplementary figure 2.

NEUROPEPTIDE	TRANSCRIPT ID	Best BLAST hit (NCBI)	IDENTITY	E - value	ACCESSION NUMBER
ADIPOKINETIC HORMONE/CORAZONINE-RELATED PEPTIDE (ACP)	AALB008450-RA				
QVTFSRDWNAa KR		adipokinetic hormone/corazonin-related peptide [*Anopheles gambiae*]	100%	0.010	ABD60145.1
ALLATOSTATIN-A	AALB015793_RA				
R SPKYNFGLa KR		hypothetical protein AND_004794 [*Anopheles darlingi*]	100%	0.007	ETN63487.1
KR RTYDFGLa KR		hypothetical protein AND_004794 [*Anopheles darlingi*]	100%	0.005	ETN63487.1
KR LPHYNFGLa KR		preproallatostatin [*Aedes aegypti*]	100%	3e-04	AAB08870.1
KR LPNRYNFGL		preproallatostatin [*Aedes aegypti*]	100%	0.020	AAB08870.1
KR ATSGNGAGGAYRYHFGLa KR		hypothetical protein AND_004794 [*Anopheles darlingi*]	100%	4e-12	ETN63487.1
KR YFEAEEFN KR		hypothetical protein AND_004794 [*Anopheles darlingi*]	100%	0.001	ETN63487.1
KR RYIIEDVPa KR		preproallatostatin [*Aedes aegypti*]	100%	0.005	AAB08870.1
ALLATOSTATIN-C	AALB007903-RA				
KR QIRYRQCYFNPISCF KK		allatostatin 2 [*Anopheles darlingi*]	100%	1e-08	ETN 61475.1
ALLATOTROPIN	AALB004051-RA				
R SIRAPFRNSEMMTARGFa KR		allatotropin [*Aedes aegypti*]	100%	4e-06	AAB06179.1
ADIPOKINETIC HORMONE I (AKHI)	AALB015568-RA				
QLTFTPAWa KR		adipokinetic hormone preproprotein [*Periplaneta americana*]	91%	0.36	AAV41425.1
BURSICON	AALB003562-RA				
***Sequence too long to be given here.**		partner of bursicon [*Anopheles darlingi*]	100%	9e-96	ETN65201.1
CORAZONIN	AALB010867-RA				
QTFQYSRGWTNa KR		preprocorazonin (*Drosophila melanogaster*)	100%	3e-04	AAB32283.1
CRUSTACEAN CARDIOACTIVE PEPTIDE (CCAP)	AALB000113-RA				
KR PFCNAFTGCa KK		cardioacceleratory peptide 2a [*Manduca sexta*]	100%	0.082	AAC24871.1
DIURETIC HORMONE 31 (Dh31)	AALB009260-RA				
KR TVDFGLSRGYSGAQEAKHRMAMAVANFAGGPa RK		diuretic hormone 31 [*Anopheles darlingi*]	100%	3e-16	ETN65540.1
DIURETIC HORMONE 44 (Dh44)	AALB009504-RA				
RR QMRENTRQVELNKALLREIa KR		diuretic hormone 44, isoform A [*Drosophila melanogaster*]	71%	6e-08	NP_649922.2
ECLOSION HORMONE	AALB007602-RA				
***Sequence too long to be given here.**		eclosion hormone [*Anopheles darlingi*]	94%	3e-23	ETN65246.1

ECDYSIS TRIGGERING HORMONE	AALB004998-RA				
TESPGFFIKLSKSVPRIa RR		ecdysis-triggering hormone [*Anopheles darlingi*]	100%	1e-10	ETN66573.1
RR GDLENFFLKQSKSVPRIa RR		ecdysis-triggering hormone [*Anopheles darlingi*]	100%	3e-13	ETN66573.1
INSULIN LIKE PEPTIDE (ILP 1, 3, 7)	AALB010411-RA				
RR GHYCGRILSETLAKVCNSYNGMR KK		insulin-like peptide 1 precursor (*Anopheles stephensi*)	68%	2e-04	AEA51303.1
***Sequence too long to be given here.**		insulin-like peptide 7 precursor [*Anopheles gambiae*]	42%	1.9	AAQ89699.1
***Sequence too long to be given here.**		insulin-like peptide 3 precursor [*Anopheles stephensi*]	53%	0.061	AEA51303.1
KR YCGAELVKVLSFLCDEFPDLHSTN KK		insulin-like peptide 3 precursor [*Anopheles darlingi*]	100%	3e-16	ETN61522.1
***Sequence too long to be given here.**		insulin-like peptide 3 precursor [*Anopheles darlingi*]	100%	2e-43	ETN61522.1
***Sequence too long to be given here.**		insulin-like peptide 3 precursor [*Anopheles darlingi*]	96%	1e-10	ETN61522.1
KK AGSFFGDAFDPTETGLSFPIPARF RR		insulin-like peptide 3 precursor [*Anopheles darlingi*]	100%	2.00E-43	ETN61522.1
INSULIN LIKE PEPTIDE 2 (ILP-2)	AALB010410-RA				
TSTPKGDALISTLTRSRYCa RR		insulin-like peptide 2 precursor [*Anopheles darlingi*]	95%	6e-09	ETN61520.1
***Sequence too long to be given here.**		insulin-like peptide 2 precursor [*Anopheles sinensis*]	97%	3e-31	KFB49854.1
INSULIN LIKE PEPTIDE 5 (ILP-5)	AALB008758-RA				
***Sequence too long to be given here.**		insulin-like peptide 5 precursor [*Anopheles gambiae*]	90%	4e-29	AAQ89696.1
***Sequence too long to be given here.**		insulin-like peptide 5 precursor [*Anopheles gambiae*]	70%	2e-04	AAQ89696.1
***Sequence too long to be given here.**		insulin-like peptide 5 precursor [*Anopheles sinensis*]	**97%**	**2e-17**	KFB46716.1
MYOSUPRESSIN	AALB009255-RA				
KR TDVDHVFLRFa KR		myosuppressin, isoform A [*An. gambiae*]	100%	1.4	NP 536772.1 and AGAP001474-PA
NEUROPEPTIDE F	AALB002883-RA				
LTAARPQDGDAASVAAAIRYLQELETKHAQHARPRLa		neuropeptide F [*Culex quinquefasciatus*]	81%	1e-09	AFS50167.1
ORCOKININ	AALB009966-RA				
KR NFDEIDRFARFNa KR		orcokinin protein coding [*An. gambiae*]	100%	1.5	AGAP012220-PA
KR NFDEIDRFNAGFNYRLNGDEVAAIa KR		orcokinin protein coding [*An. gambiae*]	83%	0.065	AGAP012220-PA
KR NFDEIDRFGRFSNFa KR		orcokinin protein coding [*An. gambiae*]	83%	0.45	AGAP012220-PA
KR SLNNSDRRTLLYNYYSRGLAPMYE KR		orcokinin protein coding [*An. gambiae*]	95%	2e-10	AGAP012220-PA
KR NLDYEPSYVGTMDHGYSGRMS KR		orcokinin protein coding [*An. gambiae*]	72%	0.009	AGAP012220-PA
PARTNER OR BURSICON (PBURS)	AALB003562-RA				
***Sequence too long to be given here.**		partner of bur [*Anopheles darlingi*]	100%	3e-81	ETN65201.1
PIGMENT DISPERSING HORMONE (PDH)	AALB006094-RA				
KR NSELINSLLSLPKSMNDAa K		pigment dispersing hormone [*Anopheles darlingi*]	100%	9e-13	ETN58114.1
PYROKININ/CAPA	AALB006405_RA				

KR GPTVGLFAFPRVa R (PVK-1)		cardioacceleratory peptide 2b isoform X1 [*Aedes aegypti*]	100%	9.00E-07	XP_021696180.1
KR QGLVPFPRVa R (PVK-2)		cardioacceleratory peptide 2b splicing variant b [*Nilaparvata lugens*]	92%	0.004	BAO00941.1
KR ASGSGANGGMWFGPRLa KR (PK)		CAPA [*Periplaneta americana*]	73%	3e-04	AIK03055.1
PK/PBAN	AALB008609-RA				
KR AAAMWFGPRLa KR (PK-1)		pyrokinin [*Anopheles darlingi*]	100%	0.012	ETN67561.1
RK PQPLFYHTAAPRLa RR		pyrokinin [*Anopheles darlingi*]	100%	2e-05	ETN67561.1
R NLPFSPRLa R (PK-3)		pyrokinin [*Anopheles darlingi*]	100%	0.054	ETN67561.1
RR DSVGENHQRPPFAPRLa R (PK-2)		pyrokinin [*Anopheles darlingi*]	100%	2e-08	ETN67561.1
RR EDDSGLEGNGVS KR		pyrokinin [*Anopheles darlingi*]	81%	0.003	ETN67561.1
SIFamide	AALB005004-RA				
RK PPFNGSIFa KR		SIFamide [*Drosophila melanogaster*]	100%	2.5	XP 001246496.1
TACHYKININ	AALB000888-RA				
RR VPSGFNGVRa KK		tachykinin, isoform A [*Drosophila melanogaster*]	100%	0.58	NP 650141.1
KR APSGFLGMRa KK		preprotachykinin B [*Panulirus interruptus*]	100%	2e-04	BAD 06363.1
KR APTGFTGMRa RR		tachykinin, isoform A [*Drosophila melanogaster*]	100%	0.58	NP 650141.1
KR VPNGFMGLRa KK		hypothetical protein AND_006811 [*Anopheles darlingi*]	100%	0.30	ETN61527.1
GLICOPROTEIN GPA2	AALB001547-RA				
*Sequence too long to be given here.		glycoprotein hormone alpha 2 [*Anopheles darlingi*]	100%	2.00E-68	ETN58907.1
PROTHORACICOTROPIC HORMONE (PTH)	AALB007627-RA				
*Sequence too long to be given here.		prothoracicotropic hormone preproprotein [*Culex pipiens*]	79%	2.00E-59	ADO51754.1
NEUROPEPTIDE LIKE PRECURSOR 1 (NPLP1)	AALB001239-RA				
*Sequence too long to be given here.		PREDICTED: neuropeptide-like 1 [*Aedes albopictus*]	74%	1.00E-38	XP_019547010.1
TRISSIN	AALB015467-RA				
ALSCDSCGRECASACGTRHFRTCCFNYLR KR		trissin 1 splicing variant A and B [*Spodoptera exigua*]	93%	5.00E-19	AXY04300.1 and AXY04301.1
SHORT NEUROPEPTIDE F (sNPF)	AALB007863-RA				
RK AVRSPSLRLRFa RR		short neuropeptide F precursor [*Culex quinquefasciatus*]	100%	1.00E-08	AVR59281.1
R APQLRLRFa R		short neuropeptide F [*Anopheles sinensis*]	100%	0.037	KFB48121.1
R APQLRLRFa R		short neuropeptide F [*Anopheles sinensis*]	100%	0.037	KFB48121.1
***Sequence too long to be given here. Supplemetary figures show each neuropeptide sequence**					

Pk/PBAN

One transcript encoding a precursor of pk/PBAN was identified (AALB008609-RA) with 199 amino acid residues. It has a predicted signal peptide of 22 amino acid residues in the N-terminus, and five predicted neuropeptide-coding sequences (Fig. 5A) [90]. One such sequence codes for a peptide of 11 amino acid residues (AAAMWFGPRLG) that is identical to the *An. darlingi*, *An. gambiae* and *Ae. aegypti* peptides. The second one codes for a peptide with 14 amino acid residues (PQPLFYHTAAPRLG), identical to that of *An. darlingi*; The third one codes for a peptide with 16 amino acid residues (DSVGENHQRPPFAPRLG), identical to peptides of *An. darlingi* and *An. gambiae*; and the fourth codes for a peptide with nine amino acid residues (NLPFSPRLG), identical to peptides of *An. darlingi*, *An. gambiae* and *Ae. aegypti*. All these have a G residue and PRL-conserved motif in their C-terminus, and are flanked by KR, RK, RR and R cleavage sites (Figs. 5A and 5B). The fifth sequence codes for a peptide with 12 amino acid residues (EDDSGLEGNGVS), which is flanked by RR and KR

cleavage sites, indicating that this peptide may be processed, but does not have the conserved motif and G residue in its C-terminus (Fig. 5A, highlighted in gray).

Corazonin

One transcript encoding a precursor of corazonin was identified (AALB010867-RA). It has 156 amino acid residues, whit a predicted signal peptide of 20 amino acid residues in the N-terminus, followed by one predicted neuropeptide-coding sequence with 12 amino acid residues (QTFQYSRGWTNG). It has a G residue and KR cleavage sites in the C-terminus (Fig. 6A). This precursor is identical to the corazonin of, *An. gambiae*, *D. melanogaster*, *Ae. aegypti*, *M. domestica*, *B. mori*, *D. pulex* and *N. vitripennis* (Fig. 6B) [91].

Neuropeptide brain expression in*P. berghei* experimental infection.

We observed a significant increased expression of ACP (3.94-fold), pk/PBAN (3.40-fold), and corazonin (0.75-fold) in ($p < 0.001$) in the infected group. Tachykinin-related peptide, SIFamide, myosuppressin, and ILP-5 were consistently elevated in infected mosquitoes, 0.34-fold, 0.64-fold, a 0.54-fold and 0.46-fold, respectively) compared to uninfected mosquitoes, but the differences were not significant. No differential expression was observed for ILP2 and ILP3 (Fig. 7).

4. Discussion

In this work, we report the expression of critical neuropeptides in *Ny. albimanus*, with increased expression during infection with a malaria parasite. Of the 29 putative neuropeptide transcripts, that predicted at least 60 possible biopeptides, 27 had coding sequence (CDS).

In addition, we describe transcripts mainly associated with translation, oxidation-reduction process, protein binding, ATP binding, integral components of membrane and ribosomes. Previously, we reported 19 brain proteins of this mosquito with differential expression after *P. berghei* infection, and we identified 17 transcripts of proteins previously reported [92] (Additional file 7. Figure S3). All transcripts reported herein have orthologues in other insect disease vectors [4, 16, 50, 93–95] and in relevant agricultural insect pest [5, 96, 97]. Although most of the encoded peptides are identical or similar to orthologs in other species, some of them exhibit amino acid sequence variations. Alike to other mosquito species (*An. darlingi*, *An. gambiae*, *Ae. aegypti* and *Culex quinquefasciatus*) [98]. Our results in *Ny. albimanus* indicate that the allatostatin-A transcript has the potential to generate seven biopeptides; similar to the allatostatin-A transcripts of *D. melanogaster* and *M. domestica* that can generate six and five biopeptides, respectively. Whereas the tachykinin-related peptide transcript of *Ny. albimanus* is identical to that of *Ny. darlingi* and has the potential for generating four biopeptides, in *Ae. aegypti* this is limited to only one biopeptide. Other organisms, such as *Procambarus clarkii* (Decapoda: Cambaridae) and *Phenacogrammus interruptus* (Characiformes; Alestiidae) can generate three biopeptides. Insulin-like peptides presented the most remarkable amino acid sequence variation. This results could be related to the long size of these neuropeptides and the diversity of its functions [99–101].

The *Ny. albimanus* myosuppressin sequence is identical to that of other insects, including several anophelines orthologs [85] (Fig. 3F). A close analysis of the *An. albimanus* AALB009255-RA, transcript revealed that the first two exons, coding for a signal peptide, are highly similar to that of *An. gambiae* myosuppressin precursor (Fig. 2B), but the downstream sequence lacked myosuppressin homology (Fig. 3A). Full reconstruction of the myosuppressin precursor, including the first two exons of AALB009255-RA and the novel exon predicted a full ORF with the canonical structure of the myosuppressin precursor gene. This observation indicates that AALB009255-RA annotation was mischaracterized, since it codes for two or more different transcripts, one of which includes a myosuppressin (Fig. 3B-E). The predicted myosuppressin sequence was identified in the second intron of the genomic sequence (Fig. 3B and E) and the Locus_31294_Length_691 of the annotated *An. albimanus* transcriptome version 2 [56] (Fig. 3C). This locus has an ORF of 97 amino acid residues; the first 75 amino acid residues are identical to those of the peptide AALB009255-PA. This transcript encodes a signal peptide of 20 amino acid residues (Fig. 3C and D, highlighted in red) and a predicted neuropeptide-coding sequence of 11 amino acid residues corresponding to myosuppressin (TDVDHVFLRF**G**) (Fig. 3D, highlighted in green).

Despite the importance of neuropeptides in adult insects, information about their function is limited, especially for anophelines. Neuropeptides are associated with most of the physiological processes in insects, including the immune response activation [102–105] and suppression [106] after infection by different microorganisms. Interestingly, we detected a significant increase in the transcription of ACP, pk/PBAN, and corazonin in *Ny. albimanus* infected with *P. berghei*.

The study of adipokinetic hormone/corazonin-related peptide (ACP) [88], initially characterized as adipokinetic hormone II (AKH II) [107–109], structurally intermediate between corazonin and adipokinetic hormone, has provided new evidence and questions on the role of neuropeptides in insects. The structural and sequential analysis described this neuropeptide in several insects, including anophelines. ACP is primarily expressed in the nervous system and, to a lesser extent, in other insect organs and tissues [110, 111]. ACP transcripts were detected in the head and thorax of larvae, pupae, and adult of *Ae. aegypti* and *An. gambiae* [109]. Furthermore, ACP transcripts expression increases prominently in the brain and thoracic ganglia of *Ae. aegypti* after adult eclosion, suggest this neuropeptide may function in the regulation of post-ecdysis activities [112].

ACP transcripts in the brain of *Ny. albimanus* is consistent with previous results in *An. gambiae* where ACP (called AKHII) is expressed 72 h after feeding (blood or sugar) [109]. The differential expression of ACP between uninfected blood-fed and *P. berghei*-infected *Ny. albimanus* suggests that the midgut invasion by this parasite activates or modifies physiological pathways dependent on ACP. [14, 15, 17, 24]. Thus, it is possible that the increased level of this neuropeptide in *Plasmodium* infected mosquitoes is part of the immune response activation against this parasite. Further studies are necessary to unravel the function of the ACP system; currently, no definitive function for ACP has been determined and, functional studies in other insects revealed that ACP does not perform AKH and corazonin functions [112–114].

Pyrokinins have been identified in various insects [95, 110, 115–118] and are generated from the *capa* and *pk/pban* genes. *Capa* produces at least two peptides with sequence XXFPRV and XXWFGPRL, while *pk/pban* produces at least one peptide with sequence XXWFGPRL. Two genes *pk/pban-like* (*capa* and *hugin*) encode pyrokinins PK1 (XXXXWFGPRL) and PK2 (XXXXXXRPPFAPRL) respectively in *D. melanogaster*. Several functions for these peptides were described, including stimulation of pheromone biosynthesis [119], induction of melanization [120], induction of embryonic diapause [121], stimulation of visceral muscle contraction [122] and termination of pupal diapause development [123]. But no evidence exists for the participation of pyrokinins in immune response mechanisms. Here, we show that a *pk/pban* and *capa* transcripts coding for PK, PVK-1, and PVK-2 peptides (Table 2) increase in *Plasmodium*-infected *Ny. albimanus*; suggesting that the pyrokinins PK-1, PK-2, and PK-3 could be involved in signaling or activation mechanisms of the immune response of mosquitoes to these parasites.

Corazonin is widely conserved across insect genera [124] with various functions including cuticular melanization [32]. Its increased expression in brains of *P. berghei*-infected *Ny. albimanus* could be related to these two functions, but other not yet identified roles of this neuropeptide in mosquito defenses await identification.

Insulin-like peptides are involved in several processes that are conserved among vertebrates and invertebrates, including immunity [125–127]. ILPs have been identified in *Ae. aegypti* [128], *An. gambiae* [129], and *An. stephensi* (42). In *An. stephensi*, the ingestion of *P. falciparum*-infected blood, increased ILPs expression (42), and the inhibition of this induction reduced parasite development, indicating the possibility that ILP induction is a parasite mechanism to avoid elimination by the mosquito.t [106]. However, while inhibition of ILP4 induced the expression of immune genes prior to parasite invasion of the mosquito midgut, the inhibition of ILP3 increased immune gene expression at 24 hours after infection, when parasites had already invaded the midgut, indicating that the relationship between ILP mediated mechanism and immunity could be multiple. Our results documented an increase in of ILP-5 transcription in *Ny. albimanus* at times when *P. berghei* parasites had initiated development on the mosquito midgut. Although this increase was consistent among mosquito samples, it was not statically significant. On the other hand, no difference in ILP 3 transcription was observed between infected and uninfected mosquitoes. These results require further studies with a bigger sample size, but they may indicate that ILPs also participate in *P. berghei* infection, and that the induction of ILP is a generalized mechanism of *Plasmodium* parasites survival in mosquitoes. Our results on Tachykinin-related peptide transcription were also inconclusive.

The neuropeptide receptors identified in this work, have characteristic domains, almost all are G-protein coupled receptors rhodopshin like (GPCRs) (Table 1). However, the functional characterization of these receptors to understand the neuropeptide-receptor interaction and the role they play in the mosquito-parasite interaction awaits investigation.

In summary, we provide a brain neuropeptidome repertoire composed by 29 transcripts coding for at least 60 potential biopeptides in *Ny. albimanus*. Particularly we report the increased expression of the adipokinetic hormone/corazonin-related peptide (ACP), pk/pban pirokinin, and corazonin transcripts after *P. berghei* infection. At present, the functions of neuropeptides in insects during infection with parasites or viruses are partially understood. Further investigation is required as to whether significant changes in ACP and pk/PBAN transcription in the brain reflect an increase in the production or release of the respective biopeptides.

Most of the neuropeptides identified here showed high similarity with previously reported in other insects and other mosquito vectors. However, it is still necessary to explore the mechanisms triggered by neuropeptides up-regulated in the *Ny. albimanus* brain by a *Plasmodium* infection.

Conclusions

Most of the neuropeptides identified here showed high similarity with previously reported in other insects and other mosquito vectors. Our results indicate that *P. berghei* promoted a modification of transcripts neuropeptides expression in the mosquito brain at 24 hours post-infection. The pattern of differential expression in adipokinetic hormone/corazonin-related peptide, pk/PBAN, and corazonin indicates that the invasion of midgut tissue by *Plasmodium* triggered a brain response. However, it is still necessary to explore the mechanisms activated by neuropeptides up-regulated in the *Ny. albimanus* brain by a *Plasmodium* infection. Whether this change is due to stress or immune response remains unclear since the function of these neuropeptides is practically unknown in anophelines. Nevertheless, these findings provide insights on the behavior and immune response of *Anopheles* during a *Plasmodium* invasion and contribute with initial leads for the understanding of its neuroregulation.

Abbreviations

NPLP1
neuropeptide-like precursor 1; sNPF:short neuropeptide F; CCAP; crustacean cardioactive peptide; PBURS:partner of bursicon; ETH:ecdysis triggering hormone, Dh44:diuretic hormone 44, ILP:insulin-like peptides; AKH:adipokinetic hormone; EH:eclosion hormone; PDH:pigment dispersing hormone; GPA2:glycoprotein 2; qPCR:quantitative polymerase chain reaction; CNS:central nervous system; K:lysine; R:arginine; GFP:green fluorescent protein; GO:Gene ontology; BLAST:Basic Local Alignment Search Tool; CDS:Coding sequence.

Declarations

Acknowledgments

Alejandro Alvarado-Delgado, a doctoral student in PROGRAMA DE DOCTORADO EN CIENCIAS BIOMEDICAS-UNAM, appreciates the support given by CONACYT (Beca-98230). We thank R. E. Sinden for kindly supplying the *Plasmodium berghei* ANKA strain clone 2.34. The authors would like to thank Rosa

Elena Goméz Barreto, Jorge Luis Ocampo Bahena, Guillermo Perales Ortiz, Lilia Juarez Palma and Laura Salgado Albarran for technical assistance during the development of this work.

Dedication:

The authors dedicate this work to **Rosa Elena Goméz Barreto**[†], rest in peace.

Funding

The research described herein was supported by Conacyt, Grant 243171 to HLM and Beca-98230 to AAD.

Availability of data and materials

The datasets supporting the conclusions of this article are included within

the article and its additional files. The datasets supporting the results

are available at http://201.131.57.23:8080/nyssorhynchus_albimanus/

Authors' contributions

AAD: Study design and experimental work; mosquito infection, mosquito tissue extraction, analysis results and manuscript writing. JMB: Study design, analysis results, interpretation data and helped draft the manuscript. JTS: Preparation of cDNA library and 454 pyrosequencing experiments. VSN: Parasites culture. MHR: Study design and manuscript writing. EGM website design, EGM and FAZE: design of tables and figures, analysis and interpretation results. MCR: Parasites culture. ATL: mosquito infection, mosquito tissue extraction. HLM: Experimental design and manuscript writing. All authors read and approved the final version of the manuscript.

Competing interests

The authors declare that they have no competing interests.

Consent for publication

Not applicable.

Ethics approval and consent to participate

Not applicable.

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Figures

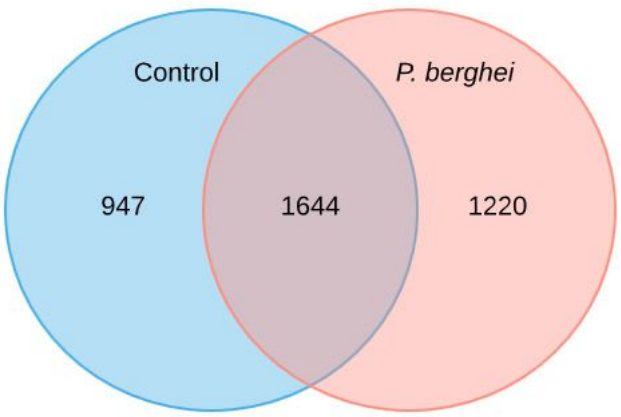

Figure 1

Venn diagram depicting total transcripts detected in control (947) and Plasmodium berghei-infected brains (1220) of Ny. albimanus: 1,220 transcripts were identified in infected mosquitoes and 947 transcripts were identified in the control group.

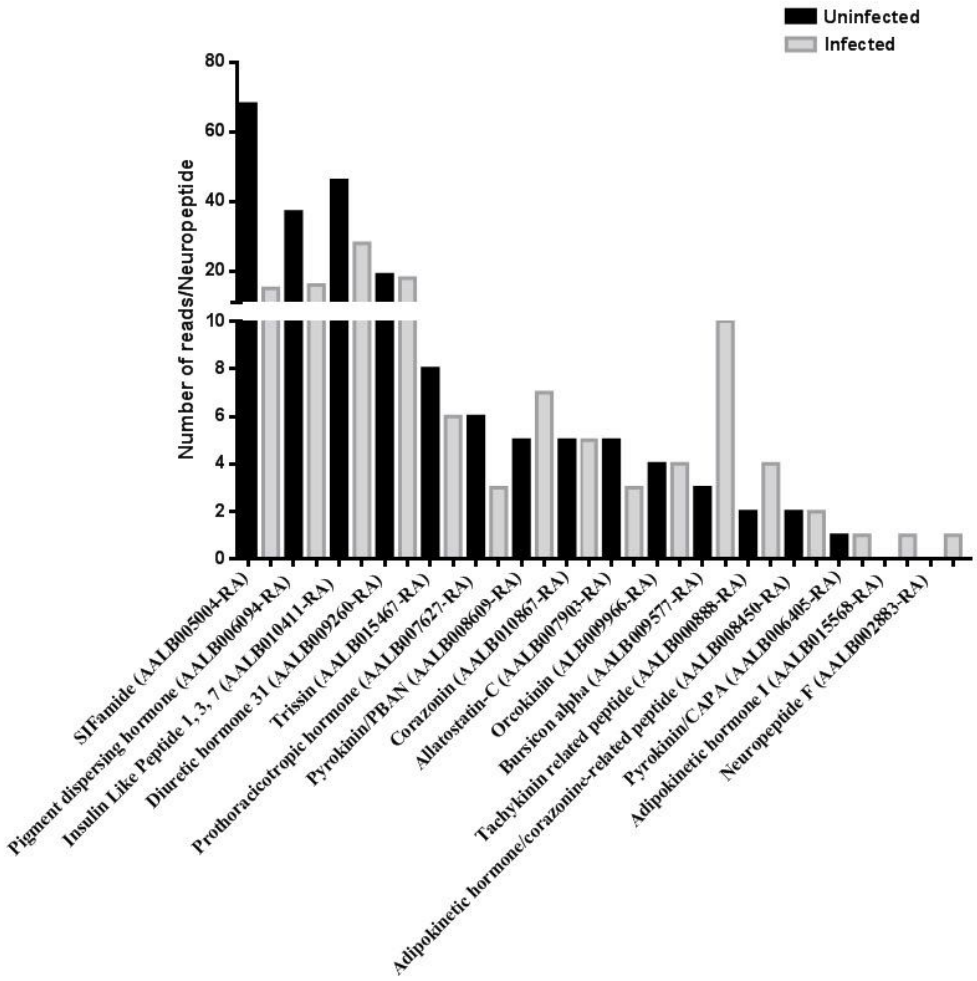

Figure 2

Neuropeptides transcripts identified in Ny. albimanus brain transcriptome. SIFamide (AALB005004-RA), pigment dispersing hormone (AALB006094-RA) and insulin like peptide 1, 3, 7 (AALB010411-RA) transcripts with more represented.

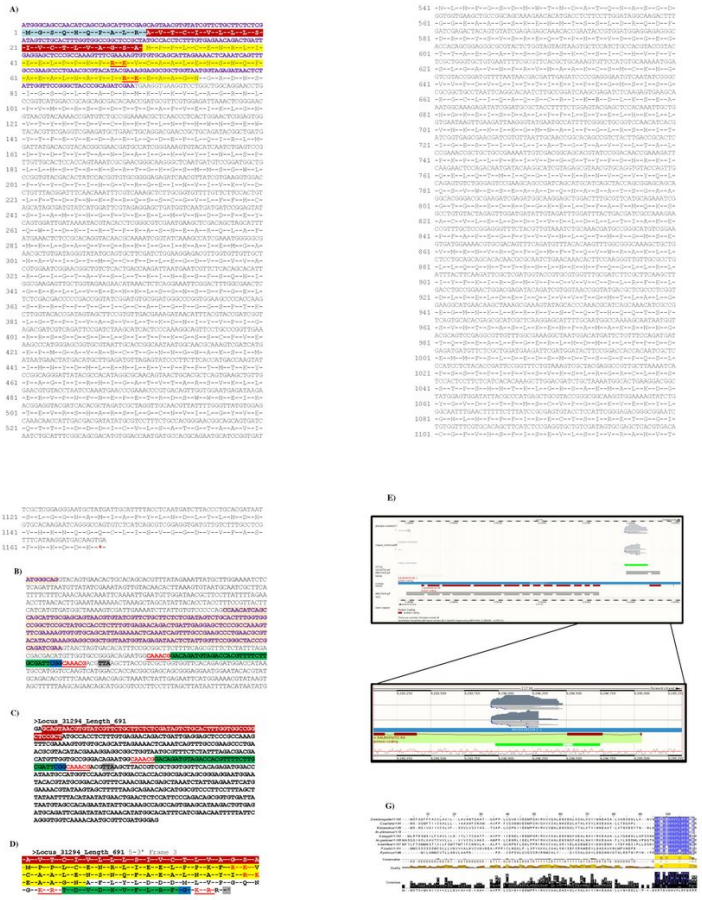

Figure 3

Myosuppressin identification. A) Mapping sequences of the brain Ny. albimanus transcriptome (gray) that align to the AALB009255-RA transcript on the genome region previously annotated as intron 3. B) Multiple sequence alignment of the Ny. albimanus myosuppressin peptide and the myosuppressin sequences with the conserved motif XDXDHVFXRFamide of D. melanogaster, C. capitata, M. domestica, Ae. aegypti, An. gambiae, A. mellifera, P. clarkii and R. prolixus..

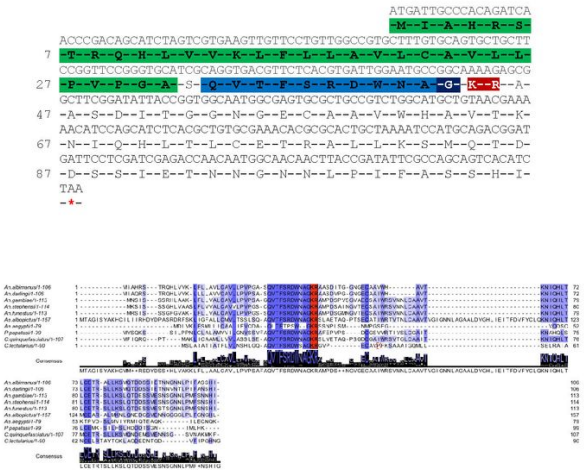

Figure 4

Adipokinetic hormone/corazonin-related peptide (ACP). Nucleotidic, amino acidic and multiple alignment of the Ny. albimanus ACP peptide with ACP sequences, with the conserved motif QVTFSRDWNX.amide motif, of Ny. darlingi, An. gambiae, A. aegypti, C. pipiens and T. castaneum.

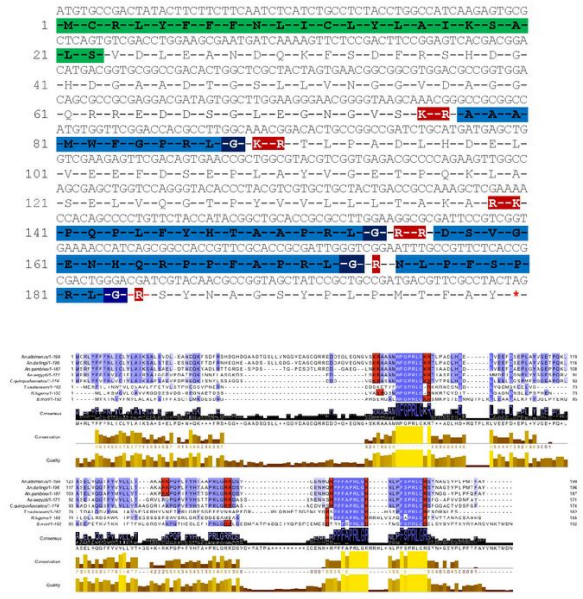

Figure 5

Pk/PBAN. Nucleotidic, amino acidic and multiple alignment of the Ny. albimanus Pk/PBAN peptide and Pk/PBAN peptide, with conserved.XXXXXPRL amide motif, of Ny. darlingi, An. gambiae, C. quinquefasciatus, B. germanica, P. clarkii, D. melanogaster, M. domestica, A. mellifera and B. mori.

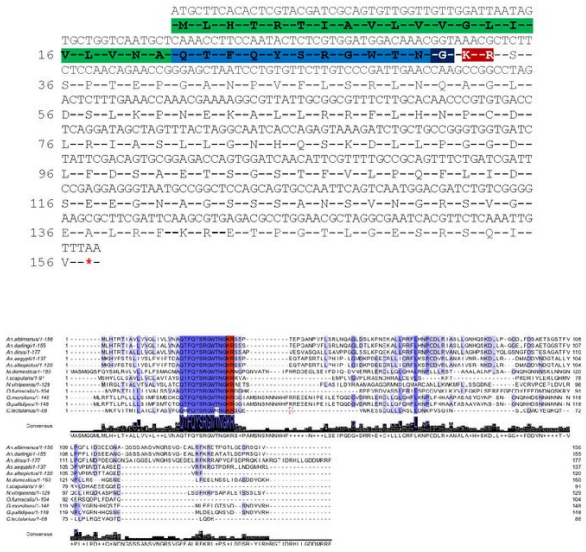

Figure 6

Corazonin. Nucleotidic, amino acidic and multiple alignment of the Ny. albimanus corazonin peptide and Corzonin with the conserved motif QTFQYSRGWTNamide, except C. capitata. Of M. domestica, D. melanogaster, N. vitripennis, D. pulex, C. capitata, Ae. aegypti, An. gambiae and B. mori.

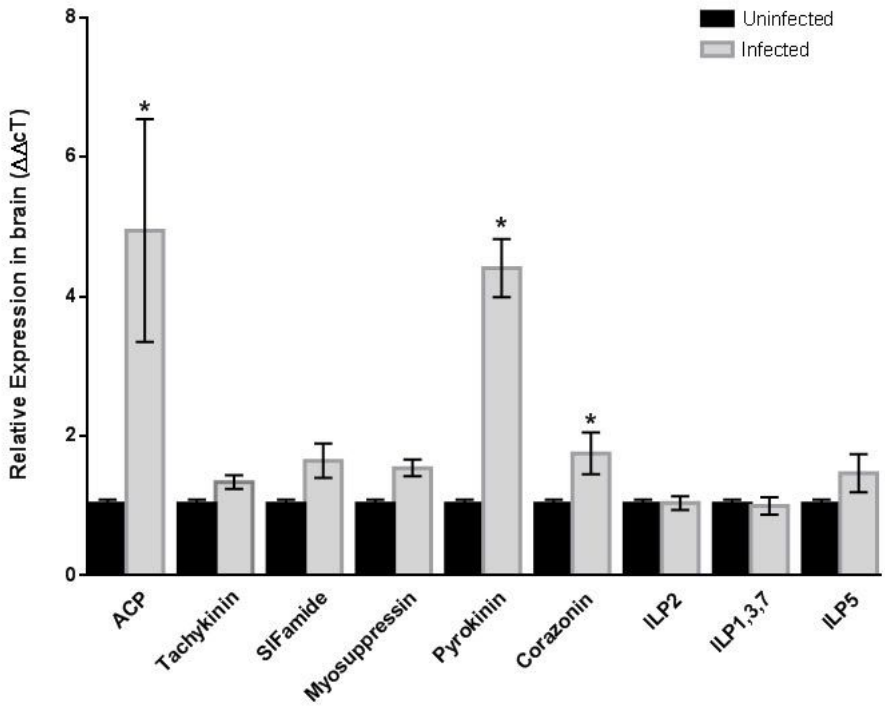

Figure 7

Relative expression of neuropeptide transcripts in the brain of Ny. albimanus mosquitoes infected with P. berghei ookinetes. Expression of neuropeptide transcripts in the uninfected group was normalized to a fold change of 1, to which the other condition (infected) was compared. ACP (3.94*), Tachykinin (0.34), SIFamide (0.64), Myosuppressin (0.54), Pyrokinin (3.41*), corazonin (0.75*), ILP-2 (0.04), ILP-3 (0.00) and ILP-5 (0.46). For statistical analysis, data were analyzed by ANOVA followed by Kruskal-Wallis post-test. () = Fold change vs uninfected group, * = significative fold change Kruskal-Wallis post-test (α= 0.05)

Supplementary Files

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