Journal of Insect Physiology 108 (2018) 31–39

Contents lists available at ScienceDirect

Journal of Insect Physiology

journal homepage: www.elsevier.com/locate/jinsphys

Orcokinins regulate the expression of neuropeptide precursor genes related to ecdysis in the hemimetabolous insect *Rhodnius prolixus*

Juan Pedro Wulff, Natalia Capriotti, Sheila Ons*

Laboratory of Insects Neurobiology, Regional Center for Genomic Studies, Faculty of Exact Sciences, National University of La Plata Bvd 120, 1459 (1900) La Plata, Buenos Aires, Argentina

ARTICLE INFO

Keywords:
Corazonin
Ecdysis triggering hormone
Eclosion hormone
Crustacean cardioactive peptide
Ecdysone
Chagas' disease

ABSTRACT

Ecdysis is a vital process for insects, during which they shed the old cuticle in order to emerge as the following developmental stage. Given its relevance for survival and reproduction, ecdysis is tightly regulated by peptidic hormones that conform an interrelated neuromodulatory network. This network was studied in species that undergo a complete metamorphosis, but not in hemimetabola. In a recent work, we demonstrated that orcokinin neuropeptides are essential for ecdysis to occur in the kissing bug *Rhodnius prolixus*. Here we performed gene silencing, quantitative PCR and *in vitro* treatments in order to study the interrelationships between *RhoprOKs* and hormones such as ecdysis triggering hormone, corazonin, eclosion hormone, crustacean cardioactive peptide and ecdysone. Our results suggest that *RhoprOKs* directly or indirectly regulate the expression of other genes. Whereas RhoprOKA is centrally involved in the regulation of gene expression, RhoprOKB is implicated in processes related to midgut physiology. Therefore, we propose that the different transcripts encoded in *RhoprOK* gene could integrate signaling cues, in order to coordinate the nutritional state with development and ecdysis. Given the emerging data that point to OKs as important factors for survival and reproduction, they could be candidates in the search for new insect management strategies based on neuroendocrine targets.

1. Introduction

Considering their abundance, insects are a prevalent animal life form on our planet. A hard cuticle preserves these animals from physical damage and desiccation, contributing to the success of insect species in the colonization of a wide range of ecological niches, with the only exception of marine ecosystems. In order to grow and develop, insects go through successive stages of feeding and molting. Each molt culminates in ecdysis, an innate behavior tightly controlled by peptidic hormones (Zitnan and Adams, 2012). During ecdysis, the animal sheds the external cuticle and the lining of the foregut, hindgut and tracheae to emerge as the following developmental stage. Based mainly on studies in *Manduca sexta* and *Drosophila melanogaster*, the accepted model proposes that the steroid hormone 20-hydroxyecdysone (20E) initiates the molting process. This hormone controls the expression and release of neuropeptides that conform an interrelated neuromodulatory network (Diao et al. 2017; Kim et al., 2004, 2006; Kingan et al., 1997, 2015; Mena et al., 2016; Zitnan et al., 1999, 2003). Briefly, in *M. sexta* the neuropeptide corazonin (CZ) stimulates the release of ecdysis triggering hormone (ETH) from epithracheal Inka cells. ETH elicits the expression and release of eclosion hormone (EH), which acts back on Inka cells in a positive feedback loop to further stimulate the release of ETH (reviewed in Zitnan and Adams (2012)). The massive release of EH and ETH to the hemolymph leads to the activation of a peptidergic network producing crustacean cardioactive peptide (CCAP), among other neuropeptides that control the ecdysis sequence. Even though the proposed model is based on studies performed in holometabola, recent reports suggest that major components of this network would be conserved also in hemimetabola (Lee et al., 2013; Lenaerts et al., 2017). Given their characteristics, neuropeptides and their membrane receptors have been proposed as a promising target for a novel generation of insecticides (Audsley and Down, 2015; Verlinden et al., 2014). Insecticide strategies specifically targeting the neuropeptides that control molting and/or ecdysis would be safe for vertebrates and the environment, when compared to neurotoxic insecticides.

Rhodnius prolixus (Hemiptera: Reduviidae) is a hemimetabolous species that was used as an experimental model since the pioneer studies on insects' physiology (see Wigglesworth, 1934, 1939, 1953, 1954, 1959, 1964). This species is a convenient model to study molting and ecdysis, given that these events occur in a predictable manner after a blood meal, allowing an accurate timing for experiments. The availability of genomic information (Mesquita et al., 2015) facilitates genetic

* Corresponding author.
E-mail address: sheila.ons@presi.unlp.edu.ar (S. Ons).

https://doi.org/10.1016/j.jinsphys.2018.05.006
Received 12 February 2018; Received in revised form 15 May 2018; Accepted 17 May 2018
Available online 17 May 2018

Table 1
Sequence of the primers used in this study.

Primer name	5′-3′ Sequence
qPCR_CCAP Fwd	CTGCAAAAAAGGCTTTATTTTCC
qPCR_CCAP Rev	TCCCATAACTTCGCTTCAGAC
qPCR_CZ Fwd	TGCCCTTTACTAGACAGGGG
qPCR_CZ Rev	CTGAAATGTTTGGCCAAAGACA
qPCR_EH Fwd	TGTGCCCAGTGTAAGAAAATGT
qPCR_EH Rev	TGTCCTCACAGTCTGGAATCA
qPCR_ETH Fwd	GCAGAGATGAGTTCCACGAG
qPCR_ETH Rev	GTGATCCGCTGAACTGTCAC
qPCR_OKA Fwd	GATGATCAACATGCTGTCGT
qPCR_OKA Rev	AGGTTTGGATGAGCCCTGAGT
qPCR_OKB Fwd	GAGCGAAATTATGGACCAGCA
qPCR_OKB Rev	ATGACCTCCACCCAAACCAT
qPCR_RpEF-1 Fwd	GATTCCACTGAACCGCCTTA
qPCR_RpEF-1 Rev	GCCGGGTTATATCCGATTTT
qPCR_TUB Fwd	TGTGCCCAAGGATGTGAACG
qPCR_TUB Rev	CACAGTGGGTGGTTGGTAGTTGAT
dsOKA Fwd	TAATACGACTCACTATAGGGGAAGCGGTTTTGATGGTTTTGT
dsOKA Rev	TAATACGACTCACTATAGGGGGGATTCTTTGCATAAATGGTCA
dsOKB Fwd	TAATACGACTCACTATAGGGTAGACGGTGTTATCGTAGAG
dsOKB Rev	TAATACGACTCACTATAGGGAATGACCTCCACCCAAACCAT
dsOKs Fwd	TAATACGACTCACTATAGGGTCACTATCGCTGCGTCTCTGT
dsOKs Rev	TAATACGACTCACTATAGGGCTAAAGTATCCAAATTTCGGCCCTC
dsAmpi Fwd	TAATACGACTCACTATAGGGGAACTGGATCTCAACAG
dsAmpi Rev	TAATACGACTCACTATAGGGGGATCTTCACCTAGATC
T7-Promotor Primer	ATAGAATTCTCTCTAGAAGCTTAATACGACTCACTATAGGG

and molecular approaches. Importantly, like other triatomines, *R. prolixus* is a vector of the protozoan parasite *Trypanosoma cruzi*, the causative agent of Chagas' disease, which is a neglected life-threatening disease that affects about 7 million people around the world (World Health Organization, 2017***). Hence, the advantage of *R. prolixus* as a convenient experimental model is reinforced by its medical relevance.

Orcokinins (OK) are conserved neuropeptides in arthropods; their physiological role, however, has not been extensively studied to date. In insects, the *OK* gene undergoes alternative splicing, generating two precursors with different conserved core sequences: OKA (expressed mainly in the nervous system) and OKB (expressed in the nervous system and midgut) (Sterkel et al., 2012). Recently, we demonstrated that *R. prolixus* also presents a third isoform (RhoprOKC). This isoform encodes OKB-type neuropeptides, with an extra exon encoding three nonconserved peptides, and is expressed exclusively in the anterior midgut. Hence, as in other species, only OKA-type or OKB-type conserved neuropeptides are expressed in *RhoprOK* gene (Wulff et al., 2017).

Scattered information has been published regarding OK physiological role, even though its high conservation suggests an involvement in the regulation of vital processes. Studies on OKA pointed to a prothoracicotropic action in *Bombyx mori* (Yamanaka et al., 2011) and the regulation of the circadian locomotor activity in the cockroach *Leucophaea maderae* (Hofer and Homberg, 2006). Both OKA and OKB demonstrated to be involved in oogenesis in *Blattella germanica* (Ons et al., 2015) and in the regulation of an innate escape behavior in *Tribolium castaneum* (Jiang et al., 2015). Our recent results revealed that OKA expression is necessary for ecdysis to occur, whereas the role of OKB seems to be less critical (Wulff et al., 2017). We observed that RhoprOKA and RhoprOKs RNAi-silenced insects suffered a lethal arrest at the expected ecdysis moment, whereas RhoprOKB/C RNAi silenced animals performed a successful ecdysis, even though with a significant delay respect to controls. In the present work, we aimed to further investigate the mechanisms of RhoprOKs to regulate the occurrence of ecdysis. The results presented here suggest that RhoprOK affects the expression of neuropeptide genes that were previously related to the neuromodulation of ecdysis behavior in insects. Furthermore, in certain physiological conditions, the expression of RhoprOKA seems to be regulated by 20E. We also show that RhoprOKB could be involved in processes related to feeding. The results reinforce the hypothesis of a central involvement of RhoprOKs in the regulation of post-embryonic development, and give cues about their physiologic action mechanisms.

2. Material and methods

2.1. Insect rearing

A colony of *R. prolixus* was maintained in our laboratory in a 12 h light/dark schedule at 28 °C. Insects were weekly fed on chickens, which were housed, cared, fed and handled in accordance with resolution 1047/2005 (National Council of Scientific and Technical Research, CONICET) regarding the National Reference Ethical Framework for Biomedical Research with Laboratory, Farm, and Nature Collected Animals. This framework is in accordance with the standard procedures of the Office for Laboratory Animal Welfare, Department of Health and Human Services, NIH, and the recommendations established by the 2010/63/EU Directive of the European Parliament, related to the protection of animals used for scientific purposes. Biosecurity considerations are in agreement with CONICET resolution 1619/2008, which is in accordance with the WHO Biosecurity Handbook (ISBN 92 4 354 6503).

2.2. RNA interference

To generate dsRNAs targeting RhoprOKA (dsOKA), RhoprOKB/C (dsOKB/C) or the three isoforms together (dsOKs), specific fragments were PCR-amplified using Pegasus Taq polymerase (Productos Bio-Lógicos, Argentina) and primers containing T7 sequence at 5′ end, designed for each target (see sequences in Table 1). The dsOKB/C will silence as much RhoprOKB as RhoprOKC isoforms of the gene (both encoding OKB-type mature neuropeptides). One µl of the PCR product was used for a secondary PCR using T7-promotor primer (Table 1). Double strand RNAs (dsRNA) were generated by *in vitro* transcription using T7-RNA polymerase (Promega, USA). The products of transcription were heated for 10 min at 95 °C, cooled down slowly to room temperature, and treated with DNAse and RNAseA (Fermentas, USA), in order to eliminate DNA and single strand RNA. The formation of dsRNAs was confirmed by running a 1.5% agarose gel and quantified

from an image of the gel using the software ImageJ. The dsRNAs were stored at −20 °C until use. A heterologous 808-fragment from the β-lactamase gene was PCR-amplified from the pBluescript plasmid and used as a control (dsCont) (see primers in Table 1). Fourth instar larvae *R. prolixus* 1–2 weeks after molt and starved since ecdysis were ventrally injected into the abdomen with 2 μg dsRNA (2 μl) diluted in saline solution. Forty-eight hours after injection, the insects were fed on chickens. Either on day 6 or on day 11 post-blood meal (PBM) the insects were individually collected in Tri reagent (Sigma-Aldrich, Darmstadt, Germany). In our previous work (Wulff et al., 2017) we discarded an off-target effect on ecdysis regulation of the dsOKs fragment used here.

2.3. In vitro treatments with OKA, OKB or 20E

Fourth instar larvae were treated as described above ("RNA interference"). On day 11 PBM insects were microdissected in sterile saline solution. The ventral cuticle was removed to expose the CNS, tracheas and fat body; the remaining organs were set aside. The preparations were preincubated for 30 min in 500 μl of Grace's insect medium (Sigma-Aldrich, Darmstadt, Germany). After incubation they were transferred for 3 h to 500 μl of medium containing 10^{-6} M of either synthetic OKA (NFDEIDRSGFGSFI; GenScript, USA), RhoprOKB (EFLDPLGGGHLIC; GenScript, USA) or 20E (Santa Cruz Biotechnology, Dallas, USA). Finally, Grace's medium was replaced with Tri reagent (Sigma-Aldrich, Darmstadt, Germany) and kept at −80 °C until use.

2.4. qRT-PCR

Total RNA from a whole individual insect was isolated separately using Trizol (Ambion, São Paulo, Brazil) and treated with DNAse (Promega, USA). cDNA was synthesized using MMLV Reverse Transcription Kit (Applied Biosystems, São Paulo, Brazil), following the manufacturer's instructions. cDNA amplifications were performed in technical triplicates (3 wells/cDNA sample), in a 20 μl final volume (primers detailed in Table 1). Every individual insect was considered an individual sample (pooled samples were not used). cDNA levels were quantified using FastStart SYBR Green Master (Roche) in iQ single color in a MiniOpticon Real-Time PCR Detection System (Bio-Rad). The schedule used for the amplifying reaction was as follows: (i) 95 °C for 5 min; (ii) 95 °C for 30 sec; (iii) 58 °C for 30 sec; (iv) steps (i) and (ii) were repeated for 40 cycles. Real-time data were collected through the iQ5 optical system software v.2.0 (Bio-Rad). A control without a template was included in all batches. We used both elongation factor 1 (RhoprEF1) and tubulin (RhoprTub) as housekeeping genes for all qRT-PCR determinations, with the exception of percentages of gene silencing, where only *Rhopr EF1* was used as reference gene. As much *RhoprEF1* as *RhoprTub* were previously validated as stable genes in *R. prolixus* (Majerowicz et al., 2011; Omondi et al., 2015). All primer pairs used for qRT-PCRs (Table 1) were tested for dimerization, efficiency, and amplification of a single product. The statistical significance of the results (transcript abundance) for the different transcripts under study was analyzed using one-way ANOVA (pairwise comparisons). Log transformation of the data was performed to achieve variance homogeneity when necessary. In the case of EH on day 6 PBM nonparametric statistic was performed.

2.5. Heart rate and anterior midgut contraction assays

Unfed fifth instar *R. prolixus* two weeks after ecdysis were used. Each insect was fixed with the dorsal side up in a Petri dish covered with paraffin wax. The dorsal cuticle was carefully removed under physiological saline solution using a binocular microscope with a video camera adapted. Internal organs, including the intact dorsal aorta, were exposed, and covered with 50 μl saline solution. Four washes with fresh saline solution were performed in order to stabilize the tissues for 20 min. The tissue contractions under the microscope were recorded. Growing concentrations of synthetic OKA and/or OKB in 50 μl were exchanged for 50 μl of saline solution every 3 min. The video recordings were analyzed to count the frequency (beats/min) under each condition. The frequency under each peptide was relativized to the frequency under saline. Statistical analysis was performed using one-way ANOVA followed by Bonferroni's post-hoc test.

2.6. Immunohistochemistry

Starved fifth instar *R. prolixus* 4–5 weeks post-emergence were used. The insects were sacrificed either unfed, 1 h or 24 h after the beginning of blood feeding on chickens (N = 4–6 for each experimental group). The anterior midguts were dissected under a binocular microscope and IHC was performed as follows with anti-RhoprOKB9 rabbit antiserum produced by GenScript, Piscataway, NJ, USA (see Wulff et al., 2017). Anterior midguts were fixed in 2% paraformaldehyde (pH7) for 12 h at 4 °C. They were washed with PBS, transferred to 4% Triton X-100 with 2% BSA and 10% normal goat serum (NGS) in PBS for 1 h at room temperature, and then washed several times with PBS at room temperature. Primary antiserum was preincubated at a dilution of 1:5000 in a 0.4% solution of Triton X-100 in PBS with 2% BSA and 2% NGS for 12 h at 4 °C prior to use. Tissues were incubated in primary antiserum for 48 h at 4 °C, and then washed 18–24 h at 4 °C in PBS. Tissues were then incubated in Cy3-labelled goat anti-rabbit immunoglobulin solution (Jackson, USA) at a dilution 1:500 with 10% NGS in PBS for 12 h at 4 °C, and finally rinsed several times in PBS at room temperature. Control experiments omitting the primary antiserum or in which the primary antiserum was preincubated for 24 h at room temperature with 1 mM of the specific synthetic peptide (GenScript, Piscataway, NJ, USA) were also performed. Tissues were mounted on microscope slides in Mowiol. Preparations were viewed and images were obtained using a confocal microscope and LSM510 viewing software (LSM510-Zeiss, Germany).

Four to six images were obtained for each individual, covering the complete anterior midgut, keeping the settings on the microscope constant. ImageJ software was used to quantify the number of immunoreactive cells in every image. This number was averaged for all the images derived from a particular individual. One-way ANOVA was performed to evaluate statistical differences in the number of OKB immunoreactive cells.

3. Results

3.1. RhoprOKA affects the expression of neuropeptide precursor genes related to ecdysis

Regarding our previous results (Wulff et al., 2017), we hypothesized that RhoprOKs expression could influence the expression of neuropeptides controlling ecdysis. To test this hypothesis, we injected either saline solution (control group), dsRhoprOKA RNA (dsOKA group) or dsRhoprOKB/C RNA (dsOKB/C group) into starved fourth instar *R. prolixus*. Three days after injections the insects were allowed to feed (see Methods). We studied two main time points during the molting cycle: day 6 PBM, when the 20E peak in hemolymph occurs, and one day before the expected ecdysis (day 11 PBM), when 20E level returns to basal (Wulff et al., 2017). With this approach, we obtained a significant and specific decay in mRNA levels for both treatments, in the two time points studied. dsRNAOKA injection caused 84% of decay in RhoprOKA expression on day 6 ($p < 0.0001$; N = 4 insects/group), and 82% of decay on day 11 PBM ($p < 0.05$; N = 4 insects/group) (Fig. 1A). dsRNAOKB injection caused 82% of decay in RhoprOKB expression on day 6 ($p < 0.001$; N = 4 insects/group), and 83% of decay on day 11 PBM ($p < 0.01$; N = 4 insects/group) (Fig. 1B). The expression levels of RhoprCZ, RhoprETH, RhoprEH and RhoprCCAP were determined by qRT-PCR in the insects belonging to the control, dsOKA

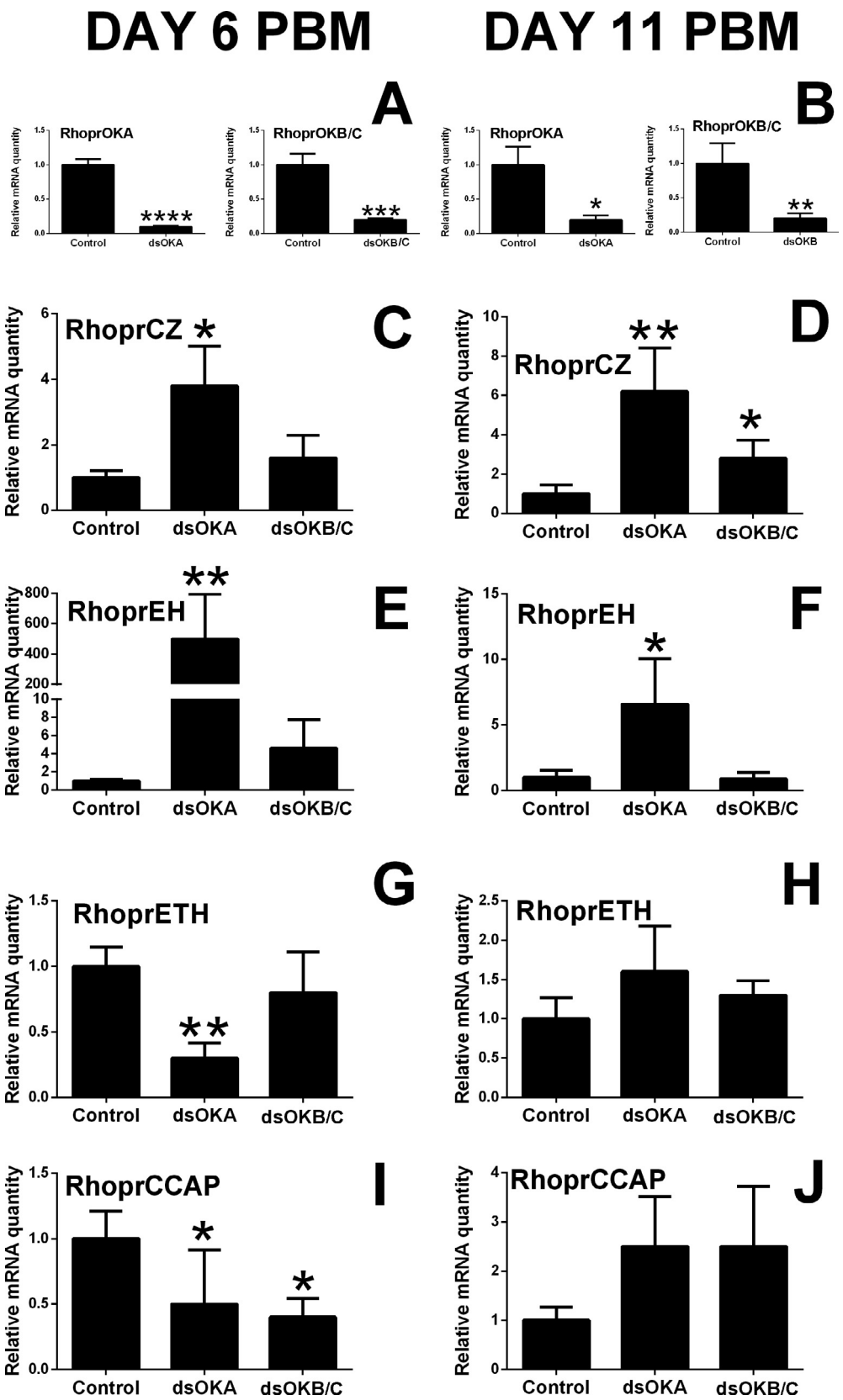

Fig. 1. Effect of dsRNAOKA and dsRNAOKB injections on expression levels of RhoprOKA and RhoprOKB on (A) day 6 PBM, (B) day 11 PBM; (C) expression levels of RhoprCRZ on day 6 PBM and (D) day 11 PBM; expression levels of RhoprEH on day 6 PBM and (F) day 11 PBM; (G) expression levels of RhoprETH on day 6 PBM and (H) day 11 PBM; (I) expression levels of RhoprCCAP on day 6 PBM and (J) day 11 PBM. n = 4–5; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$; **** = $p < 0.001$ with respect to control group.

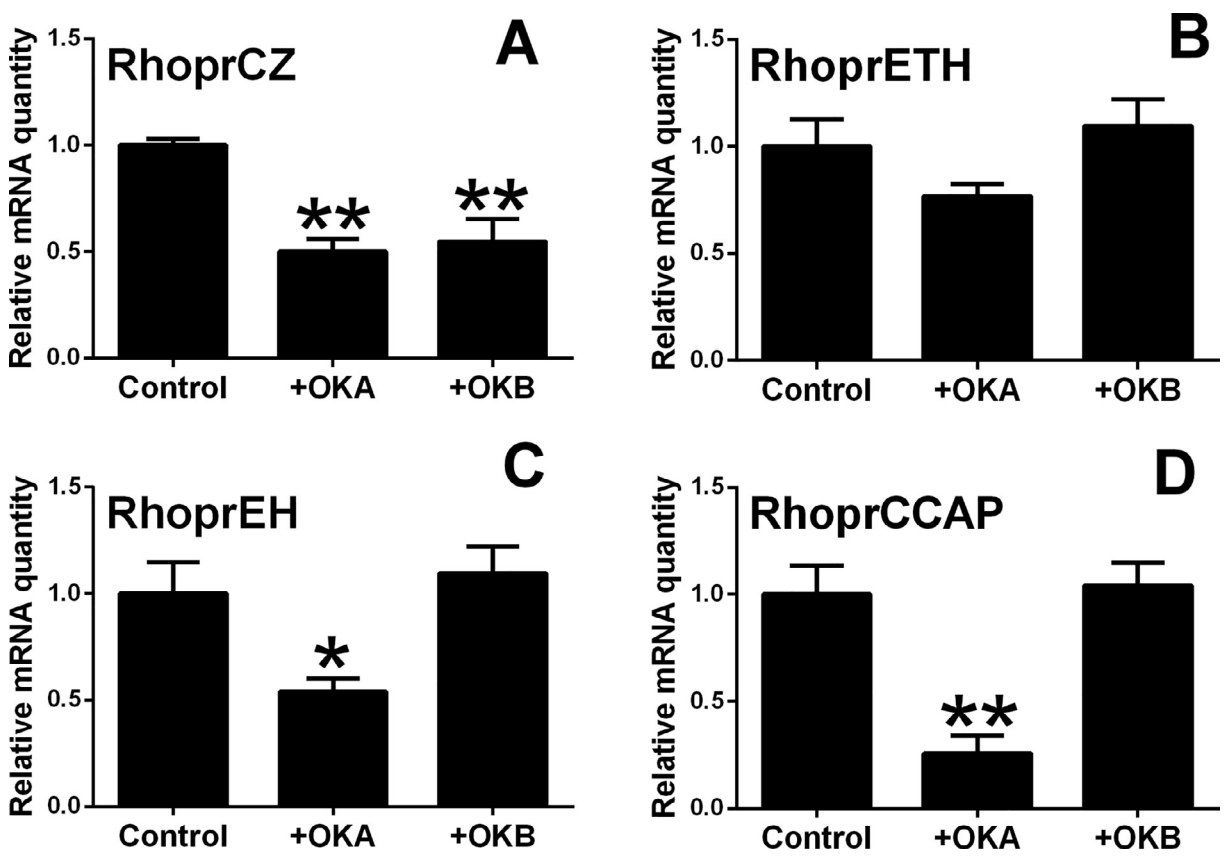

Fig. 2. Effect of the administration of synthetic OKA or OKB on gene expression in dsRNAOKs/dsRNAOK treated insects on day 11PBM. (A) OKA and OKB downregulate the expression of RhoprCRZ; (B) expression levels of RhoprETH are not affected; (C) OKA downregulates the expression of RhoprEH; (D) OKA downregulates the expression of RhoprCCAP. n = 6; * = $p < 0.05$; ** = $p < 0.01$ with respect to control group.

and dsOKB/C groups.

RhoprCZ was significantly upregulated in dsOKA animals with respect to controls, as much on day 6 ($p < 0.05$; N = 5 insects/group) (Fig. 1C) as on day 11 PBM ($p < 0.01$; N = 5 insects/group) (Fig. 1D). Besides, RhoprEH was almost 500-fold upregulated in dsOKA group on day 6 PBM ($p < 0.01$; N = 5 insects/group) (Fig. 1E), and almost 7-fold upregulated on day 11 PBM ($p < 0.01$; N = 5 insects/group) (Fig. 1F) compared to the control group. On the other hand, RhoprETH ($p < 0.01$; N = 5 insects/group, Fig. 1G) and RhoprCCAP ($p < 0.05$ insects; N = 5 insects/group, Fig. 1I) levels were downregulated in dsOKA insects with respect to the control group on day 6 PBM. RhoprETH levels were similar in the control and dsOKA groups on day 11 PBM (Fig. 1H). RhoprCCAP levels were 2.5-fold upregulated on day 11 in dsOKA animals, even though the results did not reach statistical significance (Fig. 1J).

The dsOKB/C treated insects expressed lower RhoprCCAP levels compared to controls on day 6 PBM ($p < 0.05$; N = 5 insects/group; Fig. 1I); no statistical differences were detected on day 11 PBM for RhoprCCAP, even though a 2.5-fold upregulation was observed (Fig. 1J). Besides, no differences in the expression of RhoprCZ were detected on day 6 PBM for dsOKB/C compared to the control group (Fig. 1C), but dsOKB/C expressed higher levels of this neuropeptide on day 11 PBM ($p < 0.05$; N = 5 insects/group, Fig. 1D). RhoprEH and RhoprETH were not affected by dsOKB/C silencing in the time points analyzed (Fig. 1E–H).

In order to obtain further evidence for the conclusions suggested by gene silencing, we used an alternative experimental approach. In this experiment, we tested whether the administration of synthetic peptides encoded in RhoprOKA or RhoprOKB/C precursors were able to revert the effects of *RhoprOKs* silencing on the expression of ecdysis-related neuropeptide precursor genes. RhoprOKA and RhoprOKB/C transcripts were silenced together in starved fourth instar nymphs by the injection of dsOKs fragment spanning all the isoforms of the gene (Wulff et al., 2017). Three days after injections the insects were allowed to feed (see Methods). The treatment significantly decreased RhoprOKA (84%; $p < 0.001$; N = 4 insects/group) and RhoprOKB/C levels (82%; $p < 0.001$ N = 4 insects/group) on day 11 PBM when compared to saline injected animals. On day 11 PBM we dissected the dorsal cuticle of the insects to expose the central nervous system and tracheae. This preparation was incubated for three hours/3 h in Grace's insect medium with either saline solution (control), synthetic OKA-type (OKA group) or synthetic OKB-type (OKB group) peptide at a final concentration of 10^{-6} M. Gene expression levels were measured in the control, OKA and OKB groups by qRT-PCR. RhoprCZ transcript levels were downregulated as much with the addition of synthetic OKA ($p < 0.001$; N = 6 insects/group) as OKB ($p < 0.001$; N = 6 insects/group) (Fig. 2A). The addition of synthetic peptides did not change the expression levels of *RhoprETH* on day 11 PBM (Fig. 2B). *RhoprEH* expression was downregulated by the addition of synthetic OKA ($p < 0.05$; N = 6 insects/group), but not OKB (Fig. 2C). In a similar way, *RhoprCCAP* expression was downregulated by the addition of synthetic OKA ($p < 0.01$; N = 6 insects/group), but not OKB (Fig. 2D). The results obtained confirmed the observations presented in Fig. 1, given that downregulation in the expression of genes on day 11PBM was reverted by the addition of synthetic peptides (Fig. 2).

3.2. RhoprOKA expression is upregulated by 20E

The results presented here and in a previous report (Wulff et al., 2017) suggested that ecdysone might regulate the expression of RhoprOKs, even though the injections of 20E in starved fourth instar insects did not elicit changes in RhoprOKs gene expression 6 days later (Wulff

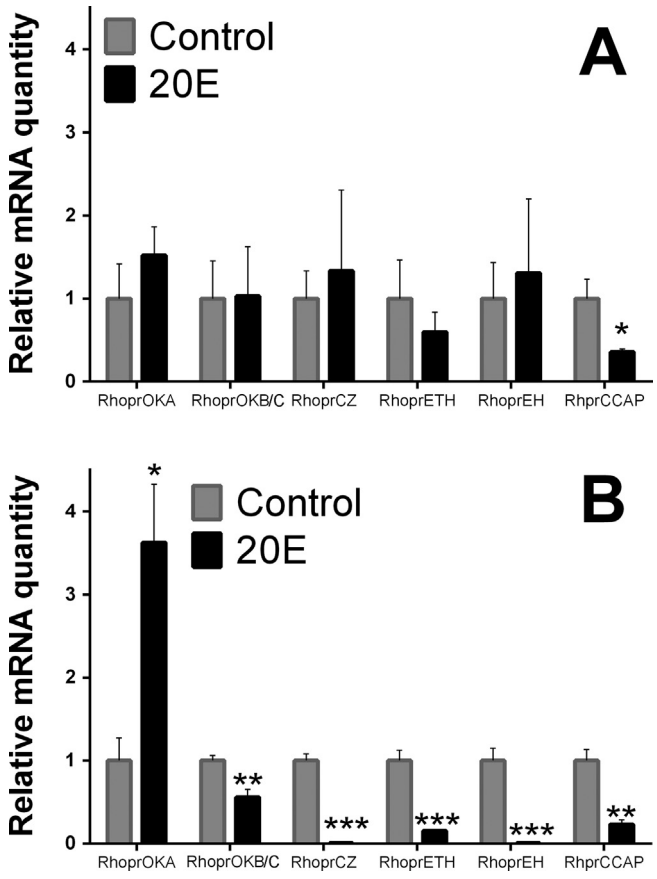

Fig. 3. Effect of the administration of 20E on gene expression in (A) control and (B) dsRNAOKs/dsRNAOK treated insects on day 11 PBM. n = 6; * = $p < 0.05$; ** = $p < 0.01$; *** = $p < 0.005$ with respect to control group.

et al., 2017).

We performed the *in vitro* preparation described above using fourth instar nymphs 11 days PBM. The preparations were incubated 3 h with either Grace's medium (control group) or with Grace's medium supplemented with 20E (10^{-6} M). We measured RhoprOKA, RhoprOKB/C, RhoprCZ, RhoprETH, RhoprEH and RhoprCCAP expression levels in the control and 20E treated groups using qRT-PCR. The incubation with 20E downregulated the expression of RhoprCCAP ($p < 0.01$; N = 6 insects/group); the other transcripts analyzed did not change their expression levels compared to controls (Fig. 3A).

We repeated the previous experiment, but using insects where the expression of RhoprOKs gene was silenced by a dsOKs injection three days before blood meal; we obtained a > 80% downregulation from both RhoprOKA and RhoprOKB/C ($p < 0.001$; N = 4 insects/group). In these conditions, 20E in the incubation medium elicited a 3.6-fold upregulation in the expression of RhoprOKA ($p < 0.05$; N = 6 insects/group) and a downregulation in the expression of RhoprOKB/C ($p < 0.01$; N = 6 insects/group) compared to saline (Fig. 3B). In agreement with the results observed when synthetic RhoprOKA was added to the incubation medium (see Fig. 2), the 20E treatment also downregulated the expression of RhoprCZ, RhoprEH ($p < 0.001$; N = 6 insects/group) and RhoprCCAP ($p < 0.01$; N = 6 insects/group). In this case, the reduction of ETH expression also reached statistical significance ($p < 0.001$; N = 6 insects/group) (Fig. 3B).

3.3. RhoprOKs peptides slightly increased heart contraction frequency

Neuropeptides related to ecdysis, such as CCAP and CZ, increased the heartbeat frequency in *R. prolixus* (Lee and Lange, 2011; Patel et al., 2014). We studied the activity of OKA and OKB in the frequency of

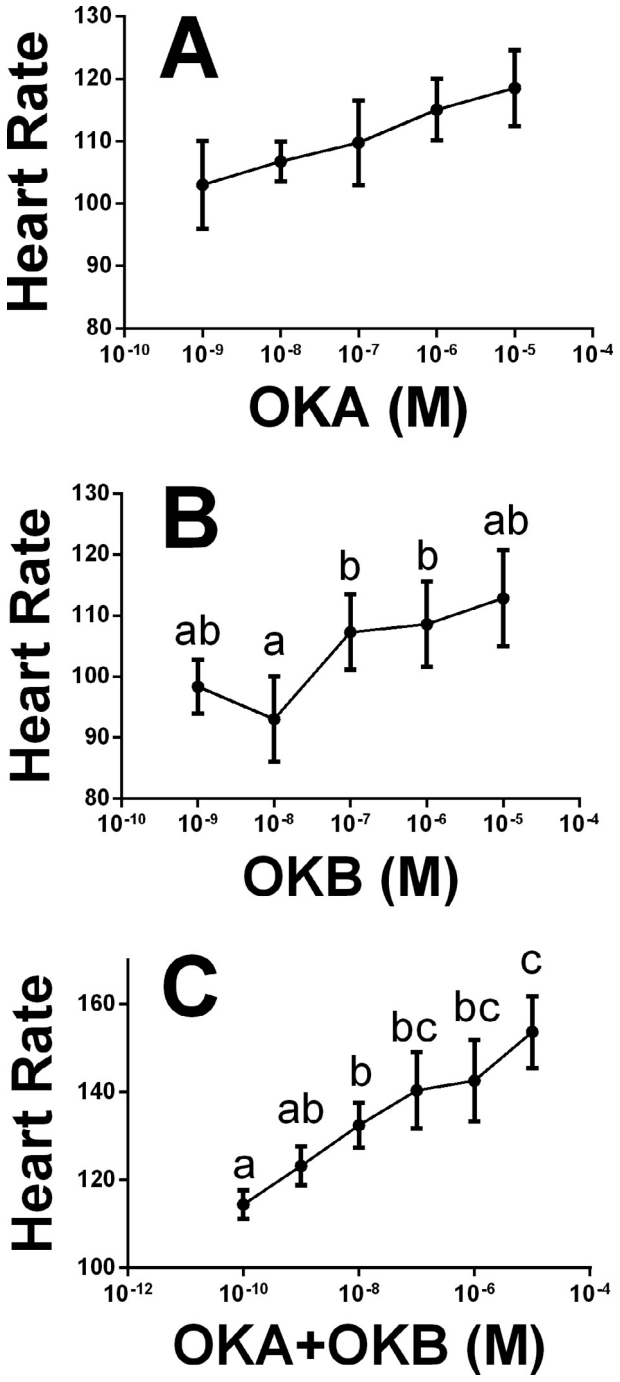

Fig. 4. Effect of (A) OKA, (B) OKB and (C) OKA + OKB on heartbeat frequency of 5th instar *R. prolixus*. N = 7–10. Different characters denote significant differences ($p < 0.05$). Results are expressed as percentage of heartbeat rate compared to the average heartbeat rate in saline.

aorta contractions. A conserved peptide encoded in RhoprOKA (OKA) did not cause a significant increase in heartbeat contractions (N = 7 insects), whereas a peptide encoded in RhoprOKB and RhoprOKC (OKB) transcripts only produced small but significant increases at 10^{-7} and 10^{-6} M (N = 7 insects; $p < 0.05$) (Fig. 4A, B). When both peptides were supplemented together, the increase in the frequency of contractions was significant higher than saline solution at 10^{-8}, 10^{-7}, 10^{-6} and 10^{-5} M (N = 7 insects; $p < 0.05$). The latter concentration increased the rate of contractions around 50% compared to saline solution (Fig. 4C). However, it should be noted that this increase was considerably smaller than the reported for cardioactive neuropeptides

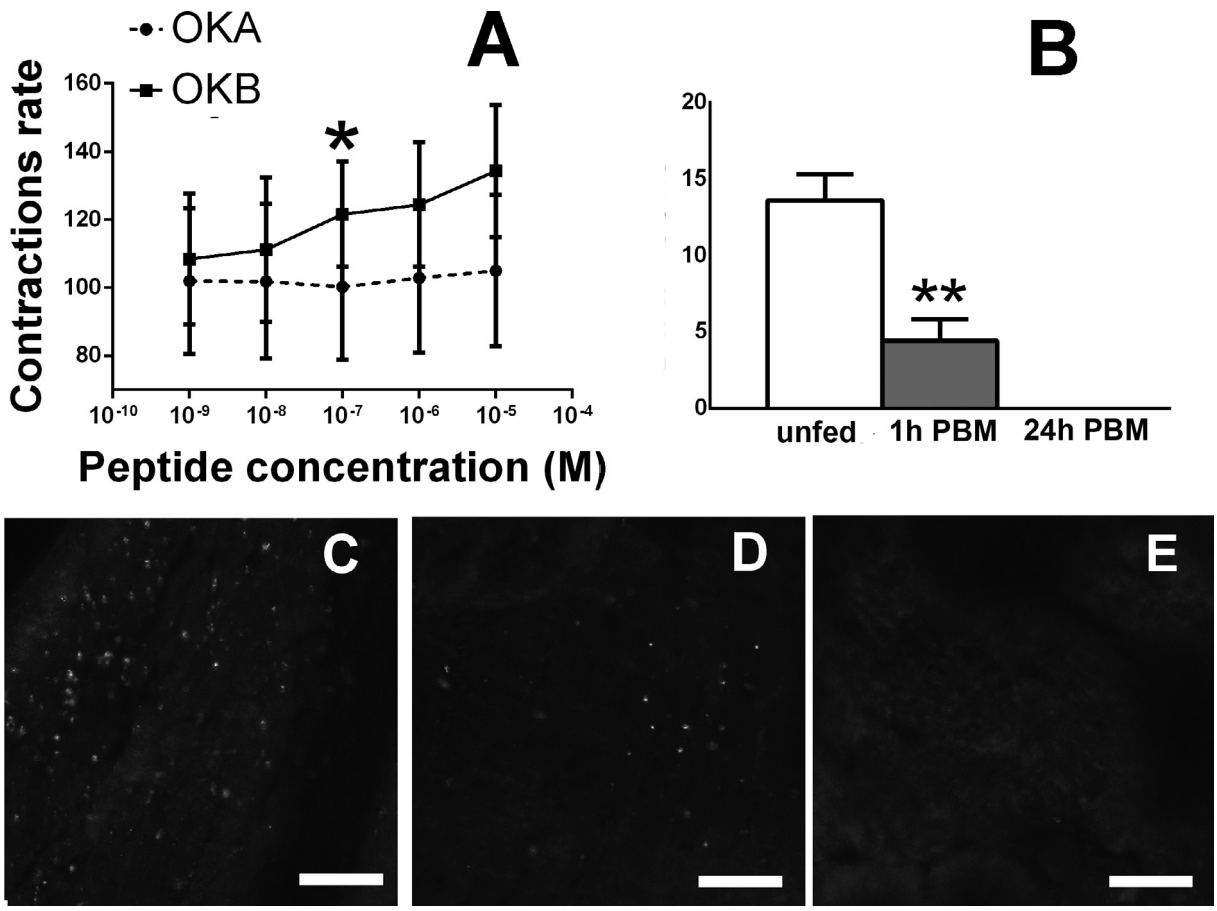

Fig. 5. (A) Effect of OKA or OKB on the frequency of anterior midgut contractions in 5th instar *R. prolixus*. N = 7–10. * = $p < 0.05$ vs. 10^{-9} M. (B) Change in the number of OKB/C-like immunoreactive cells in *R. prolixus* 5th instar nymph anterior midgut after blood intake. Neuroendocrine-like cell abundance in the different groups is represented (means ± SEM; N = 4–6). ** = $p < 0.01$ vs. unfed group. Twenty-four hours post-blood meal no immunoreactive cells were detected. (C) Representative micrograph of an anterior midgut from an unfed insect. (D) Representative micrograph of an anterior midgut from an insect 1 h PBM. (E) Representative micrograph of an anterior midgut from an insect 24 PBM. White bar: 100 μM.

such as RhoprCRZ (a maximum of 1500% reached at 10^{-7} M) or RhoprCCAP (a maximum of 380% reached at 10^{-10} M with) (Lee and Lange 2011; Patel et al., 2014).

3.4. Evidence for the involvement of RhoprOKB in midgut physiology

Present and previous results (Wulff et al., 2017) point to a requirement of RhoprOKA for achieving a successful ecdysis. However, RhoprOKB/C seems to be less crucial. Given that OKB are brain-gut peptides in insects, we hypothesized an involvement of this neuropeptide family in anterior midgut physiology. A small but significant activation in the frequency of spontaneous peristaltic contractions was observed in the bioassays for OKB at 10^{-7} M compared to 10^{-9} M ($p < 0.05$; N = 7 insects), but not for OKA (Fig. 5A).

In our previous work, we observed orcokinin B/C-like immunoreactivity (OBCLI) in endocrine-like cells homogeneously distributed throughout the anterior midgut (Wulff et al., 2017). This allowed the estimation of the number of RhoprOKB/C-expressing cells under different physiological conditions using semiquantitative immunohistochemistry. One hour after blood intake, the number of immunoreactive cells decreased when compared with unfed insects ($p < 0.005$; N = 6 midguts/group) (Fig. 5B). OBCLI cells were undetectable in anterior midgut 24 h PBM. Representative micrographs are presented in Fig. 5C–E. These results suggest a release of RhoprOKB/C peptides from the endocrine cells in midgut immediately after blood feeding.

4. Discussion

In a previous work, we demonstrated that RhoprOKA is necessary for a successful ecdysis in the kissing bug *R. prolixus* (Wulff et al., 2017). Here we present evidence pointing to an effect of neuropeptides encoded in RhoprOKs gene in the expression of other neuropeptide precursor genes that modulate the ecdysis in insects. Based on the results obtained, we propose that RhoprOKA (in a direct or indirect way) represses the expression of *RhoprCRZ* and *RhoprEH* genes. The results also suggest an effect of RhoprOKA stimulating the expression of *RhoprETH* and *RhoprCCAP* during the ecdysone peak (day 6 PBM), but not on the day before the ecdysis (day 11 PBM) when ecdysone levels return to basal (Wulff et al., 2017). Further experiments would be necessary to test whether the results observed in the regulation of genes are due to a direct effect of RhoprOKA, or to an interplay between RhoprOKA and ecdysone or other hormonal factors.

Here we used two complementary experimental approaches: specific transcript silencing by RNAi and *in vitro* rescue with synthetic OKA or OKB. As can be expected, we observed that the rescue in gene expressions is not complete. For example, on day 11 PBM dsOKA injections correlate with an 8-fold upregulation in the expression of RhoprEH. The rescue with synthetic OKA for 3 h in the incubation medium only partially reverted this upregulation, causing a significant but smaller reduction in the rescued group (0.5 with respect to controls). In the case of gene silencing, RhoprOKA transcript was silenced throughout the molting cycle. Hence, it could be expected that the effect on the expression of other genes will be greater than that of the

treatment with synthetic peptides for 3 h. Furthermore, the effect of gene silencing is usually difficult to revert with synthetic peptide, and is normally only reverted partially. In agreement with this, Lenaerts et al. (2017) recently reported that daily injections of both ETH1 and ETH2 peptides were necessary to rescue lethal arrest during ecdysis phenotype in dsETH injected *Schistocerca gregaria*; only 41% of the animals were rescued with this protocol. The authors reported the unsuccessful implementation of different rescue protocols by the injection of synthetic ETHs. Hence, even if we consider that the *in vitro* protocol used here can overcome several of the problems of *in vivo* injections, it is not surprising that the rescue observed was not complete.

As was suggested in our previous work (Wulff et al., 2017), the involvement of RhoprOKB/C in a successful ecdysis seems to be less crucial, restricted to affect the expression of RhoprCCAP on day 6 PBM and of RhoprCRZ on day 11 PBM. Furthermore, the results presented here point to an effect of ecdysone upregulating the expression of RhoprOKA, which in turn affected the expression of other neuropeptide precursor genes. Interestingly, this effect was only observed when OK levels were reduced by a dsOKs injection. In wild-type insects neither RhoprETH, RhoprEH, RhoprCRZ, RhoprOKA nor RhoprOKB/C expression responds to the addition of 20E to the medium just before ecdysis, on day 11 PBM. 20E exerts its physiological action by binding to a nuclear receptor (EcR), which forms a heterodimer with another nuclear receptor called ultraspiracle. This elicits a cascade of gene expressions that amplify the signal (reviewed in (King-Jones and Thummel, 2005)). Temporally and spatially diverse responses have been observed for many ecdysteroid-inducible genes. The stage and tissue specificity could be due to the differential expression pattern of different EcR isoforms (Talbot et al., 1993) or to the presence of competence factors such as the nuclear receptors E75 or HR3 (King-Jones and Thummel, 2005). The results presented here suggest for the first time that the expression of RhoprOKA, but not RhoprOKB/C, is regulated by 20E, indicating that RhoprOKA could be involved in the ecdysteroid pathway. Interestingly, a previous work from Yamanaka et al. (2011) showed that BommoOKA peptides had a prothoracicotropic effect on *B. mori*. Hence, a conserved functional interrelation between OKA and 20E could be proposed, even though further research would be necessary to test this hypothesis.

Besides the involvement of RhoprOKA in post-embryonic development, present results suggest that RhoprOKB/C is involved in feeding-related events. Feeding and molting are functionally linked events in insect's physiology (Zitnan and Adams, 2012); the success of post-embryonic development and ecdysis depends on the nutritional state of the individual. In *R. prolixus*, molting and ecdysis are coordinated and timed whenever a large blood meal is taken by the nymph. Hence, this kissing bug is an ideal model to study the link between feeding and molting/ecdysis. The results presented here suggest that the different isoforms of the *RhoprOK* gene, RhoprOKA and RhoprOKB/C, are associated with the regulation of post embryonic development and feeding related events respectively. Therefore, we propose that *RhoprOK* is a candidate gene for the integration of signaling cues, in order to coordinate the nutritional state with development and ecdysis. Even though the functional characterization of OKs in insects is still poor, the present report in conjunction with other recent papers (Ons et al., 2015; Wulff et al., 2017) point to an involvement of this neuropeptide precursor gene in processes that are essential for insect survival and reproduction. Hence, OKs could be good candidates in the search for new insect pest management strategies based on neuroendocrine targets.

5. Acknowledgments

We thank Raúl Stariolo and Patricia Lobbia from Centro de Referencia de Vectores for their generous assistance in the rearing of insects. Thanks to Flavio Francini for his kind help in qRT-PCR analysis. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET Grant PIP 2015 076 to S.O), Agencia Nacional de Ciencia y Tecnología (ANPCyT Grant PICT2014-1399 and 2015 0468 to S.O.). S.O. is investigator of the CONICET. J.P.W. and N. C are recipients of research fellowships from CONICET.

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