Insect Biochemistry and Molecular Biology 81 (2017) 91–102

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Orcokinin neuropeptides regulate ecdysis in the hemimetabolous insect *Rhodnius prolixus*

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ARTICLE INFO

Article history:
Received 10 August 2016
Received in revised form
14 December 2016
Accepted 4 January 2017
Available online 9 January 2017

Keywords:
Chagas' disease
Post-embryonic development
Molting
Brain-gut neuropeptides
RNA interference (RNAi)

ABSTRACT

To grow and develop insects must undergo ecdysis. During this process, the individual sheds the old cuticle to emerge as the following developmental stage. During ecdysis, different programed behaviors are regulated by neuropeptidergic pathways. In general, components of these pathways are better characterized in crustacean and holometabolous insects than in hemimetabola. In insects, the *orkoninin* gene produces two different neuropeptide precursors by alternative splicing: orcokinin A and orcokinin B. Although orcokinins are well conserved in insect species, their physiological role remains elusive. Here we describe a new splicing variant of the orcokinin gene in the hemimetabolous triatomine *Rhodnius prolixus*. We further analyze the expression pattern and the function of the alternatively spliced RhoprOK transcripts by means of immunohistochemistry and RNAi-mediated gene silencing. Our results indicate that orkoninis play an essential role in the peptidergic signaling pathway regulating ecdysis in the hemimetabolous insect *Rhodnius prolixus*.

1. Introduction

Animals are capable of performing programed behaviors that are stereotypic and critical for survival in the absence of previous experience, including escape strategies, nurturing and courtship (Zitnan et al., 1999; Kim et al., 2006). In insects, development occurs through successive stages of feeding and molting that culminate in an endocrine-regulated process called ecdysis. During ecdysis, insects undergo a sequence of programed behaviors to shed the cuticle surrounding the external surface and the lining of the foregut, hindgut and tracheae.

Ecdysis is coordinated by a complex network of hormones leading to a cascade of (neuro)peptides that are secreted after the decline of ecdysteroid levels (Zitnan et al., 1999; Zitnan and Adams, 2012), in a process that was studied mainly in two holometabolous species: the moth *Manduca sexta* and the fruit fly *Drosophila melanogaster* (Zitnan and Adams, 2012). In hemimetabola, the regulation of ecdysis by neuropeptides has not been thoroughly characterized, and only scattered information is available.

In insects, the *orcokinin* (*OK*) gene produces two peptide precursors by alternative splicing: orcokinin A (OKA) encoding mainly OKA peptides, and orcokinin B (OKB) encoding OKB mature neuropeptides (Sterkel et al., 2012). Even though OKs are well conserved in insect species, their role in the regulation of insect physiology has been poorly studied, compared to other neuroendocrine systems. Two reports dealt with the physiological effects of synthetic OKA. These studies showed an *in vitro* prothoracicotropic action in the lepidopteran *Bombyx mori* (Yamanaka et al., 2011) and the regulation of circadian locomotor activity in the cockroach

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http://dx.doi.org/10.1016/j.ibmb.2017.01.003
0965-1748/

Leucophaea maderae (Hofer and Homberg, 2006). Recently, studies using gene silencing by RNAi suggested a role of the *OK* gene (OKA and OKB isoforms) in oogenesis in the cockroach *Blattella germanica* (Ons et al., 2015) and in the regulation of "awakening" escape behavior in the beetle *Tribolium castaneum* (Jiang et al., 2015).

Rhodnius prolixus is a medically relevant insect species as a vector of Chagas' disease, a neglected disease affecting approximately 7 million people around the world (Rassi et al., 2010). Larvae remain in an arrested developmental state after molting; blood feeding triggers the neuroendocrinological response that conducts to molting and ecdysis to the following life stage (Maddrell, 1964, 1966). This characteristic allows experimental timing during physiological assays, making *R. prolixus* a convenient experimental model for the study of post-embryonic development and ecdysis.

In the recent years, we have structurally characterized OKs in *R. prolixus* by genomic and peptidomic approaches (Ons et al., 2009; Ons et al., 2011; Sterkel et al., 2011; Sterkel et al., 2012). In the present work, we study the *RhoprOK* encoding transcripts both structurally and functionally. The expression pattern as well as the phenotype obtained with the depletion of the transcript by RNAi point to *RhoprOKA,* and to a lesser extent to RhoprOKB, as regulators of ecdysis. This is the first study demonstrating the crucial role of OKs in peptidergic pathways regulating ecdysis, highlighting the relevance of this hormonal system in the regulation of insect physiology.

2. Material and methods

2.1. Insect rearing

A colony of *R. prolixus* was maintained in the laboratory under 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 4354 6503).

2.2. RhoprOKC transcript identification and tissue profiling

A public EST database from *R. prolixus* whole body and tissues (http://rhodnius.iq.ufrj.br/index.php?option=com_content&view=article&id=22&Itemid=34) was used for the homology-based search, performed by local TBLASTN by using BLOSUM62 and RhoprOKB sequence as query. In addition to the known *RhoprOKB* sequence, we found an EST encoding a potentially new isoform of *RhoprOK*. The latter was used to design specific primers to sequence the complete open reading frame of the *RhoprOKC* transcript by using RACE-PCR. For the 3′ region, we used the primers OKA/B fwd (Table 1) and the 3′ GeneRacer primer from the GeneRacer® Kit (Invitrogen, Argentina). One μl of this reaction was used as template for amplification with the primers OKC fwd (Table 1) and 3′GeneRacer nested primer (Invitrogen, Argentina). Template cDNA was prepared from fifth instar *R. prolixus* starved for 4–5 weeks, using Trizol Reagent (Invitrogen, Carlsbad, CA, USA). PCR fragments were purified by ethanolic precipitation and directly sequenced in Macrogen (Seoul, Korea). The 5′ region of the transcript was complete in the EST database. The sequences obtained were compared to *R. prolixus* genome (http://rprolixus.vectorbase.org/). For prediction of convertase cleavage sites, the rules described for insect neuropeptide precursors (Veenstra, 2000) were followed. Signal peptide sequence was predicted with the SignalP server (Petersen et al., 2011) (http://www.cbs.dtu.dk/services/SignalP/).

Tissues were microdissected from adult *R. prolixus* at different times postfeeding. Total RNA was isolated from tissues using Trizol (Ambion, São Paulo, Brazil). cDNA was synthesized using MMLV Reverse Transcriptase (Promega, USA), following the manufacturer's instructions. An aliquot of the cDNA from each tissue was used as template to perform the subsequent PCR reactions. The following temperature-cycling parameters were used to amplify the *RhoprOKC* specific fragment in parallel reactions: 95 °C, 5 min (1 cycle); 94 °C, 35 s; 54 °C, 35 s; 72 °C, 1.5 min (35 cycles); 72 °C, 10 min (1 cycle). For a positive control to test the quality of the template cDNA, a 300 bp fragment from *RhoprActin* was used (see primer sequences in Table 1). The procedure was repeated three times using independent biological samples.

2.3. Immunohistochemistry

The dorsal cuticle was removed from 5th instar larvae, or male and female adult *R. prolixus* starved since last eclosion (2 weeks before dissections). Cuticle removal was performed under phosphate buffered saline pH 7 (PBS), exposing nervous structures and organs. Tissues were fixed *in situ* in 2% paraformaldehyde (pH 7) for 12 h at 4 °C. Nervous system and organs were removed, 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 antisera were 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. Organs and tissues were incubated in primary antiserum for 48 h at 4 °C, and then washed for 18–24 h at 4 °C in PBS. Primary rabbit antiserum anti-Asn13-orcokinin (OKA) was generously provided by Dr. H. Dircksen, (Department of Zoology, Stockholm University). The primary rabbit antiserum against OKB9 peptide (present in RhoprOK-B and RhprOK-C isoforms) was produced by Genscript (Piscataway, NJ, USA). The antiserum was generated in rabbits injected with a peptide with sequence EFLDPLGGGHLIC and T-Max™ as adjuvant (Genscript, Piscataway, NJ, USA). Once produced, the antiserum was affinity purified to the antigen. Finally, the sensitivity of the antiserum was characterized by ELISA (reported titer = 1:512000).

Tissues were incubated in Cy3-labeled goat anti-rabbit immunoglobulin IgG solution (Jackson, USA) at a dilution of 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. Images were obtained using a confocal microscope and LSM510 viewing software (LSM510-Zeiss, Germany).

2.4. RNA interference

To generate dsRNAs targeting *RhoprOKs*, 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). One μl of the PCR product was used for a secondary PCR using T7-promoter primer (Table 1). RNAs were generated by *in vitro* transcription using T7-RNA polymerase (Promega, USA). To obtain dsRNAs, the

Table 1
Sequence of the primers used for qRT-PCR.

Primer name	5′-3′ Sequence
PCR_OKC Fwd	TGCGGCTAGAGTTAAGAGGA
PCR_OKC Rev	TGACATATTTAAAAGCGCAGCA
PCR_OKA/B fwd	GATGATCAACATGCTGTCGT
GenRacer™ 3′ Primer	GCTGTCAACGATACGCTACGTAACG
GenRacer™ 3′ Nested Primer	CGCTACGTAACGGCATGACAGTG
RhoprACTIN Fwd	ACACCCAGTTTTGCTTACGG
RhoprACTIN Rev	GTTCGGCTGTGGTGATGA
qPCR_OKA Fwd	GATGATCAACATGCTGTCGT
qPCR_OKA Rev	AGGTTTGGATGAGCCCTGAGT
qPCR_OKB Fwd	GAGCGAAATTATGGACCAGCA
qPCR_OKB Rev	ATGACCTCCACCCAAACCAT
qPCR_E75a Fwd	AGAGGGAGTCAATACATAGTGGA
qPCR_E75a Rev	AATCTCCGGATCTGTCAGCC
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/C Rev	TAATACGACTCACTATAGGGAATGACCTCCACCCAAACCAT
dsOKs Fwd	TAATACGACTCACTATAGGGTCACTATCGCTGCGTCTCTGT
dsOKs Rev	TAATACGACTCACTATAGGGCTAAAGTATCCAAATTTCGGCCCTC
dsAmpi Fwd	TAATACGACTCACTATAGGGGAACTGGATCTCAACAG
dsAmpi Rev	TAATACGACTCACTATAGGGGGATCTTCACCTAGATC
T7-Promotor Primer	ATAGAATTCTCTCTAGAAGCTTAATACGACTCACTATAGGG
Oligo dT	TTTTTTTTTTTTTTTTTTTTTTTT

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). The formation of dsRNAs was confirmed by running a 1.5% agarose gel and quantified using the software ImageJ from an image of the gel. The dsRNAs were stored at −20 °C until use. Three fragments were used to generate three different dsRNAs: a 315 fragment spanning positions 389 to 703 for depleting *RhoprOKA* mRNA (*dsOKA*), a 348 fragment spanning positions 341 to 689 for depleting *RhproOKB*, and a 343 fragment spanning positions 1 to 343, a part of the molecule that is common to all isoforms of the gene (*dsRhoprOKs*). A heterologous 808-fragment from the ampicillin resistance genes, which was PCR-amplified from the pBluescript plasmid, was used as a control (dsAmpi) (see primers in Table 1). Once we confirmed that there were no differences between dsAmpi and water injected controls (Table 2), injection of water was used as control in further experiments. Depending on the experiment, 4th or 5th instar *R. prolixus* larvae 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. In all the experiments performed, the insects that died during the firsts 5 days after dsRNA injection were discarded from the analysis; early mortality equally occurred in all the experimental and control groups.

2.5. qRT-PCR

Total RNA was isolated 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 triplicate, in a 20 μl final volume (primers are shown in Table 1). cDNA levels were quantified using FastStart SYBR Green Master (Roche) in iQ single color in a Mini Opticon/Miniopticon Real-Time PCR Detection System (Bio-Rad). The schedule used for the amplification reaction was as follows: (i) 95 °C for 5 min; (ii) 95 °C for 30 s; (iii) 58 °C for 30 s; (iv) steps (i) and (ii) were repeated for 40 cycles. Real-time data were collected

Table 2
Effect of experimental treatments in *Rhodnius prolixus nymphs* ecdysis and mortality.

Experimental treatment	Larval stage	Phenotype obtained		n
		Molting (days PBM)	Mortality (days PBM)	
water	4th	100% (12,3 ± 0.63)	–	39
dsAmpi	4th	100% (12.9 ± 0.42)	–	20
dsOKs	4th	–	100% (13.6 ± 0.14)	34
dsOKA + dsOKB/C	4th	–	100% (14.67 ± 0.47)	18
dsOKA	4th	–	100% (14.18 ± 0.37)	17
dsOKB	4th	100% (14.46 ± 0.63)****		13
water	5th	100% (19.4 ± 0.35)	–	5
dsOKs	5th	–	100% (21.5 ± 1.77)	6
water + water (2 × 2μl)	4th	100% (13.5 ± 0.62)	–	10
water+20E (2 × 5 μg)	4th	100% (13.0 ± 0.37)	–	5
water+20E (2 × 10 μg)	4th	100% (12.3 ± 0.35)*	–	7
dsOKs + water (2 × 2μl)	4th	–	100% (13.2 ± 0.7)	13
dsOKs+20E (2 × 5 μg)	4th	–	100% (12.5 ± 0.49)	11
dsOKs+20E (2 × 10 μg)	4th	–	100% (12.2 ± 0.45)&	13

**** = $p < 0.001$ respect to water injected; * = $p < 0.05$ respect to water + water injected; & = $p < 0.05$ respect to dsOKs + water injected.

through the iQ5 optical system software v.2.0 (Bio-Rad). A control without a template was included in all batches. We used Elongation Factor 1 (RpEF1) as reference transcript to determine the efficiency of gene silencing. For the study of the expression of *RhoprOKa* and *RhoprOKB* at different times postfeeding and after 20E injection, we normalized the expression levels using two reference genes: RpEF1 and tubulin (RpTub). The reference primers used were previously validated as stable targets 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.

2.6. Identification of RhoprE75 transcript sequence

A TBLASTN search was performed using online BLAST tool (Altschul et al., 1990) in *Rhopr*C3.1 geneset database available on Vectorbase (www.vectorbase.org). The amino-acidic sequence of DromeE75B (CG8127) was used as query. With this strategy, we identified the transcript RPRC000853-RA as RhoprE75 (E value: 2e-87).

2.7. Ecdysteroid extraction of hemolymph samples and quantification

Hemolymph samples from control and *dsOK* treated 4th instar larvae were taken at different time points after blood feeding. Each sample was collected in 95 μl of cold methanol, the pellet was washed twice more with 100 μl of cold methanol and stored at −20 °C until further processing. The samples were extracted three times with 100% methanol by cyclic centrifugation and collection of the supernatants. The different supernatants originating from one sample were combined and dried under vacuum. When completely dry, the samples were diluted in a sample buffer for enzyme immunoassay (EIA) measurement (0.1 M phosphate buffer, pH 7.4) and stored at −20 °C.

Ecdysteroid levels were evaluated using EIA, as described in Van Wielendaele et al. (Van Wielendaele et al., 2012). A peroxidase conjugated form of 20-hydroxy-ecdysone (20E) was used as tracer. The antibody used was a rabbit polyclonal antibody against ecdysteroids. The antibody and tracer were a kind gift from Dr. De Reggi (Marseille, France) and Dr. Delbecque (Bordeaux, France). In order to quantify the ecdysteroid levels, a serial dilution (ranging from 1028 M to 10212 M) of 20E was placed on each 96-well plate. Results were determined by comparison with dose-response curves obtained using these diluted 20E standards. Hence, the values were calculated and expressed as 20E equivalents.

2.8. Treatments in vivo with 20E

Fourth instar larvae from control or *dsOK* treated groups were injected at day 4 and day 6 PBM with 20E (Sigma-Aldrich, Missouri, USA) at doses of 0 (water injected), 5 or 10 μg per specimen in 2 μl of distilled water.

In another experiment, unfed 4th instar larvae 2 weeks post-eclosion were injected with either water or 10 μg/2 μl 20E. Six days after injections the insects were processed as described above, and transcript levels were measured by qRT-PCR.

2.9. Statistical analysis

The statistical significance of qRT-PCR results (transcript abundance) for the different transcripts under study, as well as the molting/mortality day results, was analyzed using one-way ANOVA. For the EIA assays, the statistical significance of 20E concentration in hemolymph was analyzed by two-way ANOVA with "experimental treatment" and "days PBM" as the main factors. Log-transformation of data was performed to achieve homogeneity of variances when appropriate.

3. Results

3.1. Identification and characterization of a new transcript isoform encoding RhoprOK

A TBLASTN search in an EST database from *R. prolixus* whole body and tissues revealed the presence of a partial open reading frame (ORF) containing the first two exons of the gene (encoding the signal peptide) followed by a third exon, which was absent in the previously known isoforms. To obtain the complete sequence of this transcript, we performed RACE-PCR on cDNA from *R. prolixus* whole body. The amplified cDNA (*RhoprOKC*, Genbank™ accession number KF179047) spans 1803 nucleotides, with an ORF encoding a prepropeptide precursor of 422 amino acid residues (Fig. 1A). *RhoprOKC* shares two exons with *RhoprOKA* and three exons with *RhoprOKB*, but also includes a specific exon (Fig. 1B). Dibasic and monobasic cleavage sites were predicted from *RhoprOKC* sequence to generate 18 putative mature peptides. From these, 15 show the characteristic OKB core domains previously described (Sterkel et al., 2012), being actually OKB-like mature peptides. The other three peptides deduced from the *RhoprOKC* sequence are specific OKC-derived neuropeptides (Fig. 1A). These three neuropeptides lack the characteristic OKA or OKB core domains, or any other conserved sequence, being considered "spacer peptides" (Wegener and Gorbashov (2008). Therefore, all the peptides with conserved core sequence encoded in the *RhoprOK* gene are OKA or OKB peptides, similar to other species studied (Chen et al., 2015; Jiang et al., 2015; Sterkel et al., 2012; Ons et al., 2015; Veenstra and Ida, 2014).

To determine the expression pattern of the *RhoprOKC* transcript, RT-PCR tissue profiling was performed (three independent trials). Results show that *RhoprOKC* shows a peripheral expression restricted to anterior midgut, but no expression in the central nervous system (CNS), where most neuropeptides are expressed (including *RhoprOKA* and *RhoprOKB*) (Fig. 1C).

3.2. Expression pattern of OKA and OKB peptides in central nervous system and peripheral organs determined by immunohistochemistry

The immunoreactivity of OKA and OKB peptides was determined in CNS, salivary glands, anterior midgut, posterior midgut, hindgut, and reproductive organs.

3.2.1. OKA-like immunoreactivity

OKA-like immunoreactivity (OALI) was observed in neurons that were distributed extensively throughout the CNS. Fig. 2A shows a schematic representation of the more intensely stained cell groups and processes in the CNS. OALI was widely distributed in *R. prolixus* brain, as was previously observed for other insect species (Hofer et al., 2005). Approximately 80–100 big neurosecretory cells (NSCs) presented intense OALI in the protocerebrum. In the optic lobes, small neurons were profusely stained (Fig. 2B). Groups of OALI cells were located at the boundary between the protocerebrum and the optic lobes, in a cluster of NSCs characterized as the master circadian clock in the insect's brain (Steel and Vafopoulou, 2006; Reischig and Stengl, 2003; Helfrich-Forster et al., 2007). NSCs in posterior and lateral margins of the brain presented OALI, in neurons showing posteromedial processes. A cluster of paired medial NSCs stained strongly in each lobe of the brain. Posterior neurons send processes that could be traced to the subesophageal ganglion (SOG). A cluster of immunoreactive (IR)

Predicted mature peptides encoded absents in RhoprOKA and RhoprOKB isoforms:

RhoprOKC1: NLDTLDSLQNNRLNAARV (2027.06)

RhoprOKC2: NSPEIQKSNWS (1289.61)

RhoprOKC3: DGVIVEPIYGLRPD (1542.82)

Fig. 1. Structure of *RhoprOK* gene. (A) Complete nucleotide and amino acid sequence of *RhoprOKC* transcript. The predicted signal peptide sequence is underlined. The mono and dibasic post-translational cleavage sites are highlighted in black. The *RhoprOKC* exclusive exon and predicted mature peptides are in boldface. Arrows indicate intron sites. $M+H^+$ for each peptide is indicated. (B) Structure of the orcokinin precursor gene, and the three splicing variants in *Rhodnius prolixus*. The numbers into each exon (black boxes) indicate its size in nucleotides. Lines represent introns. Exon number is indicated. (C) RT-PCR of *RhoprOKC* in adult *R. prolixus*. A fragment of *RhoprOKC* is PCR amplified in cDNA from anterior midgut. CNS: central nervous system; AM: anterior midgut; PM: posterior midgut; HG: hindgut; OV: ovaries; T: testes; MW: molecular weight marker; *RhoprAmpi* was PCR amplified from tissues as a positive control to test the quality of the template cDNAs (n = 3 biological replicates). Numbers on the right indicate molecular weight in base pairs.

cells was found in the posterior margin of the protocerebral lobes. Processes from this group of cells join in a small nerve that exits the brain through the corpora cardiaca (CC), a neurohemal release site where OALI was also found (Fig. 2B).

Extensive neuropile and 20–30 neuroendocrine cells were IR in the SOG (Fig. 2C–D). There were strongly stained bilaterally paired cells in the lateral margins of the SOG, all of them sending anterior projections. The strongest stained were a pair of NSCs was situated at the posterior end, next to the connection with the prothoracic ganglion (PRO). Ventral view revealed OALI neuropile and neuronal bodies in the medial part (Fig. 2C). Dorsally, OALI neuropile traversed the SOG in an anteroposterior direction; the stained projections formed a cross in the midline. Neuronal bodies sending anterior projections were observed in the dorsal-lateral margins (Fig. 2D).

Two pairs of axonal processes could be traced from the anterior end of the SOG to the mesothoracic ganglionic mass (MTGM), traversing the PRO. Strongly stained bilateral cells were present in the anterior and in the posterior margins of the PRO (Fig. 2E). A group of cells in the anterior margin sent posterior projections, whereas cells in the posterior margin sent processes to the anterior side. Extensive IR neuropile and neurons were observed throughout all the structure, both in ventral and in dorsal view. The MTGM contained approximately 80 OALI cells (Fig. 2F). Extensive IR neuropile was present throughout all the structure. Axonal tracts could be traced running transversally in the middle line, from PRO to the posterior margin of the MTGM. The posterior lateral NSC groups contained 5 bilaterally paired strongly stained cells, sending anterior projections to a group of unpaired medial moderately stained neurons and posterior projections to abdominal nerves (ABN) 1 and 2. These NSCs contain neuropeptides that are known to regulate diuresis (Coast, 2009). OALI positive projections were also observed in mesothoracic nerves 2 and 3 (Fig. 2G).

IHC in reproductive organs revealed OALI in cells over the common oviduct and testes, and processes associated with the two former structures and the seminal vessel (Fig. 2H–L). The other organs and tissues analyzed (anterior midgut, posterior midgut, rectum, salivary glands, fat body, and Malpighian tubules) were positive neither for cells nor for neuropili.

3.2.2. *OKB/C-like immunoreactivity (OBCLI)*

OBCLI in brain and ganglia was more restricted than OALI (Fig. 3), as has been observed in *D. melanogaster* (Chen et al., 2015). The main places of OBCLI were the lateral clock neurons bilaterally paired in the boundary between the protocerebral and optic lobes (Fig. 3B). Posterior and lateral margins of the brain presented scattered OBCLI neurons (Fig. 3B). OBCLI was also found in the CC (Fig. 3C). Only one unpaired neuroendocrine cell was intensely stained in the SOG (Fig. 3C). This cell was located in the posterior lateral margin, sending an anterior projection (Fig. 3C). Even though this expression pattern is not frequent for neuropeptides, an unpaired OKB expressing cell was also found for *Drome*OKB in the abdominal ganglion (Chen et al., 2015). In the PRO, two paired neurons were stained at the posterior lateral margins, sending anterior projections (Fig. 3D). In the MTGM 16 paired cells presented intense OBLI. From these, one pair was in the anterior margin, one pair was medial-lateral, and one pair was in the posterior end (Fig. 3E). The five neurons of the posterior lateral NSC groups stained strongly, and sent projections to the ABN 1 and 2 (Fig. 3E–F).

OBCLI was observed in endocrine-like cells distributed throughout the anterior midgut (Fig. 3G), and in a few cells scattered in the posterior midgut (Fig. 3H). These cells appear to be of the open type with processes extending towards the midgut lumen. This pattern of expression is consistent with previous reports in

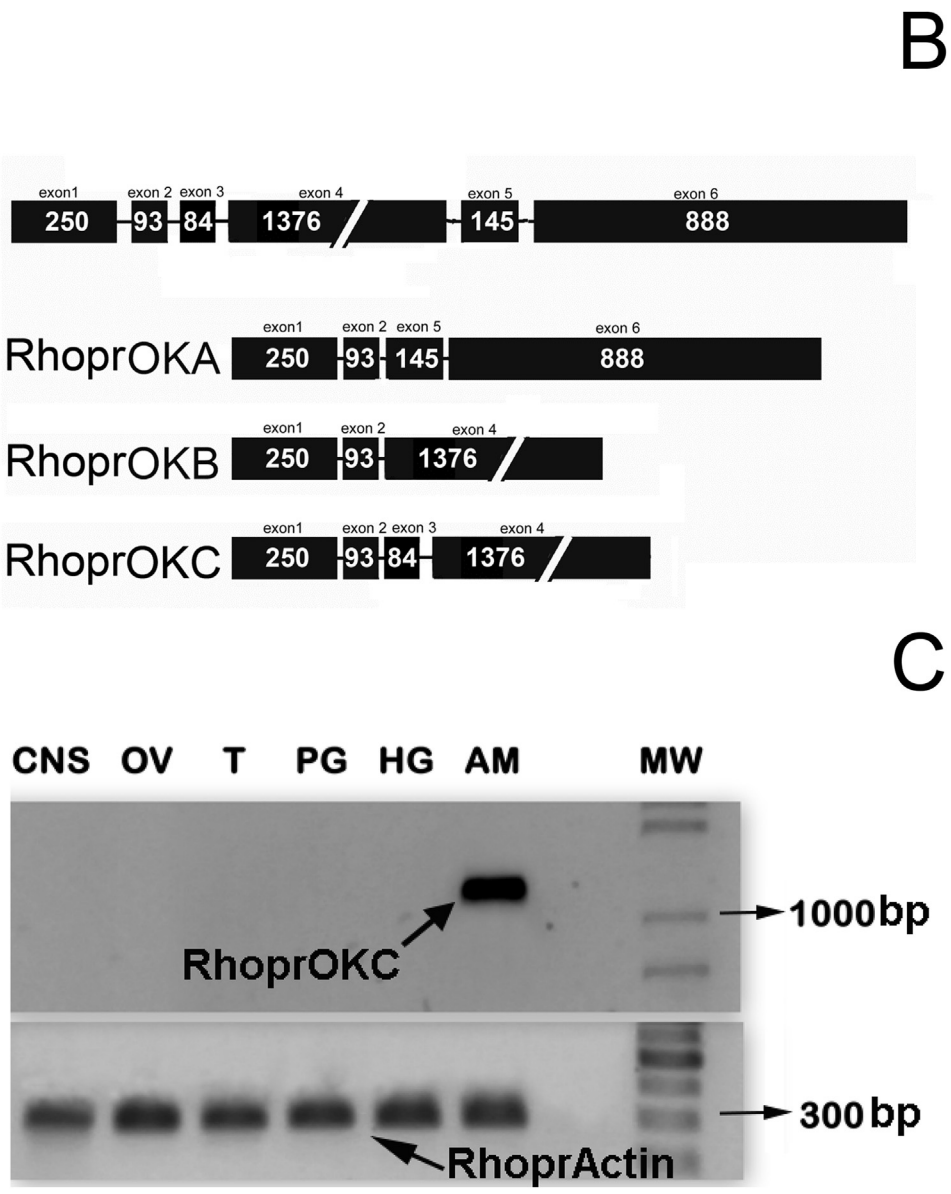

Fig. 1. (*continued*).

D. melanogaster (Veenstra and Ida, 2014; Chen et al., 2015) and differs from *T. castaneum*, where OKB (and OKA) are detected in the posterior but not in the anterior midgut (Jiang et al., 2015).

Cells positively stained for OBCLI were observed in ovaries (Fig. 3I). Processes were associated with seminal vessel (Fig. 3J), and a few IR cells were detected in testes (Fig. 3K). Rectum, salivary glands, fat body, and Malpighian tubules were negative for OBCLI.

3.3. Disruption of RhoprOKs by in vivo RNAi affects ecdysis

Insects were treated with a dsRNA spanning a region common to *Rhopr*OKA and *Rhopr*OKB/C precursors (*dsRhoprOKs*), in order to silence all these transcript isoforms. We included an experimental group injected with both *dsRNAOKA* and *dsRNAOKB/C* (2 μg each, diluted in 2 μl saline solution), to discard off-target effects. Controls were equivalently treated with either water (control water) or dsRNAAmpi (unrelated gene; control Ampi). Forty-eight hours after injection, the insects were allowed to feed *ad libitum*.

As a consequence of the treatments, the mRNA levels of *Rhopr*OKA and *Rhopr*OKB/C were dramatically reduced for *dsRhoprOKs* (85% and 92%, respectively) as well as for *dsRhoprOKA* + *dsRhoprOKB* injected insects (99.5% and 84%, respectively) (Fig. 4).

The insects belonging to the different experimental groups did not show phenotypic differences until the expected ecdysis time (12–13 days post blood meal; PBM). At this time, the insects belonging to *dsAmpi* (N = 20) and water (N = 39) control groups molted to normal 5th instar larvae. Conversely, those insects treated with *dsOKs* (n = 34) or with a conjunct injection of *dsOKA* + *dsOKB* (n = 18) did not molt at the expected time; 100% of them stopped any movement and died 1–2 days later (Table 2; Fig. 5). Treated insects did not reach the stage when they fold their heads, the first step of the ecdysis sequence in *R. prolixus* (Lee et al., 2013), which would suggest that they are arrested before ecdysis initiation.

The larvae treated with *dsOKs* or *dsOKA* + *dsOKB* showed duplicated and superimposed ectodermally derived structures, such as the proboscis (Fig. 5A). Furthermore, the manual removal of

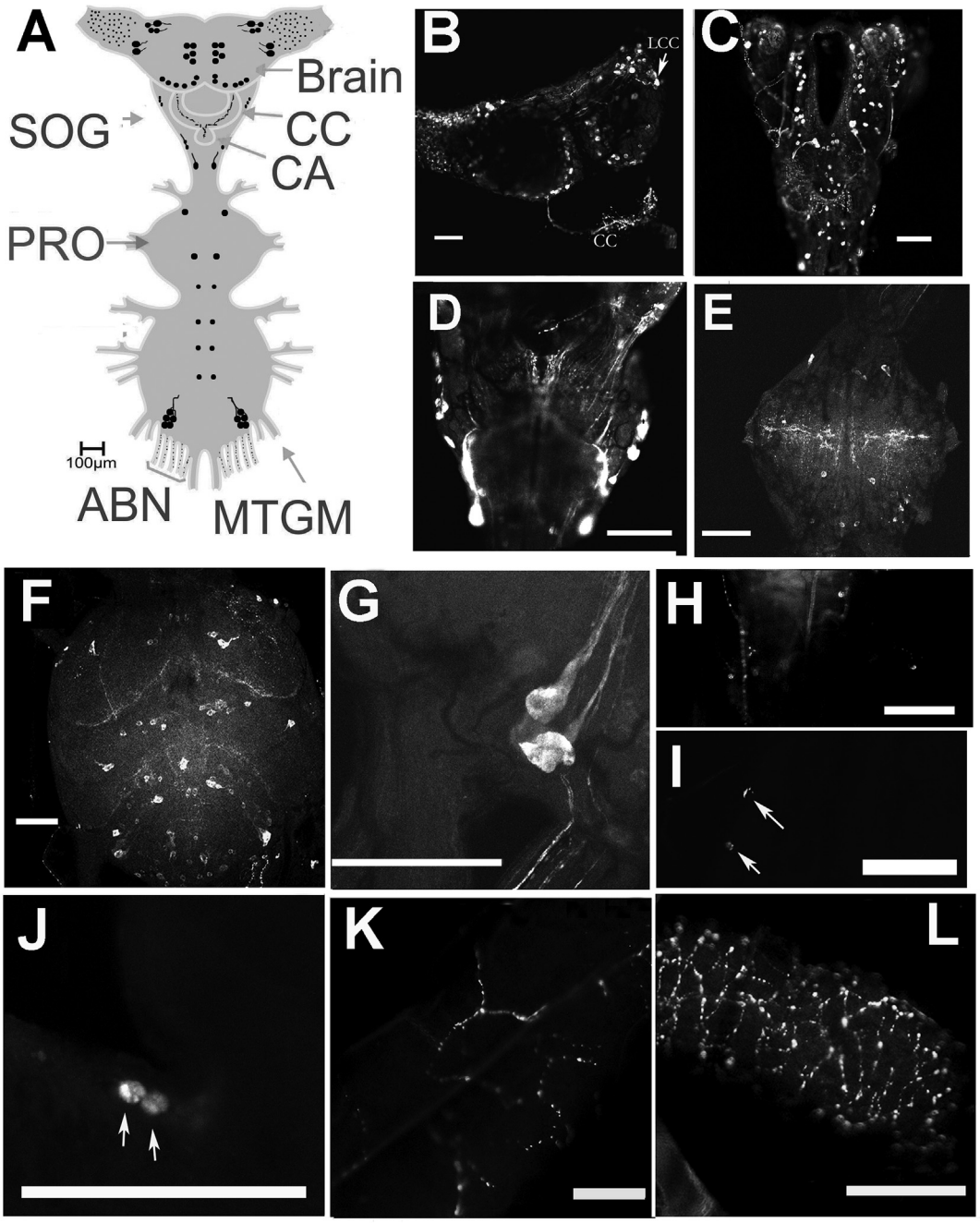

Fig. 2. Orkokinin A-like immunoreactivity (OALI) in *R. prolixus*. (A) Illustration representing the most intensely stained neurosecretory cells and processes in brain, subesophageal ganglion (SOG), prothoracic ganglion (PRO), mesothoracic ganglionic mass (MTGM), corpora cardiaca (CC), corpora allata (CA), and abdominal nerves (ABN). (B) Dorsal view of the 5th instar brain. Note the presence of OALI in the CC and lateral clock cells (LCCs). (C) Ventral view of the 5th instar brain and SOG. (D) Dorsal view of the 5th instar SOG. (E) 5th instar PTG. (F) 5th instar MTGM. (G) Detail of one of the posterior lateral neurosecretory cell groups in the 5th instar MTGM and ABN. (H) OALI processes are associated with the common oviduct in adults. (I) Positively stained cells from the common oviduct in adults (arrows). (J) Positively stained cells from the adult testes (arrows). (K) OALI processes over the adult testes. (L) OALI processes are associated with the seminal vessel in adults. Bar = 100 µm.

the cuticle revealed that the new cuticle was already sclerotized and tanned under the old one (Fig. 5B), indicating that the 5th larva was already formed but failed to shed its old cuticle.

To test whether the effects of RhoprOKs on ecdysis were restricted to the larval molt or were also necessary in the imaginal molt, we repeated the experiment using 5th (last) instar insects. As was observed for 4th instar larvae, all the insects normally survived until the expected ecdysis time. The individuals belonging to the control group normally molted to adults (Table 2), whereas 24–48 h later 100% of *dsOKs* treated larvae stopped moving and finally died (Table 2). All the treated insects showed the opened ecdysis line, but ecdysis was aborted at this stage (Fig. 5C–D).

Once we analyzed the effects of the simultaneous depletion of both *RhoprOK* types on ecdysis, we aimed to determine the involvement of isoforms RhoprOKA and RhoprOKB/C separately. We therefore used dsRNA fragments specifically targeting the RhoprOKA or RhoprOKB/C precursors transcripts. Injections provoked a marked isoform-specific depletion of both precursors (>99%) (Fig. 4E–H). Interestingly, all the *dsOKA* treated insects died 1–2 days after the expected ecdysis (Table 2). On the other hand, all

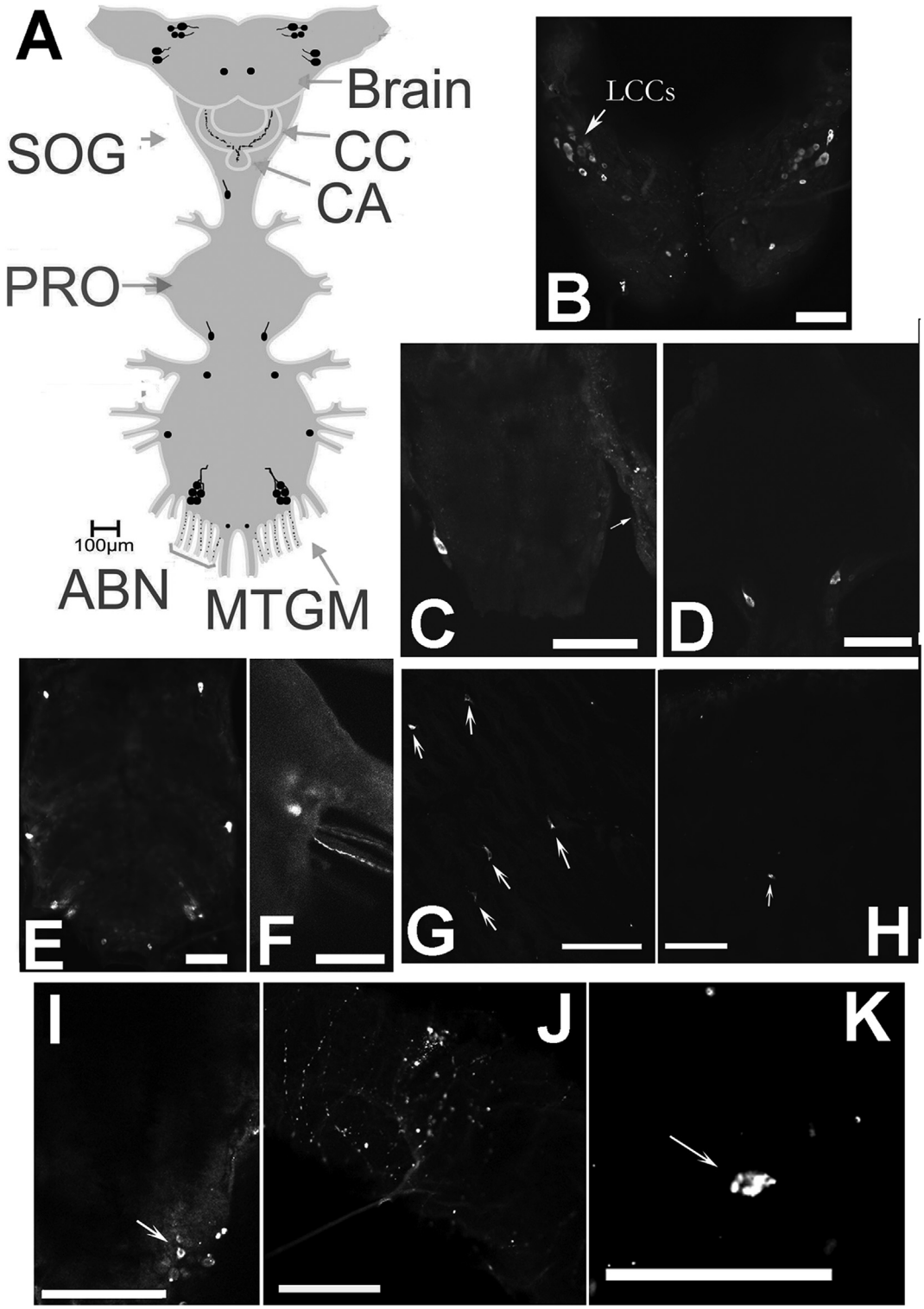

Fig. 3. Orkokinin B-like immunoreactivity (OBLI) *R. prolixus*. (A) Illustration representing the most intensely stained neurosecretory cells and processes in brain, subesophageal ganglion (SOG), prothoracic ganglion (PRO), mesothoracic ganglionic mass (MTGM), corpora cardiaca (CC), corpora allata (CA), and abdominal nerves (ABN). (B) Dorsal view of the 5th instar brain. Note the presence of OBLI in the lateral clock cells (LCCs). (C) Dorsal view of the 5th instar subesophageal ganglion and corpora cardiaca (arrow). (D) Prothoracic ganglion. (E) Mesothoracic ganglionic mass (MTGM). (E) (F) Detail of one of the posterior-lateral neurosecretory cell groups in the MTGM and abdominal nerves. (G) OBLI in endocrine-like cells from 5th instar anterior midgut (arrows). (H) OBLI in a cell from posterior midgut (arrow). (I) The arrow indicates a group of OBLI positive cells in adult lateral oviduct. (J) OBLI is observable in processes associated with the seminal vessel. (K) An immunoreactive cell present in testes (arrow). Bar = 100 µm.

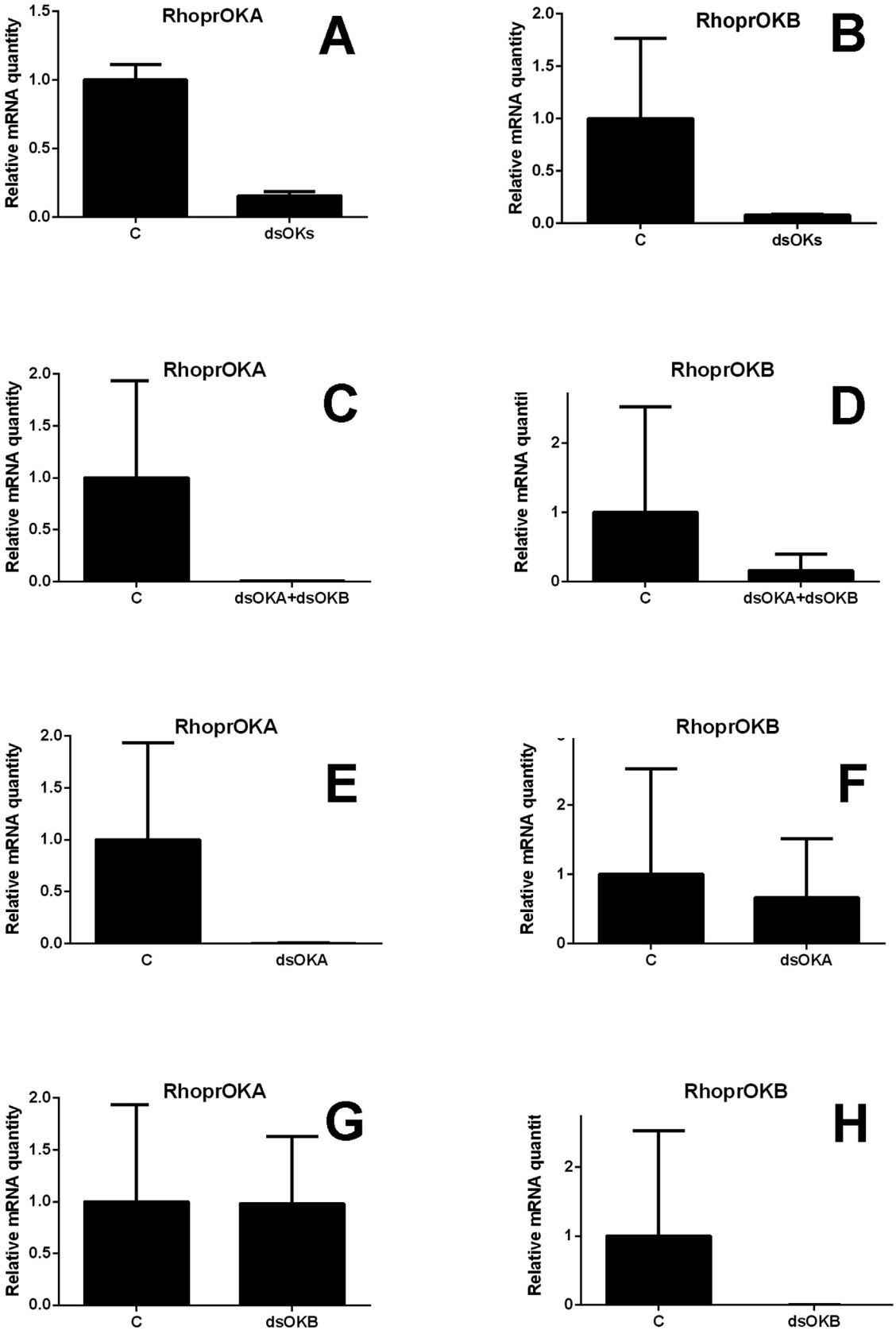

Fig. 4. Effect of dsRNA injections in/on the expression levels of *RhoprOKA* and *RhoprOKB*/C. C: control. n = 3–4. (A) *dsOK* injection effect on the expression of *RhoprOKA*. (B) *dsOK* injection effect on the expression of *RhoprOKB*. (C) *dsOKA* + *dsOKB* injection effect on the expression of *RhoprOKA*. (D) *dsOKA* + *dsOKB* injection effect on the expression of *RhoprOKB*. (E) *dsOKA* injection effect on the expression of *RhoprOKA*. (F) *dsOKA* injection effect on the expression of *RhoprOKB*. (G) *dsOKB* injection effect on the expression of *RhoprOKA*. (H) *dsOKB* injection effect on the expression of *RhoprOKB*.

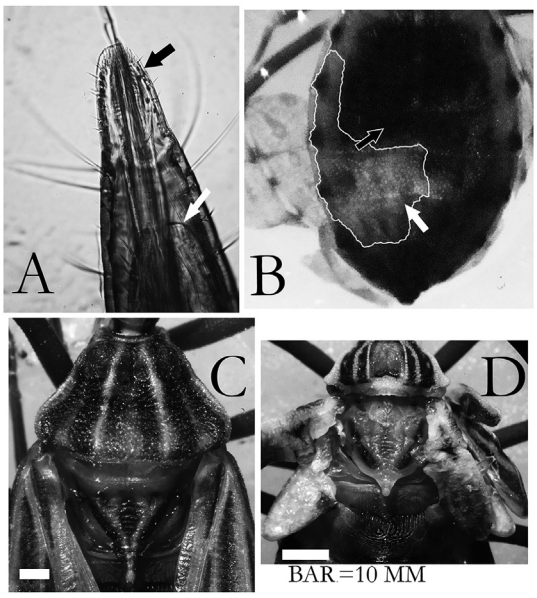

Fig. 5. *RhoprOKs in vivo* RNAi in nymphs of *R. prolixus*. (A) Arrested specimens show the larvae 4th proboscis (black arrow) above the parathe larva 5th proboscis (white arrow). (B) The old cuticle (black arrow) was manually removed in a *dsOK* treated 4th larva that died at the time of ecdysis. The new cuticle (white arrow, surrounded by a dotted line) is hardened and tanned under the old one. (C) Fifth instar larvae belonging to the control group molt at the expected time, showing the normal adult appearance. (D) Fifth instar larvae belonging to the *dsOK* group did not undergo ecdysis and died as a parathe adult. A gap is observed in the pronotum region.

the *dsOKB* injected animals reached a successful molt, albeit with a significant delay compared to the control group (14.5 days PBM) ($p < 0.001$; $n = 13$) (Table 2).

3.4. Disruption of RhoprOKs by in vivo RNAi does not affect ecdysteroidogenesis

We injected 20E to 4th instar larvae from *dsOKs* treated and control groups, to study whether this hormone would rescue the ecdysis for treated insects. The different experimental groups were injected with either water or 5 μg/10 μg of 20E. Every individual insect received two identical injections, one on day 4 and a second on day 6 PBM. While all the insects from the control group molted to 5th instar at the expected time PBM, none of the insects from the *dsOKs* group underwent ecdysis. All of them died between days 12 and 14 PBM (Table 2). The molt occurred significantly earlier in control insects injected with 10 μg of 20E (around day 12 PBM) compared to the control water injected group (around day 13.5 PBM) ($p < 0.05$, $n = 7–10$). In parallel, *dsOKs*+10 μg 20E injected animals died around day 12 PBM, significantly earlier than the *dsOKs* + water injected group, which died around day 13 PBM ($p < 0.05$, $n = 13$). The former could be explained given that RNAi treatments did not prevent molting to be initiated, but prevented shedding of the old cuticle as expected to occur during ecdysis. Ecdysteroid injection caused the earlier initiation of the molting process. Therefore, the ecdysis deficiencies caused by the RNAi treatment manifested earlier.

In an indirect way to measure ecdysteroid signaling, we evaluated the expression of *Rhopr*E75 (RPRC000853), a primary response gene encoding a transcription factor, whose expression was demonstrated to be depending on and directly correlating with the concentration of 20E in hemolymph (Mané-Padrós et al., 2008). The qRT-PCRs indicated that *RhoprE75* was not differentially expressed in water and *dsOKs* injected groups ($n = 9–10$) (Fig. 6A).

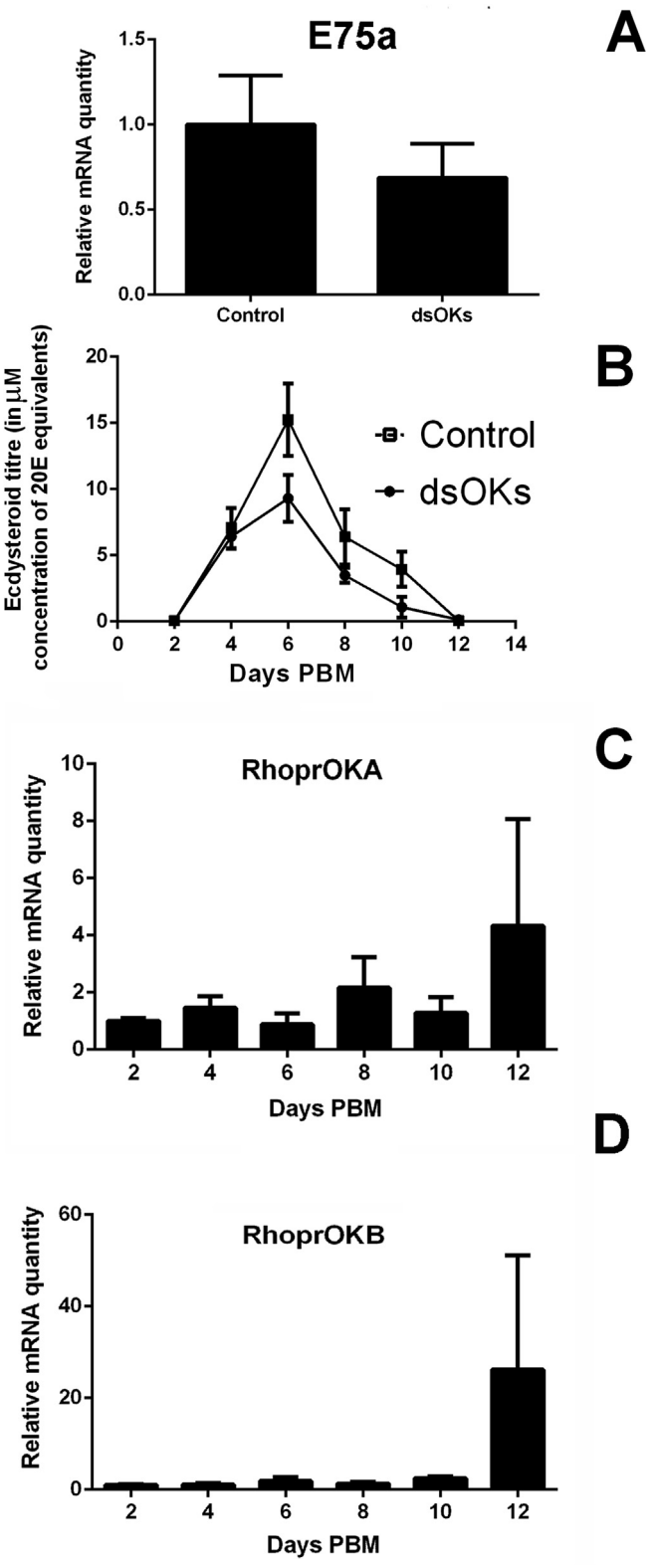

Fig. 6. (A) RhoprE75 expression levels were evaluated in control and *dsOK* treated 4th instar larvae. (B) Ecdysteroid levels were evaluated by EIA in hemolymph samples from 4th larvae *R. prolixus* belonging to control and *dsOKs* groups. Hemolymph samples were taken from insects at different times post blood meal (PBM) ($n = 4–10$/group/time point). (C) *RhoprOKA* mRNA levels were evaluated by qRT-PCR in 4th instar larvae at different times PBM. (D) *RhoprOKB/C* mRNA levels were evaluated by qRT-PCR in 4th instar larvae at different times PBM.

As a direct way to measure possible failures in ecdysteroidogenesis, we analyzed the 20E levels in the hemolymph of both water and *dsOKs* treated 4th instar larvae at 2, 4, 6, 8, 10 and 12 days PBM. The 20E equivalents in the hemolymph reached a peak around day 6 PBM in both experimental groups, recovering to basal levels at day 12, just before the expected ecdysis time. No significant differences were observed in ecdysteroid concentration throughout all the time points analyzed (Two way ANOVA; n = 4–10/group/time point (Fig. 6B)).

3.5. *RhoprOKA and RhoprOKB/C are upregulated just before ecdysis*

The expression of *RhoprOKA* and *RhoprOKB* was measured in 4th instar larvae, in the same individuals where the 20E levels were analyzed in the hemolymph. *RhoprOKA* mRNA levels increased throughout the cycle, reaching a peak on day 12, just before ecdysis (Fig. 6C). *RhoprOKB* levels were 10–20-fold overexpressed on day 12, compared to previous time points (Fig. 6D). To test whether the overexpression of *RhoprOKs* on day 12 PBM could be regulated by the 20E peak on day 6 PBM, we injected unfed 4th instar larvae with either water or 10 μg/2 μl 20E. Six days after injection the insects were processed, and the transcript levels for *RhoprOKA* and *RhoprOKB/C* were evaluated. We did not find significant differences between the experimental groups (N = 10/group).

4. Discussion

Our results indicate for the first time that RhoprOKs are essential for successful ecdysis in *R. prolixus*. The experimental evidence obtained here suggests a dramatic involvement of *Rhopr*OKA in controlling ecdysis, whereas the involvement of *Rhopr*OKB appears to be less critical. Even though the exact participation of each *Rhopr*OK isoform in the peptidergic network regulating ecdysis remains to be elucidated, we propose that these molecules are important regulators in this network.

The expression in LCCs is coherent with the observed effects of *Rhopr*OKs on controlling ecdysis, which is a circadian-regulated behavior. In addition, we observed that the pattern of neurosecretory cells presenting OKB/C-like IR in MTGM is identical to the pattern obtained for *Rhopr*CCAP, a neuropeptide involved in ecdysis in several insect species including *R. prolixus* (Lee and Lange, 2011; Lee et al., 2013). The co-expression of both neuropeptides in similar neurosecretory cells reinforces the observations regarding an involvement of *Rhopr*OKs in regulating ecdysis. Furthermore, the expression of CCAP is also elevated during ecdysis in the crab *Carcinus maenas,* the crayfish *Orconectes limosus,* and *R. prolixus* (Phlippen et al., 2000; Lee et al., 2013). Our results show that *Rhopr*OKA and *Rhopr*OKB/C expression levels are elevated just before ecdysis, supporting the hypothesis of a role of these molecules in the regulation of ecdysis behavior, as previously described for CCAP.

The physiological characterization of OKs in insects is still incipient. This is the first report involving OK neuropeptides in post-embryonic development. However, the expression of this gene in anterior midgut and adult gonads suggests that this probably is a pleiotropic (neuro) peptide system. The identification of the OK receptor(s), which has not been done in any species to date, will be fundamental in the future study of this neuronal/endocrine signaling system, which is now emerging as a central regulator in the physiology of insects.

Acknowledgments

We thank Raúl Stariolo and Patricia Lobbia from Centro de Referencia de Vectores, for their generous assistance in the rearing of insects, and to Natalia Esponda for the illustration on *Rhodnius prolixus* CNS. This work was supported by grants from the Consejo Nacional de Investigaciones Científicas y Técnicas (CONICET Grant PIP 2012-2014 049 to S.O), Agencia Nacional de Ciencia y Tecnología (ANPCyT Grant PICT2011-194 and PICT2014-1399 to S.O), the Interuniversity Attraction Poles program (Belgian Science Policy Grant IAP P7/40 to J.VdB), and the International Research Collaboration between Argentina and Flanders (MINCyT and FWO: grant number 1204/VS.045.16N and 1505 to S.O. and J.VdB). S.O. and F.F are investigators of the CONICET. J.P.W is a recipient of a research fellowship from CONICET. I.S is supported by a fellowship from CONICET. M.H. was supported by a PhD fellowship of the IWT (Agency for Innovation by Science and Technology, Flanders - Belgium).

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