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Orcokinins contribute to the regulation of vitellogenin transcription in the cockroach *Blattella germanica*

Sheila Ons [1], Xavier Bellés, José L. Maestro *

Institute of Evolutionary Biology (CSIC-Universitat Pompeu Fabra), Passeig Marítim de la Barceloneta 37-49, 08003 Barcelona, Spain

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ABSTRACT

Orcokinins (OKs) are neuropeptides that were first identified in crustacean through their myotropic activity. In insects, the *OK* gene gives rise to two mRNAs coding for two different families of conserved mature neuropeptides: OKA and OKB. Although OKs are conserved in many insect species, its physiological role in this animal class is not fully understood. Until now prothoracicotropic, regulatory of light entrainment to the circadian clock and "awakening" activities have been reported for these peptides in different insect species. Here we report the identification of OKA and OKB precursors in the cockroach *Blattella germanica*. OKA mRNA was detected in brain, whereas OKB mRNA was detected both in brain and midgut. In vivo silencing of OK precursors suggests the involvement of *OK* gene products in the regulation of vitellogenin expression in the fat body, an action that appears to be independent of juvenile hormone. This is the first time that a function of this kind has been reported for OKs.

1. Introduction

Sexual reproduction allows genetic recombination, promotes offspring survival and enables evolution to occur. To be able to reproduce, insects and other metazoans need to regulate multiple interrelated processes as nutrition, gonadal maturation, reproductive behavior, oogenesis and embryogenesis. To achieve the production and encounter of female and male mature gametes, biological processes occurring in different insect organs should be tightly coordinated. In this context, ovaries and fat body are crucial organs for oocyte maturation.

In insects, neuropeptides and lipophilic hormones play an important role in the regulation and coordination of reproductive biology (Belles and Maestro, 2005; Van Wielendaele et al., 2013). In the cockroach *Blattella germanica*, as in most insect species, juvenile hormone (JH) is the main gonadotrophic hormone, being synthesized in the corpora allata and released to hemolymph, thus activating vitellogenin (Vg) production in the fat body. Vg is then incorporated into growing oocytes as a storage protein for embryo growth and development (Belles, 2005; Raikhel et al., 2004). Vg production in the fat body and its uptake by oocytes is also regulated by neuropeptides in *B. germanica* and other insect species (Badisco et al., 2011; Brown et al., 2008; Martin et al., 1996).

Orcokinins (OKs) are arthropod neuropeptides that were first discovered in the spiny-cheek crayfish *Orconectes limosus* through its myotropic activity (Stangier et al., 1992). An OK neuropeptide was identified for the first time in insects in *B. germanica* (Pascual et al., 2004), and subsequently in species from different insect orders (Hofer et al., 2005; Hummon et al., 2006; Jiang et al., 2015; Ons et al., 2009; Roller et al., 2008). In insects, the OK gene is transcribed into two different mRNAs that code for two families of conserved mature neuropeptides: Orcokinin A (OKA) and Orcokinin B (OKB) (Jiang et al., 2015; Sterkel et al., 2012). Until now, few studies have analyzed the physiological functions of OKs. The results of these studies show that OKA has a prothoracicotropic effect in vitro in the lepidopteran *Bombyx mori* (Yamanaka et al., 2011), and that plays a role in the regulation of circadian locomotor activity in the cockroach *Leucophaea maderae* (Hofer and Homberg, 2006a). In addition, in *Tribolium castaneum*, using specific depletion of OKA and OKB mRNAs, it has been reported that both OKA and OKB have what authors called "awakening" activities that may be involved in the control of circadian rhythms (Jiang et al., 2015).

In the present paper, we report the identification and characterization of OKA and OKB transcripts in *B. germanica*, and its involvement in the control of vitellogenesis and oocyte growth in adult female of this cockroach, as shown by experiments of transcript depletion mediated by RNAi.

* Corresponding author.
E-mail addresses: sheila.ons@presi.unlp.edu.ar (S. Ons), xavier.belles@ibe.upf-csic.es (X. Bellés), joseluis.maestro@ibe.upf-csic.es (J.L. Maestro).
[1] Permanent address: Regional Center of Genomic Studies, Bvd 120 y 62, 1900 La Plata, Argentina.

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2. Materials and methods

2.1. Insects

Specimens of *B. germanica* were obtained from a colony reared on dry dog food (Panlab 125C3) and water in the dark at 30 ± 1 °C and 60–70% relative humidity. Virgin females were used for the study of gene expression levels during the first gonadotrophic cycle. Tissues were dissected under saline solution from carbon dioxide-anesthetized animals. After dissection, tissues were immediately frozen in liquid nitrogen and stored at −80 °C.

2.2. Cloning of BgOKA and BgOKB transcripts

Sequences corresponding to *B. germanica* A and B orcokinin neuropeptide precursor mRNAs were identified in transcriptomic databases obtained in our group, representing different organs and stages of development (data available at NCBI, BioProject PRJNA268902). Using these sequences, we designed specific primers (Supplementary Table 1) to obtain *B. germanica* cDNA fragments for OKA and OKB open reading frames by RT-PCR as described previously (Maestro and Belles, 2006), except that Transcriptor First Strand cDNA Synthesis kit (ROCHE) was used. The fragments were cloned in pSTBlue-1 vector (Novagen), following the manufacturer's protocol, and sequenced.

2.3. RNA extraction, cDNA synthesis, real-time PCR analyses and quantification of proteins in ovaries

The expression levels of the different genes studied were analyzed using quantitative real-time PCR (qRT-PCR) in cDNA prepared from different tissues. cDNA was synthesized from total RNA as described above. The absence of genomic contamination was confirmed using a control without reverse transcription. cDNA amplifications of OKA, OKB, Vg, 3-hydroxy-3-methylglutaryl coenzyme A synthase 1 (HMG-CoA synthase-1), juvenile hormone acid methyltransferase (JHAMT) and actin 5C were performed in duplicate or triplicate, in a 20 µl final volume (primers detailed in Supplementary Table 1). cDNA levels were quantified using iQ SYBR Green supermix (Bio-Rad) in an iQ cycler and iQ single colour detection system (Bio-Rad). The schedule used for the amplifying reaction was as follows: (i) 95 °C for 3 min, (ii) 95 °C for 10 s; (iii) 57 °C for 1 min; (iv) steps (i) and (ii) were repeated for 50 cycles. Real-time data was collected through the iQ5 optical system software v.2.0 (BioRad). For quantification of soluble proteins, ovaries were dissected and placed at −80 °C until their use. Total soluble proteins were extracted by ultrasonication and centrifugation in NaCl 0.4 M solution and quantified according to Bradford (Bradford, 1976).

2.4. RNA interference

dsRNA for RNAi experiments were prepared as previously described (Maestro and Belles, 2006). Three different fragments were used to generate three different dsRNA: a 224 bp fragment spanning positions 1–224, encompassing the signal peptide, a part of the molecule that is common to both transcripts (dsOKs); a 210 bp fragment, exclusive for depleting BgOKA mRNA, spanning positions 416–626 (dsOKA); a 211 bp fragment in the 3′-UTR of BgOKB mRNA, exclusive for this transcript, spanning positions 1484–1695 (dsOKB). A heterologous 250-bp fragment from the polyhedrin of *Autographa californica* nucleopolyhedrovirus (dsMock) was used as a control. A dose of 2 µg diluted in sterile saline was injected into the abdomen of freshly emerged penultimate (fifth) nymphal instar females, followed by a second 2-µg dose injected just after molting to the last (sixth) instar. Dissections were carried out 5 days after the adult emergence.

2.5. Juvenile hormone and synthetic peptide treatments

JH treatment was performed by topical application. Four days after adult emergence, the wings of dsOKs-treated female insects were cut and 1 µl of JH III (Sigma) diluted in analytical grade acetone at a concentration of 2 µg/µl was topically applied on the abdominal tergites using a 10 µl Hamilton syringe. Controls were equivalently treated with acetone.

OKA type peptide (NFDEIDRSGFNSFV) and OKB type peptide (ALDSIGGGNLV-NH_2) were synthetized by the Protein Chemistry Laboratory at the *Centro de Investigaciones Biológicas* (CIB, CSIC), using Fmoc chemistry, and diluted in 10% DMSO to a concentration of 1.25 µg/µl. dsOKs-treated females were injected with 2 µl (2.5 µg) of peptide solution, or the equivalent solvent (controls), 2 and 4 days after adult emergence. Fat bodies were dissected one day after the second injection.

3. Results and discussion

3.1. B. germanica has OKA and OKB precursor mRNAs

Using the sequences of OKA and OKB of *Rhodnius prolixus* as queries we screened a transcriptomic database obtained in our group, representing different organs and stages of development (BioProject PRJNA268902). This search revealed the presence of a complete open reading frame (ORF) for OKA (BgOKA), and two sequences representing a portion of OKB ORF (BgOKB). We designed specific primers in order to clone the complete ORFs for both types.

The cloned cDNA sequence for BgOKA (Genbank™ accession number: KP744806) spans 626 nucleotides, with an ORF encoding a prepropeptide of 167 amino acid residues (Fig. 1). The cloned BgOKB (Genbank™ accession number: KP744807) spans 1695 nucleotides, with an ORF encoding a prepropeptide of 479 amino acid residues (Fig. 1). Both mRNAs share a 256 nt in the 5′ region, which contains the putative first Met and a total of 54 amino acids, including the predicted signal peptide. This suggests that both precursors are alternative splicing variants expressed by the same gene, as occurs in other insect species (Sterkel et al., 2012; Veenstra and Ida, 2014). Taking into account the putative monobasic or dibasic cleavage sites that would give rise to peptides showing the characteristic N-terminal OKA motif NXDEID (X = F or L), we could find the sequence coding for three OKA peptides in the BgOKA transcript, including the peptide already biochemically purified from brain extracts NFDEIDRSGFNS (Pascual et al., 2004). The BgOKB transcript shows the sequence coding for 22 putative mature peptides showing, with very few variations, the sequence X_1DSIGGGNX_2V (X_1 and X_2 = L or I), with a Gly residue (which allows amidation) or not at the C-terminus, compatible with the characteristic sequence of OKB peptides. In addition, the BgOKA mRNA encodes a sequence (LDSIGGGHLL) that is also compatible with a mature OKB peptide. Interestingly, OKB peptides from *R. prolixus* and *Drosophila melanogaster* have a His in position 8 (Sterkel et al., 2012; Veenstra and Ida, 2014), whereas in *B. germanica* this position is occupied by an Asn.

3.2. BgOKs are expressed in brain and midgut

Specific primers were designed to measure the levels of BgOKA and BgOKB transcripts in the brain, midgut, ovaries and fat body of *B. germanica* adult females. Results showed that BgOKA is expressed only in brain at moderate levels, whereas BgOKB is

BgOKA:

*MKLLALLVVTIAATSVPSSA*SPIQSDALRESAFRDYRADSGDEENVVRHLDSIGGGHLLRE
LDGLSHFPRRTRSGLDSLSGASFGGNKRFDTLSGISFGNQKRNFDEIDRSGFNSFVKKNLD
EIDRSGFDSFVKRNFDEIDRVGFGSFVKRNAPLFLTRYYDKQENH

BgOKB:

*MKLLALLVVTIAATSVPSSA*SPIQSDALRESAFRDYRADSGDEENVVRHLDSIGELSKKED
GPKDREEELEEHKIKNLKKFLTHGQHSRLDSIGGGNIVRGIHPFNRELLKELESLRSGHIV
TRNLESIGGGNIVGRSLDSIGGNIVGRSLDPIGGGNIVGRSIDPIGGGGIVGRRIESIGGG
NIVRAIDSIGGNILGRSLDSIGGGNLVRALDSIGGGNLVGRSIDDIGGGNIVGRRIDSLGG
GNLVGRKIESIGGGNIVGRSLDSIGGGNLVRALDSIGGGNLIGRNIDGIGGGNLVRALDSI
GGGNLVGRSIDDIGGGNIVRALDSIGGGNLVGRSIDDIGGGNIVRALDSIGGGNLVGRNID
GIGGGNLVRALDSIGGGNLVGRSIDDIGGGNILGRGHSRTIESIGGDGGIVRSLDSIGGGN
LLGRGPSRTIESIGSDGGIVRDNEDNFDIYEERLFQTKHRNKQSEESLEDKS

Fig. 1. Sequences of BgOKA and BgOKB ORFs. Italics indicate the putative signal peptides. The region highlighted in gray is shared by the two transcripts. Underlined sequences correspond to putative OKA (in BgOKA) and OKB (in BgOKB) peptides. Double underlined corresponds to the putative OKB type peptide encoded by the BgOKA precursor.

expressed in brain and midgut at rather low levels (Fig. 2A). In *R. prolixus*, the OKA precursor is expressed in central nervous system (CNS) whereas that of OKB is expressed in both CNS and anterior midgut (Sterkel et al., 2012). In *D. melanogaster*, the OKA precursor is mainly expressed in CNS of both larvae and adults, whereas OKB is mainly expressed in larva and adult midgut enteroendocrine cells and in one unpaired neuron in adult abdominal ganglion (Chen et al., 2015; Veenstra and Ida, 2014). In *B. mori*, in situ hybridization using a probe which encompasses a region common to OKA and OKB precursors shows that orcokinins are found in midgut endocrine cells and different neurons of the brain and ventral nerve cord, whereas immunohistochemical analysis using an antibody against an OKA peptide localize this peptide family only in the nervous system (Yamanaka et al., 2011). In *T. castaneum*, in situ hybridization studies reveal identical staining pattern for OKA and OKB precursors in brain and midgut, while immunohistochemistry assays suggest different pools of secretory vesicles for OKA and OKB in the same enteroendocrine cells (Jiang et al., 2015).

The expression of BgOKA and BgOKB was measured in the brain (BgOKA and BgOKB) and midgut (BgOKB) of adult females throughout the first gonadotrophic cycle. Brain BgOKA mRNA levels showed a tendency to increase during the cycle, reaching a peak of around 0.8 copies per copy of actin mRNA on day 7 (Fig. 2B), whereas those of BgOKB fluctuated around 0.02 copies, although showing the highest values on day 5 (Fig. 2C). In the midgut, BgOKB also showed an expression peak of ca. 0.02 copies on day 5 (Fig. 2C). Interestingly, on day 5 of adult female it is observed maximal vitellogenic activity (Martín et al., 1995).

3.3. Depletion of both BgOK types impairs oocyte growth and vitellogenin expression

In order to assess BgOKs function, the expression of both BgOK types was depleted using RNAi and a dsRNA spanning positions 1–224, a part of the sequence that is common to OKA and OKB precursors (dsOKs). A dose of 2 μg of dsOKs was injected into the abdomen of freshly emerged fifth (penultimate) instar female nymphs, and the same dose was injected again just after the next molt. Controls were equivalently treated with dsMock. The effect of dsOKs on transcript decrease was assessed by quantifying BgOKA in brain and BgOKB in brain and midgut in 5-day-old adult females. dsOKs treatment reduced the levels of brain BgOKA mRNA by 95%, whereas BgOKB brain and midgut mRNA levels were reduced by 98% and 95%, respectively (Fig. 3A). In addition, the length and total protein contents of basal oocytes in the ovarioles of dsOKs-treated animals were lower (23% and 60%, respectively, as average) than in controls (Fig. 3B). Vitellogenin (BgVg) mRNA levels in the fat body were also lower in dsOKs-treated animals (60% as average) than in controls (Fig. 3B).

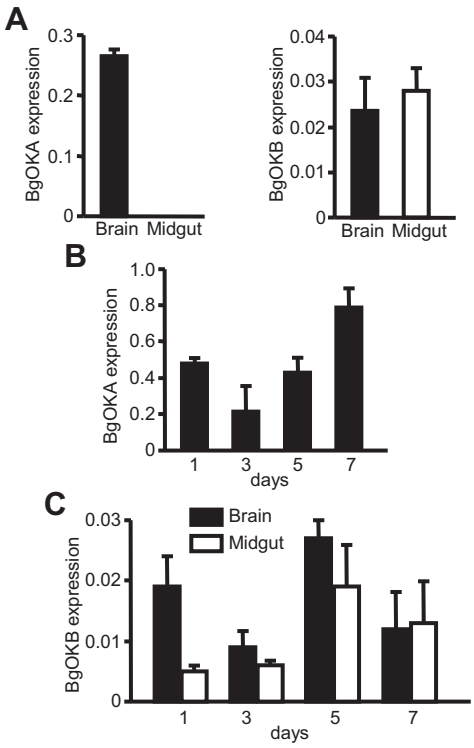

Fig. 2. BgOKA and BgOKB expression in the brain and midgut. (A) Expression levels of BgOKA and BgOKB in the brain and midgut of 5-day-old adult females ($n = 3$). (B and C) Expression profile through the first gonadotrophic cycle of BgOKA in brain (B) and BgOKB in brain and midgut (C) ($n = 3$). Results are expressed as the mean ± S.E. The *y*-axis represents copies per copy of BgActin 5C.

3.4. Depletion of both BgOK types does not affect JH production

In *B. germanica*, JH induces the expression of BgVg both in vivo (Comas et al., 1999) and in vitro (Comas et al., 2001). Thus, given that our experiments indicated that BgOKs knockdown affected vitellogenesis and oocyte growth, a possible hypothesis to explain these results is that they were due to problems related with JH production. Thus, we measured mRNA levels of two enzymes of the JH biosynthetic pathway in the corpora allata, namely HMG-CoA synthase-1 and JHAMT. Previous results had showed that mRNA levels of HMG-CoA synthase-1 were correlated with JH synthesis in *B. germanica* adult females (Maestro et al., 2009; Abrisqueta et al., 2014). Similarly, expression levels of JHAMT have also been correlated with JH synthesis in other species (Minakuchi et al., 2008; Rivera-Perez et al., 2014; Sheng et al., 2008). However, our results indicate that there are no significant differences in the mRNA levels of these enzymes between dsMock and dsOKs groups (Fig. 3C). Furthermore, on day 4 of adult life, we treated BgOK-depleted females with JH III diluted in acetone, and 24 h after the treatment, BgVg mRNA levels were measured in the fat body of dsMock-treated, dsOKs-treated plus acetone and dsOKs-treated plus JH. Results showed that JH treatment did not restore the reduced Vg expression in the dsBgOK-treated animals (Fig. 3D). These results suggest that the reduction on BgVg expression observed in dsOKs-treated females is not due to a reduction of JH levels.

3.5. Effects of BgOKA and BgOKB specific RNAi on vitellogenesis and basal oocyte growth

Once we analyzed the effects of the simultaneous depletion of both BgOK types on vitellogenesis and oocyte growth, we aimed at determining whether the effect observed was due to BgOKA or to BgOKB depletion. Thus, we used dsRNA fragments specific for

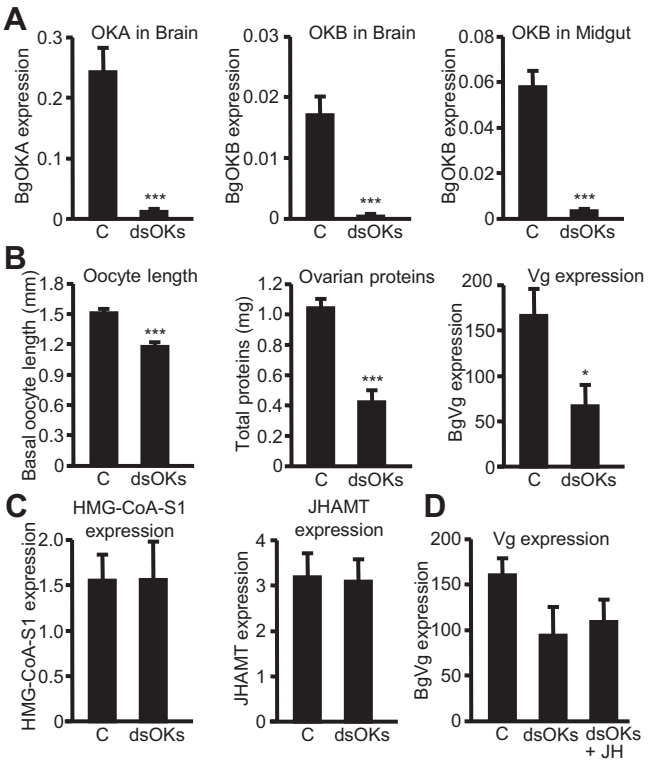

Fig. 3. Effect of BgOKs depletion on *B. germanica* vitellogenesis. dsRNA targeting a sequence common to BgOKA and BgOKB mRNAs (dsOKs) was injected on the first day of the penultimate (fifth) and last (sixth) nymph instars. Dissections were performed five days after the adult emergence. (A) BgOKA mRNA levels in brain and BgOKB mRNA levels in brain and midgut (n = 4–11). (B) Basal oocyte length, total ovarian proteins and vitellogenin (BgVg) expression in the fat body (n = 7–17). (C) HMG-CoA synthase 1 (HMG-CoA-S1) and juvenile hormone acid methyltransferase (JHAMT) mRNA levels in CA (n = 7–8). (D) Effect of the treatment of dsOKs females with juvenile hormone (JH) 24 h before the dissections (n = 6–7). C (control) corresponds to females treated with dsMock. Results are expressed as the mean ± S.E. In the expression studies, the y-axis represents copies per copy of BgActin 5C. Asterisks indicate significant differences (Student's t-test, $^{*}P < 0.05$; $^{***}P < 0.0001$).

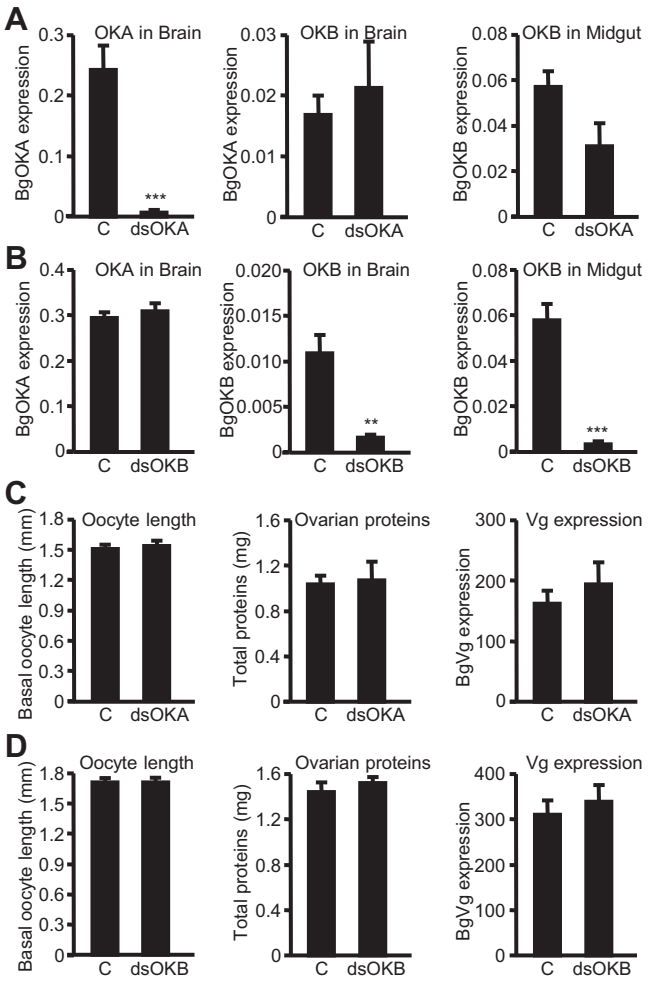

Fig. 4. Effect of specific depletion of OKA or OKB expression. dsRNA targeting a sequence specific for BgOKA (dsOKA) or BgOKB (dsOKB) was injected on the first day of the penultimate (fifth) and last (sixth) nymph instars. Dissections were performed five days after the adult emergence. (A) BgOKA mRNA levels in brain and BgOKB mRNA levels in brain and midgut in dsOKA-treated females. (B) BgOKA mRNA levels in brain and BgOKB mRNA levels in brain and midgut in dsOKB-treated females. (C) Basal oocyte length, total ovarian proteins and vitellogenin (BgVg) expression in the fat body of dsOKA-treated females. (D) Basal oocyte length, total ovarian proteins and vitellogenin (BgVg) expression in the fat body of dsOKB-treated females. C (control) corresponds to females treated with a heterologous dsRNA (dsMock) (n = 5–11). Results are expressed as the mean ± S.E. In the expression studies, the y-axis represents copies per copy of BgActin 5C. Asterisks indicate significant differences (Student's t-test, $^{**}P < 0.005$; $^{***}P < 0.001$).

each BgOKA and BgOKB precursor. A dose of 2 μg of the respective dsRNA (dsOKA or dsOKB) was injected into the abdomen of freshly emerged fifth (penultimate) instar female nymph, and the same treatment was repeated just after the next molt. Control specimens were treated with dsMock. RNAi specificity was assessed by quantifying BgOKA in brain and BgOKB in brain and midgut in 5-day-old adult females in dsOKA- and dsOKB-treated specimens. The treatment with dsOKA, dramatically reduced the levels of OKA mRNA in brain (97% reduction as average), but did not significantly affect those of OKB in brain and midgut (Fig. 4A). Conversely, the treatment with dsOKB, did not affect the levels of OKA mRNA in brain, but dramatically reduced those of OKB in brain and midgut (85% and 95% reduction as average, respectively) (Fig. 4B). In terms of phenotype, and quite unexpectedly, neither the specific depletion of BgOKA nor that of BgOKB affected basal oocyte growth, protein accumulation in the ovaries or transcription of BgVg in the fat body (Fig. 4C and D).

Different hypotheses can be proposed to explain these results. (i) we could consider the possibility that the factor operating in vitellogenesis would be comprised within the region common to both OKA and OKB precursors, but no significant motifs suggestive of special functions are present in this region, (ii) a synergistic action between OKA and OKB-type peptides could be responsible of the showed effect, and (iii) OKB-type orcokinins are responsible for the vitellogenic action, and the OKB type peptide encoded by the BgOKA precursor would play this action when BgOKB precursor is specifically depleted. Although BgOKA precursor only encodes for one putative OKB peptide, their expression levels are much higher than those of BgOKB precursor, which is in support to this hypothesis. In order to test this hypothesis we used BgOK-depleted adult females and treated them at day 2 and day 4 with 2.5 μg of synthetic OKA type peptide (NFDEIDRSGFNSFV) or OKB type peptide (ALDSIGGGNLV-NH_2) or an equal volume (2 μl) of the corresponding solvent (10% DMSO). Fat bodies from treated specimens were dissected one day after the second treatment, and BgVg mRNA levels were calculated. dsOKs treatment resulted in a reduction of basal oocyte length, measured at day 5, that was not be rescued neither with OKA nor with OKB treatments at days 2 and 4 (dsMock: 1.89 ± 0.05 mm (n = 8); dsOKs + 10% DMSO: 1.47 ± 0.18 mm (n = 6); dsOKs + OKA: 1.54 ± 0.12 mm (n = 7); dsOKs + OKB: 1.46 ± 0.07 (n = 8)). Nevertheless, OKB-treated females showed 22% and 27% higher BgVg mRNA levels than controls (solvent-treated) or OKA-treated females, respectively (Fig. 5), although differences were not statistically significant.

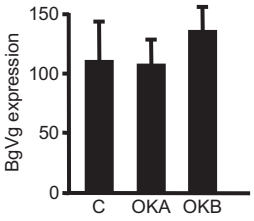

Fig. 5. Effect of OKA and OKB treatment on BgOK-depleted *B. germanica* females. dsRNA targeting a sequence common to BgOKA and BgOKB mRNAs (dsOKs) was injected on the first day of the penultimate (fifth) and last (sixth) nymph instars. Once they molted into adults, they were treated at day 2 and day 4 with 2.5 μg of synthetic OKA (OKA) or OKB (OKB) or an equal volume of the corresponding solvent (C: control). Dissections were performed one day later. Graph shows vitellogenin (BgVg) expression in the fat body ($n = 6–8$).

4. Conclusion

Before the present work, there were not many studies dealing with the function of orcokinins in insects. Previous work carried out in *B. mori* (Yamanaka et al., 2011) showed the presence of OKA in the neurons innervating the prothoracic gland (PG) as well as an ecdysteroidogenic effect of OKAs upon prothoracic glands incubated in vitro. In the cockroach *L. maderae*, previous studies localized OKA in the accessory medulla, a region located at the anterior base of the medulla in the brain (Hofer et al., 2005; Hofer and Homberg, 2006b). The accessory medulla acts as the circadian pacemaker controlling locomotor activity rhythms in this cockroach (Reischig and Stengl, 2003). Hofer and Homberg (2006a) suggest a role for OKA in light entrainment of the circadian clock of this cockroach. Jiang et al. (2015) propose for both OKA and OKB peptides from *T. castaneum* an awakening function involved in behavioral responses to stress. In this species, the only OKA peptide in the OKA precursor also shows some similarities to peptides of the OKB family (Jiang et al., 2015). The results presented herein, show that orcokinins contribute to modulate vitellogenin transcription in the fat body, an action that appears to be independent of JH. Although peptide treatments and the presence of a putative OKB-type peptide in the BgOKA may suggest that dsOKs phenotype could be due to OKB depletion, the possibility that peptides of both families were involved in vitellogenesis cannot be ruled out. Further experiments will be performed in order to unveil the mechanisms of OK action in the fat body.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at http://dx.doi.org/10.1016/j.jinsphys.2015.10.002.

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