Insect Biochemistry and Molecular Biology 139 (2021) 103676

Contents lists available at ScienceDirect

Insect Biochemistry and Molecular Biology

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

Orcokinin neuropeptides regulate reproduction in the fruit fly, *Drosophila melanogaster*

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ARTICLE INFO

Keywords:
Innate behaviors
Ecdysis
Sleep
Locomotor activity
Courtship
Oviposition

ABSTRACT

In animals, neuropeptidergic signaling is essential for the regulation of survival and reproduction. In insects, Orcokinins are poorly studied, despite their high level of conservation among different orders. In particular, there are currently no reports on the role of Orcokinins in the experimental insect model, the fruit fly, *Drosophila melanogaster*. In the present work, we made use of the genetic tools available in this species to investigate the role of Orcokinins in the regulation of different innate behaviors including ecdysis, sleep, locomotor activity, oviposition, and courtship. We found that RNAi-mediated knockdown of the *orcokinin* gene caused a disinhibition of male courtship behavior, including the occurrence of male to male courtship, which is rarely seen in wildtype flies. In addition, *orcokinin* gene silencing caused a reduction in egg production. Orcokinin is emerging as an important neuropeptide family in the regulation of the physiology of insects from different orders. In the case of the fruit fly, our results suggest an important role in reproductive success.

1. Introduction

Animal survival and reproduction depend on the adjustment of physiology and behavior to internal and environmental cues. Neural and neuroendocrine systems play a central role in the coordination of different stimuli in order to generate an adaptive response (Strand, 1999). Signaling molecules involved in physiological and behavioral coordination include neurotransmitters, which are involved in fast synaptic communication, and neurohormones, which are released into the extracellular medium and exert their actions via autocrine, paracrine, and endocrine signaling pathways (Nässel and Zandawala, 2019). Of special relevance among neurohormones are neuropeptides, which are encoded as pre-propeptide precursors and post-translationally modified to exert their role on physiology and behavior (Ons, 2017).

Neuroendocrinological research in insects has received much attention both for its relevance in basic insect science and because the neuroendocrine system has been proposed as a potential target for next-generation insecticidal agents for the control of harmful species (Audsley and Down, 2015; Verlinden et al., 2014). Hence, much physiological information exists for several neuropeptide systems in different species. However, the advent of "omic" technologies (proteomics, transcriptomics, and genomics) has led to the identification of neuropeptide and neuropeptide precursor genes in large databases, even before any physiological roles have been assigned to these molecules in any species. As a result, several neuroendocrine systems in insects and other invertebrates remain functionally uncharacterized.

A poorly studied invertebrate neuropeptide family is that of the Orcokinins (OKs), first discovered and characterized as myotropic

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https://doi.org/10.1016/j.ibmb.2021.103676
Received 21 September 2020; Received in revised form 25 October 2021; Accepted 28 October 2021
Available online 3 November 2021

peptides in the crustacean, *Orconectes limosus* (Stangier et al., 1992). In insects, OKs were detected for the first time in brain extracts from the cockroach, *Blattella germanica* (a species in which the corresponding OK peptide does not show myotropic activity; Pascual et al., 2004). In insects, but not in other invertebrates, two different transcripts, called *ok*-A and *ok*-B, are produced from the *orcokinin* gene (*ok*), each encoding a different family of conserved peptides (Sterkel et al., 2012). In the fruit fly, *Drosophila melanogaster*, a third isoform of *ok* is predicted to exist, which encodes OK-B-type mature neuropeptides (www.flybase.org). A conserved *ok* gene has been identified in all insect genomes sequenced to date; by contrast, the OK receptor has not yet been identified in any species (Nässel and Zandawala, 2019).

The effects of OKs are remarkably diverse and include (*in vitro*) stimulation of prothoracicotropic activity, regulation of pigmentation in the lepidopteran *Bombyx mori* (Wang et al., 2019; Yamanaka et al., 2011), and stimulation of oogenesis in *B. germanica* (Ons et al., 2015). OKs have also been implicated in the control of innate behaviors, such as ecdysis in *Rhodnius prolixus* (Wulff et al., 2017, 2018), circadian activity in the cockroach *Leucophaea maderae* (Hofer and Homberg, 2006), and escape responses in *Tribolium castaneum* (Jiang et al., 2015). Although *Drosophila* is an experimentally powerful organism for genetic, behavioral, and physiological studies, largely due to the number of genetic tools available for the species, there are currently no reports on the role of OKs in this species, even though their expression pattern in the nervous system and the midgut has been reported (Chen et al., 2015; Veenstra and Ida, 2014). In this context, we investigated in *Drosophila* the role of OKs in the regulation of several innate behaviors including, ecdysis, courtship, circadian rhythmicity, locomotor activity, sleep, and oviposition. We found that RNAi-mediated knockdown of *ok* caused marked disinhibition of male courtship behavior, including the expression of courtship towards other males, which is rarely observed between wildtype males. We also detected a significant reduction in egg production. By contrast, we found that *ok* knockdown did not cause consistent changes to the timing of ecdysis, to the levels or the circadian rhythmicity of locomotor activity or to sleep behavior. Taken together, these results suggest that OKs modulate behavioral and physiological events related to reproductive success in *Drosophila*.

2. Materials and methods

2.1. Fly strains and genetics

Flies stocks were maintained at room temperature (22–25 °C) on standard agar/cornmeal/yeast media. The GAL4 drivers for the *orcokinin* gene (*ok*; CG1565) used here, *ok*-GAL4-4 (here named *ok1*-GAL4) and *ok*-GAL4-5 (here named *ok2*-GAL4) were characterized in Chen et al. (2015), and were kindly provided by Christian Wegener (U. Würzburg, Germany). The following UAS-*ok* RNAi lines were obtained from the Vienna *Drosophila* RNAi Center (VDRC; Vienna, Austria): VDRC #106882 (called here RNAi-A1), which targets the *ok*-A isoform; and VDRC #12876 (called here RNAi-A + B), which targets all known isoforms of the gene. In addition, line #61833 (called here RNAi-A2), which targets only the *ok*-A isoform, was obtained from the Bloomington *Drosophila* Stock Center. Wildtype Canton-S (CS), *white*^{1118} (*w*^{1118}), UAS-mCD8GFP, and UAS-*dicer2* (UAS-*dcr2* for short) were also obtained from the Bloomington *Drosophila* Stock Center. UAS-*dcr2* was always co-expressed with the RNAi transgenes in order to boost the effectiveness of RNAi knockdown.

2.2. qPCR

Total RNA was extracted from adult flies (5-days-old) using Trizol reagent (Invitrogen, Carlsbad, CA, USA) following manufacturer's instructions and resuspended in 20 μL RNAse free water. cDNA synthesis then was performed as described in Selcho et al. (2017). RNA expression was quantified by qPCR using a Strategene Mx3000P Real-Time qPCR System (Agilent Technologies) and Maxima SYBR Green/ROX qPCR Master Mix (Thermo Fisher Scientific). Primers used showed >97% efficiency and were: for *okA*: okA_Fwd GGCTCGAAGAAGTACGATGG and okA_Rev GCTTACTGCTCCAGGTGTCC; for *okB*: okB_Fwd CGAACTGCTGGATGGAAAAT and okB_Rev GGGCTCTTGTTTTTGGACAG; *rp49* was used as reference gene; primers used were: rp49_Fwd AGCATACAGGCCCAAGATCG and rp49_Rev TGTTGTCGATACCCTTGGGC). All analyses were carried out using MxPro QPCR Software (Agilent, Santa Clara, CA, USA). Three to 4 independently isolated cDNAs were used, and each cDNA was quantified in triplicate.

2.3. Pupal ecdysis

Animals 6–10 h after pupariation were examined and those containing a bubble in the mid region of the puparium (late stage p4(i); Bainbridge and Bownes, 1981) were selected, placed dorsal side up on a microscope slide, and video-recorded at room temperature (ca. 22 °C) under dim transmitted light using a Leica dissecting microscope. Experimental and control animals were recorded simultaneously on the same slide. Videos were then scored noting the start and end of both pre-ecdysis and ecdysis, as previously described (Lahr et al., 2012). Scoring was done blind with respect to genotype.

2.4. Courtship behavior

Courtship assays were performed as described in McBride et al. (1999) using standard courtship chambers in a humidified room kept at ca. 22 °C and illuminated with dim light. One experimental 5-day-old virgin male was carefully aspirated into the arena and left undisturbed for 5 min. For assays testing courtship towards a female target, a 5-day--old wildtype (Canton-S: CS) virgin female fly was then introduced into the arena. Two couples (in separate courtship chambers), one experimental and one control, were recorded at the same time. Videos were then scored noting the duration of the different courtship elements starting with the orientation of the male towards the female, and including: following, tapping, wing extension, licking, attempted copulation (bending of abdomen), and copulation. For assays testing courtship towards a male target, a decapitated 5-day-old virgin male was introduced into the arena with the tester male (decapitated flies are used in these assays in order to avoid interference from the behavior of the target fly; Iftikhar et al., 2019; Liu et al., 2008). The target male was either a wildtype CS fly, or was of the same genotype as that of the tester male. For preference experiments, a decapitated pair consisting of a 5-day-old virgin male and female was simultaneously introduced into the arena with the tester male. For all tests, a courtship index (CI) was calculated, which is the percentage of time spent courting a subject during a 10 min test session, or until copulation, whichever occurred first. Successful copulation was recorded as 1 if copulation occurred during the 10 min session and 0 if it did not.

2.5. Locomotor activity rhythms and levels

One to 3 day-old adult flies were entrained to a 12L:12D light:dark regime (12L:12D) for 3 days at 25 °C, placed individually in Trikinetics monitors (Trikinetics, USA), and their activity measured every 30 min for 7–10 days under conditions of constant darkness (DD). Resulting records were analyzed using a Matlab-based analysis software package (Levine et al., 2002). The strength of rhythmicity was quantified using the rhythmicity index (RI) derived from autocorrelation analysis. By this measure, records are considered rhythmic when RI is greater than 0.3, weakly rhythmic for RI values in the range of 0.1–0.3, and arrhythmic when RI is less than 0.1 (or when record is obviously aperiodic) (Sundram et al., 2012).

In order to measure the levels of locomotor activity, readings were made every minute for 3 days under 12L:12D conditions. From these records we derived the total activity during the day and the night, as

well as a measure of the instantaneous level of activity, based on the number of beam crossings per minute.

2.6. Sleep

Drosophila sleep is defined as periods of 5 or more minutes of quiescence (Shaw et al., 2000). Sleep was measured at 25 °C using the same monitors used for locomotor activity except that readings were made every minute. Sleep profiles (amount of sleep per 30 min), total amount of sleep, and number of sleep episodes and their length, were derived from records collected over 3 consecutive days using the Matlab-based SCAMP software package (Donelson et al., 2012). Flies that had sleep episodes longer than 350s were discarded (<2.5% of the population).

2.7. Oviposition

Three virgin females of the genotype of interest and 4 wildtype (*Canton-S*) males were placed in a standard food vial. Twenty four hours later they were transferred to a new vial and the old one discarded. They were then transferred daily to a new vial and the eggs laid during each 24h period were counted for 4 consecutive days. On days 13 the flies were again transferred daily to a new vial and the number of eggs laid on day 15 were counted, to serve as an end point of the experiment. Nine to 11 independent vials were analyzed per genotype.

2.8. Histochemistry

The central nervous system (CNS) and gut from *ok*-GAL4>UAS-*mcd8GFP* adults were dissected in cold PBS (phosphate-buffered saline) and fixed for 1 h in 4% paraformaldehyde at room temperature (RT). Fixed tissues were rinsed 3 × 10 min in PBS at RT and mounted in Fluoromount-G mounting medium (SouthernBiotech) on poly-lysine coated coverslips. Images were obtained using a Spinning disk microscope (Olympus DSU) and analyzed using ImageJ (Schindelin et al., 2009).

2.9. Statistics

If data were normally distributed and had equal variance they were analyzed by one-way ANOVA followed by Tukey (for comparisons between all genotypes) or Dunnett (for comparisons to control) *post hoc* multiple comparison analyses. Otherwise Kruskal Wallis followed by Dunn test for multiple comparison analyses was used. Contingency tables were analyzed using Chi-square tests. Locomotor activity and sleep during day and night were analyzed by ordinary or repeated measurement (RM) two-way ANOVA followed by Tukey analyses for multiple comparisons. The exact values for each comparison are shown in Supplementary Table S1.

3. Results

3.1. Validation of GAL4 drivers and UAS-RNAi lines

Given that the extent of knockdown effected by the different *ok*-RNAi lines used here has not been documented, we first measured the changes in *ok* transcript levels caused by expression of the UAS-*ok* RNAi transgenes used, named here RNAi-A1 and RNAi-A2 (both of which target the *ok*-A isoform) and RNAi-A + B (which targets all known transcripts of the gene). For this, we used two different *ok*-Gal4 drivers, named here *ok1*-GAL4 and *ok2*-GAL4 (see Materials and Methods for details on these lines). Both UAS-RNAi-A1 and UAS-RNAi-A2 caused close to a 95% reduction in the levels of the *ok*-A transcript (Fig. 1A) when driven using either GAL4 driver. These transgenes caused around 40% reduction in the levels of the *ok*-B transcript using *ok1*-GAL4 driver; however, no statistically significant knockdown was observed using *ok*2-GAL4 (Fig. 1B). Surprisingly, UAS-RNAi-A + B did not cause appreciable changes to the levels of the *ok*-A transcript (despite being statistically different from control; Fig. 1A) and caused a statistically significant reduction to the levels of the *ok*-B transcripts using only the *ok*1-GAL4 driver (Fig. 1B). Nevertheless, we included here the results obtained using the UAS-RNAi-A + B transgene because in the case of courtship and oviposition it caused the same changes as those observed using the UAS-RNAi-A1 and UAS-RNAi-A2 constructs (see below).

We also examined the pattern of expression driven by the *ok1*-GAL4 and *ok2*-GAL4 drivers in the adult CNS using a GFP reporter (Supplementary Fig. S1). Consistent with the results reported by Chen et al. (2015), we found that both drivers caused reporter expression in large bilateral neurons that project extensively throughout the protocerebrum (called orcoA-PLP neurons by Chen et al., 2015), as well as in four accessory medulla neurons (called orcoA-AME neurons by Chen et al., 2015). In the adult ventral nervous system (VNS), both *ok1*-GAL4 and *ok2*-GAL4 drove reporter expression in five cell bodies (whereas Chen et al., 2015 reported two pairs of neurons expressing OK-A, but in a similar location). Finally, both *ok1*-GAL4 and *ok2*-GAL4 drivers drove reporter expression in cells of the anterior midgut, as previously reported.

3.2. Pupal ecdysis

Knockdown of *ok* expression using the different UAS-*ok*-RNAi transgenes and the two GAL4 drivers did not cause consistent changes to the duration of the pre-ecdysis phase. Indeed, whereas a significant shortening in the duration of pre-ecdysis was observed when the UAS-*ok*-RNAi-A2 transgene was expressed using either *ok* GAL4 driver (Fig. 2A–B), no such shortening was observed using the UAS-*ok*-RNAi-

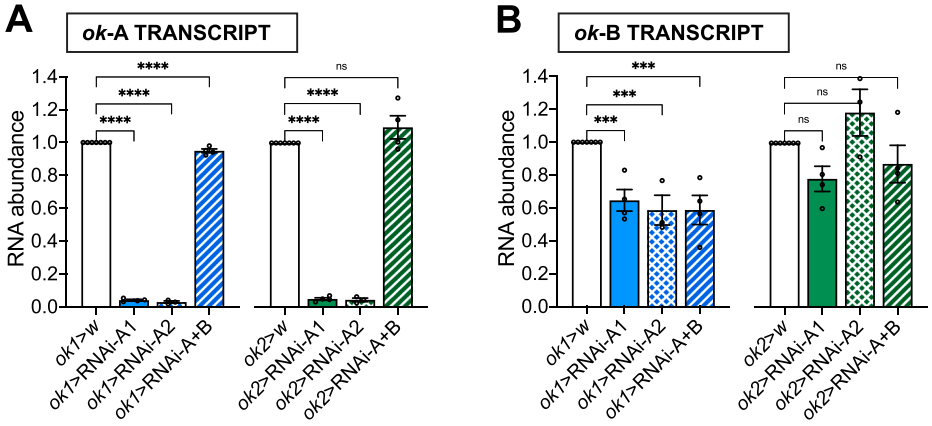

Fig. 1. Knockdown efficiency of RNAi transgenes. Levels of *ok*-A (A) and *ok*-B (B) transcripts in 5-day-old adult flies, relative to those measured in controls (white bars); genotypes are indicated along the X-axes. N = 3–4 independent biological replicates were quantified in triplicate for each group. The number of flies used per replicate ranged between 18 and 24 per genotype. ns = non-significant, * = $p < 0.0332$; ** = $p < 0.0021$; *** = $p < 0.0002$; **** = $p < 0.0001$; ordinary one-way ANOVA followed by Dunnett *post-hoc* analyses. See Supplementary Table S1 for exact values for all statistical analyses.

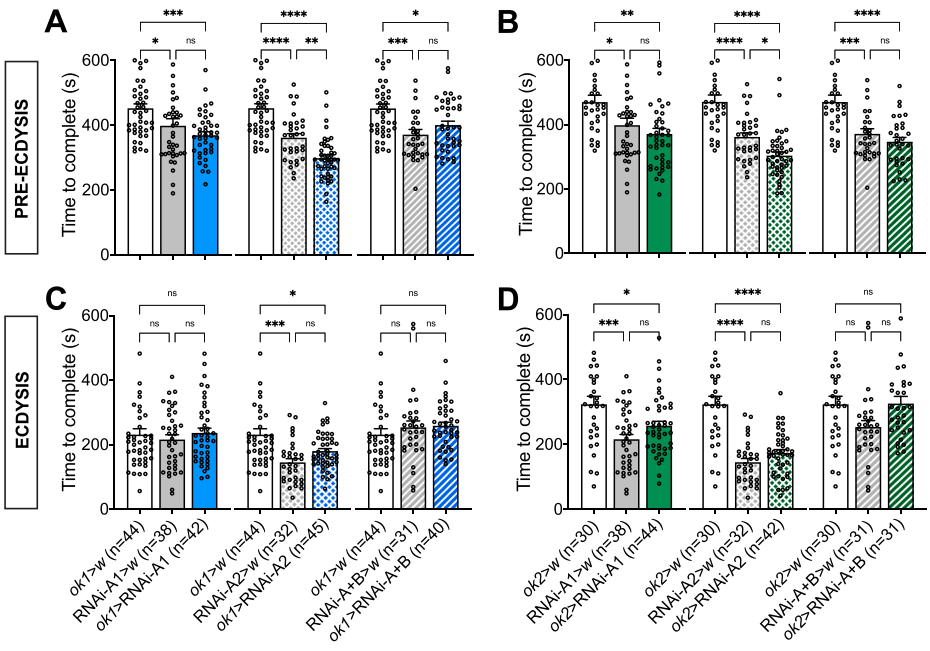

Fig. 2. Effect of *ok* knockdown on the duration of ecdysis behaviors. Duration of pre-ecdysis (A and B) and ecdysis (C and D) when *ok* was knocked down using the *ok1*-GAL4 (A and C) and *ok2*-GAL4 (B and D) drivers. Times are averages ±SEM. ns = non-significant, * = $p < 0.0332$; ** = $p < 0.0021$; *** = $p < 0.0002$; **** = $p < 0.0001$; ordinary one-way ANOVA followed by Tukey *post-hoc* analyses. The number of flies tested is indicated in parenthesis. See Supplementary Table S1 for exact values for all statistical analyses.

A1 or the UAS-*ok*-RNAi-A + B (Fig. 2A–B) transgenes. No effects of *ok* knockdown were observed on the duration of the ecdysis phase of the ecdysial sequence with any of the UAS-*ok*-RNAi lines tested (Fig. 2C–D). In addition, both pre-ecdysis and ecdysis behaviors appeared normal, and both females and males bearing *ok* knockdown emerged as adults without noticeable defects.

3.3. Locomotor activity and sleep

We then examined the consequences of *ok* knockdown on locomotor activity. As shown in Fig. 3 A, C and quantified in Fig. 3 B, D, the effect of *ok* knockdown was not consistent among the different genotypes, and different conclusions could sometimes be drawn depending on the control genotype used for comparison. Thus, whereas expression of UAS-

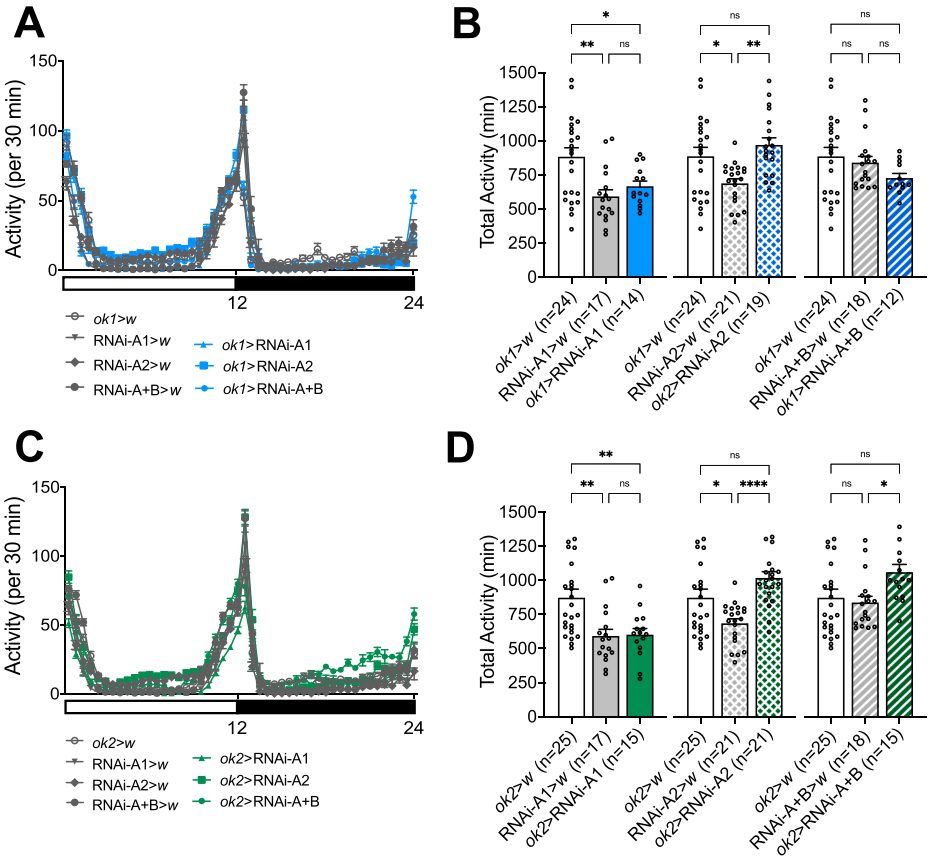

Fig. 3. Effect of *OK* knockdown on the daily pattern of locomotor activity. (A and C) Profile of locomotor activity under 12h:12 h L:D regime in flies bearing *ok* knockdown using *ok1*-GAL4 (A) and *ok2*-GAL4 (C) drivers. (B and D) Corresponding total activity (±SEM) under 12h:12 h L:D regime in flies bearing *ok* knockdown using *ok1*-GAL4 (B) or *ok2*-GAL4 (D) drivers. ns = non-significant, * = $p < 0.0332$; ** = $p < 0.0021$; *** = $p < 0.0002$; **** = $p < 0.0001$; ordinary one-way ANOVA and Tukey *post-hoc* analyses. The number of flies tested is indicated in parentheses. See Supplementary Table S1 for exact values for all statistical analyses.

ok-RNAi-A2 caused a small but significant increase in total activity using both GAL4 drivers, this increase was not observed with UAS-*ok*-RNAi-A1. Knockdown using UAS-*ok*-RNAi-A + B also caused a significant increase in total activity, but only using the *ok2*-GAL4 driver. Similarly, no consistent effect on locomotor activity speed was observed. Indeed, whereas UAS-*ok*-RNAi-A1 and UAS-*ok*-RNAi-A + B reduced the speed of nighttime activity, UAS-*ok*-RNAi-A2 increased the speed of both daytime and nighttime locomotor activity (Supplementary Fig. S2). We also investigated whether *ok* might regulate the circadian rhythmicity of locomotor activity. As shown in Fig. 4 A and quantified in Fig. 4 B–C, knockdown of *ok* did not consistently affect the periodicity or the strength of this circadian rhythm.

Drosophila sleep is operationally defined as an episode of inactivity lasting at least 5 min (Shaw et al., 2000), and shares the core clinical features of mammalian sleep (Beckwith and French, 2019). No consistent effects of *ok* knockdown were observed on total sleep (Fig. 5A–D), nor when daytime and nighttime sleep were quantified separately (considering number and duration of sleep episodes; Supplementary Fig. S3).

3.4. Courtship behavior

We next tested the involvement of *ok* in the control of courtship behavior and copulation. No consistent effects of *ok* knockdown were observed in courtship latency, courtship index, or in the percentage of males performing different courtship actions when a wildtype male was tested with an experimental female (Fig. 6 A-C for *ok1*-GAL4 and Supplementary Fig. S4 A-C for *ok2*-GAL4). By contrast, when an experimental male was tested with a wildtype female (Fig. 6 D-F for *ok1*-GAL4 and Supplementary Fig. S4 D-F for *ok2*-GAL4), a tendency to a shorter courtship latency was observed; this tendency was accentuated when both male and female animals were experimental (Fig. 6 G-I for *ok1*-GAL4 and Supplementary Fig. S4 G-I for *ok2*-GAL4); in both cases this tendency reached statistical significance for several *ok* knockdown genotypes. By contrast, the percentage of males performing the different elements of the courtship sequence did not differ among experimental groups.

Given that the results suggested a possible role of *ok* in courtship inhibition, we then assayed male-male courtship, which is a behavior

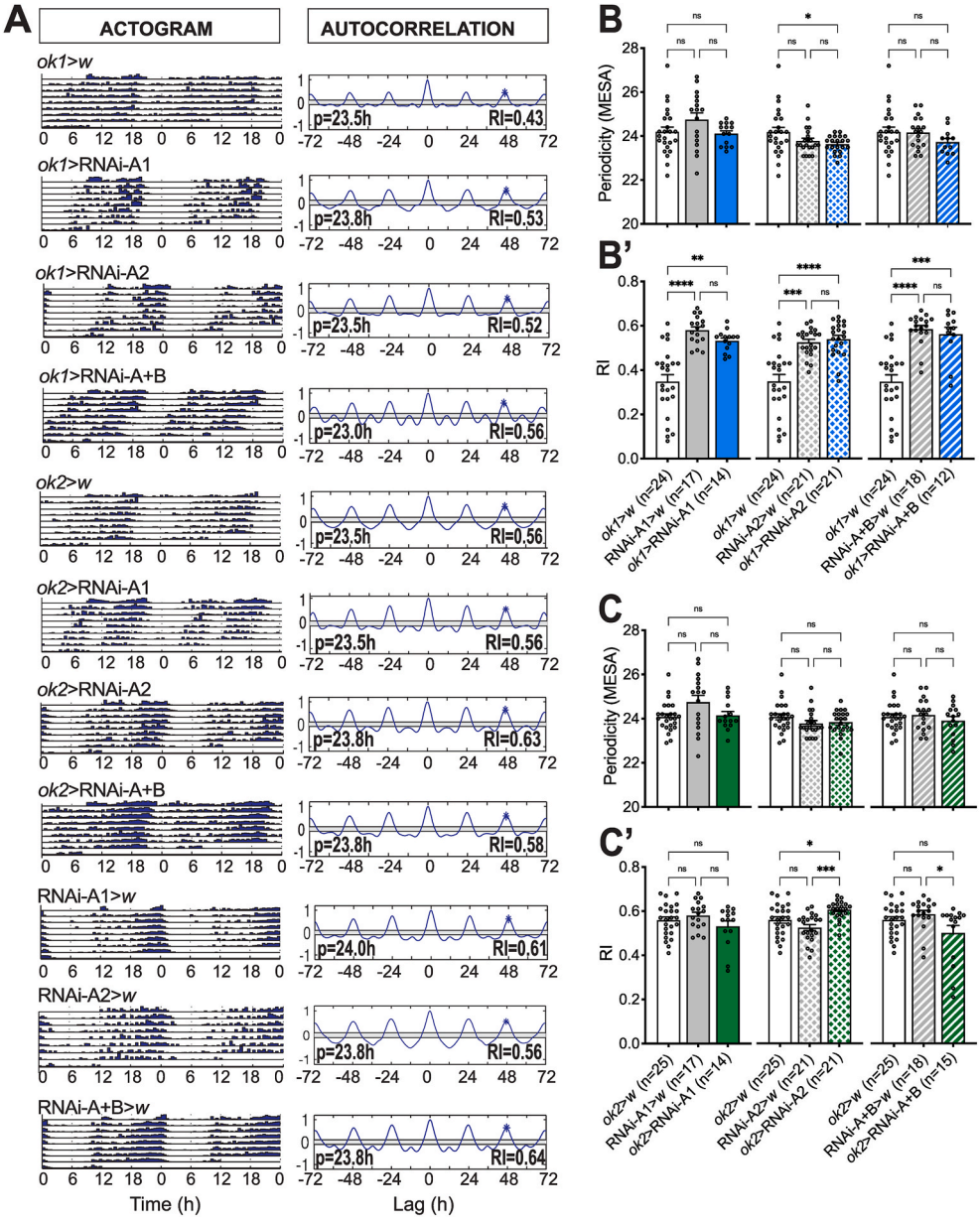

Fig. 4. Circadian rhythmicity of locomotor activity was not affected by *ok* knockdown. (A) Actograms (left panels) and corresponding autocorrelograms (right panels) of flies bearing *ok* knockdown using *ok1*-GAL4 and *ok2*-GAL4 GAL4 drivers. (B, B′ and C, C′) Corresponding average periodicity (±SEM) obtained from MESA analyses (B, C), and rhythmicity index (±SEM) (B′, C′) using the *ok1*-GAL4 (B, B′) and *ok2*-GAL4 drivers (C, C′). ns = non-significant, * = $p < 0.0332$; ** = $p < 0.0021$; *** = $p < 0.0002$; **** = $p < 0.0001$; ordinary one-way ANOVA followed by Tukey multiple comparison analyses. The number of flies tested is indicated in parenthesis. See Supplementary Table S1 for exact values for all statistical analyses.

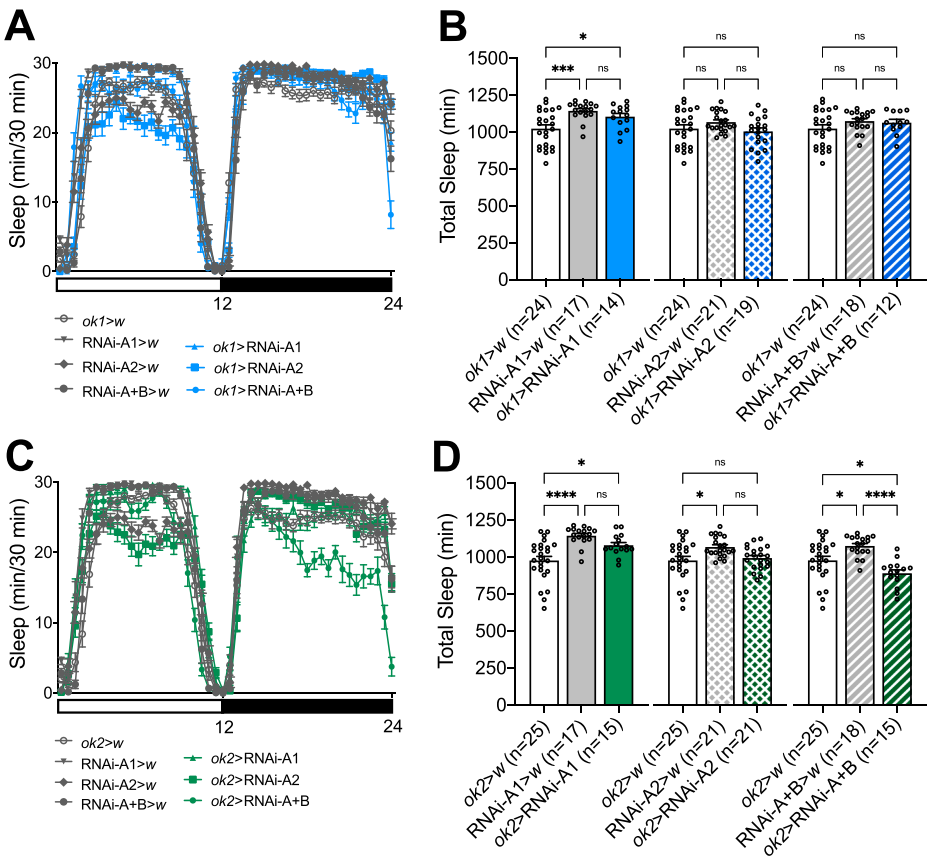

Fig. 5. Sleep behavior after *ok* knockdown. (A and C) Sleep profiles under 12h:12 h L:D regime when *ok* was knocked down using *ok1*-GAL4 (A) or *ok2*-GAL4 (C) drivers; the corresponding total sleep (±SEM) was quantified in B and D. The number of flies tested is indicated in parenthesis. ns = non-significant, * = $p < 0.0332$; ** = $p < 0.0021$; *** = $p < 0.0002$; **** = $p < 0.0001$; ordinary one-way ANOVA followed by Tukey *post-hoc* analyses. See Supplementary Table S1 for exact values for all statistical analyses.

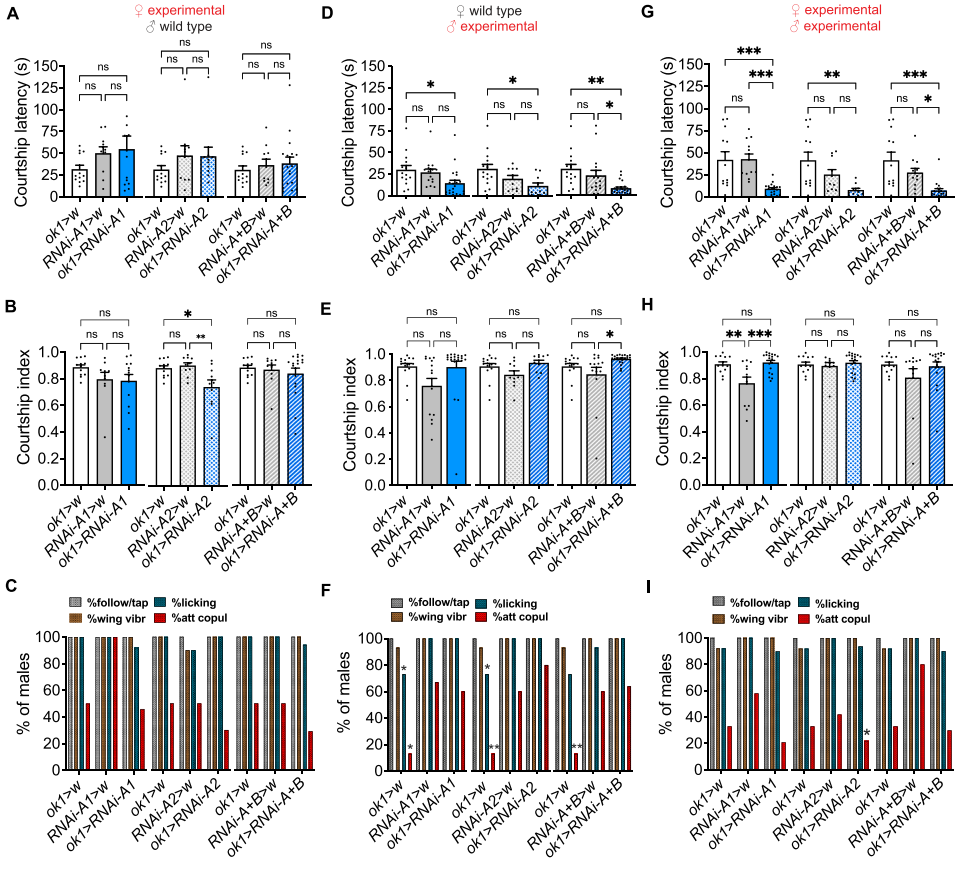

Fig. 6. Courtship behavior of males towards females after *ok* knockdown. Courtship latency (A, D and G), courtship index (B, E and H), and percentage of males performing different steps of the courtship sequence (C, F and I) when paired with females. Pairs consisted of a wildtype male with an experimental female (A to C); an experimental male with a wildtype female (D to F); and an experimental male with an experimental female (G to I). The results obtained using the *ok1*-GAL4 driver are shown here; results obtained using *ok2*-GAL4 driver are shown in Supplementary Fig. S4. The number of pairs of flies used ranged between eight and twenty-two per genotype. Values indicated in A, B, D, E, G, and H are averages ±SEM. ns = non-significant, * = $p < 0.0332$; ** = $p < 0.0021$; *** = $p < 0.0002$; **** = $p < 0.0001$; ordinary one-way ANOVA followed by Tukey *post-hoc* analyses. For figures C, F and I color bar represents the percentage of males that execute following/tapping (gray box), wing vibration (brown box), licking (calypso box) and attempted copulation (red box) behavior. Contingency tables were analyzed using Chi-square test, * = $p < 0.05$; ** = $p < 0.01$. See Supplementary Table S1 for exact values for all statistical analyses (except for data shown in panels C, F and I, for which values were 100% for all genotypes). (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

that does not usually occur between wildtype male flies. We found a highly significant increase in the levels of courtship towards wildtype males by males bearing *ok* knockdown with any UAS-*ok*-RNAi and *ok*-GAL4 driver (Fig. 7 A and D). This increased male to male courtship was also observed when both males were of the same *ok* knockdown genotypes (Fig. 7 B and E). Despite the occurrence of male to male courtship, preference tests showed that *ok* knockdown males preferred courting a female vs. a male, regardless of the genotype of the tester flies (Fig. 7 C and F).

3.5. Oviposition

We evaluated daily egg laying by females bearing *ok* knockdown mated to wildtype (CS) male flies first during days 1–4 and then on day 15. We observed a consistent reduction in egg production during the initial period for all knockdown genotypes (Fig. 8 A, C). Nevertheless, the average number of eggs laid during the first 4 days was significantly different from controls only in females for which UAS-*ok*-RNAi-A1 was driven by the *ok2*-GAL4 driver and when UAS-*ok*-RNAi-A + B was driven by either *ok1*-GAL4 or *ok2*-GAL4 drivers (Fig. 8 B, D). The tendency for knockdown females to lay fewer eggs was no longer observed by 15 days (Fig. 8 A, C), suggesting a slower maturation. No differences were observed in the percentage of egg hatching among the different genotypes (not shown).

4. Discussion

Here we investigated the involvement of the Orcokinin neuropeptides (OKs) in the regulation of different *Drosophila* behaviors: ecdysis, locomotor activity and its circadian control, and sleep. We also studied their involvement in sex-specific behaviors including courtship and egg-laying. Overall, we did not observe consistent changes caused by the knockdown of the *orcokinin* gene, with the exception of courtship, where *orcokinin* knockdown males tended to show a reduction in courtship latency toward either a wildtype or an *orcokinin* knockdown female partner. Most interestingly, we observed robust courtship of knockdown males toward other males (wildtype or *orcokinin* knockdown), a behavior that is rarely observed between mature wildtype males; nevertheless, sexual preference of males toward females was not affected. Overall, these results suggest that OKs may play an inhibitory role in *Drosophila* courtship behavior. In addition, females expressing *orcokinin* gene knockdown tended to lay fewer eggs, but only during the first days after mating. Interestingly, defects in oogenesis were also reported for the cockroach *B. germanica* expressing low BlageOK levels (Ons et al., 2015), indicating that this could be a conserved function in insects.

The results obtained here are reminiscent of those reported for ETH, a neuropeptide centrally involved in ecdysis regulation in insects, including *Drosophila* (Zitňan and Adams, 2012), and recently shown to also regulate male-male courtship as well as reproductive development (Deshpande et al., 2018; Meiselman et al., 2017). One possibility for explaining the role of OKs in courtship is that these peptides could act as additional ligands of the ETH receptor. Nevertheless, *in vitro* receptor activity assays did not support this hypothesis (P. Taghert, personal communication). The effects of OK knockdown on courtship are also similar to those reported for ecdysone receptor mutants, which show elevated male-male courtship behavior (Ganter et al., 2007; Schwedes and Carney, 2012). Considering that in *B. mori* OK peptides stimulate the production of ecdysteroids *in vitro*, it is possible that the increase in male-male courtship activity in *ok* knockdown flies could, at least in part, be due to a deficit in ecdysone signaling. In this regard, a possible point of convergence might be the P1 group of *fruitless* central brain neurons, which play a key role in regulating male courtship (Pan et al., 2012). Indeed, the ecdysone receptor isoform, EcR-A, is required in P1 neurons for male courtship and for the establishment of male-specific neuronal architecture (Dalton et al., 2009). It has also been reported that P1 neurons regulate sleep and courtship in a hierarchical manner, with moderate activation of these neurons inhibiting sleep, whereas stronger activation causes an increase in sleep and promotes courtship (Zhang et al., 2018). According to Pan et al. (2012), P1 male-specific neurons that project across the anterior dorsal commissure of the brain would be responsible for motion-based courtship behavior. It is

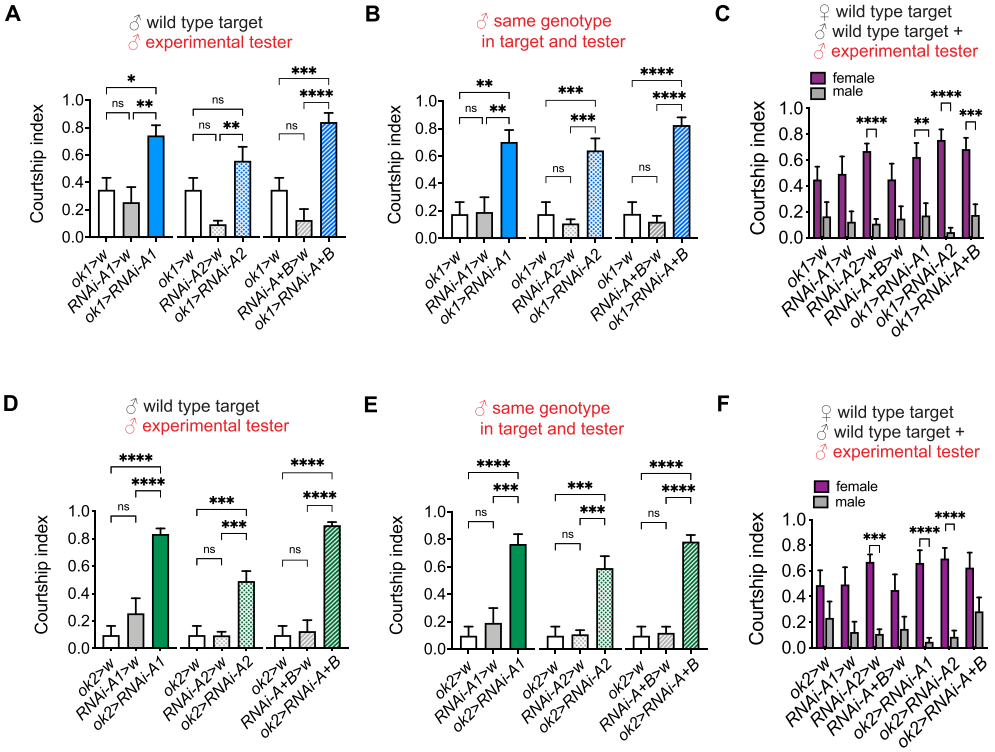

Fig. 7. Courtship behavior of a male towards another male was disinhibited by *ok* knockdown without affecting sexual preference. Courtship index for male bearing *ok* knockdown towards: a CS male (A and D) and towards a male of the same *ok* knockdown genotype (B and E). (C and F) Preference of male bearing *ok* knockdown toward a pair consisting of a wildtype (CS) female (indicated in purple) and a wildtype (CS) male (indicated in gray). The number of pairs of males used ranged between eight and eighteen per genotype. ns = non-significant, * = $p < 0.0332$; ** = $p < 0.0021$; *** = $p < 0.0002$; **** = $p < 0.0001$; ordinary one-way ANOVA followed by Tukey *post-hoc* analyses (A, B, D and E) and Sidák's multiple comparisons test (C and F). The exact values for each comparison are shown in Supplementary Table S1. (For interpretation of the references to color in this figure legend, the reader is referred to the Web version of this article.)

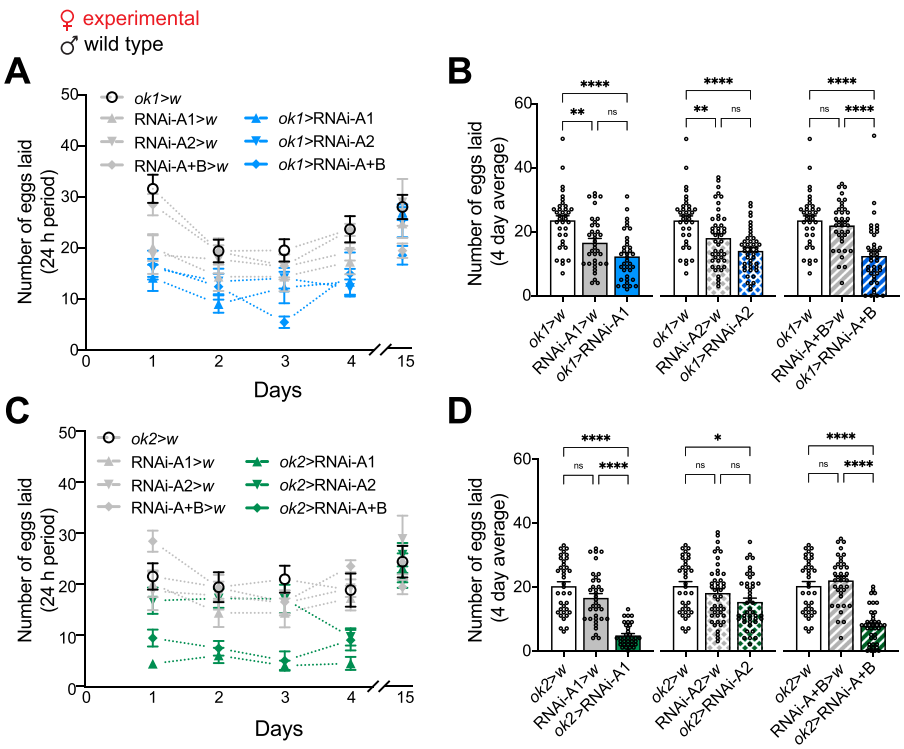

Fig. 8. Effect of *ok* knockdown on the number of eggs laid by mated females. Number of eggs laid daily by females during days 1–4 and on day 15 after mating (A, C) when *ok* was knocked down using *ok1*-GAL4 (upper panel) or *ok2*-GAL4 (lower panel). Mean (±SEM) of eggs laid during 24 h during days 1–4 (B, D). Twenty-seven to 33 females were tested per condition. ns = non-significant, * = $p < 0.0332$; ** = $p < 0.0021$; *** = $p < 0.0002$; **** = $p < 0.0001$; ordinary one-way ANOVA followed by Tukey *post-hoc* analyses. See Supplementary Table S1 for exact values for all statistical analyses.

thus possible to envisage that orcoA-PLP and orcoA-AME neurons, which arborize profusely in the protocerebrum, could be (directly or indirectly) inhibiting P1 neurons when two male flies meet. Direct connections between OK and P1 neurons could be explored in *Drosophila* using GRASP (GFP Reconstitution Across Synaptic Partners), and functional connections between these OK neurons and P1 neurons could be investigated by recording neuronal activation of P1 neurons following stimulation of OK neurons using chromogenetic or thermogenetic approaches. Neuronal activity in some fruM or DOUBLESEX circuitries in the ventral nerve cord (VNC) is sufficient to induce courtship behaviors in headless males (Pan et al., 2012) so *ok*-expressing neurons in the VNC might also play a role in the regulation of courtship toward males.

The effects of *ok* knockdown on locomotor activity and sleep were small and inconsistent among the different genotypes used. Likewise, no change to the circadian rhythmicity of locomotor activity was observed in flies bearing *ok* knockdown. This result contrast with those obtained in the cockroach *L. maderae*, where injection of synthetic OK-A into the accessory medulla (the location of the master circadian clock in the insect brain) during the active period (subjective night), but not during the inactive phase, caused a time-dependent phase shift in circadian activity (Hofer and Homberg, 2006).

We found that both male and female expressing lower levels of *ok* successfully reached the adult stage, without showing any visible defects. We observed a slight shortening in the duration of the pupal pre-ecdysis phase of the ecdysial sequence, although this effect was not consistent across all *ok* knockdown genotypes. Similarly, the duration of the ecdysis phase was not consistently altered following these manipulations. Overall, these results differ significantly from those observed in the hemimetabolous model, *R. prolixus* (Wulff et al., 2017, 2018), where knockdown of RhoprOK completely blocked the expression of ecdysis. Thus, our results suggest that OKs' role in ecdysis is not conserved in these two species from different orders; it will be interesting to determine whether this is due to their differing post-embryonic development strategies (holo-vs. hemimetabolous).

Our results do not allow us to determine whether the A, the B or both the A and the B products of the *orcokinin* gene are involved in the control of the courtship and egg-laying defects observed following *orcokinin* knockdown because the efficiency of gene knockdown was not consistent and did not correlate with the severity of the defects observed. This was especially apparent for the effect on male-male courtship, which was increased significantly for all knockdown genotypes compared to the relevant controls, including for those bearing the UAS-*ok*-RNAi-A + B transgene, which, nevertheless, appears to cause little if any reduction in *orcokinin* transcript levels. We do not understand the bases for such inconsistencies; they could, for example, reflect the occurrence of effective knockdown during a critical developmental period. Regardless of their cause, the exact roles of OK peptides in the control of behavior merits further investigation using tools that allow for the complete knockout of either or both transcripts (e.g. Diao et al., 2015; Port et al., 2020).

Regarding neuropeptides in insects, OK is emerging as an important family in the regulation of vital processes, such as reproduction and post-embryonic development. Hence, its use as a target for next-generation insecticides to control harmful species could be further explored. More studies will be necessary to reveal the molecular mechanisms and neuronal circuits involved in OK signaling. In particular, efforts towards the identification of OK receptor in insects will be crucial in order to fully understand the roles of OK peptides in the regulation of insect physiology. To our knowledge, this is the first report on the role of OK neuropeptides in *Drosophila*.

Declaration of interest

The authors declare no conflict of interest.

5. Author contributions

Conceptualization VS, AP-M, JE, SO; Data curation VS, AP-M, MV, JE, SO; Formal analysis VS, AP-M, MV, JE, SO; Funding acquisition JE, SO; Investigation VS, AP-M, JE, SO; Methodology VS, AP-M, JE, SO; Project administration JE, SO; Resources JE, SO; Software JE, SO; Supervision JE, SO; Validation VS, AP-M, MV, LF, JE, SO; Visualization VS,

AP-M, MV; Roles/Writing - original draft JE, SO; Writing - review & editing VS, AP-M, MV, LF, JE, SO.

Acknowledgements

This work was supported by a grant from the International Brain Research Organization (IBRO) thanks to the collaborative IBRO-LARC-RPOLAB program to S.O. and J.E. Other funding was provided by Agencia Nacional de Promoción de la Investigación, el Desarrollo Tecnológico y la Innovación (PICT-2018 0862 to S.O.) and FONDECYT INICIACIÓN grant (#11190601) to A.P.M., CONICYT Doctoral fellowship #21191720 to V.S., and FONDECYT grant #1180403 and the Centro Interdisciplinario de Neurociencia de Valparaiso (CINV) Millennium Institute grant P09-022-F, supported by the Millennium Scientific Initiative of the Ministerio de Economia, Fomento y Turismo to J. E. S.O. and L.F. are researcher of Consejo Nacional de Investigación Científica y Tecnológica (CONICET). M.V. is recipient of a doctoral fellowship from CONICET. We thank Christian Wegener for *orco*-GAL4 drivers, Joel Levine for the locomotor activity analyses software, Christopher Vecsey and Leslie Griffith for the SCAMP program, and the Bloomington and Vienna *Drosophila* stock centers for stocks.

Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.ibmb.2021.103676.

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