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Adipokinetic hormone signalling system in the Chagas disease vector, *Rhodnius prolixus*

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Abstract

Neuropeptides and their G protein-coupled receptors are widespread throughout Metazoa and in several cases, clear orthologues can be identified in both protostomes and deuterostomes. One such neuropeptide is the insect adipokinetic hormone (AKH), which is related to the mammalian gonadotropin-releasing hormone. AKH has been studied extensively and is known to mobilize lipid, carbohydrates and proline for energy-consuming activities such as flight. In order to determine the possible roles for this signalling system in *Rhodnius prolixus*, we isolated the cDNA sequences encoding *R. prolixus AKH* (*Rhopr-AKH*) and its receptor (*Rhopr-AKHR*). We also examined their spatial expression pattern using quantitative PCR. Our expression analysis indicates that *Rhopr-AKH* is only expressed in the corpus cardiacum of fifth-instars and adults. *Rhopr-AKHR*, by contrast, is expressed in several peripheral tissues including the fat body. The expression of the receptor in the fat body suggests that AKH is involved in lipid mobilization, which was confirmed by knockdown of *Rhopr-AKHR* via RNA interference. Adult males that had been injected with double-stranded RNA (dsRNA) for Rhopr-AKHR exhibited increased lipid content in the fat body and decreased lipid levels in the haemolymph. Moreover, injection of Rhopr-AKH in *Rhopr-AKHR* dsRNA-treated males failed to elevate haemolymph lipid levels, confirming that this is indeed the receptor for Rhopr-AKH.

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Introduction

Adipokinetic hormone (AKH) was the first insect neurohormone to be isolated, sequenced and synthesized (Stone *et al.*, 1976; Broomfield & Hardy, 1977) and is one of the most studied neuropeptides in insects. To date there are over 50 members of the now-termed AKH/red-pigment concentrating hormone (RPCH) family (Gäde & Marco, 2013). First identified for their ability to mobilize lipid, carbohydrate or proline from the fat body during locomotory activities such as flight, AKHs have also been shown to have additional functions, such as stimulating heartbeat rate (Rosiński & Gäde, 1988; Keeley *et al.*, 1991; Noyes *et al.*, 1995; Malik *et al.*, 2012; Gäde & Marco, 2013), inhibiting protein synthesis (Carlisle & Loughton, 1986) and extending life span during starvation in *Drosophila melanogaster* (Isabel *et al.*, 2005). Recently, AKH signalling has also been shown to play a role in oxidative stress (Bednarova *et al.*, 2013).

It was over 25 years since the discovery of AKH that the first AKH receptors (AKHRs) were identified in the fruit fly *D. melanogaster* and the silkworm *Bombyx mori* (Park *et al.*, 2002; Staubli *et al.*, 2002). These receptors are rhodopsin-like G protein-coupled receptors (GPCRs) and have been found to be structurally related to the vertebrate gonadotropin-releasing hormone (GnRH) receptor in vertebrates. AKHRs were subsequently identified in *Manduca sexta* (Ziegler *et al.*, 2011), *Periplaneta americana* (Hansen *et al.*, 2006), *Anopheles gambiae* (Kaufmann & Brown, 2006) and *Aedes aegypti* (Kaufmann *et al.*, 2009), and many more have been deduced from genomic sequences of other insect species (see Grimmelikhuijzen & Hauser, 2012; Hauser & Grimmelikhuijzen, 2014). Owing to the growth in genomic data and availability of extensive amino acid sequences of GPCRs, Hauser & Grimmelikhuijzen (2014) used an *in silico* approach to propose a scenario for the evolution of

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AKH and its receptor along with those of two other insect peptide signalling pathways, namely those of corazonin (CRZ) and AKH/CRZ-related peptide (ACP). In their scenario, these three ligands and their receptors originated from an ancestral GnRH-like ligand and receptor, and duplication occurred before the emergence of Mollusca and Annelida, leading to two branches; one producing a CRZ-like receptor/ligand and the other an AKH-like receptor/ligand. Further duplication of the AKH hormonal system led to the AKH and ACP systems that are present today in Arthropoda (see Roch *et al*., 2014). A recent study by Patel *et al*. (2014) examined these three peptide signalling pathways to determine if the co-evolution of these three peptide families with partially conserved amino acid sequences also resulted in shared physiological activities in the blood-gorging bug *Rhodnius prolixus*. Their study determined that Rhopr-AKH was capable of elevating haemolymph lipid levels in a dose-dependent manner in adult male insects (but had no effect on heartbeat frequency) whereas Rhopr-CRZ was capable of increasing heartbeat frequency in a dose-dependent manner (but had no effect on lipid mobilization). Rhopr-ACP had no effect on either haemolymph lipid levels or heartbeat frequency. ACP appears to have a widespread presence in insects and yet no physiological function has been identified in any insect. Although all three peptides share some amino acid sequence similarity, none of these peptides in *An. gambiae* cross-react with the receptors for the other ligands (Hansen *et al*., 2010). These data suggest that each peptide family has retained a distinct physiological function.

AKHR transcript expression has been shown in the fat body, central nervous system (CNS) and ovaries of mosquitoes (Kaufmann & Brown, 2006; Kaufmann *et al*., 2009). In *P. americana*, *AKHR* is expressed in several tissues, including the brain, ovaries, flight muscles and digestive system (Wicher *et al*., 2006). Interestingly, the fact that AKHR is structurally related to the GnRH receptor in mammals, coupled with AKHR transcript expression in the fat body and ovaries, supports the notion that nutrient metabolism in females might be regulated to meet the demands not only for flight but also for reproduction. A role in reproduction in insects has yet to be proven, although there are several lines of indirect evidence. AKHR knockdown in the tsetse fly *Glossina morsitans* affects ‘milk’ production during tsetse pregnancy (Attardo *et al*., 2012) and AKH has been found to inhibit egg production indirectly in the cricket *Gryllus bimaculatus* (Lorenz, 2003). However, AKH has been shown to be involved in reproduction in the nematode *Caenorhabditis elegans*, with AKH-GnRH knockdown leading to a delay in the timing of egg-laying and a decrease in the number of total progeny (Lindemans *et al*., 2009). Recent studies have shown that knockdown of *AKHR* in *G. bimaculatus* using Grybi-AKHR double-stranded RNA (dsRNA) decreased levels of 1,2-diacylglycerol and trehalose in the haemolymph whilst increasing the levels of triacylglycerol in the fat body (Konuma *et al*., 2012). This knockdown also enhanced starvation resistance, decreased locomotory activity and increased food intake in *G. bimaculatus*. This increase in food intake was the result of an increase in feeding frequency, indicating that AKH signalling is involved in nutritional control. Similar conclusions were obtained in experiments in which AKH injection or topical application activates locomotory and flight activity in orthopteran species, suggesting that AKH signalling might contribute to the regulation of feeding-related behaviours (Lorenz & Anand, 2004; Wicher *et al*., 2006).

Previously, AKH has been sequenced in the kissing bug *R. prolixus* (Ons *et al*., 2011; Marco *et al*., 2013) and shown to be present in a group of neurosecretory cells in the corpus cardiacum (CC) (Patel *et al*., 2014). Injection of Rhopr-AKH into adult males elevated haemolymph lipid levels in a dose-dependent manner (Patel *et al*., 2014). Haemolymph lipid levels also increase during flight in *R. prolixus* and this elevation in lipid levels may be the result of the release of Rhopr-AKH (Ward *et al*., 1982). Rhopr-AKH does not alter heartbeat rate in *R. prolixus* although it has been shown to alter heart rate frequency in other insects (Rosiński & Gäde, 1988; Keeley *et al*., 1991; Noyes *et al*., 1995; Malik *et al*., 2012). In the present study, the cDNA sequences were cloned for *Rhopr-AKH* and *Rhopr-AKHR* and quantitative PCR (qPCR) was used to determine their spatial expression profiles. As expected, *Rhopr-AKH* was only expressed in the CC and *Rhopr-AKHR* was enriched in the fat body as well as in adult reproductive tissues. RNA interference (RNAi) experiments were performed to knockdown *Rhopr-AKHR* and the effect on lipid mobilization from the fat body was examined.

Results

Rhopr-AKH *and* Rhopr-AKHR

First, we cloned and sequenced the complete cDNA encoding *Rhopr-AKH* (Fig. 1A). The sequence is at least 393 bp long and includes a 216-bp open reading frame (ORF). The 5′ and 3′ untranslated regions (UTRs) are at least 108 and 69 bp long, respectively. There is an in-frame stop codon upstream of the start codon, confirming that the ORF is complete. Within the prepropeptide sequence, a signal peptide cleavage site is present between the alanine residue at position 21 and glutamine at position 22. The mature peptide is flanked by the signal peptide at the N-terminus and a lysine and arginine dibasic cleavage site at the C-terminus. Molecular organization of *Rhopr-AKH* shows that the gene comprises three

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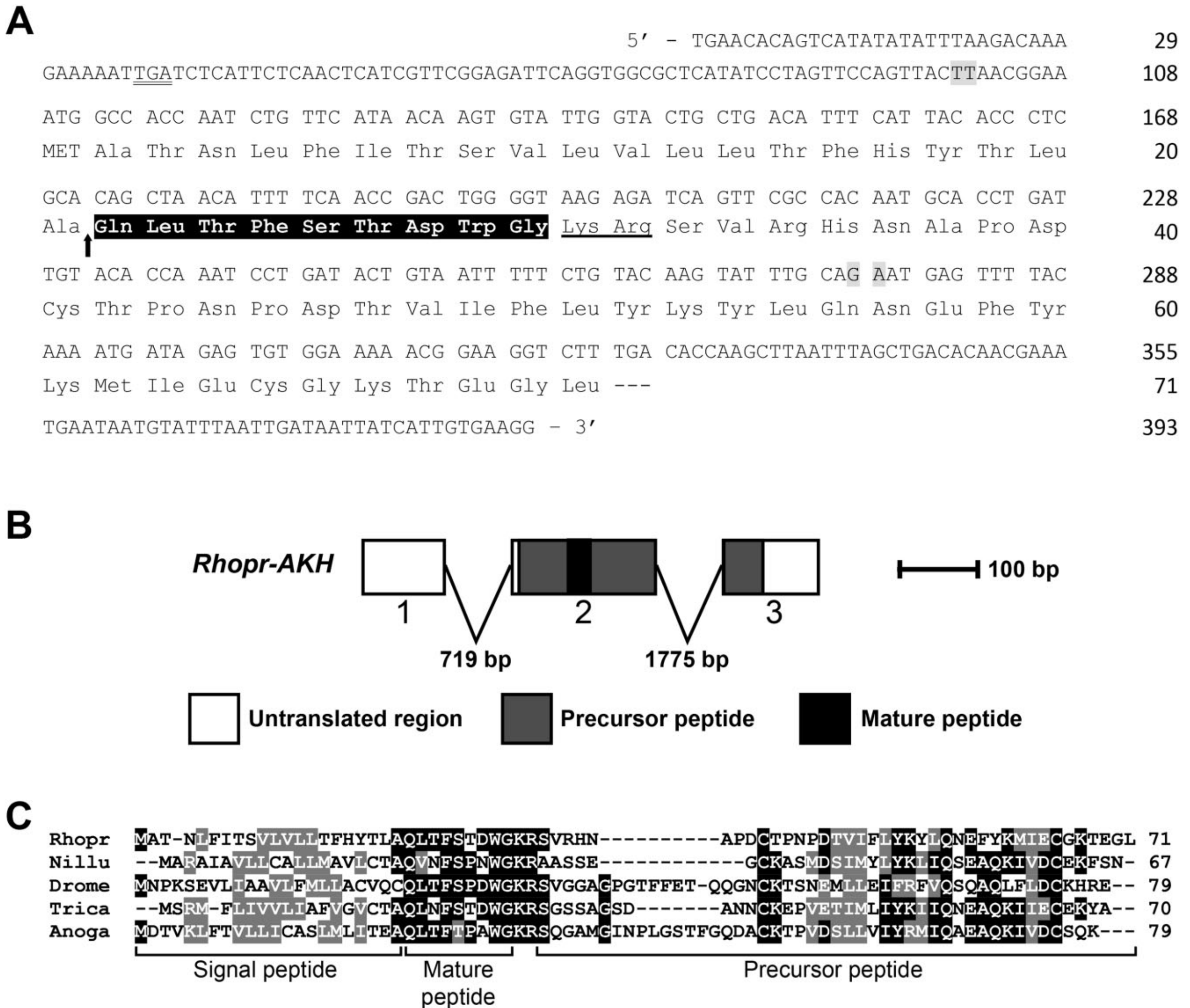

Figure 1. *Rhodnius prolixus* adipokinetic hormone (Rhopr-AKH) sequence and structure. (A) Rhopr-AKH cDNA sequence and the deduced amino acid sequence. The numbering for each sequence is shown on the right. Within the nucleotide sequence, the exon–exon boundaries are shaded in grey and an in-frame stop codon upstream of the start codon is double underlined. Within the amino acid sequence, the start codon has been capitalized, the mature peptide is highlighted in black, the predicted site for signal peptide cleavage is marked by an arrow and the dibasic cleavage site has been underlined. (B) Rhopr-AKH gene structure. The boxes represent exons that have been drawn to scale. (C) Multiple sequence alignment of select AKH prepropeptides. Rhopr-AKH prepropeptide was aligned with its orthologues from *Nilaparvata lugens* (Nillu), *Drosophila melanogaster* (Drome), *Tribolium castaneum* (Trica) and *Anopheles gambiae* (Anoga). Identical amino acids have been highlighted in black and similar amino acids in grey if at least 50% of the sequences are similar.

exons, which are 100, 176 and 117 bp long (Fig. 1B). Two small introns (719 and 1775 bp) separate these exons. The mature peptide is encoded by exon 2 and is very well conserved across other insects (Fig. 1C).

Next, we isolated the complete cDNA sequence encoding *Rhopr-AKHR* (Fig. 2A). This sequence is 1384 bp long. The ORF is 1062 bp long and encodes a receptor comprising of 353 amino acids. The 5′ and 3′ UTRs are at least 101 and 221 bp long, respectively. The 5′ UTR also contains an in-frame stop codon upstream of the start codon, confirming that this ORF is also complete. Rhopr-AKHR has all the characteristics of a GPCR: an extracellular N-terminus, seven transmembrane domains and an intracellular C-terminus. It is also predicted to contain one N-linked glycosylation site in its N-terminus and 10 phosphorylation sites. Moreover, Rhopr-AKHR is highly predicted (99% probability) to couple with the G stimulatory/G other alpha subunit by two different algorithms. PRED-COUPLE 2.0 also predicted the receptor to couple with the Gq/11 alpha subunit, although with a much

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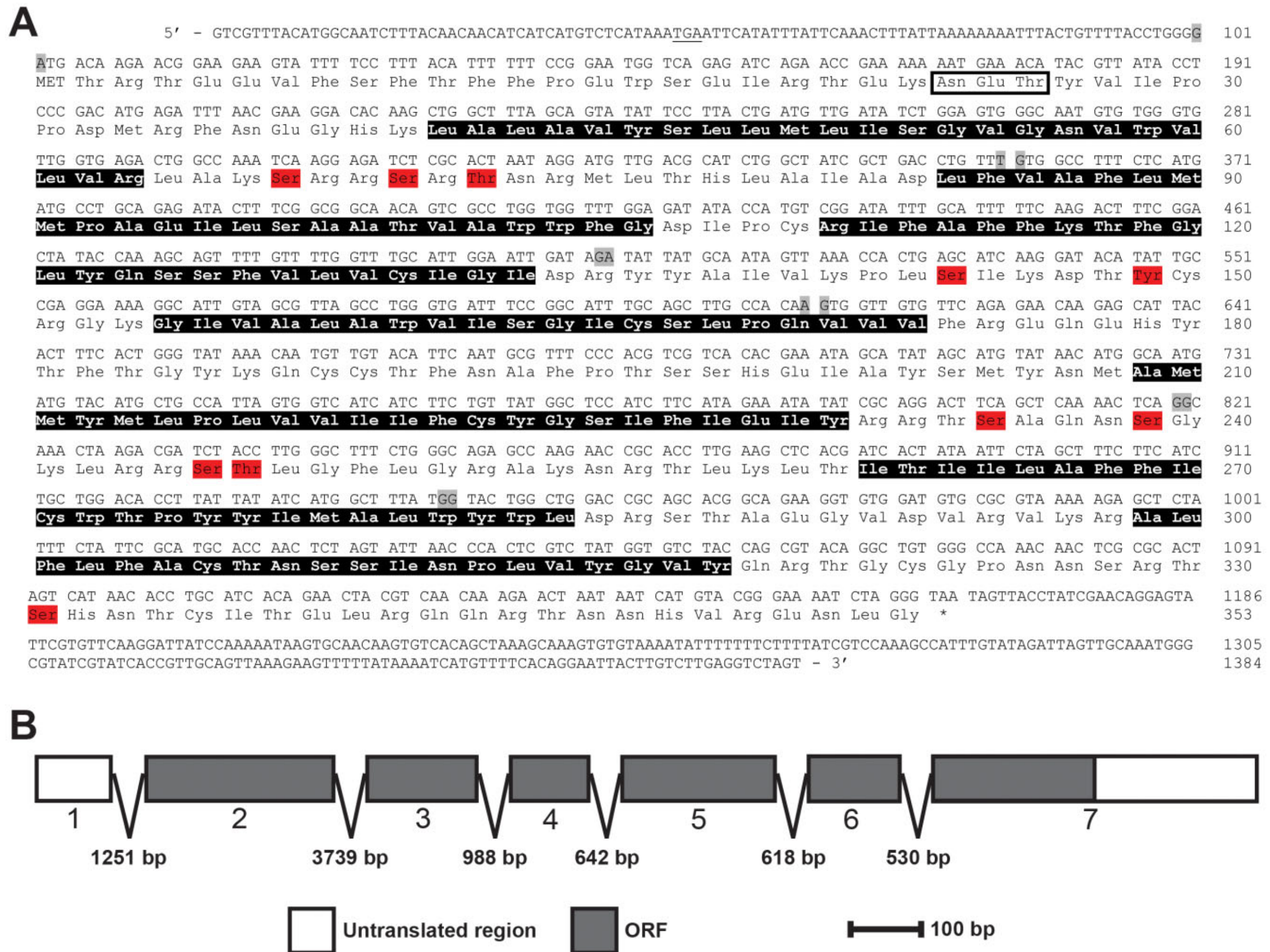

Figure 2. *Rhodnius prolixus* adipokinetic hormone receptor (Rhopr-AKHR) sequence and structure. (A) Rhopr-AKHR cDNA sequence and the deduced amino acid sequence. The numbering for each sequence is shown on the right. Within the nucleotide sequence, the exon–exon boundaries are shaded in grey. Within the amino acid sequence, the initial methionine start codon has been capitalized, the seven predicted transmembrane domains are highlighted in black, one predicted N-linked glycosylation site has been boxed and 10 predicted phosphorylation sites have been highlighted in red. (B) Rhopr-AKHR gene structure. The boxes represent exons that have been drawn to scale. ORF, open reading frame.

lower probability (53%). Lastly, *Rhopr-AKHR* comprises seven exons and the ORF spans exons 2 to 7 (Fig. 2B).

Sequence and phylogenetic analysis

We aligned Rhopr-AKHR with other insect AKHRs and *Homo sapiens* GnRH-R1 to examine the conservation across various species (Fig. 3). The alignment shows that the region between the seven transmembrane domains is well conserved; however, the N- and C-termini are less conserved. The putative N-linked glycosylation site in the N-terminus is also conserved across all of the receptors examined. Moreover, several functionally important residues in *H. sapiens* GnRH-R1 are also conserved in Rhopr-AKHR and other orthologues. These include residues that are involved in receptor activation, binding pocket formation, ligand binding, protein kinase C phosphorylation, G stimulatory alpha coupling and Gq/11 alpha coupling (see Millar *et al.*, 2004).

Phylogenetic analysis of AKH, ACP, CRZ and GnRH receptors confirmed the evolutionary relatedness amongst these hormonal systems (Fig. 4). Hence, AKHRs and ACPRs in Protostomia are sister to a clade of GnRHRs in Deuterostomia that includes human GnRH-R1 and amphioxus (*Branchiostoma floridae*) GnRH-R1 and R2. However, CRZRs are sister to *Br. floridae* GnRH-R3 and R4. Rhopr-AKHR is closely related to other insect receptors that have previously been functionally characterized as AKHR. These include receptors from *D. melanogaster* (Staubli *et al.*, 2002) and *An. gambiae* (Hansen *et al.*, 2010), thus supporting the claim that the receptor isolated from *R. prolixus* is an AKHR.

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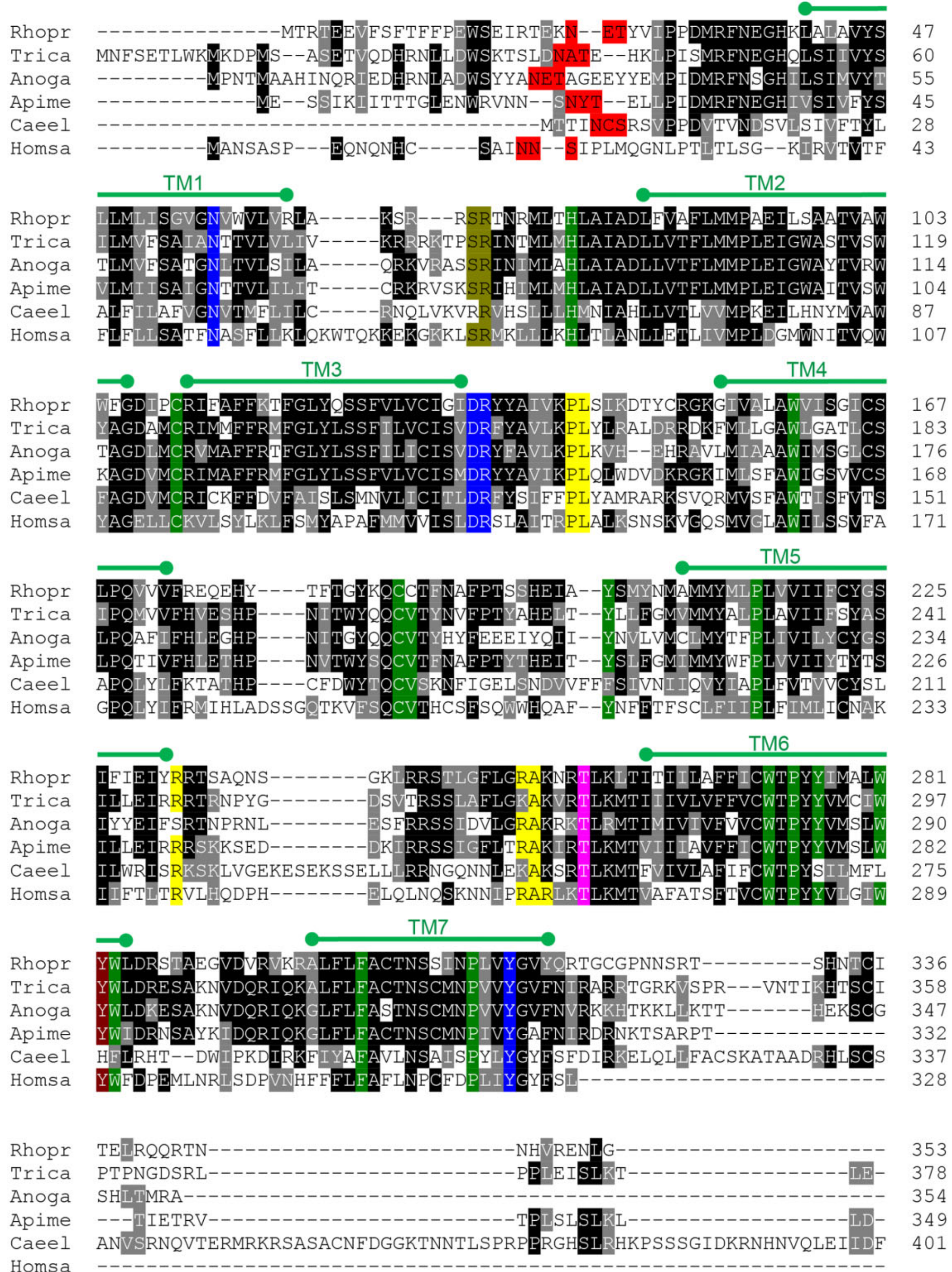

Figure 3. Multiple sequence alignment of select invertebrate adipokinetic hormone (AKH) receptors and *Homo sapiens* gonadotropin-releasing hormone receptor 1 (GnRH-R1). *Rhodnius prolixus* AKH receptor (Rhopr-AKHR) was aligned with its orthologues from *Tribolium castaneum* (Trica), *Anopheles gambiae* (Anoga), *Apis mellifera* (Apime), *Caenorhabditis elegans* (Cael) and *H. sapiens* (Homsa). Identical amino acids have been highlighted in black and similar amino acids in grey if at least 50% of the sequences are similar. The predicted locations of the seven transmembrane domains of Rhopr-AKHR have been indicated using green lines and the predicted N-linked glycosylation site has been highlighted in red. Functionally important amino acid residues in human GnRH-R1 and their putative homologues in the other sequences have been highlighted using the following scheme: residues important in receptor activation (blue), binding pocket formation (green), ligand binding (dark red), protein kinase C (PKC) phosphorylation (pink), Gs alpha coupling (olive green) and Gq/11 alpha coupling (yellow) (see Millar *et al.*, 2004).

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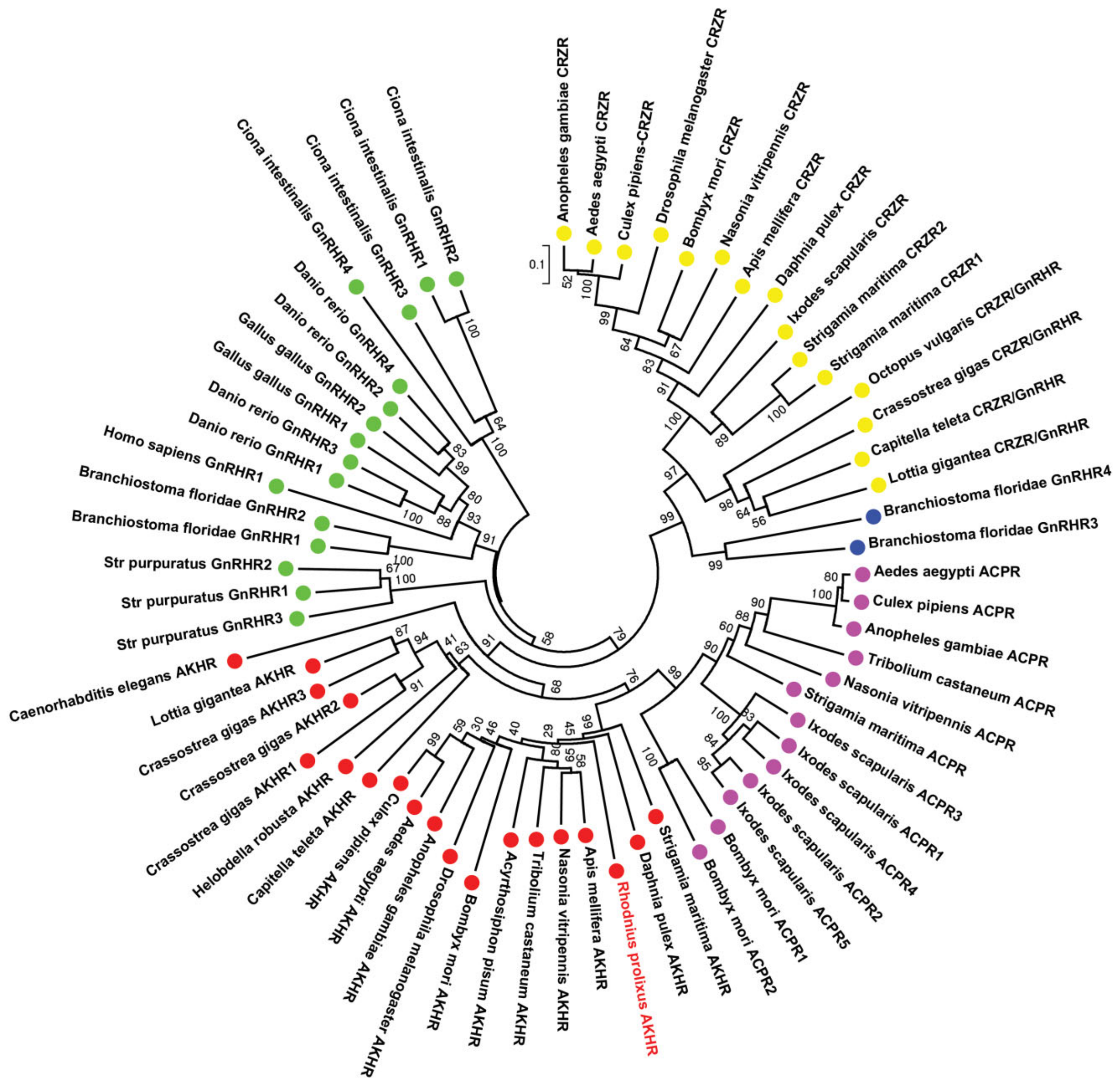

Figure 4. A phylogram of adipokinetic hormone (AKH), AKH/corazonin (CRZ)-related peptide (ACP), CRZ and gonadotropin-releasing hormone (GnRH) receptors obtained following a neighbour-joining analysis (1000 bootstrap replicates). The taxa are labelled using species names. Str purpuratus refers to *Strongylocentrotus purpuratus*. Note that the AKH receptors in protostomes are sister to the GnRH receptors in deuterostomes, which include human GnRH-R1 and amphioxus (*Branchiostoma floridae*) GnRH-R1 and R2.

Spatial expression profile of Rhopr-AKH *and* Rhopr-AKHR

We examined *Rhopr-AKH* expression in the CNS and the CC/corpora allata (CA) complex of both fifth-instars and adults using qPCR. Our analysis indicates that *Rhopr-AKH* is expressed in the CC/CA complex but not in the CNS of either fifth-instars or adults (Fig. 5A). Moreover, the expression in fifth-instars is almost twofold higher than that seen in adults. By contrast, *Rhopr-AKHR* is expressed in both the CNS and the CC/CA complex of fifth-instars and adults (Fig. 5B).

Spatial expression profiling of *Rhopr-AKHR* was performed via qPCR to identify possible target tissues of Rhopr-AKH. Within the fifth-instar, *Rhopr-AKHR* was highly expressed in a pool of tissues comprising the fat bodies, diaphragm and abdominal nerves, and also in prothoracic glands and associated fat bodies (Fig. 6). The

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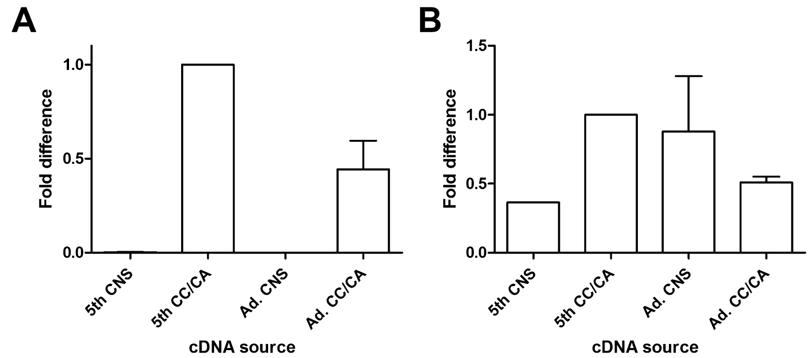

Figure 5. Spatial expression analysis of (A) *Rhodnius prolixus* adipokinetic hormone (Rhopr-AKH) and (B) Rhopr-AKH receptor (Rhopr-AKHR) in the *R. prolixus* central nervous system (CNS) determined using quantitative PCR. Expression was analysed in the following tissues: fifth-instar CNS (5th CNS), fifth-instar corpora cardiaca and corpora allata (5th CC/CA), adult CNS (Ad. CNS) and adult corpora cardiaca and corpora allata (Ad. CC/CA). Expression is shown relative to transcript levels in fifth instar CC/CA cDNA ($n = 3$). Data are mean ± SEM.

transcript was expressed at moderate levels in the dorsal vessel and at lower levels in the CNS and female reproductive tissues (Fig. 6). Within the adult reproductive tissues, the highest expression for *Rhopr-AKHR* was observed in testes and the second highest abundance in a pool of female reproductive tissues comprising the bursa, oviducts, spermatheca and cement gland (Fig. 7). The receptor was also expressed, albeit at lower levels, in the ovaries and a pool of male reproductive tissues comprising the vas deferens, seminal vesicle, accessory glands and ejaculatory duct.

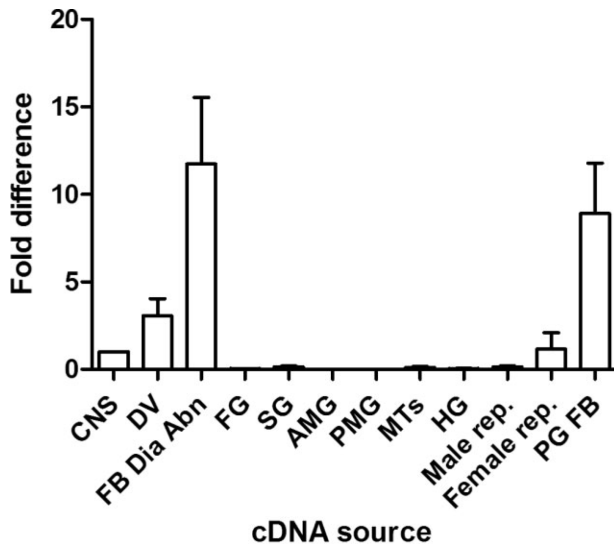

Figure 6. Spatial expression analysis of *Rhodnius prolixus* adipokinetic hormone receptor (Rhopr-AKHR) in fifth instar *R. prolixus* determined using quantitative PCR. Expression was analysed in the following tissues: central nervous system (CNS), dorsal vessel (DV), fat bodies, diaphragm and abdominal nerves (FB Dia Abn), foregut (FG), salivary glands (SG), anterior midgut (AMG), posterior midgut (PMG), Malpighian tubules (MTs), hindgut (HG), male reproductive tissues (Male rep.),. female reproductive tissues (Female rep) and prothoracic glands and associated fat body (PG FB). Expression is shown relative to transcript levels in CNS cDNA ($n = 2$). Data are mean ± SEM.

Rhopr-AKHR in lipid mobilization

Adult male *R. prolixus* were injected 10 days after blood-gorging with 2 μg of either ampicillin-resistance gene (ARG) or Rhopr-AKHR dsRNA. Rhopr-AKHR dsRNA-injected insects exhibited a greater than 97% knockdown 4 days post-injection (Fig. 8). Four days post-injection of Rhopr-AKHR dsRNA, ie 14 days post blood-gorging, the haemolymph lipid levels were significantly reduced (Fig. 9A) and the ventral abdominal fat body lipid content was significantly increased compared with controls (Fig. 9B).

To verify the importance of Rhopr-AKHR in lipid mobilization from the fat body, we investigated if Rhopr-AKH was still capable of mobilizing lipid in adult male *R. prolixus* after knocking down the *Rhopr-AKHR* using dsRNA (Fig. 10). Rhopr-AKH (5 pmol) injected into

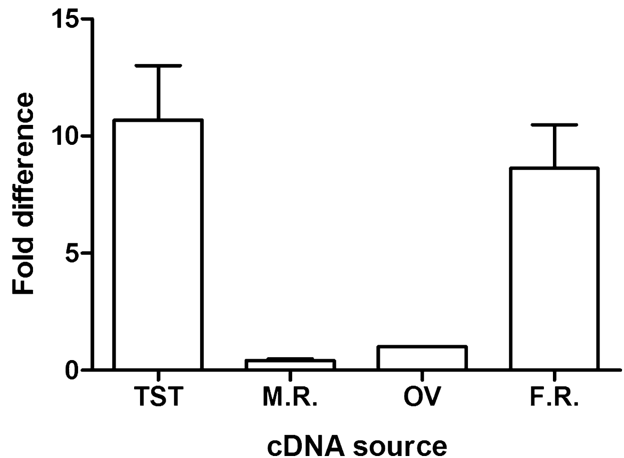

Figure 7. Spatial expression analysis of *Rhodnius prolixus* adipokinetic hormone receptor (Rhopr-AKHR) in *R. prolixus* adult reproductive tissues determined using quantitative PCR. Expression was analysed in the following tissues: testes (TST), rest of the male reproductive tissues (M.R.), ovaries (OV) and rest of the female reproductive tissues (F.R). Expression is shown relative to transcript levels cDNA of ovaries ($n = 3$). Data are mean ± SEM.

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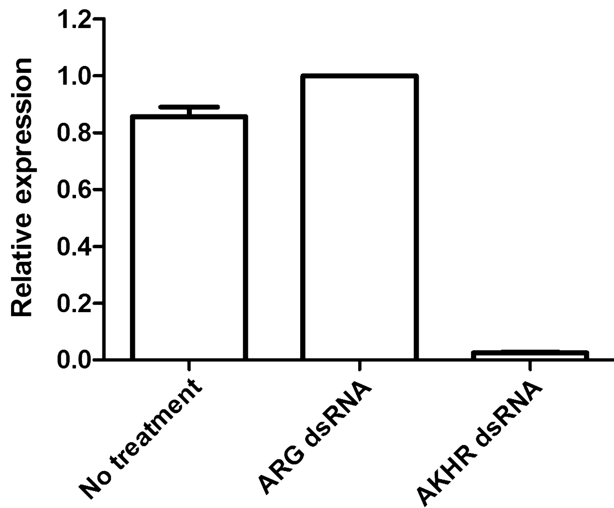

Figure 8. Effect of double-stranded RNA (dsRNA) injection on *Rhodnius prolixus* adipokinetic hormone receptor (Rhopr-AKHR) transcript levels in adult male ventral abdominal fat body. Adult males were left untreated, or injected with either ampicillin-resistance gene (ARG) dsRNA or Rhopr-AKHR dsRNA. Transcript levels were determined in fat body 4 days post-injection. Results are presented as means of three independent biological replicates. Rhopr-AKHR dsRNA-injected insects exhibited >97% knockdown compared with ARG dsRNA injected insects ($n = 3$). Data are mean ± SEM.

R. prolixus that had received no treatment (NT) led to a significant increase in haemolymph lipid levels as it did in those insects previously injected with ARG dsRNA (Fig. 10). Insects injected with Rhopr-AKHR dsRNA did not significantly increase their haemolymph lipid levels in response to Rhopr-AKH injection (Fig. 10).

Discussion

In the present study, we isolated and characterized cDNA sequences encoding AKH and AKHR from the Chagas

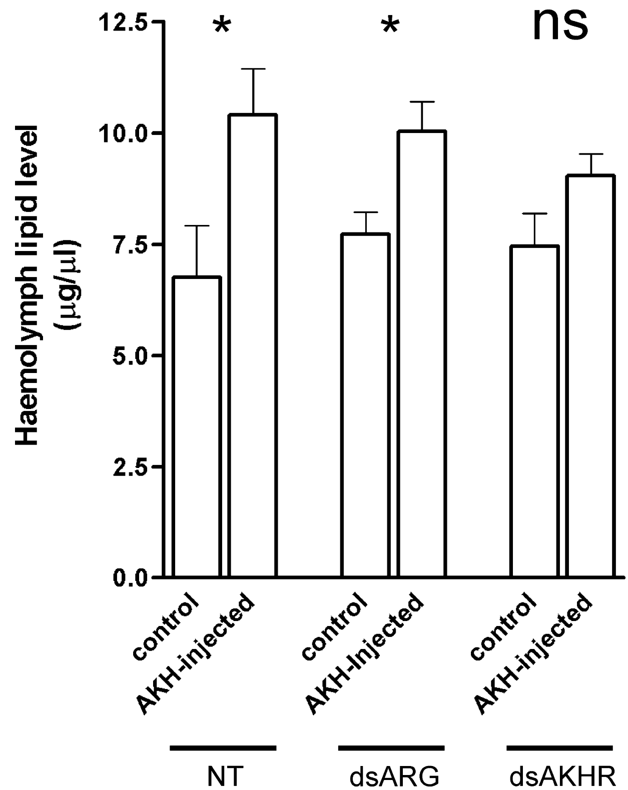

Figure 10. Functional characterization of *Rhodnius prolixus* adipokinetic hormone receptor (Rhopr-AKHR) using RNA interference. Changes in haemolymph lipid levels following injection of saline (control) or Rhopr-AKH (AKH-injected). Knockdown of Rhopr-AKHR with AKHR double-stranded RNA (dsAKHR) resulted in a reduction in the increase in haemolymph lipid levels 90 min after injection of 5 pmol Rhopr-AKH compared with no treatment (NT) insects [not treated with double-stranded RNA (dsRNA)] and insects injected with ampicillin-resistance gene (ARG) dsRNA (dsARG). Data were analysed using a one-way ANOVA ($P = 0.063$) followed by Bonferroni's multiple comparison test (*, $P < 0.05$; ns, not significant). Data are mean ± SEM ($n = 7$ for NT; $n = 14$–16 for ARG dsRNA and AKHR dsRNA).

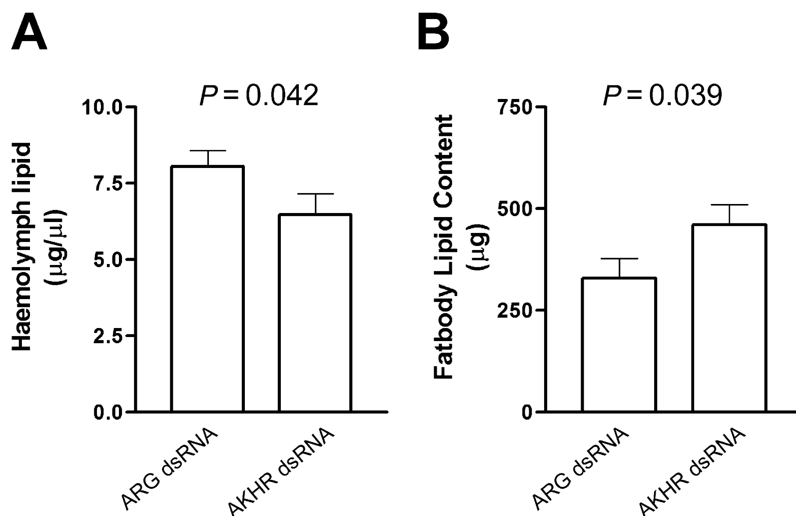

Figure 9. Effect of *Rhodnius prolixus* adipokinetic hormone receptor (Rhopr-AKHR) knockdown on (A) haemolymph lipid levels and (B) fat body lipid content of adult males. Rhopr-AKHR knockdown caused a decrease in haemolymph lipid levels and an increase in fat body lipid content compared with ampicillin-resistance gene (ARG) double-stranded RNA-injected insects. Statistical analysis was performed using a one-tailed *t*-test ($n = 11$–13). Data are mean ± SEM.

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disease vector, *R. prolixus* (*Rhopr-AKH* and *Rhopr-AKH-R*, respectively). Two sequences for *Rhopr-AKH* are found in GenBank. These sequences are 291 bp (GU062794.1) and 320 bp long (GQ888667.1) and encode prepropeptides comprising 96 amino acids and 71 amino acids, respectively. In order to clarify this discrepancy, we cloned and sequenced the complete cDNA encoding *Rhopr-AKH*. The sequence presented here is more similar to the sequence under the accession number GQ888667.1. The ORF presented here differs by only one nucleotide (an adenine at position 314 in place of guanosine). This also alters the amino acid from glutamate to glycine. The sequence presented here also has the additional 5′ UTR. This sequence verification and extension was essential as it allowed for the design of suitable primers to perform qPCR and thereby examine the expression pattern of *Rhopr-AKH*. A previous report examining the distribution of AKH in *R. prolixus* using immunohistochemistry was unable to conclusively determine the expression pattern as a result of the anti-AKH antiserum possibly cross-reacting with an ACP-like substance (Patel *et al.*, 2014). Hence, in order to clarify these data, we examined the expression of *Rhopr-AKH* in CNS and the CC/CA complex. Not surprisingly, *Rhopr-AKH* was expressed in the CC/CA complex but not in the CNS. Interestingly, the expression in fifth-instar CC/CA was almost twofold higher than that seen in adult CC/CA. The significance of this difference is still unclear as one might have predicted higher *AKH* expression (and the subsequent AKH production) in adults because of energy demands associated with flight and reproduction. This study shows that in the CNS, *AKH* is solely expressed in the neurosecretory cells of the *R. prolixus* CC as has been shown for several other insects (see Schooley *et al.*, 2012; Audsley *et al.*, 2014). Nonetheless, there are a few studies that have reported the presence of AKH-like material in insect brains via immunohistochemical or mass spectrometric analyses (Siegert, 1999; Kaufmann & Brown, 2006; Kaufmann *et al.*, 2009). With the recent discovery of the ACP signalling system, it is now evident that this is ACP and not AKH (Hansen *et al.*, 2010). Caution must therefore be exercised when interpreting results of studies prior to the discovery of the ACP signalling system.

Several lines of evidence confirm that the putative Rhopr-AKHR isolated here is indeed an AKHR. Firstly, several functionally important residues that are involved in receptor activation, binding pocket formation and ligand binding in *H. sapiens* GnRH-R1 are also conserved in Rhopr-AKHR (Millar *et al.*, 2004). Secondly, our phylogenetic analysis shows that the receptor is orthologous to other insect receptors that have been functionally characterized as AKHR. Lastly, and most importantly, Rhopr-AKH injection failed to significantly increase the haemolymph lipid levels in insects in which the receptor was knocked down via RNAi. Furthermore, insects with *Rhopr-AKHR* knocked down exhibited reduced haemolymph lipid levels and increased fat body lipid content in comparison with control insects. Similar results have been obtained in *G. bimaculatus* wherein knockdown of *AKHR* resulted in decreased haemolymph 1,2 diacylglycerol and trehalose levels and an increase in triacylglycerol in the fat body (Konuma *et al.*, 2012). Clearly, the AKH signalling pathway is important for maintaining metabolic homeostasis.

Spatial expression analysis of *Rhopr-AKHR* demonstrated that it is highly expressed in fat bodies of fifth-instar *R. prolixus*. This is not surprising as Rhopr-AKH has previously been shown to mobilize lipids, most probably from fat bodies in *R. prolixus* (Marco *et al.*, 2013; Patel *et al.*, 2014). *Rhopr-AKHR* transcript was also highly expressed in the sample comprising prothoracic glands (PGs) and associated fat bodies. It can be assumed that *Rhopr-AKHR* has little to no expression in PGs because preliminary results reveal that Rhopr-AKH has no effect on ecdysteroid synthesis by PGs (X. Vafopoulou, pers. comm.). Thus, most of the expression in the sample in the present study can probably be attributed to the fat bodies. *Rhopr-AKHR* is also moderately expressed in the dorsal vessel; this is intriguing because although Rhopr-AKH does not alter heartbeat frequency (Patel *et al.*, 2014), it may increase the flow of haemolymph by modulating the stroke volume without affecting the heart rate via a Frank–Starling-like mechanism (da Silva *et al.*, 2011). *Rhopr-AKHR* is expressed at low levels in the CNS and female reproductive tissues. *Rhopr-AKHR* transcript expression was observed in both the CNS and the isolated CC/CA complex of fifth-instars and adults. Hence Rhopr-AKH, which is produced by the CC, may also act on the CC/CA complex to affect the synthesis and/or release of other hormones. Under this scenario, Rhopr-AKH would act as a releasing hormone, and thus be functionally conserved with GnRH. With regards to the receptor expression in the CNS, AKH has been shown to cross the ganglionic sheath in *P. americana* (Wicher *et al.*, 2006). Thus, Rhopr-AKH, which is released into the haemolymph, may have access to Rhopr-AKHR in the CNS, modifying behaviour associated with energy demands. *Rhopr-AKHR* expression was found in immature and adult reproductive tissues. The receptor transcript was highly expressed in the adult testes and female reproductive tissues, which include the bursa, oviducts, spermatheca and cement gland. Although a role for AKH in reproduction in insects has not yet been established, these data point towards additional functional conservation between AKH and GnRH. The presence of the receptor transcript in female reproductive tissues indicates a possible role for Rhopr-AKH in egg production

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and/or egg-laying behaviour as has been shown in *G. bimaculatus* (Lorenz, 2003) and *C. elegans* (Lindemans *et al.*, 2009), respectively. The expression in testes is intriguing, although not unique to *R. prolixus.* AKHR has also been shown to be expressed in *B. mori* testes (Yamanaka *et al.*, 2008). There is no clear indication of the role that AKH may play in this tissue. It may, of course, have something to do with energy requirements during growth and development. Clearly, future experiments are required to examine these possibilities.

Experimental procedures

Animals

Unfed fifth-instar and adult *R. prolixus* were taken from a longstanding colony at the University of Toronto Mississauga. Insects were raised in incubators maintained at 60% humidity and 25 °C, and were artificially fed on defibrinated rabbit blood (Hemostat Laboratories, Dixon, CA, USA; supplied by Cedarlane Laboratories Inc., Burlington, ON, Canada).

Isolation of cDNA sequences encoding R. prolixus *AKH and its receptor*

Supercontig sequences representing the *R. prolixus* genome assembly (January 2012 release) were downloaded from VectorBase (https://www.vectorbase.org/) and imported into GENEIOUS 4.7.6 in order to perform local BLAST searches.

The *A. gambiae* AKHR (AAQ63187.1) amino acid sequence was used to mine the *R. prolixus* genome for putative AKHR. Primers specific to the hit regions were designed (Table S1) and used to amplify the partial cDNA sequence encoding a putative Rhopr-AKHR. Plasmid DNA isolated from a fifth-instar *R. prolixus* CNS cDNA library was used as the template for PCR (Paluzzi *et al.*, 2008). The complete cDNA sequence encoding Rhopr-AKHR was obtained using a modified 5′ and 3′ rapid amplification of cDNA ends (RACE) PCR approach described previously (Zandawala *et al.*, 2011, 2012). Gene-specific primers for 5′ and 3′ RACE PCRs were designed using the partial Rhopr-AKHR sequence. For 5′ RACE, two gene-specific reverse primers and two plasmid-specific forward primers were used (Table S2). For 3′ RACE, two gene-specific forward primers and two plasmid-specific reverse primers were used (Table S3). A nested PCR approach was used to amplify the specific products, which were cloned using pGEM-T Easy vector (Promega Corporation, Madison, WI, USA) and sequenced at the SickKids DNA Sequencing Facility (The Centre for Applied Genomics, Hospital for Sick Children, Toronto, ON, Canada). All the PCR reactions were performed using a s1000 thermal cycler (Bio-Rad Laboratories, Mississauga, ON, Canada) using the following temperature-cycling profile: initial denaturation at 95 °C for 3 min, followed by 39 cycles of 94 °C for 30 s, 50–60 °C (depending on the primers used) for 30 s and 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. Lastly, the complete cDNA sequence encoding Rhopr-AKHR was amplified with a high-fidelity Taq polymerase using the primers listed in Table S4.

Previously, Ons *et al.* (2011) reported the Rhopr-AKH prepropeptide sequence. This sequence was used to perform a tBLASTn search. Primers were designed based on the hits (Table S1) and used to verify the complete ORF of *Rhopr-AKH*. In order to ensure that there were no additional methionine residues upstream of this sequence, and thus rule out the possibility of a larger ORF, 5′ RACE PCRs were performed with the primers listed in Table S2. The largest cDNA fragment for this gene was amplified with a high-fidelity Taq polymerase using the primers listed in Table S4. The verification and extension of this sequence were necessary in order to design suitable primers for qPCR (see below).

Sequence and phylogenetic analysis

The intron–exon boundaries of *Rhopr-AKH* (submitted to GenBank under accession number KM283242) and *Rhopr-AKHR* (submitted to GenBank under accession number KF534791) were predicted using BLAST and confirmed with a splice site prediction software (Reese *et al.*, 1997). The potential signal peptide cleavage site and dibasic cleavage site in the Rhopr-AKH prepropeptide were predicted using the SignalP 4.1 (Petersen *et al.*, 2011) and ProP 1.0 (Duckert *et al.*, 2004) servers, respectively. With regards to Rhopr-AKHR, the membrane topology was predicted using the TMHMM server v. 2.0 (Krogh *et al.*, 2001), whereas potential intracellular phosphorylation sites and extracellular N-linked glycosylation sites were predicted using the NetPhos 2.0 (Blom *et al.*, 1999) and NetNGlyc 1.0 servers (http://www.cbs.dtu.dk/services/NetNGlyc/ – last accessed on 22 September 2014), respectively. GRIFFIN (G protein and receptor interaction feature finding system; Yabuki *et al.*, 2005) and PRED-COUPLE 2.0 (Sgourakis *et al.*, 2005) were used to predict the coupling selectivity of Rhopr-AKHR to different G-proteins.

Rhopr-AKH prepropeptide was aligned with its orthologues from *Nilaparvata lugens* (BAO00932.1), *D. melanogaster* (NP_523918.1), *Tribolium castaneum* (ABB58739.1) and *An. gambiae* (ABD43194.1) using Clustal Omega (http://www.ebi.ac.uk/Tools/msa/clustalo/ – last accessed on 22 September 2014). Rhopr-AKHR was aligned with its orthologues from *T. castaneum* (NP_001076809), *An. gambiae* (AAQ63187.1), *Apis mellifera* (NP_001035354.1), *C. elegans* (NP_001249720.1) and *H. sapiens* (NP_000397.1). Phylogenetic relationships between various AKH and GnRH receptors were inferred using neighbour-joining analysis in MEGA6 (Tamura *et al.*, 2013). Bootstrap values are based on 1000 iterations. All the sequences used for phylogenetic analysis are presented in Appendix S1.

Spatial expression analyses using qPCR

Various reports indicate that in most insects, AKH is expressed solely in the endocrine cells of the CC (see Schooley *et al.*, 2012). To confirm that this is the case in *R. prolixus*, *Rhopr-AKH* expression was examined in the following tissues: fifth-instar CNS, fifth-instar CC/CA complex, adult CNS and the adult CC/CA complex. CNS samples included the brain (minus the CC/CA complex) and all of the ganglion. *Rhopr-AKHR* expression was also examined in these same tissues. To identify the target tissues of Rhopr-AKH, *Rhopr-AKHR* expression was examined in various tissues from fifth-instar *R. prolixus* and reproductive tissues from adult *R. prolixus.* The following tissues were dissected from fifth-instars: CNS (including the CC/CA complex), dorsal vessel, pool of fat bodies, diaphragm and abdominal nerves, foregut, salivary glands, anterior midgut, posterior midgut, Malpighian tubules,

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hindgut, male reproductive tissues, female reproductive tissues and PGs with its associated fat bodies. The following tissues were dissected from adults: testes, rest of the male reproductive tissues, ovaries and rest of the female reproductive tissues. Methods described previously were used to perform qPCR (Zandawala *et al*., 2013). Briefly, total RNA was extracted from the above tissues using a PureLink RNA Mini Kit (Life Technologies Corporation, Carlsbad, CA, USA), which was followed by cDNA synthesis with iScript Reverse Transcription Supermix for RT-qPCR (Bio-Rad Laboratories Ltd). The resulting cDNA was diluted 10-fold and used as template for qPCR. Target genes (*Rhopr-AKH* and *Rhopr-AKHR*) and three housekeeping genes (*alpha-tubulin*, *beta-actin* and *ribosomal protein 49*) (Paluzzi & O'Donnell, 2012) were amplified using the primers listed in Table S5 and their expression levels were determined using the delta-delta threshold-cycle method. A no-template negative control was also included for each experiment. Expression levels of *Rhopr-AKH* and *Rhopr-AKHR* were normalized via the geometric averaging of the transcript levels of the three housekeeping genes. At least two biological replicates (see each figure caption for the exact number) for each experiment and two technical replicates per reaction were used.

RNAi

Rhopr-AKHR (845 bp) and ARG (575 bp) fragments were amplified via PCR using the primers listed in Table S6. Plasmids containing the full-length *Rhopr-AKHR* and pGEM-T Easy vector (Promega, Madison, WI, USA) were used as the respective templates for these PCRs. T7 promoter sequence was added to these fragments via PCR using the primers listed in Table S6. The following temperature-cycling profile was used for all PCRs: initial denaturation at 94 °C for 3 min, followed by seven cycles of 94 °C for 30 s, 58 °C for 30 s and 72 °C for 90 s, 30 cycles of 94 °C for 30 s, 62 °C for 30 s and 72 °C for 90 s, and a final extension at 72 °C for 10 min. The final PCR product was purified and used as template to synthesize dsRNA with T7 Ribomax Express RNAi System (Promega). The manufacturer-supplied protocol was followed to synthesize and purify dsRNA, which was stored at –80 °C until injection.

Male adult *R. prolixus* (10 days postfed as adults) were anaesthetized by briefly exposing them to CO_2. Hamilton syringes were used to inject 1 µl of 2 µg/µl Rhopr-AKHR dsRNA. Two sets of controls were utilized in this experiment. One group was injected with 1 µl of 2 µg/µl ARG dsRNA whereas the other group was left untreated. Following injections, insects were allowed to recover for about 30 min and kept in an incubator at 28 °C on a 16 h: 8 h light/dark cycle. Lipid assays and qPCR to determine knockdown efficiency were performed 4 days post-injection (or 14 days postfeeding). Knockdown efficiency in the ventral abdominal fat body sheets was determined using qPCR as described above.

Fat body lipid content

The fat body sheet lining the ventral abdominal segments was removed under physiological saline (150 mM NaCl, 8.6 mM KCl, 2 mM $CaCl_2$, 4 mM $NaHCO_3$ and 8.5 mM $MgCl_2$, 5 mM HEPES, pH 7.0), placed in 500 µl isopropanol, and then sonicated and centrifuged. A 50 µl sample was removed from the supernatant, placed in 400 µl isopropanol and the lipid content was measured as previously described (Patel *et al*., 2014).

Haemolymph lipid levels

The haemolymph lipid content was measured in a 5 µl sample withdrawn from the clipped wing buds using a marked glass capillary (Drummond Scientific Company, Broomall, PA, USA). The lipoprotein in the haemolymph was precipitated in 10% trichloroacetic acid (TCA; Sigma, Oakville, ON, Canada), centrifuged and the resulting pellet was processed to measure lipid levels as previously described (Patel *et al*., 2014).

AKH lipid mobilization assay

Insects were immobilized by a short exposure to CO_2 and then injected with either 2 µl of 5 pmol of Rhopr-AKH (pQLTFSTDWamide) or saline (control). These injections were made between the meso- and metathoracic segments mid-ventrally using a 10 µl Hamilton syringe. Rhopr-AKH was purchased from Genscript Laboratories (Piscataway, NJ, USA), dissolved in 50% CHROMASOLV (Sigma) and then stored at –20 °C in 10 µl of 10^{-3} M aliquots. The insects were left for 90 min and then a 5 µl haemolymph sample was removed from the clipped wing base using a marked glass capillary (Drummond Scientific Company). Care was taken to ensure that no fat body droplets were withdrawn with the haemolymph. The haemolymph was then placed in 50 µl of 10% TCA to precipitate the lipoprotein. All samples were then centrifuged for 10 min at 8800 *g* using an Eppendorf Centrifuge 4513 (Mississauga, ON, Canada). The pellets were then processed to determine the lipid levels as previously described (Patel *et al*., 2014). To ensure that the vehicle used to solubilize Rhopr-AKH had no effect on haemolymph lipid levels, controls were run in which insects were injected with appropriately diluted CHROMASOLV. These insects did not show any change in haemolymph lipid levels, indicating that CHROMASOLV did not interfere with this experiment.

Statistical analysis

Graphical representations and statistical analysis were performed using GraphPad Prism (Version 5) (http://www.graphpad.com).

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Supporting Information

Additional Supporting Information may be found in the online version of this article at the publisher's web-site:

Table S1. Primers used to amplify the complete ORF of *Rhopr-AKH* and partial cDNA sequence of *Rhopr-AKHR*.

Table S2. Primers used to perform 5′ RACE PCR reactions.

Table S3. Primers used to perform 3′ RACE PCR reactions.

Table S4. Primers used to amplify the largest cDNA fragments.

Table S5. Primers used for qPCR reactions.

Table S6. Primers used to generate Rhopr-AKHR and ampicillin resistance gene (ARG) dsRNA. (Note: The T7 promoter sequence is bolded).

Appendix S1. Sequences used for phylogenetic analysis.

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