Adipokinetic hormone signaling through the gonadotropin-releasing hormone receptor modulates egg-laying in *Caenorhabditis elegans*

Marleen Lindemans[a], Feng Liu[b], Tom Janssen[a], Steven J. Husson[a], Inge Mertens[a], Gerd Gäde[c], and Liliane Schoofs[a,1]

[a]Functional Genomics and Proteomics Research Unit, Katholieke Universiteit Leuven, Naamsestraat 59, B-3000 Leuven, Belgium; [b]Data Analysis and Modeling Group, Transportation Research Institute, Hasselt University, Building D, B-3590 Diepenbeek, Belgium; and [c]Zoology Department, University of Cape Town, Private Bag, Rondebosch 7701, South Africa

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In mammals, hypothalamic gonadotropin-releasing hormone (GnRH) is a neuropeptide that stimulates the release of gonadotropins from the anterior pituitary. The existence of a putative functional equivalent of this reproduction axis in protostomian invertebrates has been a matter of debate. In this study, the ligand for the GnRH receptor in the nematode *Caenorhabditis elegans* (*Ce*-GnRHR) was found using a bioinformatics approach. The peptide and its precursor are reminiscent of both insect adipokinetic hormones and GnRH-preprohormone precursors from tunicates and higher vertebrates. We cloned the AKH-GnRH-like preprohormone and the *Ce*-GnRHR and expressed the GPCR in HEK293T cells. The GnRHR was activated by the *C. elegans* AKH-GnRH-like peptide (EC_{50} = 150 nM) and by *Drosophila* AKH and other nematode AKH-GnRHs that we found in EST databases. Analogous to both insect AKH receptor and vertebrate GnRH receptor signaling, *Ce*-AKH-GnRH activated its receptor through a $G\alpha_q$ protein with Ca^{2+} as a second messenger. Gene silencing of *Ce*-GnRHR, *Ce*-AKH-GnRH, or both resulted in a delay in the egg-laying process, comparable to a delay in puberty in mammals lacking a normal dose of GnRH peptide or with a mutated GnRH precursor or receptor gene. The present data support the view that the AKH-GnRH signaling system probably arose very early in metazoan evolution and that its role in reproduction might have been developed before the divergence of protostomians and deuterostomians.

C. elegans | G protein-coupled receptor | molecular evolution | neuropeptide | neuroendocrinology

In vertrebrates, key hormones of the hypothalamic-pituitary-gonadal axis control reproduction. The hypothalamus is capable of sensing environmental cues and regulates the production of gonadotropin-releasing hormone (GnRH) (1). At present, the chordate GnRH family consists of 23 members: 14 variants have been found in vertebrates and 9 in tunicates (2, 3). These GnRHs signal through G protein-coupled receptors (GPCRs), which have been reported in tunicates, agnathans, and various vertebrates (4). The high conservation of GnRH signaling within the chordate phylum (deuterostomians) raises the important question as to whether the appearance of this system might date back prior to the divergence of protostomian and deuterostomian lineages, between 670 MYA and 1 billion years ago. As a first step toward demonstrating the early emergence of GnRH, GnRH-related peptides have been established in 2 protostomian phyla: mollusks and annelids (5–9). This leads to several important questions regarding the evolution of the GnRH family. Has GnRH been retained in most protostomian lineages and was regulation of reproduction already a function of ancestral GnRH? The presence of a GPCR in *Octopus* (10), which is orthologous to the vertebrate GnRHR, indicates that *ov*-GnRH is a true vertebrate GnRH ortholog (11).

Tsai and Zhang recently proposed that chordate and protostomian GnRHs likely share a common ancestor but that GnRH has been lost in the ecdysozoan lineage (nematodes and arthropods) but preserved in lophotrochozoans (annelids and mollusks) (9). According to their view, GnRHR-like receptors have been retained in Ecdysozoa but they have recruited other ligands to bind. The ligand of the GnRH receptor of *Drosophila melanogaster* and *Bombyx mori* has been identified as adipokinetic hormone (AKH) (12). AKH is an insect neurohormone that is synthesized in the corpora cardiaca (CC) (13), a neurohemal organ that is considered the functional equivalent of the vertebrate pituitary gland. AKH is involved in mobilizing energy-containing substances such as sugar and lipids from the fat body during flight and locomotion (14).

Because the basic concepts of sexual reproduction are similar in most animal phyla, it is tempting to hypothesize that the regulation of reproduction must have been highly conserved and may date back to the common ancestor of protostomians and deuterostomians. Based on its expression pattern, it has been proposed that mollusk GnRH plays a role in reproduction (6, 7). However, because there is no solid physiological evidence for this suggestion, the role of GnRH (GnRH I) in reproductive activation is still considered a phenomenon unique to chordates.

To investigate whether GnRH and AKH signaling systems are related and may have been derived from a common ancestral signaling system, we looked for functional GnRH-AKH orthologues in *Caenorhabditis elegans*. A GnRH receptor orthologue has been reported in this nematode (*Ce*-GnRHR) (15). Its nervous system contains 302 neurons, but no hypothalamus, pituitary-like structures, or pars intercerebralis-CC complex can be defined. As such, its nervous system appears at first sight incommensurable with those of higher organisms. However, a large number of similarities among the classic small-molecule neurotransmitters and neuropeptidergic signaling pathways with mammalian counterparts have been demonstrated in *C. elegans* (16, 17). Although >250 neuropeptides have been identified in *C. elegans* (18–20), none of them displays sequence similarity to either GnRH or AKH. Consequently, *Ce*-GnRHR remained an orphan receptor. Routine BLAST searches appeared to be insufficient to find a GnRH or AKH-like ligand. Therefore, we used combined *in silico* searches and retrieved an AKH-GnRH-like preprohormone from our assembled *C. elegans* secretome. We further show that an AKH-GnRH-related peptide, which appears to have been highly conserved within the nematode

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[1]To whom correspondence should be addressed. E-mail: liliane.schoofs@bio.kuleuven.be.

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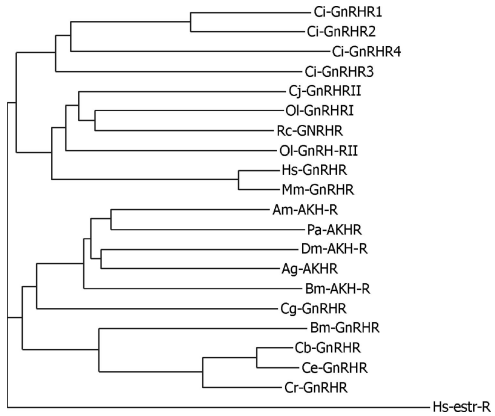

Fig. 1. Phylogenetic representation of the relationship between the *Ce*-GnRHR, insect AKH receptors, a mollusk GnRH receptor and tunicate and vertebrate GnRH receptors. The tree was generated by using the alignment in Vector NTI. The human estrogen receptor (P0337) has been chosen as outer group. Accession numbers in EMBL/GenBank; *Ciona intestinalis*: *Ci*-GnRHR1 (AAW70560), *Ci*-GnRHR2 (AAW70561), *Ci*-GnRHR3 (AAW70562), *Ci*-GnRHR4 (AAW70563), *Callithrix jacchus*: *Cj*-GnRH-RII (AF368286), *Oryzias latipes* (Japanese medaka): *Ol*-GnRH-RI (AB057675) and *Ol*-GnRH-RII (AB057674), *Rana catesbeiana*: *Rc*-GnRHR (AAL11631), *Homo sapiens*: *Hs*-GnRHR (NP_000397), *Mus musculus*: *Mm*-GnRHR (NM_010323), *Apis melifera*: *Am*-AKH-R (NM_001040264), *Periplaneta americana: Pa*-AKHR (ABB20590), *Drosophila melanogaster*: *Dm*-AKH-R (AAC61523), *Anopheles gambiae: Ag*-AKH (ABD60146), *Bombyx mori*: *Bm*-AKH-R (AF403542), *Crassostrea gigas* (oyster): *Cg*-GnRH-R (AJ890150), *Brugia malayi*: *Bm*-GnRHR (XP_001898606), *Caenorhabditis briggsae: Cb*-GnRHR (NC_009842), *Caenorhabditis elegans*: *Ce*-GnRHR (NP_491453), and *Caenorhabditis remanei*: *Cr*-GnRHR (cr01.sctg370.wum.2).

phylum, is the ligand of *Ce*-GnRHR. In addition, we provide evidence for a role in reproduction of the GnRH signaling system in protostomes by showing that gene silencing of *Ce*-GnRHR and its AKH-GnRH-related ligand or both results in a significant delay in egg laying.

Results

Identification and Cloning of the *Ce*-GnRH Receptor. *Ce*-GnRHR (gene F54D7.3 or *gnrr-1*) (15) is structurally related to the mammalian GnRH receptor (GnRHR1). The overall amino acid sequence identity with human GnRHR1 is only 21%, with the highest degree of conservation in the region involved in receptor activation and G_{q11} coupling (83% and 62% conserved amino acids, respectively) (15). Less conserved are the ligand-binding sites (36% conserved amino acids). For comparison, the overall amino acid sequence identity between *Ce*-GnRHR and the GnRH-like *Drosophila* AKH receptor (AAC61523) is 28%, which is not much higher, although both are invertebrates. A phylogenetic analysis shows the relationship between *Ce*-GnRHR, insect AKH receptors, the mollusk *Crassostrea gigas* GnRH receptor, and tunicate and vertebrate GnRH receptors (Fig. 1). The resulting tree obviously exhibits 2 distinct evolutionary lineages: the protostomian invertebrate group and the deuterostomian tunicate-vertebrate group. In the protostomian lineage, *Ce*-GnRHR clusters with the insect AKH receptor and mollusk GnRH receptor subgroups.

***Drosophila* AKH Activates Ce-GnRHR.** Transiently transfected HEK293T cells were challenged with a library containing approximately 175 synthetic *C. elegans* FLP (FMRFamide-like) and NLP (other neuropeptide-like precursors) (17) and with 80 HPLC fractions from an acid methanolic extract of whole worms. None of these peptides and fractions were able to elicit a response. Because AKH activates the GnRH receptor of *Drosophila* (12), we tested whether *Drosophila* AKH (pQLTFSPDW-NH_2) is able to activate *Ce*-GnRHR. In addition, human GnRH (pQHWSYGLRPG-NH_2) was tested, based on the sequence similarity of the human GnRH receptor with *Ce*-GnRHR. The ancient chicken GnRH-II (pQHWSHGWYPG-NH_2) was tested as well. Only *Drosophila* AKH was able to activate *Ce*-GnRHR, which led us to hypothesize that an AKH-like peptide might be present in *C. elegans* and that this peptide might be the cognate ligand of *Ce*-GnRHR.

***In Silico* Search for an Adipokinetic Hormone Peptide Precursor in *C. elegans*.** To search for an AKH-related peptide in the *C. elegans* genome, we used a combination of search programs according to (21). This search procedure is illustrated in Scheme S1 and takes into account the average length of a neuropeptide precursor (< 500 aa), the presence of an N-terminal signal peptide, general characteristics of adipokinetic hormone neuropeptides and their prepropeptide precursors, and the presence of a dibasic cleaving site after the peptide sequence in the precursor (21). The 6 retained putative neuropeptide precursors from this selection, listed in Dataset S1, were used for comparison with a pattern from the conserved domain database PROSITE, that is, 'Q-[LV]-[NT]-[FY]-[ST]-x (2)-W' that characterizes the AKH family. One single protein hit (O76722 or AAC26928) matches this pattern at all sites except one where Leu (or Val) is replaced by Met in the *C. elegans* protein. However, a search based on the chordate GnRH-motif revealed no hits in *C. elegans*.

This newly predicted *C. elegans* precursor displays a lot of similarities at the structural level with the AKH prepropeptides of arthropods and with the GnRH precursors of vertebrates (Fig. S1). In all prepropeptides, the GnRH or AKH peptide sequence is located immediately after the signal peptide and is flanked at the C terminus by a basic cleavage site. The C-flanking peptides contain 2 cysteine residues in all protostomian AKH-GnRH prepropeptides, except for locust AKH-I and II (but not locust AKH-III). Associated peptides in the chordate precursors have one or no Cys residue (Fig. S1). All listed AKH and GnRH peptides count 9 to 12 amino acids (Fig. 2). All AKH and GnRH peptides start with an N-terminal pyroglutamate residue and all processed peptides are amidated except the nematode ones, suggesting that the nonamidated AKH is most likely the most ancient form that amidation arose later in evolution. This agrees with the presence of a nonamidated AKH in the painted lady butterfly *Vanessa cardui* (22) and the fall armyworm *Spodoptera frugiperda* (23). The F(S/T) signature in the middle of the AKH-GnRH peptide sequences is common to all protostomian peptides and, remarkably, also in the echinoderm (deuterostomian) *Strongylocentrotus purpuratus*. The chordates are characterized by a middle WS motif. Further on in the peptide sequence, a tryptophan residue has been well conserved in the protostomian lineage, in the more ancient deuterostomians in the ancient chicken GnRH-II, but has been lost in the amphibians (24) (Fig. 2). To prove the existence of the AKH-like precursor in *C. elegans*, a gene-specific amplification reaction on the worm's cDNA was performed, resulting in a 270-bp fragment, exactly corresponding to its predicted length on www.wormbase.org (data not shown).

***C. elegans* AKH-GnRH-like Peptide Activates the Ce-GnRHR.** Because of the triple basic site (KKR) located downstream of the AKH-related peptide sequence within the *C. elegans* precursor (Fig. S1), it cannot be predicted with certainty where the precursor is actually cleaved to give rise to the fully processed peptide. Therefore, both pQMTFTDQWT and pQMTFTDQWTK were tested in a concentration range from 10^{-10} to 10^{-5} M on transfected HEK293T cells. Both of them were able to activate *Ce*-GnRHR in a dose-dependent manner (Fig. 3 *A* and *B*), although with different potencies. The calculated EC_{50}

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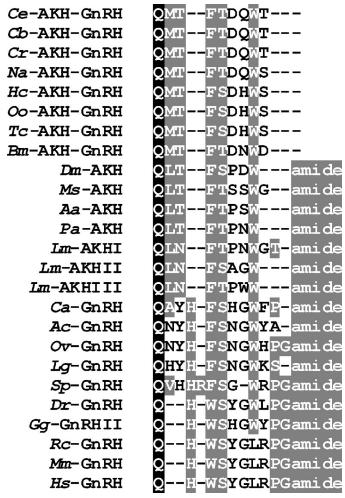

Fig. 2. Amino acid sequence alignment of AKH and GnRH peptides of different protostomian and deuterostomian representatives. In line with *Ce*-AKH-GnRH, other nematode peptides are also designated as AKH-GnRH-like. Alignment was generated by using the program Vector NTI. Identical amino acids are highlighted in black and similar amino acids in gray. Accession numbers of the peptide precursors in EMBL/GenBank; *Caenorhabditis elegans*: *Ce*-AKH-*GnRH* (NLP-47) (AAC26928), *Caenorhabditis briggsae*: *Cb*-AKH-*GnRH* (CBG19970), *Caenorhabditis remanei: Cr*-AKH-*GnRH* (cr01.sctg44.wum.78), *Necator americanus*: *Na*-AKH-*GnRH* (EST BU666660), *Haemonchus contortus*: *Hc*-AKH-*GnRH* (EST BF186675), *Osteragia osteragi*: *Oo*-AKH-*GnRH* (EST BQ625695), *Teladorsagia circumcincta*: *Tc*-AKH-*GnRH* (EST CB043182), *Brugia malayi: Bm*-AKH-*GnRH* (XP_00189879), *Drosophila melanogaster*: *Dm*-AKH (P61855), *Manduca sexta*: *Ms*-AKH (P67788), *Aedes aegypti: Aa*-AKH (XP_001655817), *Periplaneta americana: Pa*-AKH (AAV41425), *Locusta migratoria*: *Lm*-AKH I (P55319), *Lm*-AKH II (P08379), *Lm*-AKH III (P19872), *Capitella* sp. (polychaete annelid), *Ca*-GnRH (EY629959), *Aplysia californica* (mollusk): *Ac*-GnRH (ABW82703), *Octopus vulgaris*: *Ov*-GnRH (5), *Lottia gigantea* (mollusk), *Lg*-GnRH (FC805608), *Strongylocentrotus purpuratus*, *Sp*-GnRH (XP_800179), *Danio rerio*: *Dr*-GnRH (AAL99294), *Gallus gallus*: *Gg*-GnRH-II (ACF97836), *Rana catesbeiana*: *Rc*-GnRH (AAL05972), *Mus musculus*: *Mm*-GnRH (NP_032171), and *Homo sapiens*: *Hs*-GnRH (NP_000816). Notice the FS(T) signature conserved in all protostomes sequences and in the echinoderm (deuterostome). In most insect sequences, a carboxyterminal GW or WG motif is present. In mollusks, this is always GW as in some vertebrate sequences.

value for pQMTFTDQWT was 150 nM, whereas receptor-activity not even reached a plateau at 10 μM for pQMTFTDQWTK (EC_{50}>970 nM). Therefore, we conclude that pQMTFTDQWT is most likely the mature AKH-related peptide in *C. elegans*. When transfection with $G\alpha_{16}$ was omitted, an equally strong calcium response was obtained.

The *in silico* AKH pattern-based search and the sequence comparisons indicate that the newly found peptide is AKH-like. However, the present pharmacological data (activation of a GnRH-related GPCR) and the functional data (see next sections) are in favor of designating it a GnRH-like peptide. Therefore, we propose to designate pQMTFTDQWT from here onwards as the *C. elegans* AKH-GnRH-like peptide (*Ce*-AKH-GnRH). This AKH-GnRH precursor is the next in the list of formerly predicted or biochemically identified NLPs (neuropeptide-like precursors) in *C. elegans* (NLP-47, AAC26928).

Comparison of AKH-GnRH-like Peptide Sequences in Other Nematodes. To elucidate whether AKH-GnRH-like peptides are conserved in the phylum of Nematoda, we performed a NemaBLAST search with the *Ce*-AKH-GnRH precursor as a query in the EST libraries available on the web (http://www.nematode.net/BLAST/). This search revealed several orthologous neuropeptide precursor sequences in the hookworm *Necator americanus*, the intestinal parasite in sheep and goats *Haemonchus contortus*, in an endoparasite of cattle *Ostertagia ostertagi*, and in the stomach worm of sheep *Teladorsagia circumcincta*. In addition, a National Center for Biotechnology Information BLAST search yielded AKH-GnRH-like sequences in the genomes of *C. briggsae*, *C. remanei*, and the filarial *Brugia malayi* (Fig. 2). In all these nematodes, the predicted AKH-GnRH-like peptide is not amidated. Both pQMTFTDQWS (*N. americanus*) and pQMTFSDHWS (*H. contortus*, *O. ostertagi*, and *T. circumcincta*) were tested in the same cellular assay and are able to activate *Ce*-GnRHR in the cellular receptor assay with EC_{50} values of 315 nM and 624 nM, respectively (Fig. S2).

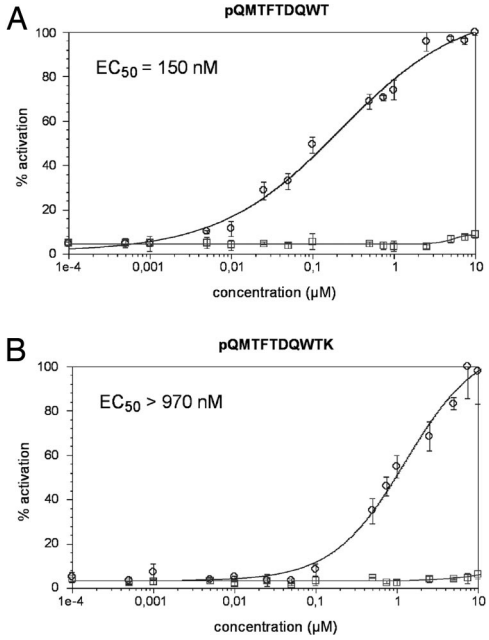

Fig. 3. Concentration-dependent calcium responses obtained by a fluorescence assay. Dose-response curves for (*A*) pQMTFTDQWT (EC_{50} = 150 nM) and (*B*) pQMTFTDQWTK (EC_{50}>970 nM) on *Ce*-GnRHR expressed in HEK293T cells. Fluorescent responses of the cloned cell line expressing *Ce*-GnRHR are expressed in % activation. Receptor responses (pcDNA/*Ce*-GnRHR) are represented by ○ and negative control responses (pcDNA3) are represented by □. These are the collected data from 3 independent measurements. The vertical bars represent standard deviations. Data were processed using Softmax Pro software (Molecular Devices).

AKH-GnRHR Signaling in *C. elegans* Modulates Egg-Laying Behavior. Because of the resemblance between *Ce*-GnRHR and vertebrate GnRHRs, we investigated whether *Ce*-GnRHR and its ligand play a role in reproduction of the worm. RNAi was performed by feeding the worms with HT115 bacteria, expressing the dsRNA of interest (25). Worms were observed every 12 h, starting at 72 h until 144 h, and their progeny was counted at different time points, as depicted in Fig. 4. We could clearly observe that 72 h, wild-type worms lay 27 eggs on average, whereas only 1-third of the normal number was laid by the RNAi knockdowns of the receptor (F54D7.3) and by the knockdowns of the AKH-GnRH precursor (F36H12.1). Knockdowns of receptor and precursor simultaneously did not result in any additional effect. This decrease in the amount of progeny remained significant for 84-h-old worms, at least for the receptor knockdowns and the double knockdowns. We also observed that the 120-h receptor knockdowns and the 120-h double knockdowns continued to lay eggs significantly longer than their wild-type counterparts. The total amounts of progeny counted in the whole experiment for each condition were on average 83.8

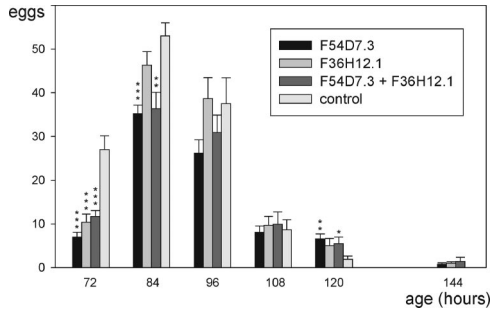

Fig. 4. Time course presenting the amount of progeny at different worm ages in RNAi knockdowns of the *Ce*-AKH-GnRH precursor (F36H12.1), *Ce*-GnRHR (F54D7.3) and in double knockdowns (F36H12.1 + F54D7.3) compared with wild-type worms (control). For every 4 conditions, progeny of 18 worms was counted; this is the result of 2 independent measurements. Error bars, s.e.m. *, $P < 0.05$; **, $P < 0.005$; ***, $P < 0.0005$.

per worm for the receptor knockdowns, 111.1 for the knockdowns of the peptide precursor, 95.7 for the double knockdowns, and 128 for the control group. We conclude that there is a delay in time of the egg-laying process and a decrease in the number of progeny when knocking down *Ce*-GnRHR and/or the AKH-GnRH precursor in *C. elegans.*

***Ce*-AKH-GnRH Increases Carbohydrate Levels.** The cockroach *Periplaneta americana* and the migratory locust *Locusta migratoria* were used as physiological model organisms to investigate the role of *Ce*-AKH-GnRH in an insect AKH bioassay as described in ref. 26. As depicted in Table 1, injection of either 250 or 500 pmol synthetic *Ce*-AKH-GnRH caused a significant increase in the levels of carbohydrates in *P. americana* haemolymph whereas the injection of distilled water as negative control had no effect. The endogenous hypertrehalosaemic hormone I of the cockroach (*Pa*-CAH-I) was used as positive control. If the increase by injection of 20 pmol of *Pa*-CAH-I is scored as maximal (100%) response, then 500 pmol of *Ce*-AKH achieved ≈40%. Injection of 250 pmol of *Ce*-AKH-GnRH into *L. migratoria* did not result in a significant increase of the lipids in the haemolymph although a small increase was observed when the experimental result (*Ce*-AKH-GnRH injection) was compared with the control (water injection).

Quantification of Fat Content in *Ce-AKH-GnRH* and *Ce*-GnRHR Knock-Downs. As insect AKH is known for its lipid-mobilizing activity, we also analyzed the intestinal fat content of worms in which either the *Ce*-GnRHR or the *Ce*AKH-GnRH precursor gene were knocked down. However, neither receptor nor precursor knockdowns differed significantly in fat content relative to wild-type worms, as visualized using the lipophilic vital dye Nile Red on L4 larvae (data not shown).

Discussion

Here, we report the existence of a biologically active adipokinetic hormone-like neuropeptide in nematodes that is able to activate an orphan *C. elegans* GPCR (*Ce*-GnRHR), which displays homology to AKHRs of insects and GnRHRs of vertebrates. We have designated this peptide *Ce*-AKH-GnRH. The phylogenetic tree of the various GnRHR sequences exhibits a clear distinction between the protostomian invertebrate group and the deuterostomian tunicate-vertebrate group (Fig. 1). This subdivision suggests that an ancestral receptor-encoding gene already existed before the divergence between Protostomia and Deuterostomia. The hypothesis arose that the ligand of the orphan *Ce*-GnRHR would either be an AKH or GnRH-related peptide. By means of genome-wide bioinformatics studies and biochemical methods, a total of 33 FLP peptide precursors and 46 NLPs have been identified in *C. elegans* (18), but none of these peptides display sequence resemblance with GnRH or AKH and none were able to activate the *Ce*-GnRHR in our cellular assay.

Having found that *Drosophila* AKH was able to activate *Ce*-GnRHR is in line with the previous cited hypothesis, that the the ligand of *Ce*-GnRHR might be a GnRH or AKH-like peptide and prompted us to conduct an in depth *in silico* search in the *C. elegans* genome. This revealed 1 protein (AAC26928), encoded by F36H12.1, that aligned nicely with the arthropod AKH prepropeptides, mollusk and annelid GnRH prepropeptides—although to a lesser extent—with vertebrate GnRH prepropeptides (Fig. S1). This difference between AKHs and GnRHs is reflected in the observation that *Drosophila* AKH could activate *Ce*-GnRHR, whereas human GnRH and chicken GnRH-II could not. Omission of $G\alpha_{16}$ in the receptor assay suggests that *Ce*-GnRHR—just as insect AKH receptors and vertebrate GnRH receptors (27)—signals through a $G\alpha_q$ protein with IP_3 and DAG as second messengers.

The newly discovered *Ce*-AKH-GnRH appears to be highly conserved in the nematode phylum as shown by a NemaBLAST. Four parasitic nonamidated orthologues very similar to *Ce*-AKH-GnRH were also able to activate *Ce*-GnRHR in a dose-dependent manner (Fig. S2). The ability of nematode AKH-GnRH-related peptides and of *Drosophila* AKH to activate *Ce*-GnRHR on one hand and the cross reactivity of *Ce*-AKH-GnRH in the cockroach assay on the other hand further indicate that *Ce*-AKH-GnRH is the nematode orthologue of insect AKH and suggests a common evolutionary origin for the respective AKH-GnRH precursors. Also from sequence comparisons of the entire prepropeptides, it becomes clear that *Ce*-AKH-GnRH, insect AKHs, mollusk GnRHs, and annelid GnRH display more similarities among each other than to chordate GnRHs. However, it is clear that the deuterostomian Echino-

Table 1. Heterologous assay for adipokinetic activity and for hyperglycaemic activity of synthetic *Ce*-AKH-GnRH (acceptor insects: *L. migratoria* and *P. americana*, measurement of total lipid and carbohydrate concentration respectively in the haemolymph)

		Haemolymph lipids (mg·ml^{-1})					Haemolymph carbohydrates (mg·ml^{-1})				
Acceptor insect	Treatment	*n*	0 min	90 min	Difference	Pa	*n*	0 min	90 min	Difference	Pa
P. americana	Control (10 μl distilled water)						10	11.0 ± 1.9	11.1 ± 1.7	0.1 ± 0.5	NS a
	10 μl *Ce*-AKH-GnRH 250 pmol						10	10.9 ± 1.7	18.0 ± 3.6	7.1 ± 2.4	0.0000001b
	10 μl *Ce*-AKH-GnRH 500 pmol						10	12.0 ± 1.6	23.2 ± 6.1	11.2 ± 5.0	0.0004 c
	10 μl *Pa*-CAH-I 20 pmol						10	11.7 ± 1.6	37.6 ± 2.5	25.9 ± 2.5	0.0000001d
L. migratoria	Control (10 μl distilled water)	6	21.2 ± 5.0	20.4 ± 4.0	−0.8 ± 2.9	NS					
	10 μl *Ce*-AKH-GnRH 250 pmol	6	17.5 ± 9.2	15.5 ± 5.7	−2.0 ± 3.8	NS					

Data are presented as mean ± SD. Paired *t*-test was used to calculate the significance between pre- and post-injection. NS indicates not significant. Significance of difference between control and experimental group (for the cockroach) was calculated using ANOVA (post-hoc: Duncan test). Different lower-case letters indicate statistical difference ($P = 0.05$).

derm *Strongylocentrotus purpuratus* GnRH resembles more the protostomian GnRHs from annelids and mollusks than the deuterostomian GnRHs. This, together with the overall structural similarities between AKHs and GnRHs, provides strong evidence for their common origin and contradicts the recently put forward hypothesis that ecdysozoans have lost GnRH during the course of evolution (9). Our data indicate that nematodes and arthropods (ecdysozoans) have an AKH-GnRH-like precursor. Edysozoan AKH-GnRH, however, seems to have diverged substantially from chordate GnRH during the course of evolution.

In locusts, all 3 prohormones present form homodimeric precursors but only the AKH-III prohormone has 2 Cys residues (28, 29). This locust AKH-III prohormone, in contrast to locust AKH-I and AKH-II prohormones (which have only a single cysteine residue), resembles best the other insect AKH prohormones and all other protostomian AKH-GnRH prohormones, which also contain 2 cysteines (Fig. S1). This suggests that the AKH-III-type precursor is reminiscent of the most ancient prohormone precursor and that AKH-I and AKH-II precursors in locusts probably arose later by gene duplication.

Because insect AKHs are involved in energy mobilization during periods of high energy demand, a similar role for *Ce*-AKH-GnRH seems plausible. Comparing the intestinal fat content—a measure for fat and energy mobilization—of L4 *Ce*-GnRHR and AKH-GnRH precursor knockdowns with wild-type L4 worms yielded no significant difference. This is in agreement with the data resulting from the genome-wide RNAi analysis of *C. elegans* fat regulatory genes (30). Remarkably, however, the list of RNAi targets that produces an increased fat content, contains an estrogen-type nuclear hormone receptor (F33D4.1). The fact that a plausible *C. elegans* orthologue of the vertebrate reproduction axis is also involved in fat storage may provide a link between energy mobilization and reproduction. Our RNAi experiments of *Ce*-GnRHR and the AKH-GnRH precursor indicated that both may play an important role in reproduction, which is in accordance to the situation in vertebrates (15). It is clear that *Ce*-GnRHR and *Ce*-AKH-GnRH affect the same pathway, because their knock-downs yielded the same phenotype: a delay in timing of the egg-laying process and a decrease in total number of progeny (Fig. 4). The similar noncumulative phenotype observed when silencing either receptor or neuropeptide precursor also provides evidence for *Ce*-AKH-GnRH being the cognate ligand for *Ce*-GnRHR in vivo. The clearest loss-of-function phenotype is observed for the receptor knock-down. *Ce*-GnRHR is localized in the nucleus of maturing oocytes, intestinal cells and on the pharyngeal musculature (15), similar to the mollusk *Crassostrea gigas*, where GnRHR transcripts are mainly expressed in the gonads (31). Although expression of *Ce*-GnRHR in the gonads favors a direct effect of *Ce*-AKH-GnRH on egg laying, *Ce*-AKH-GnRH may have an indirect effect by modulating the release of gonadotropins, analogous to the hypothalamic-pituitary-ovary axis in vertebrates. A leucine-rich GPCR (LGR), called FSHR-1 (follicle-stimulating hormone receptor 1), with sequence similarity to vertebrate pituitary gonadotropin receptors (32) and estrogen binding proteins (33), have been reported in *C. elegans*. The FSHR has a role in the control of germ-cell differentiation, proliferation, and worm survival (34). The exact working mechanism of AKH-GnRH signaling in *C. elegans* remains to be investigated, although the effect of a knockdown of this signaling system on the worm's phenotype is clear and analogous to vertebrates where disruption in the chain of events in the hypothalamic-pituitary-gonadal axis causes a deficiency of the sex hormones responsible for normal sexual development in puberty and halts normal sexual maturation. Mutations in the vertebrate GnRH receptor result in hypogonadotropic hypogonadism (35). The delay in egg laying in *C. elegans* can be considered as analogous to a delayed oocyte maturation and puberty in mammals. Taken into account that a wild-type *C. elegans* starts laying eggs at the age of 3 days, a delay in the egg laying of 12 h is enormous.

It is reasonable to suggest that the hypothalamic-pituitary-gonadal axis has also been conserved in insects and that the most ancient function of AKH might be related to reproduction. AKH deficient fruit flies seem to display normal reproductive capabilities, but, to our knowledge, an investigation toward delayed egg laying has not yet been performed. It is interesting to note that in the fall armyworm *S. frugiperda* AKH mRNAs occur in ovaries, midgut, fat body, accessory glands, and muscle tissues, suggesting that *akh* genes may play a role in the regulation of oocyte maturation (23). Also in *Anopheles gambiae* females, AKH receptor transcripts were abundant in the head, thorax, dorsal and ventral abdomen walls to which most of the fat body is attached and in ovaries as well (36). Taken together, we conclude that ecdysozoan AKHs, lophotrozoan GnRHs and chordate GnRHs derive from a common ancestor that may represent one of the most ancient hormones to persist through metazoan evolution. The present research opens a lot of questions for future research, not in the least whether the AKH-GnRH pathway targets the LH-FSH pathway and downstream gonadal steroidogenesis, not only in *C. elegans* but also in other invertebrates. In addition, the presence of AKH-GnRH-like neuropeptides in various parasitic worm species opens perspectives for practical applications. Nematode specific antagonists for the GnRH receptor having a therapeutic value in domestic animals and even in humans can now be developed.

Materials and Methods

Molecular Cloning of the *Ce*-GnRH Receptor and Transfection in Mammalian Cells. The ORF of the F54D7.3 gene was amplified by PCR performed on the cDNA (SuperScript First-Strand Synthesis System for RT-PCR, Invitrogen), synthesized from mRNA (QuickPrep Micro mRNA Purification Kit, Amersham Biosciences) extracted from mixed stage *C. elegans* N2. Specific oligonucleotide PCR primers (Sigma), based on the predicted sequence (www.wormbase.org) were used (forward primer 5′ CACCATGACAACGATCAACTGCTC 3′; reverse primer 5′ TCAAAAATCAATAATTTCTAATTGAAC 3′). The full-length cDNA of the *Ce*-GnRHR was then amplified by means of PCR (Advantage 2 PCR kit, Clontech). The resulting PCR product was directionally cloned into the eukaryotic expression vector pcDNA3.1D (Invitrogen) and sequenced. Human embryonic kidney (HEK293T) cells were transiently transfected with the *Ce*-GnRHR/pcDNA3.1 expression construct and the promiscuous G protein $G\alpha_{16}$ using FuGene 6 (Roche) (37).

Fluorescence Assay. The transfected HEK293T cells were loaded with Fluo-4-AM (Molecular Probes) for 1 hour. Excitation of the fluorophore was done at 488 nm. The Ca^{2+} response was measured for 2 min at 525 nm using a FLEXstation (Molecular Devices) at 37 °C. Data were analyzed using Softmax Pro (Molecular Devices).

***In Silico* Peptide Precursor Search.** All *C. elegans* proteins that are <500 aa in length were extracted from the Uniprot protein database (release 12.1). The first 70 aa of each protein sequence were put into a text file, which is used as input for the SignalP program (http://www.cbs.dtu.dk/services/SignalP/). This resulted in a dataset of all *C. elegans* proteins having an N-terminal signal peptide and no other transmembrane regions as verified by TMpred (http://www.ch.embnet.org/software/TMPRED_form.html). This dataset was compared by means of standalone BLAST with another text file containing all adipokinetic peptide sequences and their precursors as assembled from our recently constructed Metazoan peptide database (www.peptides.be/) (21). The scoring matrix PAM30 was used and the parameter word size is set to two to find short but strong similarities. Finally, the retained proteins were compared with the PROSITE pattern 'Q-[LV]-[NT]-[FY]-[ST]-x (2)-W,' which characterizes the adipokinetic family (http://ca.expasy.org/PROSITE). Only one remaining protein, O76722, matched the adipokinetic peptide pattern at all but one site.

Peptide Synthesis. Based on *in silico* predictions and in-house peptidomics data (18), a library of 175 synthetic *C. elegans* peptides was composed and custom-synthesized by Sigma-Genosys, and GL Biochem Ltd. (GLS) (17). In addition, the

most plausible hits, which resulted from the *in silico* peptide search described above were custom synthesized. All peptides were initially tested at a concentration of 10 μM. Receptor activating peptides were further tested in a final concentration range from 10^{-10} to 10^{-5} M.

Expression Evidence of the *Ce*-AKH Precursor by RT-PCR. The ORF of the F36H12.1 gene was amplified by reverse transcriptase PCR, performed analogous to the F54D7.3 gene on *C. elegans* cDNA. Specific oligonucleotide PCR primers (Sigma), based on the predicted sequence (www.wormbase.org), were used (forward primer 5′ CTTCCTGCTAGGTCATTGC 3′; reverse primer 5′ GCTTCTGAAAGGATTTTGG 3′).

RNA Interference by Feeding. RNAi was achieved by feeding synchronous groups of first stage (L1) larval *C. elegans* HT115 (DE3) bacteria expressing the dsRNA of interest as described in ref. 25. To obtain the *Ce*-GnRHR dsRNA expression vector, both the *Ce*-GnRHR/pcDNA3.1 expression construct and the L4440 plasmid were digested with the restriction enzymes *Bam*HI and *Xho*I and ligated to each other (Rapid DNA ligation kit, Roche). RNAi bacterial strains targeting *unc-22 (ZK617.1*) were obtained from Geneservice (Geneservice Ltd). All RNAi feeding experiments were performed with 18 replicas using wild type N2 Bristol *C. elegans* and on nematode growth media (NGM) plates. *Unc-22* was used as a positive control (twitching phenotype) and the empty L4440 vector as a negative control. Worms, reared at 20 °C, were observed every 12 h, starting at 72 h of age until 144 h of age, and their progeny were counted.

Heterologous Bioassay in *Periplaneta americana and Locusta migratoria.* The heterologous bioassay in the cockroach *Periplaneta americana* and in the migratory locust *Locusta migratoria* was performed as described in ref. 26.

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