**Structure-activity studies of *Drosophila* adipokinetic hormone (AKH) by a
cellular expression system of dipteran AKH receptors**

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Abstract

Structure-activity studies for the adipokinetic hormone receptor of insects were for the first time performed in a cellular expression system. A series of single amino acid replacement analogues for the endogenous adipokinetic hormone of *Drosophila melanogaster* (pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH₂) were screened for activity with a bioluminescence cellular assay, expressing the G-protein coupled receptor. For this series of peptide analogues, one amino acid of the N-terminal tetrapeptide was successively replaced by alanine, while those of the C-terminal tetrapeptide were successively substituted by glycine; other modifications included the blocked N- and C-termini that were replaced by an acetylated alanine and a hydroxyl group, respectively. The analogue series was tested on the AKH receptors of two dipteran species, *D. melanogaster* and *Anopheles gambiae*. The blocked termini of the AKH peptide probably play a minor role in receptor interaction and activation, but are considered functionally important elements to protect the peptide against exopeptidases. In contrast, the amino acids at positions 2, 3, 4 and 5 from the N-terminus all seem to be crucial for receptor activation. This can be explained by the potential presence of a β -strand in this part of the peptide that interacts with the receptor. The inferred β -strand is probably followed by a β -turn in which the amino acids at positions 5-8 are involved. In this β -turn, the residues at positions 6 and 8 seem to be essential, as their substitutions induce only a very low degree of receptor activation. Replacement of Asp⁷, by contrast, does not influence receptor activation at all. This implies that its side chain is folded inside the β -turn so that no interaction with the receptor occurs.

Key words: Adipokinetic hormone; Structure-activity relationship; *Drosophila melanogaster*; *Anopheles gambiae*; Adipokinetic hormone receptor; Cellular expression system.

1. Introduction

Insects are one of the most diverse groups of animals inhabiting our planet. They are spread over almost every terrestrial ecological niche available and, in general, they show a high mobility. This requires the presence of a fine-tuned energy metabolism to cope with the acquirement of energy during highly energy-demanding processes like flight. These metabolic processes in insects are regulated by the adipokinetic hormone (AKH). The synthesis and storage of the AKH peptides take place in the *corpus cardiacum* (CC). When energy is required, AKHs are released from the CC into the haemolymph. Next, these neuropeptides will bind to their G-protein coupled receptors (GPCRs), which are located in the plasma membranes of adipocytes. This will trigger a number of intracellular pathways which finally result in the mobilization of carbohydrate or lipid reserves and, in some insects, the release of proline. Oxidation of these energy rich substrates by muscle tissue will yield the necessary energy [9,28,36]. In addition to the main function of AKHs in energy metabolism, they also act as ‘anti-stress’ hormones (*e.g.* oxidative stress situations) [19,23,37]. These stress related actions include cardiostimulation and the inhibition of synthesis of RNA, fatty acids and proteins in the fat body [22]. There are also indications that AKHs have an effect on some immune responses [10,15,17,22] and that they are involved in the regulation of the reproductive process [27,31].

The AKH peptides are members of a large group of structurally related peptides, the adipokinetic hormone/red pigment-concentrating hormone (RPCH) family. They typically occur as octa-, nona- or decapeptides with the N-terminus blocked by pyroglutamate and the C-terminus blocked by amidation, which make the peptide only accessible by endopeptidases. The presence of aromatic amino acids at positions 4 (Phe or Tyr) and 8 (Trp) are conserved as well. Most of the identified AKH peptides are uncharged, only some AKHs contain an

76 aspartic acid at position 7, which gives the peptide a net negative charge. There is also ample
77 evidence that AKHs possess secondary structures in the form of a β -strand and a β -turn
78 [5,32,41].

79 Neuropeptides and their GPCRs have been proposed as a promising option for the
80 development of a new generation of species-specific insecticides [4,10,33]. To be able to
81 design proper peptidomimetics that can be useful in insecticide applications, it is helpful to
82 gather all possible information about the binding characteristics between the ligand and its
83 receptor [1,33]. So far, almost 55 AKH isoforms have been described [8,12,13,14,29,30]. The
84 question was raised whether only the conserved features of the AKH peptides are absolutely
85 necessary for activation of their receptors or whether other, non-conserved amino acids, are
86 also required. In the past, several structure-activity relationship (SAR) studies have been
87 performed using *in vivo* and *in vitro* assays to determine the essential amino acids of the AKH
88 peptides. Most studies were done on the AKHs of the migratory locusts *Locusta migratoria*
89 and *Schistocerca gregaria*, the moth *Manduca sexta*, and of the cockroaches *Periplaneta*
90 *americana* and *Blaberus discoidalis*. In general, these studies revealed that aromatic amino
91 acids at positions 4 (containing a phenyl ring) and 8 (containing an indole ring) and the entire
92 N-terminal pentapeptide of the AKH peptide are necessary to activate the AKH receptor. The
93 side chains of the amino acids at positions 6 and 7 have a minor role in activating the receptor
94 just as the modified N- and C-terminal ends which protect the peptide from exopeptidases
95 [6,7,11,,25,26,38,39]. But, despite the fact that the first AKH receptors (AKHRs) (*D.*
96 *melanogaster* AKHR and *Bombyx mori* AKHR) were already characterized in 2002 [34], true
97 functional activation assays in which the receptor is expressed in mammalian cells, have
98 never been performed with members of the AKH family [10].

99 In this study we report the first SAR assays on AKH peptides using a cellular expression
100 system. An analogue series of AKH peptides was designed based on the endogenous *D.*

melanogaster AKH peptide (Drome-AKH). These analogues were screened on the AKHR of the fruit fly *D. melanogaster* and the AKHR of the malaria mosquito *A. gambiae*. Based on the potential of a synthetic analogue to activate the AKH receptors, we could determine the residues in the Drome-AKH peptide that are crucial for receptor activation.

2. Materials and methods

2.1. Insects

D. melanogaster (Canton S strain) flies were raised at 25 °C on a 12:12 light:dark cycle and maintained on a diet of standard corn meal-yeast-agar medium. The adult *A. gambiae* male and female mosquitoes were a kind gift of the Malaria Entomology Research Unit of the Wits University (Johannesburg, South Africa).

2.2. Cloning and sequencing of receptor cDNA

Adult fruit flies and malaria mosquitoes of unspecified age of both sexes were collected for total RNA extraction using the RNeasy® Lipid Tissue Mini Kit (Qiagen) (respectively 20 flies and 10 mosquitoes were pooled). cDNA synthesis was performed with the SuperScript™ III First-Strand Synthesis System for RT-PCR (Invitrogen). The primers applied for the amplification of the *D. melanogaster* AKH receptor were 5'-CACCATGGCAAAAGTAGCTGAG-3' (forward) and 5'-TTACTTCTGGCGGATCGG-3' (reverse), which were based on the primers used by Staubli *et al.* [34]. For the amplification of the *A. gambiae* AKH receptor we used 5'-CACCATGCCCAACACAATGGCCGCCACATCAAC-3' (forward) and 5'-CTACGCCCTCATCGTCAGATGACTGCCGCAGCT-3' (reverse) as primers, which were based on the ones used by Belmont *et al.* [3]. The Advantage 2 Polymerase PCR Kit

(Clontech) was used for the actual PCR reaction and the following conditions were used: initial denaturation for 3 min at 95 °C; amplification for 30 s at 94 °C; annealing for 1 min at 68 °C and elongation for 3 min at 68 °C for 35 cycles, followed by an incubation for 3 min at 68 °C. The PCR products were separated in a 1% agarose gel and the DNA of expected size was purified using the Agarose Gel DNA Extraction Kit (Roche). The single 3' A-overhangs for T/A cloning were obtained by mixing 15 µl of the gel extracted PCR product with 4.5 µl 10x PCR Rxn Buffer (- MgCl₂; Invitrogen), 1 µl dATP (10 mM; Roche), 0.5 µl MgCl₂ (50 mM; Invitrogen), 0.2 µl Taq Polymerase (Invitrogen) and 28.8 µl Milli-Q H₂O and incubating this solution at 72 °C for 20 min. These products were T/A cloned with the pcDNATM 3.1/V5-His TOPO® TA Expression Kit (Invitrogen), followed by the isolation of the plasmids with the GenEluteTM Plasmid Miniprep Kit (Sigma-Aldrich). Sequence analysis was performed with the BigDye® Terminator v1.1 Cycle Sequencing Kit (Applied Biosystems).

2.3. Cell culture and transfections

Chinese hamster ovary (CHO) cells (CHO-K1), stably overexpressing the mitochondrially targeted apoaequorine, the promiscuous human G protein G_{α16} and a zeocin resistance gene were cultured in monolayer in Dulbecco's Modified Eagles Medium nutrient mixture F12-Ham (DMEM/F12; Sigma) supplemented with 10% heat-inactivated fetal calf serum (Invitrogen), 250 mg/ml zeocin (Invitrogen), 100 IU/ml penicillin (Invitrogen), 100 µg/ml streptomycin (Invitrogen) and 2.5 µg/ml fungizone (Amphotericin B; Invitrogen). The cells were cultured in a humid atmosphere with a constant supply of 5% CO₂ at 37 °C and passaged every 3 days (1:10).

Cells were transiently transfected with the receptor expression constructs using the transfection reagent LipofectamineTM LTX Reagent (Invitrogen), according to the

manufacturer's instructions. After an incubation period of 24 h complete cell medium was added to allow an additional growth period before the bioluminescence assay.

2.4. Bioluminescence assay

Intracellular calcium was monitored as previously described [2,20]. Briefly, CHO/mtAEQ/Gα₁₆ cells expressing the receptor were collected 2 days post-transfection in bovine serum albumin (BSA) medium (DMEM/F12 without phenol red [Gibco], with L-glutamine and 15 mM HEPES supplemented with 0.1% BSA [Sigma]) and loaded with 5 μM coelenterazine h (Invitrogen) for 4 h. After a 10-fold dilution, cells (25,000/well) were challenged with potential peptide ligands reconstituted in BSA medium. The calcium response was monitored for 30 s on a Mithras LB940 (Berthold Technologies) and recorded in triplicate for dose-response measurements. Triton X-100 (0.1%) and BSA medium were included as a positive and negative control, respectively. ATP (1 μM) was used to check the functional response. EC₅₀ values were calculated from dose-response curves, constructed using a computerized nonlinear regression analysis with a sigmoidal dose-response equation (SigmaPlot 12.0; Systat Software, Inc.) based on three independent measurements.

2.5. Peptides

The synthetic peptides representing the endogenous AKH peptides of *D. melanogaster* (pQLTFSPDWa) and *A. gambiae* (pQLTFTPAWa) were synthesized by GL Biochem Ltd. (Shanghai). The peptides for the SAR analyses were synthesized by LifeTein LLC (South Plainfield, USA). To dissolve the peptides, 80% acetonitrile was used. Purification of the peptides was performed with reversed-phase high performance liquid chromatography (HPLC). The bicinchoninic acid (BCA) assay was used to determine the concentrations of the HPLC-purified peptides [35].

The purity of the HPLC fractions was verified with a matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF, Ultraflex II Bruker Daltonics) mass spectrometer. One microlitre of each HPLC sample was spotted on a MALDI target plate and covered with 1 μ l of saturated matrix solution (α -cyano-4-hydroxycinnamic acid [10 mg/ml] in 50% acetonitrile containing 0.1% TFA). The spots were dried at room temperature before the plate was loaded in the mass spectrometer which operated in the positive reflectron, single mass spectrometry mode. The calibration was carried out with a calibration standard (Bruker Daltonics) containing the following peptides: ACTH clip 1-17, ACTH clip 18-39, angiotensin I and II, bombesin, somatostatin 28 and substance P. Every spot was shot 3000 times, and the resulting spectra were analysed with the Flex Analysis software (Bruker Daltonics). It should be kept in mind that AKH peptides exhibit a characteristic ion signature with MALDI-MS (ion pair $[M+Na]^+/[M+K]^+$) [24].

3. Results

3.1. Dose-response analysis

We performed a dose-response study with the Drome-AKH peptide on its receptor and calculated the corresponding EC_{50} value to determine whether we had successfully cloned and expressed the Drome-AKHR transiently in the CHO-K1 cells, and to determine the optimal ligand concentration to be used in the SAR assay. An EC_{50} value of 0.23 ± 0.11 nM (Figure 1) was found. The same was done for the Anoga-AKH peptide and its receptor, for which we found an EC_{50} value of 54 ± 0.07 nM (Figure 1) in our aequorine based bioluminescence assay. To define the optimal concentrations for the SAR analyses on the Anoga-AKHR with the Drome-AKH peptide analogues, we also carried out a dose-response study with Drome-AKH on the Anoga-AKHR. This resulted in an EC_{50} value of 562 ± 0.04 nM (Figure 1).

3.2. SAR analysis

A series of synthetic analogues of the Drome-AKH peptide was designed for SAR analyses to determine the relative importance of each side chain and of the blocked termini for the activation of the Drome-AKHR and the Anoga-AKHR. Each analogue had only one substitution (Table 1). Alanine and glycine were the amino acids chosen for the replacements because they mimic the effect of deleting the side chain of the replaced amino acid without interrupting the peptide backbone. Alanine was used to replace the amino acids in the N-terminal tetrapeptide. Glycine was used to replace the amino acid residues located in the C-terminal tetrapeptide where a β -turn has been predicted, because glycine is the most flexible amino acid and as such especially used for the presumed turn positions [5,11]. The importance of the N-terminus was tested by replacing the pyroglutamyl residue (pGlu) with a blocked alanine (N-acetyl-Ala). The acetylation of alanine avoids a free amino group, but it causes a difference in the hydrogen bonding potential (*i.e.*, *trans* vs. *cis* amide bond) and in flexibility (*i.e.*, cyclic vs. acyclic side chain) of the N-terminus [11,39]. To test the importance of the C-terminus, the amide was replaced with a carboxyl group, which adds an extra negative charge to the analogue in comparison with the endogenous Drome-AKH where the only negative charge is due to the aspartic acid at position 7 [11].

The analogue series was tested in concentrations that fit in the magnitude of the EC₅₀ values of the Drome-AKH peptide for the Drome- and the Anoga-AKHR (1 nM and 500 nM, respectively).

3.3. Drome-AKHR SAR analysis

Compared with the endogenous Drome-AKH (pGlu¹Leu²Thr³Phe⁴Ser⁵Pro⁶Asp⁷Trp⁸amide), no difference in activation of the Drome-AKHR was seen when Asp⁷ was replaced by a

glycine residue. When the analogues for the N-terminus (pGlu¹ replaced by an acetylated alanine residue) and the C-terminus (the amidation replaced by a hydroxyl group) were tested, a 55-70% decrease in activation of the receptor was noticed. Testing the analogues in which the amino acids Leu², Thr³, Phe⁴ were replaced by an alanine residue and where the amino acids Ser⁵, Pro⁶ and Trp⁸ were substituted by a glycine residue, resulted in a very pronounced decrease of activation between 85% and 95% (Figure 2A).

3.4. Anoga-AKHR SAR analysis

To perform the SAR studies on the Anoga-AKHR, we carried out the bioluminescence assay with the analogues of the Drome-AKH peptide in a final concentration of 500 nM. Substituting the Asp⁷ residue of the Drome-AKH peptide by a glycine resulted in a more efficacious activation of the Anoga-AKHR, as indicated by the 46% increase in activation compared with the Drome-AKH. A slight reduction in activation by only 26% was observed when the Pro⁶ was replaced by glycine. The analogues for the N- and C-terminus decreased receptor activation by 70-80%. Substitutions of Leu², Thr³, Phe⁴ by alanine and Ser⁵ and Trp⁸ by glycine led to an almost complete lack of response by the Anoga-AKHR with reductions in activation of 85-97% (Figure 2B).

4. Discussion

In the past, various attempts were made to define the structural features of the AKH peptides necessary for the activation of the AKHRs of *M. sexta*, *L. migratoria*, *S. gregaria*, *P. americana* and *B. discoidalis* [7,11,16,18,25,26,38,39]. These studies include functional assays like the *in vivo* and *in vitro* lipid mobilization assays and the *in vitro* acetate uptake

assay. Drawbacks of these experiments are the fact that *in vivo* experiments are hard to control for the influence of, for example, the degradation of injected peptides by peptidases or the release of other possibly interfering endogenous peptides. Another uncertainty with the *in vivo* and *in vitro* assays used, is the possibility that the tested peptides can bind and activate other receptors [11,16,25,38].

We report the usage of a cellular expression system to perform structure-activity relationship assays to determine the essential amino acids of AKH. The AKH receptors of *D. melanogaster* and *A. gambiae* were successfully recloned and expressed in CHO-K1 cells. The EC₅₀ value of 0.23 ± 0.11 nM found for the Drome-AKH peptide on the Drome-AKHR was of the same magnitude as the 0.8 nM EC₅₀ value reported previously [34]. For the Anoga-AKH peptide tested on the Anoga-AKHR, an EC₅₀ value of 54 ± 0.07 nM was recorded, which is a magnitude higher than the 3 nM EC₅₀ value found earlier [3]. The Drome-AKH peptide was also tested on the Anoga-AKHR receptor and showed to evoke a dose-related response with an EC₅₀ value of 562 ± 0.04 nM. This allowed us to carry out SAR analyses by testing a series of peptide analogues, based on the Drome-AKH, in the range of the stated EC₅₀ values for both receptors. However, the EC₅₀ value of Drome-AKH on the Anoga-AKHR is about 18 times higher compared with the endogenous Anoga-AKH peptide. This is probably due to the fact that Drome-AKH and Anoga-AKH differ in two amino acids. The Drome-AKH peptide has a serine residue at position 5 and an aspartic acid at position 7, whereas Anoga-AKH has a threonine at position 5 and an alanine at position 7 (Table 1).

The results of the SAR analyses with the N- and C-terminal Drome-AKH analogues confirmed that these structures are not essential for activation of the Drome-AKHR and Anoga-AKHR, although these blocked termini are conserved in all natural AKHs. The substantial activation of both receptors when pGlu¹ is substituted with a blocked acetylated Ala residue is in agreement with the findings of previous studies. These showed that the

AKHRs of *P. americana*, *L. migratoria* and *M. sexta* could still be activated when the N-termini of their AKH peptides were replaced by other residues of which the free amino terminus was blocked (*e.g.* [N-Ac-Ala], [N-Ac-Tyr], [N-Ac-Pro], [N-Ac-Gly] or [biotin-Gly]) [11,16,25,40]. However, in *B. discoidalis* the pGlu¹ replacement by [N-Ac-Ala] was only very slightly active. This could be due to important differences between the AKH receptors of different insects [6,16]. So, in general, when pGlu¹ is replaced by residues of which the free amino terminus is blocked, these analogues are still capable of activating the receptor to a certain level. This indicates that the conserved N-terminal pyroglutamyl residue is not crucial for activation of the receptor, but is probably necessary to protect the peptide from degradation by exopeptidases [25]. It should be stated here that we have not tested any non-blocked N-terminal analogue yet (thus a simple Ala residue at position 1, for example); such analogue may shed light to the fact whether the blockage at the N-terminus does indeed protect against proteolytic breakdown via exopeptidases.

For the C-terminal analogue, the amide was replaced by a hydroxyl group [Trp-OH] which gives the analogue an extra negative charge (besides the one of Asp⁷). This resulted in a sharp decline in activation of the Anoga-AKHR, which is in accordance with the outcome of other studies testing the effect of substituting the amidation with a negative charge (the endogenous AKH peptides in these studies were also neutral peptides, so the hydroxyl group provided the only negative charge) [7,11,26]. However, when we tested this analogue on the Drome-AKHR, we still noticed a rather high level of receptor activation. A possible explanation for this smaller impact on the activation of the Drome-AKHR could lie in the fact that the endogenous AKH peptide of *Drosophila* also possesses a negative charge, so the second negative charge would not be as detrimental. This might indicate that it is rather the presence of a negative charge than the amidation at the C-terminus that is essential for receptor activity.

299 Hence, the conserved C-terminal amidation probably does not interact with the receptor, but
300 may preserve the peptide from degradation by exopeptidases [26].

301 Besides the blocked termini, each natural AKH peptide contains aromatic amino acids located
302 at positions 4 (Phe or Tyr) and 8 (Trp). Ala-substitution of Phe⁴ or Gly-substitution of Trp⁸
303 led to an almost complete lack of response by the Drome- and Anoga-AKHR, which was also
304 the case in former SAR studies [11,38,39]. Thus, our studies confirm the earlier conclusions
305 that the aromatic residues at positions 4 and 8 are essential for interaction with and activation
306 of the AKH receptors.

307 The analogues for Leu², Thr³ and Ser⁵ also led to an almost complete lack of response by both
308 the Drome- and the Anoga-AKHR with reductions in activity ranging from 85% to 97%. This
309 is not surprising as the sequences of the endogenous Drome- and Anoga-AKH peptides
310 indicate the existence of a β -strand within the five N-terminal residues (Supplementary Data)
311 [11]. This assumption is supported by the presence of alternating hydrophilic (pGlu¹, Thr³ and
312 Ser⁵) and hydrophobic (Leu² and Phe⁴) amino acids which result in an amphiphilic orientation
313 typical for the presence of a β -strand [5,11,21,41]. This suggests that the side chains of these
314 residues would be oriented to expose the backbone amide protons and the carbonyl oxygens
315 to form H-bonds with β -strand or β -sheet regions of the receptor. The substitution of any of
316 these residues by alanine (simple methyl side chain) (pGlu¹, Leu², Thr³ and Phe⁴) or glycine
317 (no side chain) (Ser⁵), would interrupt the amphiphilicity pattern, which could disrupt the
318 strand stability and as such the proper interaction between the peptide and the receptor [11].
319 Our observations are in line with other studies as well [11,16].

320 Besides the β -strand predicted for amino acids 1-5, the presence of a β -turn can be assumed
321 for the amino acids at positions 5-8 (Supplementary Data) [11]. Hence, this would emphasize
322 the importance of the residue at position 5, Ser (*D. melanogaster*) or Thr (*A. gambiae*), which
323 is characterized by a hydroxylated side chain residue in all natural AKHs [11]. In addition to

the involvement of the residue at position 5 in intramolecular hydrogen bonding and stabilizing the secondary structure, it also acts as the *i* amino acid of the proposed β -turn [16,38]. The amino acids Pro⁶ and Asp⁷ in the Drome-AKH peptide and Pro⁶ and Ala⁷ in the Anoga-AKH peptide would also be part of the β -turn. The results of the SAR studies for these residues on the Drome-AKHR show that the Pro residue seems to be essential, but the replacement of Asp by Gly does not have any effect on the activation. For the Anoga-AKHR we see a different outcome. The substitution of Pro by Gly only leads to a slight reduction in activation of the receptor by 26%. However, when Gly is present at position 7, we notice an increase of activation by 46%. A Pro residue would be most strongly preferred for the *i* + 1 position of a β -turn [38]. Its replacement, however, by a Gly can also induce the turn as noticed from the results for the Anoga-AKHR. The Asp⁷ is probably not participating at all in essential H-bondings or interactions with the receptor, because a substitution of this residue with Gly does not show detrimental effects on Drome-AKHR receptor activation. A possible explanation could be that the side chain of the Asp residue is folded inside the β -turn [32]. Another observation may also be of significance here: whereas most positions in natural AKH octapeptides are taken up by only two (positions 3, 4 and 5) or four (positions 2 and 6) different amino acids, eight substitutions are known at position 7 [8]. This variety of substitutions at position 7 may be a good indicator that this position in the AKH molecule is not involved in receptor binding and, hence, diverse substitutions can take place. The increased potency to activate the Anoga-AKHR when Asp⁷ is replaced by Gly, is probably due to the fact that the endogenous Anoga-AKH peptide does not carry a negative charge (it has an Ala residue), and with a Gly residue at position 7, the neutral peptide is restored. It seems reasonable that the Anoga-AKHR has different binding characteristics in comparison to the Drome-AKHR, as it cannot tolerate a negative charge as the Drome-AKHR does. So probably the Anoga-AKHR co-evolved to bind its neutral ligand. Our findings that the β -turn

residues six and seven are probably not directly involved with receptor interaction are in agreement with previous studies [11,38].

In general, the results of the receptor assay do not provide more information than was previously obtained with *in vivo* biological assays. This is rather surprising, since the receptor assay effectively measures a very short pathway (namely, peptide binding and subsequent receptor activation), whereas the *in vivo* biological assays measured a more distant outcome, namely release of lipids or carbohydrates, which is further downstream from the ligand-receptor binding. It is, however, clear that despite the many intracellular processes that occur between the initial step of ligand-receptor binding and the final cellular output of the translated, transduced signal (hyperlipemia or hypertrehalosemia), the data obtained with the *in vivo* biological assays that were common-place earlier, are quite accurate.

5. Conclusions

Several SAR studies using organ preparations or *in vivo* bioassays have already been performed in the past to determine the essential amino acids of the AKH peptides. This time a cellular expression system was used to conduct these studies on the AKH peptide. An advantage of this system to perform the SAR analyses is that one does not have to take into account that the peptides could be degraded by peptidases, which often is the case in the *in vivo* and *in vitro* assays as described earlier. Another advantage is the fact that with the cellular expression assay, one can be sure that only one receptor is involved in the outcome of the results. Despite these seeming advantages, the presented results are in accordance with previous general findings that not only the well conserved residues but actually most amino acids of the AKH peptides are of critical importance for a proper activation of their receptors.

Further SAR assays combined with the quickly developing bioinformatical modeling techniques like protein-ligand docking to predict the conformation of receptors and the binding sites of their ligands can now enable us to start the development of peptidomimetics that can be used in drug design.

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Reference list

- [1] M. Altstein, Novel insect control agents based on neuropeptide antagonists. The PK/PBAN family as a case study, J. Mol. Neurosci. 22 (2004) 147-157.
- [2] I. Beets, M. Lindemans, T Janssen, P. Verleyen, Deorphanizing G protein-coupled receptors by a calcium mobilization assay, Methods Mol. Biol. 789 (2011) 377-391.
- [3] M. Belmont, G. Cazzamali, M. Williamson, F. Hauser, C.J.P. Grimmelikhuijzen, Identification of four evolutionary related G protein-coupled receptors from the malaria mosquito *Anopheles gambiae*, Biochem. Biophys. Res. Commun. 344 (2006) 160-165.

- 399 [4] E. Clynen, A. Reumer, G. Baggerman, I. Mertens, L. Schoofs, Neuropeptide biology
400 in *Drosophila*, in: T. Geary, A. Maule (Eds.), Neuropeptide systems as targets for parasite and
401 pest control, Springer-Verlag, Berlin, 2010, pp. 192-210.
- 402 [5] O. Cusinato, A.F. Drake, G. Gäde, G.J. Goldsworthy, The molecular conformations of
403 representative arthropod adipokinetic peptides determined by circular dichroism
404 spectroscopy, *Insect Biochem. Molec. Biol.* 28(1) (1998) 43-50.
- 405 [6] M. Ford, T. Hayes, L. Keeley, Structure-activity relationships for insect
406 hypertrehalosaemic hormone: The importance of side chains and termini, in: G.R. Marshall
407 (Eds.), *Peptides chemistry and biology*, ESCOM, Leiden, 1988, pp. 653-655.
- 408 [7] G. Gäde, Structure-function studies on hypertrehalosemic and adipokinetic hormones:
409 activity of naturally occurring analogues and some N- and C-terminal modified analogues,
410 *Physiol Entomol.* 15 (1990) 299-316.
- 411 [8] G. Gäde, Peptides of the adipokinetic hormone/red pigment-concentrating hormone
412 family. A new take on biodiversity, *Ann. N. Y. Acad. Sci.* 1163 (2009) 125-136.
- 413 [9] G. Gäde, L. Auerswald, Mode of action of neuropeptides from the adipokinetic
414 hormone family, *Gen. Comp. Endocrinol.* 132 (2003) 10-20.
- 415 [10] G. Gäde, G.J. Goldsworthy, Insect peptide hormones: a selective review of their
416 physiology and potential application for pest control, *Pest Manage. Sci.* 59 (2003) 1063-1075.
- 417 [11] G. Gäde, T.K. Hayes, Structure-activity relationships for *Periplaneta americana*
418 hypertrehalosemic hormone I: The importance of side chains and termini, *Peptides* 16(7)
419 (1995) 1173-1180.
- 420 [12] G. Gäde, P. Simek, A novel member of the adipokinetic peptide family in a “living
421 fossil”, the ice crawler *Galloisiana yuasai*, is the first identified neuropeptide from the order
422 Grylloblattodea, *Peptides* 31 (2010) 372-376.

- 423 [13] G. Gäde, P. Simek, H.G. Marco, The first identified neuropeptide in the insect order
424 Megaloptera: A novel member of the adipokinetic hormone family in the alderfly *Sialis*
425 *lutaria*, *Peptides* 30 (2009) 477-482.
- 426 [14] G. Gäde, P. Simek, H.G. Marco, An invertebrate [hydroxyproline]-modified
427 neuropeptide: Further evidence for a close evolutionary relationship between insect
428 adipokinetic hormone and mammalian gonadotropin hormone family, *Biochem. Biophys.*
429 *Res. Commun.* 414 (2011) 592-597.
- 430 [15] G. Goldsworthy, S. Chandrakant, K. Opoku-Ware, Adipokinetic hormone enhances
431 nodule formation and phenoloxidase activation in adult locusts injected with bacterial
432 lipopolysaccharide, *J. Insect Physiol.* 49 (2003) 795-803.
- 433 [16] G.J. Goldsworthy, M.J. Lee, R. Luswata, A.F. Drake, D. Hyde, Structures, assays and
434 receptors for locust adipokinetic hormones, *Comp. Biochem. Physiol.* 117B(4) (1997) 483-
435 496.
- 436 [17] G. Goldsworthy, K. Opoku-Ware, L. Mullen, Adipokinetic hormone enhances
437 laminarin and bacterial lipopolysaccharide-induced activation of the prophenoloxidase
438 cascade in the African migratory locust, *Locusta migratoria*, *J. Insect Physiol.* 48 (2002) 601-
439 608.
- 440 [18] T.K. Hayes, L.L.J. Keeley, Structure-activity relationships on hyperglycemia by
441 representatives of the adipokinetic/hyperglycemic hormone family in *Blaberus discoidalis*
442 cockroaches, *J. Comp. Physiol. B.* 160(2) (1990) 187-194.
- 443 [19] J-H. Huang, X. Bellés, H-J Lee, Functional characterization of hypertrehalosemic
444 hormone receptor in relation to hemolymph trehalose and to oxidative stress in the cockroach
445 *Blattella germanica*, *Front. Endocrinol.* 2 (2012) 1-11.

- 446 [20] T. Janssen, E. Meelkop, M. Lindemans, K. Verstraelen, S.J. Husson, L. Temmerman,
447 R.J. Nachman, L. Schoofs, Discovery of a cholecystokinin-gastrin-like signaling system in
448 nematodes. *Endocrinology*. 149(6) (2008) 2826-2839.
- 449 [21] E.T. Kaiser, F.J. Kézdy, Amphiphilic secondary structure: design of peptide hormone,
450 *Science* 223(4633) (1984) 249-255.
- 451 [22] D. Kodrík, Adipokinetic hormone functions that are not associated with insect flight,
452 *Physiol. Entomol.* 33 (2008) 171-180.
- 453 [23] D. Kodrík, N. Krishnan, O. Habušťová, Is the titer of adipokinetic peptides in
454 *Leptinotarsa decemlineata* fed on genetically modified potatoes increased by oxidative
455 stress?, *Peptides* 28 (2007) 974-980.
- 456 [24] S. König, C. Albers, G. Gäde, Mass Spectral signature for insect adipokinetic
457 hormones, *Rapid Commun. Mass Spectrom.* 19 (2005) 3021-3024.
- 458 [25] M.J. Lee, O. Cusinato, R. Luswata, C.H. Wheeler, G.J. Goldsworthy, N-terminal
459 modifications to AKH-I from *Locusta migratoria*: assessment of biological potencies *in vivo*
460 and *in vitro*, *Regul. Pept.* 69 (1997) 69-76.
- 461 [26] M.J. Lee, G.J. Goldsworthy, C.P. Poulos, A. Velentza, Synthesis and biological
462 activity of adipokinetic hormone analogues modified at the C-terminus, *Peptides* 17(8) (1996)
463 1285-1290.
- 464 [27] M.W. Lorenz, Adipokinetic hormone inhibits the formation of energy stores and egg
465 production in the cricket *Gryllus bimaculatus*, *Comp. Biochem. Physiol., Part B: Biochem.*
466 *Mol. Biol.* 136 (2003) 197-206.
- 467 [28] M.W. Lorenz, G. Gäde, Hormonal regulation of energy metabolism in insects as a
468 driving force for performance, *Integr. Comp. Biol.* 49(4) (2009) 380-392.

469 [29] H.G. Marco, G. Gäde, Biological activity of the predicted red pigment-concentrating
 470 hormone of *Daphnia pulex* in a crustacean and an insect, Gen. Comp. Endocrinol. 166(1)
 471 (2010) 104-110.

472 [30] H.G. Marco, P. Simek, G. Gäde, The first decapeptide adipokinetic hormone (AKH) in
 473 Heteroptera: a novel AKH from a South African saucer bug, *Laccocoris spurcus* (Naucoridae,
 474 Laccocorinae), Peptides 32 (2011) 454-460.

475 [31] P. Moshitzky, S.W. Applebaum, The role of adipokinetic hormone in the control of
 476 vitellogenesis in locusts, Insect Biochem. 20(3) (1990) 319-323.

477 [32] M.M. Nair, G.E. Jackson, G. Gäde, Conformational study of insect adipokinetic
 478 hormones using NMR constrained molecular dynamics, J. Comput-Aided Mol. Des. 15
 479 (2001) 259-270.

480 [33] J. Scherkenbeck, T. Zdobinsky, Insect neuropeptides: Structures, chemical
 481 modifications and potential for insect control, Bioorg. Med. Chem. 17 (2009) 4071-4084.

482 [34] F. Staubli, T.J.D. Jørgensen, G. Cazzamali, M. Williamson, C. Lenz, L. Søndergaard,
 483 P. Roepstorff, C.J.P. Grimmelikhuijzen, Molecular identification of the insect adipokinetic
 484 hormone receptors, Proc. Natl. Acad. Sci. 99(6) (2002) 3446-3451.

485 [35] C.M. Stoscheck, Quantitation of protein, Methods Enzymol. 182 (1990) 50-68.

486 [36] D.J. Van der Horst, Insect adipokinetic hormones: release and integration of flight
 487 energy metabolism, Comp. Biochem. Physiol., Part B: Biochem. Mol. Biol. 136 (2003) 217-
 488 226.

489 [37] J. Večeřa, N. Krishnan, G. Alquicer, D. Kodrík, R. Socha, Adipokinetic hormone-
 490 induced enhancement of antioxidant capacity of *Pyrrhocoris apterus* hemolymph in response
 491 to oxidative stress, Comp. Biochem. Physiol., Part C: Toxicol. Pharmacol. 146(3) (2007) 336-
 492 342.

- 493 [38] A. Velentza, S. Spiliou, C.P. Poulos, G.J. Goldsworthy, Synthesis and biological
494 activity of adipokinetic hormone analogues with modifications in the 4-8 region, *Peptides* 21
495 (2000) 631-637.
- 496 [39] R. Ziegler, A.S. Cushing, P. Walpole, R.D. Jasensky, H. Morimoto, Analogs of
497 *Manduca* adipokinetic hormone tested in a bioassay and in a receptor-binding assay, *Peptides*
498 19(3) (1998) 481-486.
- 499 [40] R. Ziegler, K. Eckart, R.D. Jasensky, J.H. Law, Structure-activity studies on
500 adipokinetic hormones in *Manduca sexta*, *Arch. Insect Biochem. Physiol.* 18(4) (1991) 229-
501 237.
- 502 [41] I.Z. Zubrzycki, G. Gäde, Conformational study on an insect neuropeptide of the
503 AKH/RPCH family by combined ¹H NMR spectroscopy and molecular mechanics, *Biochem.*
504 *Biophys. Res. Commun.* 198(1) (1994) 228-235.

Table 1
Sequence of Drome-AKH analogues

Peptide sequence		Name
pGlu	- Leu - Thr - Phe - Ser - Pro - Asp - Trp - NH ₂	Drome-AKH
<u>[N-Ac-Ala]</u>	- Leu - Thr - Phe - Ser - Pro - Asp - Trp - NH ₂	[N-Ac-Ala ¹]Drome-AKH
pGlu	- <u>Ala</u> - Thr - Phe - Ser - Pro - Asp - Trp - NH ₂	[Ala ²]Drome-AKH
pGlu	- Leu - <u>Ala</u> - Phe - Ser - Pro - Asp - Trp - NH ₂	[Ala ³]Drome-AKH
pGlu	- Leu - Thr - <u>Ala</u> - Ser - Pro - Asp - Trp - NH ₂	[Ala ⁴]Drome-AKH
pGlu	- Leu - Thr - Phe - <u>Gly</u> - Pro - Asp - Trp - NH ₂	[Gly ⁵]Drome-AKH
pGlu	- Leu - Thr - Phe - Ser - <u>Gly</u> - Asp - Trp - NH ₂	[Gly ⁶]Drome-AKH
pGlu	- Leu - Thr - Phe - Ser - Pro - <u>Gly</u> - Trp - NH ₂	[Gly ⁷]Drome-AKH
pGlu	- Leu - Thr - Phe - Ser - Pro - Asp - <u>Gly</u> - NH ₂	[Gly ⁸]Drome-AKH
pGlu	- Leu - Thr - Phe - Ser - Pro - Asp - Trp - <u>OH</u>	[Trp-OH]Drome-AKH
pGlu	- Leu - Thr - Phe - Thr - Pro - Ala - Trp - NH ₂	Anoga-AKH

Fig. 1. Dose-response curves and EC₅₀ values of Drome-AKH and Anoga-AKH, tested on Drome-AKHR and Anoga-AKHR expressed in CHO-K1/mtAEQ/Gα₁₆ cells. EC₅₀ values for each receptor-ligand couple (means ± SEM) are indicated in the top left corner. Each data point represents the mean ± SEM of an experiment performed in triplicate.

Fig. 2. Structure-activity relationship study of a series of analogues based on the Drome-AKH peptide, tested on both the Drome-AKHR and Anoga-AKHR. All peptides were tested at a concentration of 0.1 nM in A and 500 nM in B. Each bar represents the relative activation (%) compared to the Drome-AKH peptide (=100% activation) and were corrected for the negative control (BSA).

A

B

Supplementary data. A model for the secondary structure deduced from the Drome-AKH (A, B and C) and the Anoga-AKH (D, E and F) sequence. (A, D) Illustration of the alternating pattern of hydrophilicity (black lettering) and hydrophobicity (white lettering). (B, E) Illustration of the N-terminus which contains a β -strand ending with proline. (C, F) Illustration of the potential β -strand together with the predicted β -turn at residues 5-8.

A **pGlu-Leu-Thr-Phe-Ser-Pro-Asp-Trp-NH₂**

D **pGlu-Leu-Thr-Phe-Thr-Pro-Ala-Trp-NH₂**