Identification and Functional Characterization of Two Orphan G-Protein Coupled Receptors for Adipokinetic Hormones from Silkworm, *Bombyx mori*

Ying Shi¹#, Haishan Huang²#, Xiaoyan Deng¹, Xiaobai He¹, Jingwen Yang³, Huipeng Yang¹, Liangen Shi³, Lijuan Mei¹,², Jimin Gao²,* and Naiming Zhou¹,*

¹Institute of Biochemistry, College of Life Sciences, Zijingang Campus, Zhejiang University, Hangzhou, Zhejiang, 310058, China; ²Zhejiang Provincial Key Laboratory for Model Organisms, School of Life Sciences, Wenzhou Medical College, Wenzhou, Zhejiang, 325035, China; ³College of Animal Sciences, Zijingang Campus, Zhejiang University, Hangzhou, Zhejiang, 310058, China

Both authors contributed equally to this work.

Running title: Identification of two new AKHRs in Bombyx

*Address correspondence to: College of Life Sciences, Zhejiang University, Zijingang Campus, 866 Yuhang Tang Road, Hangzhou, 310058, China. Tel: +86-571-88206748; Fax: +86-571-88206134-8000; Email address: znm2000@yahoo.com; Jimin Gao, Zhejiang Provincial Key Laboratory of Medical Genetics, School of Laboratory Medicine and Life Science, Wenzhou Medical College, Wenzhou, Zhejiang, 325035, China, Tel.: +86-577-86689805; fax: +86-577-86689771; Email address: jimingao@yahoo.com

Background: AKH/AKHR system is of importance for insect to control energy homeostasis.

Results: Bombyx BNGR-A28 and BNGR-A29 were activated in signaling and internalization by AKH3 at high affinity, but not by corazonin.

Conclusion: AKH3 was a specific ligand for two orphan GPCRs, AKHR 2a and AKHR2b.

Significance: Our findings will lead to a better understanding of AKH/AKHR system in regulation of fundamental physiological processes.

Adipokinetic hormones (AKHs) are the best studied insect neuropeptides with function of mobilizing lipids and carbohydrates during energy-expensive activities, and modulating fundamental physiological processes, such as sugar homeostasis, lipid metabolism and reproduction. Three distinct cDNAs encoding the prepro-Bombyx AKH1, 2, and 3 have been cloned, and confirmed by mass spectrometric methods. Our previous research suggested Bombyx AKH receptor is activated by AKH1 and AKH2 with high affinity, but by AKH3 with quite low affinity. In this present study, using stable functional expression of the receptors in HEK293 cells, we have now identified AKH3 as a specific ligand for two orphan GPCRs, and we therefore named AKHR2a and AKHR2b, respectively. We demonstrated that both AKHR2a and AKHR2b were activated by AKH3 at high affinity and by AKH1 and AKH2 at low affinity, leading to an increase of intracellular cAMP level, activation of ERK1/2, and receptor internalization, but not by Bombyx corazonin, vice versa, Bombyx corazonin receptor was activated by corazonin, but not by AKH1, 2, 3. Quantitative RT-PCR revealed that both of AKHR2a and AKHR2b were highly expressed in the testis, but also detected at low level in other tissues. These results will lead to a better understanding of AKH/AKHR system in regulation of fundamental physiological processes.

Adipokinetic hormones / hypertrehalosaemic hormones (AKH/HrTHs) are the best studied insect neuropeptides with energy-mobilizing function, and comprise almost 40 family members from most of the major insect orders have been identified (1-5). AKHs are initially known to mobilize carbohydrates and lipids during energy-expensive activities like long-distance flight. Recent studies have highlighted the critical role of AKH in modulating fundamental physiological processes, such as sugar homeostasis, lipid metabolism and...
The structure of AKH was first identified from the locusts *Locusta migratoria* and *Schistocerca gregaria* (8). AKH structurally consists of 8-10 amino acids with a pyroglutamate at the N-terminus and an amidated C-terminus. The first cDNA encoding AKH precursor in Lepidoptera has been cloned from *Manduca* (9). In silkworm, *Bombyx mori*, a nonapeptide identical with *Manduca* AKH has been isolated (10). Recently, three distinct cDNAs encoding the prepro-*Bombyx* AKH1, 2, and 3 have been cloned (11), and confirmed by mass spectrometric methods (12). Bombyx AKH1 is identical to nonapeptides found only in moths, while *Bombyx* AKH2 is closely related to many other AKH/HrTH decapeptides, both are produced by the *Corpora cardiaca*. *Bombyx* AKH3 produced by the silkworm brain is another decapeptide sharing sequence homologies with nonlepidopteran AKHs (11,12).

The physiological function of AKH peptides is mediated via adipokinetic hormone like receptor (AKHR), a typical G protein-coupled receptor (GPCR), which was first identified from the fruit fly *Drosophila melanogaster* and the silkworm *Bombyx mori* (13), and then from the cockroach *Periplaneta Americana* (14) and African malaria mosquito *Anopheles gambiae* (15). Previous biochemical characterization with isolated fat body suggested that AKH binds to its receptor and activates adenylyl cyclase via the Gs protein, which results in an increase of intracellular cAMP levels. In addition, AKH activates phospholipase C (PLC) to induce the release of Ca$^{2+}$ from intracellular Ca$^{2+}$ stores (16,17). Our previous studies have demonstrated that AKH stimulation not only led to a ligand concentration-dependent mobilization of intracellular Ca$^{2+}$ and cAMP accumulation, but also elicited PKA and PKC pathway-mediated transient activation of the extracellular signal-regulated kinase 1 and 2 (ERK1/2) pathways in HEK293 cells stably expressing AKHR (18). We have further demonstrated that AKH2 exhibit comparable activities in intracellular cAMP accumulation to AKH1, but *Bombyx* AKH3 was much less effective in activating *Bombyx* AKHR, strongly implying that there is more likely other intrinsic AKHRs exist as a high affinity receptor for AKH3 in *Bombyx* (19).

By genomic data mining and phylogenetic analysis, *Bombyx* neuropeptide GPCR A28 and A29 (BNGR-A28 and A29) have been identified as adipokinetic hormone like receptors in *Bombyx* (20,21). Therefore, in the present study, we report on the cloning of cDNAs encoding the putative adipokinetic hormone like receptors BNGR-A28 and BNGR-A29 from silkworm, *Bombyx mori*, and functional expression of both receptors in HEK293 cells. Further characterization in signaling and internalization by comparing with a known *Bombyx* AKH receptor and a putative *Bombyx* coronadin receptor led us to conclude that the orphan receptors-BNGR-A28 and BNGR-A29 are specific receptors for *Bombyx* AKH3. Our findings provide a foundation for future studies of AKH3 and its receptors in physiological processes.

**EXPERIMENTAL PROCEDURES**

**Materials**—Cell culture media and fetal bovine serum (FBS) was bought from Hyclone (Beijing, China). G418 and Lipofectamine2000 were purchased from Invitrogen (Carlsbad, CA). The pEGFP-N1 and pCMV-Flag vectors were purchased from Clontech Laboratories, Inc. (Palo Alto, CA) and Sigma (St. Louis, MO), respectively. RIPA lysis buffer were obtained from Beyotime (Haimen, China). Anti-phospho-ERK1/2 (Thr202/Tyr204) and ERK1/2 antibodies, and horseradish peroxidase-conjugated secondary antibody, were bought from Cell Signaling Technology (Danvers, MA).

**Molecular cloning, plasmid construction**—To construct the *Bombyx* AKHR plasmid, RT-PCR was performed as described previously (19). For RT-PCR, total RNA was isolated from testes and brains using TRIzol reagent (TAKARA). To amplify the full-length sequence encoding BNGR-A28 (A28), BNGR-A29 (A29) and Coronadin receptor (BNGR-A21, A21), two pairs of primers for each receptor were designed based on the sequence of GenBank Accession No.AB330449, AB330450 and AB330442 and on the corresponding genomic data (10). Therefore, in the present study, we report on the cloning of cDNAs encoding the putative adipokinetic hormone like receptors BNGR-A28 and BNGR-A29 from silkworm, *Bombyx mori*, and functional expression of both receptors in HEK293 cells. Further characterization in signaling and internalization by comparing with a known *Bombyx* AKH receptor and a putative *Bombyx* coronadin receptor led us to conclude that the orphan receptors-BNGR-A28 and BNGR-A29 are specific receptors for *Bombyx* AKH3. Our findings provide a foundation for future studies of AKH3 and its receptors in physiological processes.

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The coding sequence of the A28 was amplified for pCMV-Flag vector using the sense primer 5’-AAGCTTATGGATGAATCGACGC AAATGGAT-3’ and the antisense primer 5’-GATCCCTAAGCGGAATACCATTTTTTTGG -3’. The coding sequence of the A29 was amplified for pCMV-Flag vector using the sense primer 5’-AAGCTTATGGAAATGTTGAATT TGATTTC-3’ and the antisense primer 5’-GGATCCTAGGCGGGCCCGTGCCGCCC.
another pair of primer also be designed for pEGFP-N1,sense primer 5'\text{-}AAGCTTGCCACCATGGACAACG
AAGGCAACAGTACG
and antisense primer 5'\text{-}GGATCCCGTAACAAACTAATGTTCTG
TCATTTCG-3'.

The corresponding PCR products were cloned into pMD19-T (Invitrogen) using the TA cloning kit (TAKARA), followed by sub-cloning into the pCMV-Flag and pEGFP-N1 expression vectors respectively using the Rapid DNA Ligation Kit (Beyotime), and named these vectors as Flag-A28, Flag-A29, Flag-A21 and A29 -EGFP, A21 -EGFP, respectively. All constructs were sequenced to verify the correct sequences, orientations and reading frame.

Cell culture and transfection-The human embryonic kidney cell line (HEK293) was maintained in Dulbecco’s Modified Eagle Medium (DMEM, Invitrogen) supplemented with 10% heat-inactivated fetal bovine serum (Hyclone) and 4 mM L-Glutamine (Invitrogen). The AKHR, A28, A29 and Corazonin receptor cDNA plasmid constructs were transfected or co-transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer’s instructions. 1-2 days after transfection, selection for stable expression was initiated by the addition of G418 (800µg/ml) for 1h at room temperature and then incubated with rabbit monoclonal anti-pERK1/2 antibody (Cell Signaling) and anti-rabbit HRP-conjugated secondary antibody (Cell Signaling) according to the manufacturers’ protocols. Total ERK1/2 (Cell Signaling) was assessed as a loading control after p-ERK1/2 chemiluminescence detection using an HRP substrate purchased from Cell Signaling (Cat#7003).

Confocal microscopy-For receptor surface-expression analysis and internalization assay, HEK293 cells transiently or stably expressing receptor-EGFP were seeded onto glass coverslips coated with 0.1 mg/ml of poly-L-lysine and allowed to attach overnight under normal growth conditions. After treatment with AKH or corazonin peptide at 37°C for 60 min, the cells were fixed with 2-4% paraformaldehyde in PBS for 10 min at room temperature. The cells were mounted in mounting reagent (Beyotime) and visualized by fluorescence microscopy on a Zeiss LSM510 laser-scanning confocal microscope attached to a Zeiss Axiovert 200 microscope using a Zeiss Plan-Apo 63× 1.40 NA oil immersion lens. Measurement of cell surface receptors by ELISA- HEK-293 cells stably transfected with the pCMV-BNGR-A28 or pCMV-BNGR-A29 construct were seeded in 48-well dishes coated with poly-L-lysine. The next day, the cells were stimulated with the indicated concentrations of the agonist for the indicated times. Medium was aspirated and the cells were washed once with Tris-buffered saline (TBS). After fixing the cells for 5 min at room temperature with 3.7%
formaldehyde in TBS, the cells were washed 3 times with TBS and then blocked for 45 min with 1% bovine serum albumin/TBS. Cells were then incubated for 1 h with an alkaline phosphatase-conjugated monoclonal antibody directed against the Flag epitope and diluted 1:1,000. Cells were washed 3 times, and antibody binding was visualized by adding 0.25 mL of an alkaline phosphatase substrate (Bio-Rad). Development was stopped by adding 0.1 mL of the substrate to a 96-well microtiter plate containing 0.1 mL of 0.4 M NaOH. Plates were read at 405 nm in a microplate reader (Bio-Rad) using Microplate Manager software.

Arrestin translocation - For the β-arrestin translocation assays, the HEK-293 cells were transfected with the β-arrestin2-GFP and the corresponding Flag-receptor constructs (AKHR, A28, A29 or A21). One day after transfection, the cells were seeded onto glass coverslip and allowed to recover for 24-36h in 6-well plate. The cells were incubated with 2 mL DMEM without FBS, if necessary, corresponding ligands were added to stimulate the receptors for 15min, and examined using a Zeiss laser scanning confocal microscope.

Quantitative real-time PCR (QRT-PCR) - QRT-PCR was performed as described previously, with slight modifications(20). Total RNA was extracted from tissues samples as detailed in the manufacturer’s protocol (Qiagen). Reverse transcription was completed using PrimeScript first Strand cDNA Synthesis Kit (Takara). cDNA from samples was quantified on a 7500 Real-Time PCR Machine (Applied Biosystems, Foster City, CA) instrument using SYBR Premix Ex Taq (Takara). The following A28, A29 receptors and actin primers were used as previous reports. The possibility of genomic DNA contamination was excluded by DNAase treatment and by measurement of actin levels in RNA samples (which were not reversing transcribed). Differential expression of the cell lines was compared using the ΔΔCT method.

Peptide synthesis - The AKH peptides were prepared by solid-phase synthesis using the Fmoc strategy on a 430A peptide synthesizer (Applied Biosystems, Foster City, CA) and a 9050 Pepsynthesizer Plus (Perceptive Biosystems, Cambridge, MA). Crude peptides were purified by preparative reverse-phase high-performance liquid chromatography using a Dynamax-300 Å C18 25 cm x 21.4 mm ID column with a flow rate of 9 ml/min and two solvent systems of 0.1% TFA/H2O and 0.1% TFA/acetonitrile. Fractions containing the appropriate peptide were pooled together and lyophilized. The purity of the final product was assessed by analytical reverse-phase high-performance liquid chromatography, capillary electrophoresis, and matrix-assisted laser desorption/ionization time-of-flight mass spectrometry.

Data Analysis - All results are expressed as the Mean ± S.E.M. of n. Data were analyzed using non-linear curve fitting (GraphPad PRISM version 5.0) to obtain pEC50 values. Statistical significance was determined using Student's t-test. Probability values less than or equal to 0.05 were considered.

RESULTS

Cloning and expression of BNGR-A28 and BNGR-A29 in HEK293 cell - Three distinct cDNAs encoding the prepro-Bombyx AKH1, 2, and 3 have been identified (11,12), but our previous studies have demonstrated that Bombyx AKH3 was much less effective in activating known Bombyx AKHR (19). Bombyx neuropeptide GPCR A28 and A29 (BNGR-A28 and A29) have been identified as adipokinetic hormone like receptors in Bombyx by genomic data mining and phylogenetic analysis (20,21). The predicted genes of BNGR-A28 (GenBank Accession No. AB330449) and A29 (GenBank Accession No. AB330450) were cloned by RT-PCR from brain and testis of silkworm larva respectively. As shown in Fig. 1A, the open reading frames (ORFs) of BNGR-A28 and -A29 encode protein of 418 and 416 amino acids, which share 30.2% and 30.8% identical amino acid residues in common with the known Bombyx AKHR respectively. We, then, stably expressed BNGR-A28 and -A29 in human embryonic kidney 293 (HEK293), and static significant cell surface expression was detected by FACS analysis and fluorescent microscopy observation, although the cell surface expression level of BNGR-A28 and -A29 was much lower than AKHR (Fig. 1B and C).

Activation of BNGR-A28 and -A29 via a Gs protein-dependent pathway by AKH3 - To detect whether AKHs can bind to BNGR-A28 and -A29 and trigger second massager-cAMP signaling pathway, HEK293 cells stably co-transfected with A28 or A29 receptor and a reporter gene system consisting of the firefly luciferase coding region under control of a minimal promoter containing cAMP-response
elements (CRE) were created. As shown in Fig. 2D-F, Upon stimulation with AKHs, both BNGR-A28 and -A29 were activated to induce accumulation of intracellular cAMP by AKH3 with a high affinity with EC$_{50}$ values of $(9.9\pm 0.7) \times 10^{-9}$M and $(4.48\pm 1.03) \times 10^{-8}$M respectively, but by AKH1 and AKH2 within a range of high concentration (>1μM), vice versa, the known AKHR was activated by a low concentration of both AKH1 and AKH2, and by AKH3 with a much lower potency. As a control, no change in the cAMP level was detected in parental HEK293 cells (Fig.2A). In addition, pre-incubated with 100 ng/ml PTX overnight was found to have no effect on cAMP production in BNGR-A28 (Fig.2B) and BNGR-A29- expressing cells (Fig.2C), whereas stimulation with CTX led to a remarkable increase in the cellular levels of cAMP in response to AKH3. Taken together, these results suggest that activated BNGR-A28 and -A29 receptors are coupled to Gs-type G protein and thereby stimulate adenylyl cyclase, leading to accumulation of intracellular cAMP.

AKH3 mediates activation of MAPK pathway and receptor internalization in BNGR-A28 and -A29-expressing cells. It has been well established that extracellular-signal-regulated kinases 1 and 2 (ERK1/2) have emerged as important effectors for GPCRs, and can be used to assess the functional outcome of receptor stimulation (22,23). Next, we assessed AKHs-mediated activation of ERK1/2 in HEK293 cells stably expressing BNGR-A28 or -A29 using a phospho-specific antibody known to bind only to the phosphorylated (Thr-202 and Tyr-204 of ERK1, and Thr-185 and Tyr-187 of ERK2) and activated forms of these kinases (24). As indicated in Fig.3, stimulation with AKH3 elicited transient phosphorylation kinetics of ERK1/2 with maximal phosphorylation evident at 5 min in BNGR-A28 or -A29 transfected cells, while AKH3 treatment did not provoke any appreciable effects on ERK1/2 in the parental 293 cells or transiently mock-transfected 293 cells. A dose-response curve showed that the response to AKH3 had an EC$_{50}$ value of $(6.1\pm 1.3) \times 10^{-8}$M for BNGR-A28 and $(1.7\pm 0.34) \times 10^{-7}$M for BNGR-A29. AKH1 and AKH2 were also found to activate BNGR-A28 or -A29-mediated ERK1/2 activation in a range of higher concentrations than 100nM (Fig.4), consistent with the observation of intracellular cAMP formation. In addition, to elucidate the signaling pathways involved in activation of ERK1/2, HEK293 cells stably transfected with A28 or A29 receptor were treated with different inhibitors. We demonstrated that BNGR-A28 or BNGR-A29-mediated activation of ERK1/2 was significantly blocked by MEK inhibitor U0126 (100nM), PKA inhibitor H89 (10μM), and PKC inhibitor Go6983 (1μM) and GF109203X (1μM), but not by EGFR tyrosine kinase inhibitor AG1478 (1μM), PI3K inhibitor wortmannin (1μM), and Src-family tyrosine kinase inhibitor PP2 (10μM), suggesting that both BNGR-A28 and BNGR-A29 activate ERK1/2 via PKA and PKC–dependent but EGFR, SRC, PI3K–independent pathways (Fig. 5).

Agonist-induced internalization is a well-characterized phenomenon for most of GPCRs that is believed to contribute to receptor desensitization (25). To visualize the receptor internalization, we constructed expression of BNGR-A29 fused with enhanced green fluorescent protein (EGFP) at the C-terminal end. As shown in Fig.6, the fluorescence of A29-EGFP was mainly localized in the plasma membrane, and was dramatically internalized in response to AKH3. Observation with confocal microscopy revealed that treatment with high concentration of AKH1 (>1μM) resulted in internalization of A29-EGFP to a remarkable degree, whereas no significant endocytosis for A28 receptor was observed, if any, upon AKH2 stimulation with high concentration (Fig.6B). This is in agreement with our observation that the AKH3 peptide could induce internalization of the known AKHR at the higher concentration of 10μM (Fig.6A). Internalization of A28 and A29 stimulated by different concentration of AKH3 was quantified by ELISA experiments (Fig.6C). Moreover, Fig.6D showed that in the presence of AKH3 and AKH1, β-arrestin2-GFP was significantly translocated to the plasma membrane, while only a small amount of AKH2 was recruited to the plasma membrane in cells expressing BNGR-A28 or -A29 receptors, confirming that AKH3 is the major agonist for both BNGR-A28 and -A29, and cross functional responses are observed among AKHR, BNGR-A28 or -A29 receptors.

To confirm the role of β-arrestins in the regulation of receptor internalization, we also used specific siRNAs to knock down the expression of β-arrestin1 and β-arrestin2 in BNGR-A29-EGFP expressing HEK293 cells. Silencing β-arrestin2 effectively inhibited the internalization of BNGR-A29, whereas knockdown of β-arrestin1 exhibited no effect on
BNGR-A29 internalization (Fig. 7). We further investigated whether BNGR-A29 undergoes internalization via clathrin-coated pits using clathrin heavy chain-specific siRNA, as also shown in Fig. 7, knock-down of the expression of clathrin heavy chain led to significant inhibition of AKH3-mediated BNGR-A29 internalization. These results suggest that the BNGR-A29 receptor undergoes rapid internalization via β-arrestin2- and clathrin-dependent pathways upon AKH3 stimulation. Furthermore, we used the endosome marker Alexa Fluor 546-labeled transferrin or the lysosome marker LysoTracker Red DND-99 to assess whether internalized AKHRs are generally degraded in lysosomes or recycled back to the plasma membrane via early and recycling endosomes. Confocal microscopy analysis revealed that the internalized BNGR-A29 receptors were colocalized with transferrin in endosomes but not with DND-99 in lysosomes (Fig. 8A and B). The recycling experiments showed that the internalized BNGR-A29 receptors were recycled to the cell surface within 3 h after agonist removal (Fig. 8C). These data are highly consistent with our previous observation with AKHR internalization (26).

AKH3/BNGR-A28 and -A29 system is distinct from Corazin/Corazonin receptor systems in Bombyx mori—Phylogenetic tree analyses showed that the insect corazonin/corazonin receptor system is characterized as an intermediate group between the GnRHR and AKHR and shares a close relation between the receptors and ligands (27). To explore the relationship between AKH/AKHR and corazonin/BNGR-A21, the cDNA encoding BNGR-A21 was cloned using RT-PCR and stably expressed in HEK293 cells. Fig. 9 showed that the synthesized corazonin peptides activate the predicted corazonin receptor BNGR-A21 in the intracellular cAMP accumulation, ERK1/2 phosphorylation and internalization, but did not cross-react with AKHR, BNGR-A28, and -A29, and, vice versa, AKHs did not activate the BNGR-A21 receptor, indicating that BNGR-A28, and -A29 receptors are distinct from corazonin/corazonin receptor system, and better to be grouped into AKH/AKHR system.

Expression of BNGR-A28 and -A29 receptors in larvae Bombyx mori. We have used real-time RT-PCR to analyze the expression of BNGR-A28 and -A29 in silkworm larvae. As shown in Fig. 10, in fifth instar larvae of silkworm, for both BNGR-A28 and -A29 receptors, the highest expression was detected in testis. We could also observed expression at low level in brain, epidermis, silk gland, ovary, midgut; and fat body for BNGR-A28, but in brain and ovary for BNGR-A29, this is in agreement with observation by Yamanaka et al. (20).

**DISCUSSION**

The AKH is an insect neuropeptide hormone synthesized and released from the neurosecretory cells of the corpora cardiaxum, and is of importance for insect to control energy homeostasis. Previous studies indicated that the number of AKHs known to synthesize in a particular insect varies from one in Drosophila melanogaster (28) to four peptides identified from Locusta migratoria (29,30). In Lepidoptera, the first AKH peptide was identified from the tobacco hornworm, Manduca sexta (9). A nonapeptide chemically isolated from Bombyx has an identical amino acid sequence with Manduca and Heliothis AKH (10). Recently, three distinct cDNAs encoding the Bombyx AKH1, 2, and 3 have been cloned from the Bombyx genome by cloning-based approach combining homology search (11). Bombyx AKH2 is closely related to many other AKH/HrTH decapeptides, while AKH3 is another decapeptide but sharing sequence homologies with mosquitoes Anopheles gamae (Ag-AKH2) and Aedes aegypti (Aa-AKH2), the red flour beetle Tribolium castaneum (Tc-AKH3) and the locust Locusta migratoria (Lm-HrTH) (11,12,31). The silkworm orthologue of the Drosophila AKHR could be activated not only by Bombyx AKH1, but also by a moth AKH-like peptide, Helicoverpa zea hyperglycohalosaminic hormone and Drosophila AKH (13). In our previous research, we have demonstrated that AKH2 exhibited high activities in cAMP accumulation and ERK1/2 activation via AKHR comparable to AKH1, whereas AKH3 was much less effective, strongly implying that it is more likely that a second intrinsic AKHR exists as a high affinity receptor for AKH3 in Bombyx (19).

Recently, two putative AKH receptors, BNGR-A28 and -A29 have been identified by using the previously identified Drosophila and Bombyx AKH receptors (13) for BLAST searches of the genomic databases from Bombyx (20,21). Phylogenetic analysis has shown that the Bombyx AKHR is grouped into
“Arthropoda-AKHR cluster” together with recently identified AKHRs of Drosophila, cockroach and mosquito, while BNGR-A28 and -A29 have been grouped into “Arthropoda-AKHR like cluster” containing putative receptors of all three mosquito species, T. castaneum, and the parasitoid wasp Nasonia vitripennis (32). In the current study, we stably transfected HEK293 cells with two putative receptors, AKHR and Bombyx corazonin receptor respectively, and accessed activities of AKH1, 2, 3 and corazonin peptides on these receptors. Our results clearly showed that both putative receptors were activated in intracellular cAMP accumulation, ERK1/2 phosphorylation, and receptor internalization and β-arrestin2 recruitment to membrane by AKH3 with high affinity, also by AKH1 and AKH2 with low affinity, but not by corazonin peptide, while Bombyx corazonin receptor was only activated by corazonin peptide, but not by AKH1, AKH2 and AKH3. An orphan GPCR from the malaria mosquito Anopheles gambiae was paired with the ligand ACP (AKH/corazonin-related peptide), a neuropeptide similar to Bombyx AKH3 that is structurally intermediate between AKH and corazonin, and did not activate the Anopheles AKH and corazonin receptors, indicating that ACP/receptor couple is an independent and so far unknown neuropeptide signaling system (33). However, based on phylogenetic analysis and our current results, it is more likely that both of BNGR-A28 and -A29 receptors are specific for AKH3, and show cross-reaction with AKH1 and AKH2 to some degree, but not with corazonin peptide. We, therefore, suggest that the known AKH receptor is named as AKHR1 and BNGR-A28 and -A29 receptors are better named as AKHR2a and AKHR2b respectively, since they are activated by the same ligand, AKH3.

Phylogenetic analysis has suggested that the insect CCAP, corazonin, and AKH receptors have apparently originated from one ancestral receptor, having one ancestral ligand (20,32). Gene duplication is common in genomes, and proteins encoded by these duplicated genes reveal different functions, but in many occasions, they have the same function (34). During long evolution, following gene duplications, specializations could have occurred, resulting in a better control of the various physiological processes, but certain overlaps could still have kept (32). As in Tribolium, both the CCAP and AKH receptors have been duplicated compared to Drosophila and the honey bee (35). In the Yellow fever mosquito, Aedes aegypti, two AKHRs, AKHR-I (410 amino acids) and AKHR-II (394 amino acids) differ only at the C-terminus, which is short by 16 amino acids in AKHR-II and differs in the last three amino acids (32). In the current study, we have identified the duplicated orphan receptors AKHR2a and AKHR2b for AKH3.

The insect fat body has been identified to be the primary tissue site for AKH action. More recently, quantitative RT-PCR analysis demonstrated that for AKHR, high expression was observed in fat body, and the expression was also detectable in tissues of epidermis, muscle, malpighian tubule, testis and ovary, whereas mRNA of BNGR-28 and -29 receptors is significantly detected only in the testis in fourth instar and fifth instar day 2 (20). We have cloned AKHR2a from larval testes and AKHR2b from larval brain by RT-PCR. Further gene expression study using qRT-PCR showed that the expression of AKHR2a and AKHR2b receptors was detected at the highest level in the testis, but also at lower level in brain, epidermis, silk gland, ovary, midgut, and fat body for AKHR2a, but in brain and ovary for AKHR2b. As suggested by expression pattern, AKHR1, AKHR2a and AKHR2b are likely to have different physiological functions. Paring of BNGR-A28 and -A29 receptors with AKH3 provides the critical elements necessary for investigation of the functions of AKH3 in Bombyx mori.

The signaling of AKH peptides has been investigated in a few insects, but mostly focused on selected signaling pathways including cAMP/PKA and Ca\(^{2+}/PKC\)(36). In general, binding of AKH to its receptor leads to dissociation of the Gs protein βγ subunit, which in turn activates an adenylate cyclase, resulting in an increase in intracellular cAMP levels (16,37). cAMP stimulates lipase activity via activation of protein kinase A (PKA). The influx of extracellular Ca\(^{2+}\) via Gq-mediated phospholipase C (PLC) is also essential for the adipokinetic effect (36,37). Our previous studies have demonstrated that agonist-activated AKHRs signal to ERK1/2 pathway via PKA and calcium-involved PKC-dependent pathways (18). We have further demonstrated that AKHRs are rapidly internalized in a dose- and time-dependent manner via the clathrin-coated pit pathway upon agonist stimulation, and internalized receptors were...
slowly recovered to the cell surface after the removal of AKH peptides (26). In the current study, we document that both activated AKHR2a and AKHR2b receptors are coupled to Gs-type G protein and thereby stimulate adenyl cyclase, leading to accumulation of intracellular cAMP. AKH3 peptide stimulation elicited transient phosphorylation of ERK1/2 in dose- and time-dependent manner via PKA- and calcium-involved PKC-dependent pathways in AKHR2a or AKHR2b stably transfected HEK293 cells. Moreover, our investigation indicated that AKH3-bound AKHR2a and AKHR2b receptors were internalized via a β-arrestin2-involving clathrin-coated pit pathway, and internalized receptors were recycled back to the plasma membrane via early and recycling endosomes. These studies provide the first in-depth data defining the mechanisms of AKHR2a and AKHR2b in regulation of signaling and internalization.

In summary, we have identified Bombyx orphan receptors AKHR2a and AKHR2b as receptors for neuropeptide AKH3, and further characterization in signaling and internalization has confirmed that AKH3 is the major agonist for both AKHR2a and AKHR2b, and however, AKHR2a and AKHR2b receptors show cross-responses to AKH1 and AKH2, but not to Bombyx corazonin peptide. Further studies of AKH3/AKHR2a and AKHR2b are required to define the physiological roles in regulation of sugar homeostasis, lipid metabolism and reproduction.

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FOOTNOTES

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The abbreviations used are: AKH, adipokinetic hormone; AKHR, adipokinetic hormone receptor; GPCR, G protein-coupled receptor; EGFP, enhanced green fluorescent protein; HEK, human embryonic kidney; BNGR: Bombyx neuropeptide GPCR; ERK1/2, extracellular signal-regulated kinase1/2; CRE, cAMP response element; V-3, fifth instar day3; Br, brain; Ep, epidermis; Sig, silk gland; Te, testis; Ov, ovary; Mp, Malpighian tubule; Mg, midgut; Fb, fat body
**FIGURE LEGENDS**

**Fig.1.** Cloning and expression of BNGR-A28 and BNGR-A29 in HEK293 cell. (A) Alignment of AKHR, A28 and A29 receptor sequences. The receptors from *Bombyx mori* AKHR (No.AF403542), BNGR-A28 (No.AB330449), and BNGR-A29 (AB330450) have been cloned, expressed in HEK293 cells. Amino acid residues that are common to three receptors are highlighted. (B) The cell surface expression of the stably transfected HEK293 cells was analyzed by FACS. Stable HEK293 cells were analyzed for cell surface expression of Flag-AKHR, Flag-A28 and Flag-A29 by flow cytometry using the FITC conjugated anti-Flag mAb M2. Bars represent the mean fluorescence intensity for cells expressing Flag-AKHR. All data are shown as Means±S.E.M from at least three independent experiments. (C) HEK293 cells stably expressing AKHR-, A29-EGFP were seeded on coverslips, the location of receptors was detected by confocal microscopy, and the pictures shown represent at least three experiments. Statistical analysis was performed by a two-tailed Student’s t test (*, P<0.05; **, P < 0.01; ***, P< 0.001, versus counterpart control)

**Fig.2.** AKH3 activated BNGR-A28 and BNGR-A29 led to cAMP accumulation in BNGR-A28 or BNGR-A29 expressing cells. cAMP accumulation in HEK293 cells transiently co-transfected with firefly luciferase reporter gene system CRE-Luc and vehicle vector (A), BNGR-A28(B) or BNGR-A29 (C) was determined in response to AKH3(1μM) treatment. If necessary, cells were pre-treated with PTX (100ng/ml) or CTX (500ng/ml) overnight prior to incubation with AKH3 for 4h. CK labeled as untreated control. cAMP accumulation in HEK293 cells stably expressing AKHR/CRE-Luc (D), BNGR-A28/CRE-Luc (E) and BNGR-A29/CRE-Luc (F) was assayed in response to different doses of AKH1, AKH2 and AKH3. The cAMP level was indirectly detected using Firefly luciferase activity assay kit. Data are expressed as the means ± S.E.M (n = 3).

**Fig.3.** AKH3 mediates activation of MAPK pathway in BNGR-A28 and -A29-expressing cells. (A) AKH3 induces pERK1/2 only in transfected A28 cells, not in controls of the experiment. (B) AKH3 induces pERK1/2 only in transfected A29 cells, not in controls of the experiment. Time course of AKH3-stimulated phosphorylation of ERK1/2 in stable A28- (C) or A29- (D) expressing HEK293 cells, were incubated with 2nM AKH3 for the indicated times. Concentration-dependent activation of ERK1/2 phosphorylation by AKH3 in HEK293 cells stably expressing Flag-A28 (E) or Flag-A29 (F) stimulated with agonist for 5min. The p-ERK was normalized to a loading control (t-ERK). Cellular lysates were immunoblotted with phospho-specific (top lane) and non-specific (bottom lane) anti-ERK1/2 antibody, as described in EXPERIMENTAL PROCEDURE. The results are representative of at least three independent experiments. Data are expressed as the means ± S.E.M (n = 3).

**Fig.4.** AKH1 and AKH2 also involved in the activation of MAPK pathway in BNGR-A28 and -A29-expressing cells. ERK1/2 phosphorylation in HEK293 cells stably expressing Flag-AKHR (A), Flag-A28 (B) or Flag-A29 (C) was detected in response to different doses of AKH1, AKH2 and AKH3 for 5min, the results are shown represent three independent experiments. Cellular lysates were immunoblotted with phospho-specific (top lane) and non-specific (bottom lane) anti-ERK1/2 antibody, as described in EXPERIMENTAL PROCEDURE. The p-ERK was normalized to a loading control (t-ERK). The results are representative of at least three independent experiments. Data are expressed as the means ± S.E.M (n = 3).

**Fig.5.** Effects of pretreatment of PKA, PKC, PI3K, EGFR and Src inhibitors on AKH3-mediated ERK1/2 activation. Serum-starved HEK293 cells stably expressing BNGR-A28 (A) and BNGR-A29 (B) were pretreated with PBS (lane 2), PKA inhibitor (H89, 10μM, lane 3), PKC inhibitor (Go6983, 1μM, lane 4; GF109203X, 1μM, lane 5), PI3K inhibitor (wortmannin, 1μM, lane 6), EGFR tyrosine kinase inhibitor (AG1478, 1μM, lane 7), Src-family tyrosine kinase inhibitor (PP2, 10μM, lane 8) and MEK inhibitor (U0126,
100nM, lane 9) for 1h, and then exposed to AKH3 (2nM) for 5min. The same cells did not be treated with any inhibitors and AKH3 was used as control (lane 1). The p-ERK was normalized to a loading control (t-ERK). The data shown are representative of at least three independent experiments. Statistical analysis was performed by a two-tailed Student’s t-test (**, p < 0.01; ***, p < 0.001, versus none).

**Fig. 6.** Internalization and β-arrestin2 translocation induced by AKHs in BNGR-A28, -A29 and AKHR expressed cells. (A) HEK-293 cells stably expressing AKHR-EGFP were activated by the indicated concentrations of different agonists for 60 min. (B) Internalization of A29-EGFP in stable cells was detected in response to different doses of AKH1, AKH2 and AKH3. (C) ELISA measurement of BNGR-28 and BNGR-29 remaining on the cell surface after treatment of cells with the indicated concentrations of AKH3 for 45 min. Error bars represent S.E.M for three replicates. (D) HEK293 cells were transiently co-transfected β-arrestin2-EGFP with Flag-AKHR, Flag-A28 or Flag-A29 respectively. β-arrestin2 resided in the cytosol prior to AKHs stimulation (control), and translocated to bind receptors in the membrane in response to treatment with 10μM AKHs for 15 min. (E) Cells were co-transfected β-arrestin1-EGFP with Flag-A28 or Flag-A29 respectively. β-arrestin1-EGFP resided in the cytosol prior to AKH3 stimulation, and no different changes in the membrane in response to treatment with 10μM AKH3 for 15 min. All of experiments above were examined by fluorescence microscopy as described in the EXPERIMENTAL PROCEDURE. All pictures shown are representative of at least three independent experiments.

**Fig. 7.** β-arrestin2 and clathrin-coated pits are involved in the internalization of BNGR-A29. A. 72 h after transfection with specific arrestin siRNA, clathrin heavy chain siRNA (CHC) or nonspecific control siRNA, HEK-293 cells stably expressing BNGR-A29-EGFP were stimulated with 10 μM AKH3 for 45 min and internalization of receptors were examined with confocal microscopy. B and C. 72 h after transfection with specific arrestin siRNA, clathrin heavy chain siRNA (CHC) or nonspecific control siRNA, HEK-293 cells stably expressing BNGR-A29-EGFP were harvested and equal amounts of total cellular lysates were separated by 10% SDS-PAGE, transferred to nitrocellulose, and incubated with the indicated antibodies. Westernblot signals were quantified by densitometric analysis and normalized to cellular tubulin levels. Data were analyzed using Student’s t-test (**, p < 0.01; ***, p < 0.001). All pictures and data shown are representative of at least three independent experiments.

**Fig. 8.** Localization of internalized BNGR-A29-EGFP stably expressed in HEK-293 cells and recycling of internalized BNGR-A29 to the cell surface. A and B. HEK-293 cells stably expressing BNGR-A29-EGFP were incubated with or without 10 μM AKH3 for 45 min at 37°C in the presence of either 100g/ml Alexa Fluor546-labeled transferrin(A) or 50nM lysotracker DND-99 (B) for 45min. C. BNGR-A29-EGFP expressing cells were treated with 100 μg/ml cycloheximide and 10 μM AKH3 at 37°C for 30 min, followed by the removal of residual agonist by washing, and further incubation in the presence of cycloheximide for the indicated time periods. The internalized receptors were recycled to the plasma membrane within 3 h after agonist removal. All pictures shown are representative of at least three independent experiments.

**Fig. 9.** AKH3/A28 and A29 system is distinct from Corazonin/Corazonin receptor systems in Bombyx mori. (A) cAMP accumulation in HEK293 cells transiently co-transfected with firefly luciferase reporter gene system CRE-Luc and BNGR-A21 was determined in response to AKH1 (10μM), AKH2 (10μM), AKH3 (10μM) or corazonin (1μM) treatment. (B) ERK1/2 phosphorylation in HEK293 cells stably expressing Flag-A21 was detected in response to different doses of AKH1, AKH2, AKH3 or corazonin for 5min, the results are shown represent three independent experiments. (C) We used confocal microscopy to localize the A21-EGFP in stable cells after stimulation of indicated concentration of AKH1,
AKH2, AKH3 or corazonin peptides. (D) ELISA measurement of putative corazonin receptor (BNGR-A21) remaining on the cell surface after treatment of cells with 10μM AKH1, 10μM AKH2, 10μMAKH3 or 1μM corazonin for 60 min. (E) HEK293 cells stably expressed Flag-AKHR/CRE-Luc, Flag-A28/CRE-Luc or Flag-A29/CRE-Luc respectively. The cAMP assay was carried out after indicated AKHs (1μM) and corazonin (10μM) stimulation. (F) Flag-AKHR, Flag-A28 or Flag-A29 stable cells were incubated with indicated peptides. The ERK1/2 activation was detected by western blotting prescribed as EXPERIMENTAL PROCEDURE. The p-ERK was normalized to a loading control (t-ERK). (G) Higher concentration of crazonin (20μM) cannot induce β-arrestin2 recruitment to membrane in transiently expressed β-arrestin2-EGFP /Flag-AKHR, β-arrestin2-EGFP /Flag-A28 or β-arrestin2-EGFP /Flag-A29 HEK293 cells. While lower concentration of crazonin (1μM) will promote the β-arrestin2-EGFP binding to the A21 receptor.

Fig.10. Quantitative tissue expression analyses of BNGR-A28 and –A29. Quantitative tissue expression analyses of Bombyx neuropeptide GPCR A28 (A) and A29 (B) on larval stage at fifth instar day3. Transcript levels of the A28 and A29 mRNA was normalized to that of β-actin within each samples. Br, brain; Ep, epidermis; Sig, silk gland; Te, testis; Ov, ovary; Mg, midgut; Mp, Malpighian tubule; Fb, fat body.
Fig. 1.

A.

<table>
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<tr>
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<th>A29</th>
<th>AKHR</th>
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</table>

Cell Surface Expression (%)

B.

C.
Fig. 2.

A. 

HEK293

Vector/CRE

B. 

BNGR-A28

C. 

BNGR-A29

D. 

AKHR

E. 

BNGR-A28

F. 

BNGR-A29
Fig.3.

A. HEK293  Vector  BNGR-A28

B. HEK293  Vector  BNGR-A29

C. BNGR-A28

D. BNGR-A29

E. BNGR-A28

F. BNGR-A29

0nM  0.001nM  0.01nM  0.1nM  1nM  10nM  100nM  1μM AKH3

LogAKH3[M]
Fig. 4.

A.

B.

C.
FIG. 5

A. BNGR-A28

B. BNGR-A29

- p-ERK1/2
- t-ERK1/2

<table>
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<tr>
<th>Treatment</th>
<th>p-ERK1/2 (%)</th>
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<tr>
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<tr>
<td>H89</td>
<td>75 ± 4.0</td>
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<td>Go6983</td>
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<tr>
<td>GF109203X</td>
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<td>Wortmannin</td>
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<td>AG1478</td>
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<tr>
<td>PP2</td>
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</tr>
<tr>
<td>U0126</td>
<td>125 ± 6.0</td>
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</table>
Fig. 6.

A. AKHR-EGFP

B. BNGR-A29-EGFP

C. Receptors on Cell Surface [%] vs. LOG [AKH M]

D. AKH1
AKH2
AKH3

E. BNGR-A28/β-arrestin1-EGFP
BNGR-A29/β-arrestin1-EGFP

- AKH3
+ AKH3
Fig. 7.

A. **BNGR-A29-EGFP**

<table>
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<th>AKH3</th>
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<td>CHC</td>
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B. **Control siRNA β-arrestin1 siRNA β-arrestin2 siRNA**

- β-arrestin1/2
- α-tubulin

<table>
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<th>Control SiRNA</th>
<th>β-arrestin1 siRNA</th>
<th>β-arrestin2 siRNA</th>
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<tbody>
<tr>
<td>Arrestins Expression (%)</td>
<td><a href="image9">Graph</a></td>
<td><a href="image10">Graph</a></td>
<td><a href="image11">Graph</a></td>
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</table>

C. **Control siRNA CHC siRNA**

- CHC
- α-tubulin

<table>
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<tr>
<th></th>
<th>Control SiRNA</th>
<th>CHC siRNA</th>
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<tr>
<td>CHC Expression (%)</td>
<td><a href="image12">Graph</a></td>
<td><a href="image13">Graph</a></td>
</tr>
</tbody>
</table>

Control: PBS
β-arrestin1: AKH3
β-arrestin2: AKH3
CHC: AKH3
Fig. 8.

A

BNGR A29-EGFP  Transferin  Merge

-\(\text{AKH}3\)

+\(\text{AKH}3\)

B

BNGR A29-EGFP  DND-99  Merge

-\(\text{AKH}3\)

+\(\text{AKH}3\)

C

BNGR A29-EGFP

-\(\text{AKH}3\)  +\(\text{AKH}3\)

Recycling 1h  Recycling 3h
**Fig. 9.**

**A.**

![Graph showing cAMP Accumulation (Folds)](image)

**B.**

<table>
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<tr>
<th>Condition</th>
<th>AKH1 (0 μM)</th>
<th>AKH2 (100 nM)</th>
<th>AKH3 (1 μM)</th>
<th>Corazonin (1 μM)</th>
<th>BNGR-A21</th>
</tr>
</thead>
<tbody>
<tr>
<td>PBS</td>
<td>0</td>
<td>100 nM</td>
<td>10 μM</td>
<td>100 nM</td>
<td>10 μM</td>
</tr>
</tbody>
</table>

![Graph showing p-ERK1/2 (%)](image)

**C.**

![Images showing cell surface receptor by BNGR-A21, PBS, AKH1, AKH2, AKH3, and Corazonin](image)

**D.**

![Graph showing Receptor on cell surface (%)](image)
Fig. 10.

A. BNGR-A28 expression

B. BNGR-A29 expression
Identification and functional characterization of two orphan G-protein coupled receptors for adipokinetic hormones from silkworm, *Bombyx mori*

Ying Shi, Haishan Huang, Xiaoyan Deng, Xiaobai He, Jingwen Yang, Huipeng Yang, Liangen Shi, Lijuan Mei, Jimin Gao and Naiming Zhou

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