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Activation of *Bombyx* neuropeptide G protein-coupled receptor A4 via a Gαi-dependent signaling pathway by direct interaction with neuropeptide F from silkworm, *Bombyx mori*

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ABSTRACT

Members of the mammalian neuropeptide Y (NPY) family serve as neurotransmitters and contribute to a diversity of physiological functions. Although neuropeptide F (NPF), the NPY-like orthologs from insects, have been identified, the NPF receptors and their signaling and physiological functions remain largely unknown. In this study, we established the stable and transient functional expression of a *Bombyx* orphan G protein-coupled receptor, BNGR-A4, in both mammalian HEK293 and insect SF21 cells. We identified *Bombyx mori* NPFs as specific endogenous ligands for the *Bombyx* Neuropeptide GPCR A4 (BNGR-A4) and, accordingly, named the receptor BomNPFR. Our results demonstrated that BomNPFR was activated by synthetic BomNPF1a and BomNPF1b at a high efficacy and by BomNPF2 at a low efficacy. This activation led to a decrease of forskolin or adipokinetic hormone peptide-stimulated adenylyl cyclase activity, an increase of intracellular Ca^{2+}, the activation of ERK1/2 signaling and receptor internalization. Moreover, a Rhodamine-labeled BomNPF1a peptide was found to bind specifically to BomNPFR. The results derived from quantitative RT-PCR analysis and dsRNA-mediated knockdown experiments demonstrated the possible role of BomNPFR in the regulation of food intake and growth. Our results provide the first in-depth information on BomNPFR-mediated signaling for the further elucidation of the BomNPF/BomNPFR system in the regulation of fundamental physiological processes.
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1. Introduction

Neuropeptide Y (NPY) is widely conserved in vertebrates and enriched in the hippocampus, which is responsible for the central regulation of multiple physiological processes in vertebrates (Beck, 2001; Zimanyi et al., 1998). The identification of NPY-like orthologous peptides from invertebrates showed that these NPY homologs have a characteristic C-terminus ending with an amidated Phe rather than a Tyr residue and, hence, are designated NPFs. Among insects, NPFs have been identified by their sequence in the fruit fly *Drosophila melanogaster* (Brown et al., 1999), the yellow fever mosquito *Aedes aegypti* (Riehle et al., 2002), and the African malaria mosquito *Anopheles gambiae* (Stanek et al., 2002). Subsequent bioinformatic analyses combined with molecular techniques clearly demonstrated the existence of NPF sequences for another mosquito species (Riehle et al., 2002), locust (Clynen et al., 2006), honeybee (Hummon et al., 2006), and silkworm (Roller et al., 2008). In contrast to other vertebrates and invertebrates with a single-copy gene encoding NPF peptide in their genome (Brown et al., 1999), *Bombyx* has been shown to have two separate genes, encoding NPF1 and NPF2; the *npf1* gene encodes two splicing variants, NPF1a and NPF1b, which share an RPRFamide at the C-terminal end (Roller et al., 2008).

Abbreviation: AKH, adipokinetic hormone; cAMP, cyclic AMP; CRE, cAMP-response element; EGFP, enhanced green fluorescent protein; ERK1/2, extracellular signal-regulated kinase1/2; Gi, inhibitory GTP-binding protein of adenylyl cyclase; Gq, GTP-binding protein that activates phospholipase C; Gs, stimulatory GTP-binding protein of adenylyl cyclise; GPCR, G-protein coupled receptor; GRK, G protein-coupled receptor kinases; HEK, human embryonic kidney; MAPK, mitogen-activated protein kinase; MEK, MAPK/ERK1; NPF, neuropeptide F; NPF, neuropeptide Y; ORF, open reading frame; PTX, Pertussis toxin; SF21 cell, *Spodoptera frugiperda* cells.

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It has been well known that peptides of the NPY family elicit their biological effects through a subfamily of G protein-coupled receptors (GPCRs) termed Y-receptors (Blomqvist and Herzog, 1997). In invertebrates, two receptors displaying the strongest sequence homology with the vertebrate neuropeptide Y2 receptor subgroup have been identified functionally only for a snail, *Lymnaea stagnalis* (Tensen et al., 1998), and for *D. melanogaster* (*Garczynski* et al., *2002*). The *Drosophila* NPF receptor (DmNPFR1; CG1147) exhibited the ability to bind to radiolabeled NPF and to inhibit forskolin-induced intracellular cAMP production in CHO cells (Garczynski et al., 2002). Subsequently, several NPF receptors have been in silico cloned from the genomes of *Tribolium* (Hauser et al., 2008), *Nasonia* (Hauser et al., 2010), and *Anopheles* (Garczynski et al., 2005), but not from honeybees (Hauser et al., 2006). When expressed in CHO cells, the Anopheles NPF receptor displayed a high affinity for native NPF in a radioligand binding assay (Garczynski et al., 2005). The NPF/NPFR system provides a new paradigm for studying the central control of cooperative behavior (Wu et al., 2003). However, detailed information on the signaling cascades and physiological roles of NPFs and NPF receptors remains to be further elucidated.

Here, we describe the cloning of the cDNA encoding a *Bombyx* neuropeptide GPCR A4 (BNGR-A4) sequence, which, as based on genomic data mining and phylogenetic analysis, is closely related to Drosophila Dm-NPFR (CG1147) and the mammalian NPY receptor type 2 (Fan et al., 2010; Yamanaka et al., 2008). Further functional characterization of signaling and internalization using synthetic *Bombyx* NPFs in both mammalian and insect cell lines led us to conclude that the orphan receptor BNGR-A4 is a specific receptor for *Bombyx* neuropeptides NPF1a and NPF1b. Our findings provide a foundation for future studies of *Bombyx* NPFs and NPF receptor in physiological regulation.

2. Materials and methods

2.1. Materials

Dulbecco's Modified Eagle Medium (DMEM) and fetal bovine serum (FBS) were purchased from Hyclone (Beijing, China). TC-100 insect Medium was purchased from Applichem, (Darmstadt, Germany) G418, Lipofectamine 2000 and Opti-MEM® I reduced serum medium, nuclear dye DAPI were purchased from Invitrogen (Carlsbad, CA). SuperFectinTM II was purchased from Pufei Biotech (Shanghai, China). The pEGFP-N1 vector was purchased from Clontech Laboratories, Inc. (Palo Alto, CA) and the pCMV-Flag vector was purchased from Sigma (St. Louis, MO). Mouse monoclonal anti-FLAG® M2 antibody, mouse monoclonal anti-FLAG® M2-FITC antibody, goat horseradish peroxidase (HRP)-conjugated anti-mouse IgG and Nifedipine were purchased from Sigma (St. Louis, MO). The Fura-2 acetoxymethyl ester derivative (Fura-2/AM) was purchased from Dojindo (Japan). The membrane probe DiI, RIPA lysis buffer and horseradish peroxidase-conjugated secondary antibody were purchased from Beyotime (Haimen, China). BomNPF1a (GenBank Accession No. NM_001173412.2), BomNPF1b (GenBank Accession No. NM_001130883.2) and BomNPF2 (GenBank Accession No. NM_001130889.1) were synthesized by GL Biochem Ltd. (Shanghai, China). Anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-ERK1/2 (p44/42 MAPK) rabbit antibodies were purchased from Cell Signaling Technology (Danvers, MA). PTX were purchased from Tocris Bioscience (Bristol, United Kingdom).

2.2. Molecular cloning and plasmid construction

Total RNA was isolated from the brain of *Bombyx mori* adults using the RNAiso Plus reagent (Takara BIO, Japan) following the manufacturer's instructions. The cDNA was synthetized using a PrimeScript 1st Strand cDNA Synthesis Kit (Takara BIO, Japan) according to the manufacturer's instructions. To amplify the full-length sequence encoding *Bombyx* NPFR, two pairs of primers were designed based on the sequence of GenBank Accession No. AB330424, as follows: forward primer 5′-AAGCTTATGCCGTTTTATGATGACATGGGGC-3′ and reverse primer 5′-CGGGGTACCTCAGAAAACCTGGGAGCACTGC-3′ for pCMV-Flag and Bom-pCMV-Flag and forward primer 5′-AAGCTTGCCACCATGCCGTTTTATGATGACATGGGGC-3′ and reverse primer 5′-GGATCCCGGAAAACCTGGGAGCACTGCGAC-3′ for pEGFP-N1 and Bom-pEGFP-N1. The corresponding PCR products were inserted into the HindIII and BamHI sites of the pCMV-Flag/Bom-pCMV-Flag and pEGFP-N1/Bom-pEGFP-N1 vectors to produce Flag-BNGR-A4, BomFlag-BNGR-A4, BNGR-A4-EGFP, and BNGR-A4-BomEGFP by using the Rapid DNA Ligation Kit (Beyotime, China), respectively. All of the constructs were sequenced to verify the correct sequence and orientation.

2.3. Cell culture and transfection

The human embryonic kidney cell line (HEK293) was maintained in DMEM supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, China) and 4 mM L-glutamine (Invitrogen, CA). The *Spodoptera frugiperda* cells (SF21) was maintained in TC-100 insect medium supplemented with 10% heat-inactivated fetal bovine serum (Hyclone, China). The BNGR-A4 cDNA plasmid constructs were transfected or cotransfected into HEK293 and SF21 cells using Lipofectamine 2000 (Invitrogen, CA) and SuperFectinTM II (Pufei, China) according to the manufacturers' instructions, respectively. Selection for cell stable expression was initiated by the addition of G418 (800 μg mL^{-1}) 1–2 days after transfection.

2.4. Flow cytometry analysis

HEK293 cells or SF21 cells transiently or stably expressing Flag-BNGR-A4 were seeded in a 6-well plate and allowed to grow to approximately 2×10^5 cells. The cells were collected and then washed once with cooled phosphate-buffered saline (PBS) supplemented with 0.5% BSA [fluorescence-activated cell sorting (FACS) buffer] and incubated with 10 mg mL^{-1} of fluorescein isothiocyanate-labeled anti-Flag M2 monoclonal antibody (Sigma, St. Louis, MO) in a total volume of 100 μl. After incubating for 60 min at 4 °C, the cells were pelleted and washed three times in FACS buffer. The cells were then resuspended and fixed with 3% paraformaldehyde in FACS buffer for 15 min; after three washes, the cells were subjected to flow cytometry analysis using FACScan flow cytometer (Coulter EPICS Elite, Coolten Corp., Hialeah, FL, USA).

2.5. Luciferase activity

After seeding in a 48-well plate overnight, HEK293 cells/SF21 cells stably co-transfected with Flag-BNGR-A4/pCRE-Luc or BomFlag-BNGR-A4/BomFlag-AKHR/BompCRE-Luc, respectively, were grown to 90–95% confluence. The HEK293 cells were stimulated with 10 μM forskolin alone or 10 μM forskolin combined with various ligands in the final concentration of 100 nM (*B. mori* Adipokinetic hormones1 (BomAKH1 or AKH); *B. mori* Tachykinin1 (BomTK1); *D. melanogaster* sex peptide (DrmSP); *B. mori* short neuropeptide F1 (BomsNPF1), BomNPF1a, BomNPF1b or BomNPF2) or 10 μM forskolin combined with indicated concentration of BomNPF1a, BomNPF1b or BomNPF2 in DMEM without FBS and incubated for 4 h at 37 °C. The SF21 cells were stimulated with 10 nM AKH alone or 10 nM AKH combined with various ligands (in final concentration, 100 nM) or with 10 nM AKH combined with the

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