**Bombyx** neuropeptide G protein–coupled receptor A7 is the third cognate receptor for short neuropeptide F from silkworm

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Qiang Ma1, Zheng Cao1, Yena Yu1, Lili Yan1, Wenjuan Zhang1, Ying Shi1, Naiming Zhou1, and Haishan Huang2

From the 1Institute of Biochemistry, College of Life Sciences, Zijingang Campus, Zhejiang University, Hangzhou, Zhejiang 310058, China and the 2Zhejiang Provincial Key Laboratory for Technology and Application of Model Organisms, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China

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The short neuropeptide F (sNPF) neuropeptides, closely related to vertebrate neuropeptide Y (NPY), have been suggested to exert pleiotropic effects on many physiological processes in insects. In the silkworm (*Bombyx mori*) two orphan G protein–coupled receptors, *Bombyx* neuropeptide G protein–coupled receptor (BNGR) A10 and A11, have been identified as cognate receptors for sNPFS, but other sNPF receptors and their signaling mechanisms in *B. mori* remain unknown. Here, we cloned the full-length cDNA of the orphan receptor BNGR-A7 from the brain of *B. mori* larvae and identified it as a receptor for *Bombyx* sNPFS. Further characterization of signaling and internalization indicated that BNGR-A7, -A10, and -A11 are activated by direct interaction with synthetic *Bombyx* sNPF-1 and -3 peptides. This activation inhibited forskolin or adipokinetic hormone–induced adenylyl cyclase activity and intracellular Ca\(^{2+}\) mobilization via a G\(_{i/o}\)-dependent pathway. Upon activation by sNPFS, BNGR-A7, -A10, and -A11 evoked ERK1/2 phosphorylation and underwent internalization. On the basis of these findings, we designated the receptors BNGR-A7, -A10, and -A11 as *Bombyx*-sNPFR-1, -2, and -3, respectively. Moreover, the results obtained with quantitative RT-PCR analysis revealed that the three *Bombyx* sNPFR subtype exhibit differential spatial and temporal expression patterns, suggesting possible roles of sNPFR signaling in the regulation of a wide range of biological processes. Our findings provide the first in-depth information on sNPFR signaling for further elucidation of the roles of the *Bombyx* sNPF/sNPFR system in the regulation of physiological activities.

The vertebrate family of neuropeptide Y (NPY),3 consisting of NPY, peptide YY (PYY), and pancreatic polypeptide (PP), is widely conserved in vertebrates and is responsible for the central regulation of pleiotropic physiological processes (1, 2). The ortholog of NPY, neuropeptide F (NPF), with a characteristic C terminus ending with an amidated Phe residue instead of a Tyr residue, has been reported in most insects (3). By using an anti-NPF antibody, two shorter neuropeptide F (sNPF), sharing the C-terminal RF amide sequence, were first obtained from extracts of brains of the Colorado potato beetle, *Leptinotarsa decemlineata* (4). Subsequently, a cDNA encoding four sNPFS was identified in the fly, *Drosophila melanogaster* (5). Other insect sNPFS have been reported in the desert locust, *Schistocerca gregaria*, the African malaria mosquito, *Anopheles gambiae*, and the yellow fever mosquito, *Aedes aegypti* (6, 7).

Considering that sNPFS are expressed in numerous small neurons in the brain (3, 8, 9), it is possible that sNPFS have a conserved role as a neurotransmitter or neuromodulator, likely functioning as regulators of feeding and reproduction. Overexpression of sNPFS leads to increased food intake both in feeding larvae and adults, whereas knockdown of sNPFS displays the opposite phenotype in *D. melanogaster* (10). In *Locusta migratoria*, the injection of sNPFS stimulates ovarian development, suggesting a possible role in insect reproduction (6, 11). Previous studies have suggested that sNPFS signaling exhibits pleiotropic effects on osmotic stress, olfactory sensitivity, locomotor activity, and learning and memory (3, 12, 13).

Neuropeptides mediate their biological actions via interactions with specific receptors present on cell surface. The first insect sNPFR receptor was identified in *Drosophila*. The *Drosophila* receptor termed NPF76F (CG7395), a G protein–coupled receptor (GPCR), shows 62–66% similarity to and 32–34% identity with the vertebrate Y2 NPY receptors (14) and has been shown to be maximally activated by the four predicted sNPFS when expressed in both *Xenopus* oocytes and Chinese hamster ovary (CHO) cells in the presence of G\(_{i/o}\) (8, 14, 15). The direct interaction of this receptor with the four sNPFS has been confirmed by a radiolabeled sNPF–based binding assay (16). Subsequently, another two insect sNPFRs were identified from the fire ant *Solenopsis invicta* and the mosquito *A. gambiae* (17–19). Several sNPF recep-

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1 To whom correspondence may be addressed: College of Life Sciences, Zhejiang University, Zijingang Campus, Hangzhou, Zhejiang 310058, China. E-mail: zhounaiming@zju.edu.cn.
2 To whom correspondence may be addressed: Zhejiang Provincial Key Laboratory for Technology and Application of Model Organisms, School of Laboratory Medicine and Life Sciences, Wenzhou Medical University, Wenzhou, Zhejiang 325035, China. E-mail: haishan_333@163.com.
3 The abbreviations used are: NPY, neuropeptide Y; NPF, neuropeptide F; sNPFR, short neuropeptide F receptor; GPCR, G protein–coupled receptor; BNGR, *Bombyx* neuropeptide G protein–coupled receptor; CRE, cAMP-responsive element; Luc, luciferase; PTX, pertussis toxin; qRT-PCR, quantitative RT-PCR; JH, juvenile hormone; AKH, adipokinetic hormone.
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tors have been identified in silico from the genomes of *Apis mellifera*, *Tribolium castaneum*, *Nasonia vitripennis*, and *A. gambiae* (17, 20–22).

The cDNA encoding the sNPF precursor in *B. mori* was obtained, and the deduced precursor sequence contains three putative mature peptides (23). The presence of Bombyx sNPF-1, sNPF-2, and sNPF-3 in the brain and other larval ganglia was confirmed by direct MALDI-TOF mass spectrometric profiling and semiquantitative analyses (24). Three orphan receptor genes, *Bombyx* neuropeptide G protein-coupled receptor (BNGR)-A7, -A10 and -A11, have been identified in silico as putative sNPF receptors (23, 25). Further investigation using Ca\(^{2+}\) imaging analysis in human embryonic kidney cell line (HEK293) cells revealed that BNGR-A10 and -A11 are equally activated by *Bombyx* sNPF-1, -2, and -3 (23). However, BNGR-A7 remains to be deorphanized as a receptor for sNPFs by biochemical and pharmacological analysis. In addition, sNPF signaling is considered important in the regulation of a wide range of physiological functions. *Bombyx* sNPFs have been reported to stage-specifically inhibit juvenile hormone (JH) biosynthesis, suggesting its role in the regulation of molting and metamorphosis (23, 26). Recent studies indicate that in *B. mori* larvae, sNPF-mediated signaling is involved in the regulation of locomotor activity associated with foraging behavior (24, 27). The silkworm not only is an economically important insect, being a primary producer of silk, but also serves as a model of Lepidoptera for the investigation of the signaling and physiological functions of the sNPF/receptor system. Thus, identification of the cognate receptors for *Bombyx* sNPFs and further elucidation of the mechanism(s) involved in sNPF-mediated signaling will lead to a better understanding of its possible roles in the regulation of reproduction, osmotic stress, olfactory sensitivity, locomotor activity, and learning and memory in the silkworm (3, 6, 12, 13).

In this study, we have reported on the cloning of the cDNA encoding a *Bombyx* neuropeptide GPCR A7 (BNGR-A7) sequence, which, as based on genomic data mining and phylogenetic analysis, is closely related to *Drosophila* receptor termed NPFR76F and the mammalian Y2 NPY receptor (23, 25). Further functional expression and characterization in both mammalian and insect cells suggests that the orphan receptor BNGR-A7 is a specific receptor for *Bombyx* sNPFs. We have also investigated the spatial and temporal expression patterns of three *Bombyx* sNPF receptors. Our study provides the detailed information on the signaling and internalization of *Bombyx* sNPF receptors and perhaps aids in the interpretation of the signaling in the regulation of physiological processes.

Results

**Cloning and expression of BNGR-A7, -A10, and -A11 in HEK293 and sf21 cells**

BNGR-A7, -A10, and -A11 have been identified in silico as sNPF-like receptors by using genomic data mining and phylogenetic analysis (23, 25), and BNGR-A10 and -A11 have been confirmed as sNPF receptors by molecular and biochemical methods (23). In the present study, the full-length cDNA sequences encoding BNGR-A7 (GenBank™ accession no. AB330428), -A10 (GenBank™ accession no. AB330431), and -A11 (GenBank™ accession no. AB330432) were obtained by RT-PCR from brain tissue of *B. mori* larvae. As shown in Fig. 1A, an alignment was performed among BNGR-A7, -A10, and -A11 and the *D. melanogaster* sNPFR receptor. The amino acid sequences of BNGR-A7, -A10, and -A11 are 35.4, 38.6, and 38.6%, respectively, identical with that of *D. melanogaster* sNPFR. To confirm the accurate expression and localization, BNGR-A7, -A10, and -A11 with an N-terminal FLAG tag or with EGFP fused to the C-terminal end were constructed and stably or transiently expressed in HEK293 and insect sf21 cells. Confocal microscopy revealed that BNGR-A10 and -A11 were expressed mainly and localized to the plasma membrane in the absence of the ligand in both HEK293 and sf21 cells (Fig. 1B). However, BNGR-A7 exhibited correct localization in the plasma membrane in sf21 cells but displayed serious intracellular accumulation in HEK293 cells (Fig. 1, B and C). Significant cell surface expression was further confirmed by ELISA (Fig. 1C). Moreover, upon stimulation by sNPF peptides, FLAG–BNGR-A11 and BNGR-A11–EGFP exhibited significant inhibition of forskolin-evoked CRE–luciferase activity with EC\(_{50}\) values of 33.41 and 36.06 nm, respectively, comparable with the wild-type receptor (EC\(_{50}\) 57.09 nm) (Fig. 1D). These data suggest that N-terminally FLAG-tagged or C-terminally EGFP-fused BNGR-A7, -A10 and -A11 exhibited normal expression and membrane translocation in HEK293 and sf21 cells.

**Specific activation of BNGR-A7, -A10, and -A11 by sNPFs**

To examine whether receptors BNGR-A7, -A10 and -A11 are activated by sNPFs, Ca\(^{2+}\)-sensitive probe Fura-2–based Ca\(^{2+}\) mobilization assay was performed. As shown in Fig. 2, A and B, both sNPF-1 and sNPF-3 elicited a rapid increase in intracellular Ca\(^{2+}\) in a concentration-dependent manner in HEK293 (Fig. 2A) and sf21 (Fig. 2B) cells transfected with BNGR-A7, -A10 and -A11, respectively. Next, a reporter gene system using a pCRE (cAMP-response element)–Luc (luciferase) construct consisting of the firefly luciferase coding region under the control of a minimal promoter containing cAMP response elements was used to indirectly detect the effects of sNPFs on the intracellular calcium mobilization. As indicated in Fig. 2C, upon stimulation by sNPF-1 and sNPF-3, cells expressing BNGR-A7, -A10, and -A11, respectively, exhibited inhibitory effects on forskolin-induced CRE–luciferase activity in a dose-dependent manner with EC\(_{50}\) values of 8.71 (sNPF-1) and 2.08 nm (sNPF-3) for BNGR-A7, 0.15 (sNPF-1) and 0.98 nm (sNPF-3) for BNGR-A10, and 45.16 pm (sNPF-1) and 4.91 nm (sNPF-3) for BNGR-A11 in transfected HEK293 cells.

We further assessed the specificity of the sNPF-mediated activation of BNGR-A7, -A10, and -A11 using a CRE-driven luciferase assay system. As illustrated in Fig. 3A, BNGR-A7, -A10, and -A11 were activated to inhibit the forskolin-induced CRE–luciferase activity in response to treatment of sNPF-1 and sNPF-3, whereas other *Bombyx* neuropeptides, including *Bombyx* NPFR-1, cazonin, tachykinin-related peptide 1 (TKRP-1), and periviscerokinin-1 (PVK-1), were found unable to produce any detectable responses at a concentration of 100 nm. More-
over, sNPF-1 and sNPF-3 had no effect on the activation of the Bombyx NPF receptor (BNGR-A4), the corazonin receptor (BNGR-A21), the tachykinin receptor (BNGR-A24) or the peri-viscerokinin receptor (BNGR-A27) (Fig. 3B). Taken together, these results suggest that receptors BNGR-A7, -A10, and -A11 are specifically activated by Bombyx sNPFs.

**Direct interaction of sNPFs with receptors BNGR-A7, -A10, and -A11**

To confirm the direct binding of sNPFs to receptors BNGR-A7, -A10, and -A11, a competitive binding assay was established by using a synthesized FITC-tagged sNPF-3 at the N terminus (FITC–sNPF-3). Functional analysis using a CRE-driven luciferase assay indicated that FITC–sNPF-3 could lead to inhibition of forskolin-stimulated luciferase activity with an EC_{50} value of 27.34 nM in BNGR-A7–expressing HEK293 cells, comparable with the wild-type sNPF-3 (Fig. 4A). The competitive displacement of FITC–sNPF-3 with sNPF-3 in HEK293/BNGR-A7, -A10, and -A11 cells was measured by FACS analysis. As shown in Fig. 4B, unlabeled sNPF-3 was found to compete with FITC-labeled sNPF-3 with EC_{50} values of 85.47, 93.11, and 171.9 nM in BNGR-A7–transfected
HEK293 cells, respectively. Confocal microscopy observation revealed that the addition of unlabeled sNPF-3 resulted in a significant block of the binding of FITC–sNPF-3 to BNGR-A7, -A10, and -A11, respectively (Fig. 4C). These results strongly suggest that sNPFs directly bind to and activate receptors BNGR-A7, -A10, and -A11.

**Activation of BNGR-A7, -A10, and -A11 via G_{i/o}-dependent pathways by sNPFs**

A previous study demonstrates that BNGR-A10 and -A11 are activated to trigger intracellular Ca^{2+} mobilization in response to stimulation of sNPF (23). However, detailed information on
sNPF-mediated signaling remains largely unavailable. To elucidate the G protein coupling in the activation of BNGR-A7, -A10, and -A11, a combination of functional assays with different inhibitors was performed. As shown in Fig. 5, sNPF-induced Ca\textsuperscript{2+}/H\textsuperscript{1001} mobilization (Fig. 5, A and B) and inhibition of forskolin-stimulated CRE–luciferase activity (Fig. 5C) through receptors BNGR-A7, -A10, and -A11 were completely blocked by pretreatment with pertussis toxin (PTX), a specific inhibitor of Gi/o proteins. The direct measurement of cAMP accumulation using a cAMP ELISA confirmed that receptors BNGR-A7, -A10, and -A11 exhibited inhibitory effects on AKH-induced intracellular cAMP production in sf21 cells in the response to sNPFs (Fig. 5D). Collectively, our data further strengthen the role of pertussis toxin-sensitive Gi/o proteins in the Bombyx sNPF receptor–mediated signaling pathways.

sNPF-mediated activation of ERK1/2 and receptor internalization

It is well known that activated GPCRs signal to the mitogen-activated protein kinase (MAPK) cascades via Go\textsubscript{i/o}, Go\textsubscript{s}, and Go\textsubscript{q}-dependent signaling pathways (28). We next investigated whether sNPFs can induce phosphorylation of ERK1/2 through receptors BNGR-A7, -A10, and -A11. The sf21 cells transfected with BNGR-A7, -A10, or -A11, respectively, were seeded in 24-well plates and starved for 4 h in serum-free medium before stimulation. After stimulation with the indicated concentrations of agonist, the cell lysates were assayed using a phospho-specific antibody that binds only to the phosphorylated (Thr\textsuperscript{3202} and Tyr\textsuperscript{304} of ERK1 and Thr\textsuperscript{4185} and Tyr\textsuperscript{427} of ERK2) forms of these kinases (29). As shown in Fig. 6A, treatment with different concentrations of sNPF-1 induced a dose-dependent activation of ERK1/2 in sf21 cells. An analysis of the time course indicated that treatment of cells with sNPF-1 elicited transient phosphorylation of ERK1/2 with maximal phosphorylation evident at 5 min (BNGR-A7 and -A10) or 10 min (BNGR-A11), which returned to nearly basal levels by 60 min (Fig. 6B). Moreover, diverse specific inhibitors were used to elucidate the signaling pathways involved in sNPFR-mediated ERK1/2 phosphorylation in HEK293 cells. We found that the Gi/o inhibitor PTX, PKC inhibitor Go6983, and MEK inhibitor U0126, respectively, exhibited inhibitory effects on the sNPF-induced activation of ERK1/2 (Fig. 6C and D). In addition, to determine the biological activity of sNPFR receptors under physiological conditions, we dissected Malpighian tubules from fifth instar larvae for the detection of ERK1/2 phosphorylation. As shown in Fig. 6E, the stimulation of Malpighian tubules with sNPF-1 induced phosphorylation of ERK1/2, which could be significantly inhibited by PTX treatment. These results demonstrate that sNPFR receptors induce the activation of ERK1/2 via the Gi/o/PKC/MEK pathway in response to sNPF.

Ligand-induced internalization from the cell surface to the cytoplasm is a key process believed to contribute to the regulation of the strength and duration of GPCR signaling pathways.
In the present study, fusion expression of BNGR-A7, -A10, and -A11 with GFP at the C terminus was used to visualize the internalization and trafficking of receptors in insect sf21 cells. As shown in Fig. 7A, upon stimulation with sNPF-1 for 1 h, BNGR-A7, -A10, and -A11 underwent significant internalization from the plasma membrane to the cytoplasm in sf21 cells. Furthermore, sf21 cells co-transfected with BNGR-A7, -A10, and -A11, respectively, and BmKurtz, a novel nonvisual arrestin identified from Bombyx, fused with EGFP to the C-terminal end were exposed to sNPF-1 for 15 min and then examined using confocal microscopy. In the absence of sNPF-1, BmKurtz–EGFP was evenly distributed in the cytoplasm (Fig. 7B, top). Upon stimulation of cells with sNPF-1, BmKurtz was significantly translocated to the plasma membrane (Fig. 7B, bottom). These data provide clear evidence that Bombyx sNPFs appear to be endogenous ligands for BNGR-A7, -A10, and -A11.

Spatial and temporal expression profiles of BNGR-A7, -A10, and -A11

To better understand the physiological role of Bombyx sNPF signaling, we examined the mRNA expression patterns of BNGR-A7, -A10, and -A11. We dissected different tissues from the fifth instar larvae, pupae, and moths, respectively, and determined the sNPF receptor expression using real-time RT-PCR. Reference genes including Bombyx actin-A3, GAPDH, Rp49, and RpL3 were used for normalization of the qRT-PCR results. As shown in Fig. 8, transcripts of BNGR-A7, -A10, and -A11 were detectable in the brain, Malpighian tubules, midgut, testis, and ovary, but a higher level expression of BNGR-A10 was detected throughout development compared with BNGR-A7 and -A11. We found relatively high-level expression of the three receptors in the moth brain (Fig. 8A), in agreement with previous observations (14, 19, 31–34). We also observed obvious expression of BNGR-A7 and -A10 in moth brains and Malpighian tubules of the larvae and pupae, whereas BNGR-A11 mRNA could be detected at a relatively high level in the testis, Malpighian tubules, midgut, brain, and ovary at different developmental stages. Collectively, our data suggest that Bombyx sNPF signaling is likely to function as a pleiotropic regulator.

Discussion

In insects, sNPFS are widely expressed in the nervous system in all body regions and in some gut endocrine cells (10, 16, 19,
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Figure 5. BNGR-A7, -A10, and -A11 signals via Gi/o-dependent pathways. A and B, HEK293/BNGR-A7, -A10, and -A11 cells were pretreated with the G\textsubscript{i/o} inhibitor PTX (50 ng ml\textsuperscript{-1}) for 8 h prior to treatment with 100 nM sNPF-1 (A) and sNPF-3 (B). C, effects of PTX on sNPF-mediated induction of cAMP response. HEK293 cells expressing BNGR-A7, -A10, and -A11 were pretreated with PTX (50 ng ml\textsuperscript{-1}) overnight prior to incubation with sNPF (100 nM) for 4 h. D, accumulation of cAMP was measured using a cAMP ELISA kit. sf21 cells co-transfected with pBMFLAG–AKHR and BNGR-A7, -A10, or -A11, respectively, were pretreated with PTX (50 ng ml\textsuperscript{-1}) overnight and then stimulated by sNPF-1 (100 nM) and AKH1 (100 nM) for 15 min. Data were analyzed by using a Student’s t test (*, p < 0.05; **, p < 0.01; ***, p < 0.001; ns, not significant). All data were taken from at least three independent experiments.

24, 35) and are likely to play important roles in the regulation of feeding and growth (10, 36), reproduction (6, 11), locomotor activity (37), olfactory sensitivity (13), JH biosynthesis (23), and sleep (38). The pairing and characterization of the receptor with a given neuropeptide is a first but essential step in elucidating its action and function in vitro and in vivo. The NPF RFID receptor (CG7395) is the first and only one identified for sNPFs in Drosophila (8, 14); subsequently, other sNPF receptors have been identified and characterized in the red imported fire ant, S. invicta (18), the African malaria mosquito, A. gambiae (19), and the desert locust, S. gregaria (32). Further genes encoding putative sNPF receptors have been cloned in silico such as Am32 in the honeybee, A. mellifera (20), Tc57 and Tc58 in the flour beetle, T. castaneum (21), and NV_13003 in the parasitic wasp, N. vitripennis (22). In B. mori, three putative sNPF receptors BNGR-A7, -A10, and -A11 were identified in silico by Yamanaka et al. and our group (23, 25). BNGR-A10 and -A11 have been shown to be equally activated by Bombyx sNPF-1, -2, and -3 (23). In the present study, the orphan receptor BNGR-A7 was identified as a cognate receptor for Bombyx sNPFs. Further functional characterization clearly demonstrated that these three receptors, BNGR-A7, -A10, and -A11,
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A

B

C

D

E
were specifically activated by direct interaction with synthetic Bombyx sNPF but not by Bombyx NPF, corazonin, tachykinin, and CAPA neuropeptides; vice versa, sNPF was unable to activate the Bombyx NPF receptor, corazonin receptor, tachykinin receptor, or CAPA receptor. We, therefore, suggest that BNGR-A7, -A10, and -A11 are better designated as B. mori sNPF receptor-1 (Bommo-sNPFR-1), -2 (Bommo-sNPFR-2), and -3 (Bommo-sNPFR-3), respectively.

So far, mammalian derived-cell lines HEK293 and CHO-K1 have been widely selected as model cellular systems for the molecular and functional characterization of insect GPCRs in many studies. Interestingly, receptors BNGR-A10 and -A11 sNPF receptor-1 (Bommo-sNPFR-1), -2 (Bommo-sNPFR-2), and -3 (Bommo-sNPFR-3), respectively.

Figure 7. sNPF-induced internalization of BNGR-A7, -A10, and -A11. A, sf21 cells transiently co-transfected with BNGR-A7, -A10, and -A11–EGFP and BmKurtz, respectively, were stimulated with 1 μM sNPF-1 for 45 min, and the internalization of BNGR-A7–, -A10–, and -A11–EGFP was observed by confocal microscopy. B, sNPF-1–mediated recruitment of BmKurtz in BNGR-A7–, -A10–, and -A11–expressing cells. sf21 cells were transiently co-transfected with BmKurtz-EGFP and FLAG-BNGR-A7, -A10, or -A11, respectively, stimulated with 1 μM sNPF-1 for 15 min, and then detected by confocal microscopy. All panels shown are representative of at least three independent experiments.

Figure 8. Expression profiles of BNGR-A7, -A10, and -A11 determined by qRT-PCR. A, BNGR-A7, -A10, and -A11 expression in tissues of fifth instar larvae, pupae, and moths. B, expression of BNGR-A7, -A10, and -A11 at different developmental stages. The data were normalized to the geometric mean of four reference genes (actin-A3, GAPDH, Rp49, and RpL3). BR, brain; EP, epidermis; FB, fat body; MG, midgut; MT, Malpighian tubule; OV, ovary; TE, testis; SiG, silk gland. All panels are representative of at least three independent experiments.

Figure 6. sNPF-mediated induction of ERK1/2 phosphorylation. A, dose–response analysis of ERK1/2 phosphorylation on sf21/BNGR-A7, -A10, or -A11 cells. Serum-starved cells were incubated with an increasing dose of sNPF-1, ranging from 100 pM to 1 μM, for 10 min and harvested to detect ERK1/2 phosphorylation. B, kinetics of ERK1/2 phosphorylation initiated by sNPF-1 in sf21/BNGR-A7, -A10, or -A11 cells. Serum-starved cells were stimulated with 100 nm sNPF-1 for the indicated periods of time and harvested to detect ERK1/2 phosphorylation. C and D, effects of PTX (100 ng/ml) (C), PKC inhibitor Go6983 (10 μM), and MEK inhibitor U0126 (1 μM) (D) on sNPF-mediated activation of ERK1/2 in HEK293 cells. The cells were pretreated with or without inhibitors for 1 h and then stimulated with sNPF (100 nm). CTL, control. E, ERK1/2 phosphorylation in Malpighian tubules dissected from fifth instar larvae was determined in response to sNPF-1. All data were taken from at least three independent experiments. All panels shown are representative of at least three independent experiments (*, p < 0.05; **, p < 0.01; ***, p < 0.001).
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localized correctly in the plasma membrane, whereas BNGR-A7 displayed serious intracellular retention, although only a small portion of the expressed receptors could reach to the cell surface because signals in the intracellular cAMP formation and Ca^{2+} mobilization were detectable when expressed in HEK293 cells. The human CXCR1 chemokine receptor (39) and the murine CB2 cannabinoid receptor (40) expressed in mammalian cells and the human D2S dopamine receptor (41) and human bradykinin B2 receptor (42) expressed in insect cells were found to accumulate intracellularly for the most part, probably because of the high level of overexpression and saturation of trafficking machinery of the host cell (43). Post-translational glycosylation was demonstrated to play a crucial role in surface trafficking for the human angiotensin II type 1a receptor (43), the human bradykinin receptor (44), and the FSH receptor (45). However, the mechanism underlying the intracellular retention of the BNGR-A7 receptor expressed in HEK293 cells remains to be investigated.

Insect sNPFR receptors belong to the rhodopsin family of GPCRs and display a strong sequence resemblance to the vertebrate type 2 neuropeptide Y receptors (14). Coexpression of Drosophila NPFR76F with the promiscuous G_{16} in Xenopus oocytes revealed that the receptor is maximally activated by Drosophilas NPFRs to produce inward currents due to the activation of an endogenous oocyte calcium-dependent chloride current (14). This activation could be blocked by pretreatment with pertussis toxin, indicating that Drosophila NPFR76F signals via the G_{i/o}-dependent pathway in the Xenopus oocytes system (15). Drosophila sNPFR-1 and sNPFR-2 elicited a calcium response when the NPFR76F receptor was expressed in a CHO cell line (8). In mammalian cells expressing the sNPFR receptor of the mosquito, A. gambiae, and the desert locust, S. gregaria, cognate sNPFRs were found to potently inhibit forskolin-stimulated cAMP production, suggesting the coupling of G_{i/o} to the activated sNPFR receptor (19, 32). These results derived from heterologous systems suggest that insect sNPFR receptor couples preferentially to pertussis toxin-sensitive G_{i/o} proteins, leading to the inhibition of adenylate cyclase activity. However, the reasons for the discrepancy between our results and those of Hong et al. (47) and Chen et al. (38) are not clear but might be attributable to the different cell systems used in the original study.

Insect sNPFRs were initially identified by cross-reactivity with an anti-NPF antibody and are found in every insect genome discovered thus far (3, 48). However, information on their physiological function is still limited. sNPFR neuropeptides have been proposed to act as neuromodulator(s) and neurohormone(s) in the regulation of feeding behavior, locomotion, reproduction, sleep, and learning and memory in insects (3, 6, 10, 37, 49). In B. mori, sNPFR signaling has been suggested to be involved in the regulation of feeding behavior and JH biosynthesis and secretion (23, 26, 27). In the present study, in identifying potential target tissues of sNPFRs, we used quantitative RT-PCR to examine the tissue and developmental expression profile of three Bommo-sNPFR receptors. Overall, Bommo-sNPFR-2 showed the highest expression throughout development compared with Bommo-sNPFR-1 and -3. Additionally, three subtypes, Bommo-sNPFR-1, -2, and -3, exhibited differential spatial and temporal expression patterns. The expression of three receptors in the brain was detected at a relatively lower level in larvae and pupae but at the maximal levels in moths, consistent with previous observations that sNPFR receptor mRNA mainly appears to be limited to the nervous system in other insects (14, 19, 31–34). This suggests the possible roles of sNPFR signaling in the regulation of feeding and growth, locomotor activity, and learning and memory. Interestingly, it is noteworthy that high-level expression of Bommo-sNPFR-1, -2, and -3 was detected in Malpighian tubules as compared with other peripheral tissues such as the gut, fat body, and ovaries, suggesting a regulatory role of sNPFR signaling in the control of osmotic homeostasis in diuresis. This finding is in agreement with studies in S. invicta, Glossina morsitans, and A. gambiae (18, 19, 48). However, receptor transcript presence may not exactly reflect protein level. In addition, because of the high level of expression of the sNPFR receptors in the nervous system, the RT-PCR products potentially could be amplified from the neuronal contamination (34). Therefore, further efforts are required to examine the spatial and temporal distribution of sNPFR receptor proteins for better understanding the physiological roles of sNPFR signaling.

In conclusion, in this study, we have paired the Bombyx orphan receptor BNGR-A7 as a cognate receptor with the Bombyx neuropeptide sNPFRs. Our results on the characterization of Bommo-sNPFR–mediated signaling have demonstrated that BNGR-A7 and previously deorphanized BNGR-A10 and -A11 are activated by direct interaction with Bommo-sNPFRs via a G_{i/o}-dependent pathway, leading to the inhibition of forskolin- or AKH-mediated cAMP production, intracellular Ca^{2+} mobilization, and ERK1/2 activation in a PTX-sensitive manner. Furthermore, quantitative RT-PCR analysis has indicated that three subtypes of Bommo-sNPFR receptors show developmental stage-specific and tissue-specific expression patterns, suggesting that these three receptors may play different physiological roles in different tissues and stages. Our Bommo-sNPFR receptor–based func-
tional assay systems and molecular data provide a foundation for further characterization and validation of this signaling system in vitro and in vivo.

**Experimental procedures**

**Materials**

Cell culture media and FBS were purchased from HyClone (Beijing, China). sf21 cell lines were kindly provided by Z. Zhang, X-tremeGENE HP was purchased from Roche (Mannheim, Germany). The nuclear probe DAPI, radioimmunoprecipitation assay lysis buffer, and horseradish peroxidase-conjugated secondary antibody were purchased from Beyotime (Haimen, China). Opti®-MEM 1 reduced serum medium was purchased from Invitrogen. The pCMV–FLAG vector, forskolin Go6983, U0126, and PTX were purchased from Sigma-Aldrich, and the pEGFP-N1 vector was from Clontech Laboratories (Palo Alto, CA). Anti-phospho-ERK1/2 (Thr202/Tyr204) and anti-ERK1/2 antibodies were purchased from Cell Signaling Technology (Danvers, MA).

**Molecular cloning and plasmid construction**

Total RNA was isolated from the brain of *B. mori* larvae using the RNAsiso Plus reagent (Takara, Tokyo, Japan) following the manufacturer’s instructions. cDNA was synthesized using a PrimeScript first strand cDNA synthesis kit (Takara) according to the manufacturer’s instructions. The entire coding region of the BNGR-A7, -A10, and -A11 genes were cloned and sequenced. The primers used for the PCR cloning of BNGR-A7, -A10, and -A11 see [supplemental Table 1](#). The PCR products obtained were directly cloned into the pcDNA-3.1 vector, the pCMV–FLAG vector, and the pEGFP-N1 vector for expression in mammalian cells. For expression in insect cells, the immediate-early gene promoter (IE1) and homologous region 3 (Hr3) of *B. mori* nucleopolyhedrovirus (BmNPV) and the promoter of BmActin A3 were used to replace the corresponding sites of pCMV–FLAG and pEGFP-N1 as described previously (50). pBmCRE–Luc was also reconstituted using the promoter of BmHsp20.4 (B. mori heat shock protein 20.4, GenBank™ accession no. EU350577) and BmFibL (B. mori fibroin light chain, GenBank™ accession no. NM_001044023) polyadenylation signal to replace pVIP (promoter of vasointestinal peptide) and the SV40polyadenylation signal of pCRE–Luc, respectively. All constructs were verified by sequencing.

**Cell culture and transfection**

The insect *Spodoptera frugiperda* ovarian cell line sf21 cells were maintained in insect cell culture medium TC100 from Applichem (Darmstadt, Germany) supplemented with 10% FBS (HyClone) at 28 °C and seeded onto a 6-well tissue culture plate 2 h prior to transfection. The BNGR-A7, -A10, and -A11 cDNA plasmid constructs were transfected into sf21 cells using an X-tremeGENE HP protocol (Roche) according to the manufacturer’s instruction. HEK293 cells were maintained in DMEM (HyClone) supplemented with 10% heat-inactivated FBS (HyClone) and were incubated at 37 °C in a humidified atmosphere with 5% CO₂, 95% air. The BNGR-A7, -A10, and -A11 cDNA plasmid constructs were transfected into HEK293 cells using Lipofectamine 2000 (Invitrogen) according to the manufacturer's instructions. Selection for cell-stable expression was initiated by the addition of G418 (800 mg/ml, Invitrogen) 1–2 days after transfection.

**Luciferase activity assay**

A reporter gene assay was performed to investigate changes in the intracellular levels of cAMP. In the present study, the pCRE–Luc or pBmCRE–Luc reporter gene systems, consisting of a minimal promoter containing CREs, were used to measure the intracellular cAMP levels. HEK293 and sf21 cells co-expressing BNGR-A7, -A10, or -A11, respectively, with the reporter protein were seeded into 96-well plates and incubated overnight. Cells were then stimulated with different concentrations of sNPF in serum-free medium and incubated for 4 h. Luciferase activity was detected using a firefly luciferase assay kit (Kenreal, Shanghai, China). When required, cells were treated with PTX (50 ng ml⁻¹) for 16 h prior to the start of the experiment. The cAMP concentration was assessed using a commercially available cAMP detection kit (R&D Systems, Minneapolis, MN).

**Intracellular calcium measurement**

The fluorescent Ca²⁺ indicator Fura-2/AM (fura-2-acetoxymethylester, Dojindo Laboratories, Kumamoto, Japan) was employed to monitor the changes in intracellular calcium. HEK293/BNGR-A7, -A10, or -A11 cells were harvested with a cell scraper and resuspended in 37 °C. For sf21/BNGR-A7, -A10, or -A11 cells, the experiment was performed at 28 °C in Hepes-buffered medium (HBM: 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.2 mM Na₂HPO₄, 5 mM NaHCO₃, 10 mM glucose, 20 mM HEPES-NaOH, and CaCl₂ (1 mM) (pH 6.2)) instead of Hanks’ balanced salt solution (Dongsheng, Hangzhou, China). The cells were then loaded with 3 μM Fura-2/AM for 30 min at 37 °C. For sf21/BNGR-A7, -A10, or -A11 cells, the experiment was initiated by the addition of G418 (800 mg/ml, Invitrogen) 1–2 days after transfection.

**ERK1/2 activation assay**

sf21 cells expressing BNGR-A7, -A10, or -A11, respectively, were seeded in 24-well plates and starved for 4 h in serum-free medium to reduce background ERK1/2 activation and eliminate the effects of the change of medium. If required, the cells were pretreated with PTX for 10–16 h before activation. After stimulation with the indicated agonist, the cells were lysed in 80 μl of lysis buffer (20 mM HEPES (pH 7.5), 10 mM EDTA, 150 mM NaCl, and 1% Triton X-100) with protease inhibitors (Roche) at 4 °C for 30 min. Equal amounts of total cell lysate were size-fractionated by SDS-PAGE (10%) and transferred to a PVDF membrane (Millipore, catalog no. SCHV01RE). The membranes were blocked in TBS containing 0.05% Tween 20 and 5% nonfat dry milk for 1 h at room temperature and then incubated with rabbit monoclonal anti-pERK1/2 antibody (Cell Signaling Technology, Danvers, MA). The fluorescent Ca²⁺ indicator Fura-2/AM (fura-2-acetoxymethylester, Dojindo Laboratories, Kumamoto, Japan) was employed to monitor the changes in intracellular calcium. HEK293/BNGR-A7, -A10, or -A11 cells were harvested with a cell scraper and resuspended in 37 °C. For sf21/BNGR-A7, -A10, or -A11 cells, the experiment was performed at 28 °C in Hepes-buffered medium (HBM: 140 mM NaCl, 5 mM KCl, 1 mM MgCl₂, 1.2 mM Na₂HPO₄, 5 mM NaHCO₃, 10 mM glucose, 20 mM HEPES-NaOH, and CaCl₂ (1 mM) (pH 6.2)) instead of Hanks’ solution. Calcium flux was measured using excitation wavelengths of 340 and 380 nm in a fluorescence spectrometer (Infinite F200Pro, Tecan, Männedorf, Switzerland). When required, cells were treated for 16 h with PTX prior to the initiation of the experiment.
Identification of Bombyx sNPF receptors

Technology and anti-rabbit horseradish peroxidase-conjugated secondary antibody (Beyotime) according to the manufacturers’ protocols. The blots were stripped and relabeled using an anti-β-actin (1:2000) monoclonal antibody as a control for protein loading. The levels of ERK1/2 phosphorylation were normalized to β-actin. All immunoblots were quantified using a Bio-Rad Quantity One imaging system.

Malpighian tubules from fifth instar larvae were dissected and placed in cold HBM buffer. Following dissection, the HBM buffer was replaced with fresh TC100 medium (with or without the G1 inhibitor PTX) for a 6-h preincubation with or without the G1 inhibitor PTX. Then the Malpighian tubules were rapidly transferred to fresh medium containing 100 μM sNPF-1 and incubated for 1 h. All treatment samples (n > 5) were collected and homogenized in 300 μl of radioimmune precipitation assay lysis buffer and centrifuged. Supernatant of the lysate was diluted and boiled in an equal volume of SDS buffer for 10 min. Lysates were loaded onto 12% SDS gels according to the total protein content determined by a BCA protein assay kit, and then phosphor-ERK and β-actin levels were detected.

Internalization assay

To detect the expression of receptors on the cell membrane, BNGR-A7, -A10, or -A11/EGFP cells were seeded in glass-bottom 6-well plates. Cells were washed with PBS and fixed with 3% paraformaldehyde in PBS for 10 min at room temperature. The cells were then incubated with DAPI (Beyotime) for 10 min to stain the cell nuclei. For the internalization assay, the cells were treated with sNPF1 at 28 °C for 60 min. After fixation with 3% paraformaldehyde, the cells were mounted in 50% glycerol and visualized by fluorescence microscopy using a Zeiss LSM510 laser-scanning confocal microscope attached to a Zeiss Axiovert 200 microscope with a Zeiss Plan-Apo 63×1.4 NA oil immersion lens. Images were collected using an LSM 5 Image Examiner and processed with Adobe Photoshop.

Binding assay

The binding assay for HEK293 cells that had been transiently transfected with BNGR-A7, -A10, or -A11/EGFP cells was seeded in glass-bottom 6-well plates. Cells were washed with PBS and fixed with 3% paraformaldehyde in PBS for 10 min at room temperature. The cells were then incubated with DAPI (Beyotime) for 10 min to stain the cell nuclei. For the internalization assay, the cells were treated with sNPF1 at 28 °C for 60 min. After fixation with 3% paraformaldehyde, the cells were mounted in 50% glycerol and visualized by fluorescence microscopy using a Zeiss LSM510 laser-scanning confocal microscope attached to a Zeiss Axiovert 200 microscope with a Zeiss Plan-Apo 63×1.4 NA oil immersion lens. Images were collected using an LSM 5 Image Examiner and processed with Adobe Photoshop.

Quantitative real-time PCR

Quantitative RT-PCR was performed as described previously with slight modifications (48). Tissues from the fifth instar larvae, pupae, and moths were dissected under a binocular microscope, collected in tubes containing MagNAlyzer Green Beads (Roche), and homogenized with the MagNA Lyser® (Roche). Total RNA was then extracted using the RNeasy® lipid tissue mini kit (Qiagen, Duesseldorf, Germany) in combination with a DNase digestion (RNase-free DNase set, Qiagen). The total RNA (0.5 μg) was converted to cDNA using a PrimeScript first strand cDNA synthesis kit (TaKaRa). The cDNA from the samples were quantified on a real-time PCR machine (CFX-Touch, Bio-Rad) using SYBR Premix ExTaq (TaKaRa). The possibility of genomic DNA contamination was excluded by DNase treatment. All samples were measured in three technical replicates and contained a no-template control for all primer pairs to check for the presence of unwanted genomic DNA. Reference genes, including Bombyx actin-A3, GAPDH, Rp49, and RpL3, were determined with geNorm software (52) and used for normalization of the qRT-PCR results (see supplemental Table 1 for primer sequences). Results were expressed using the comparative cycle threshold (ΔΔCt) method as described previously (53). Briefly, data were normalized by subtracting the Ct value of the geometric average of the reference genes from that of the target gene. ΔΔCt was calculated as the difference in the normalized Ct value (ΔCt) of the different tissue samples. The comparative expression level of target genes is equal to 2−ΔΔCt.

Peptide synthesis

The Bombyx neuropeptides sNPF-1 (SVRSPSRLRF–NH2) (3, 54), sNPF-3 (SDERAVPHIFQEQDRAVRPSMLRF–NH2) (3, 54), and FITC–sNPF-3 (FITC–Akh×–SDERAVPHIFQEQDRAVRPSMLRF–NH2) used in this study were synthesized commercially by GL Biochem Ltd. (Shanghai, China). All peptides were purified by reverse-phase high performance liquid chromatography (HPLC). The purity of the obtained peptides was then verified with a matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF, Ultraflex II Bruker Daltonics) mass spectrometer. All peptides were lyophilized and diluted to the desired concentrations for experiments.

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