Characterization of a neuropeptide F receptor in the tsetse fly, *Glossina morsitans morsitans*

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ARTICLE INFO

Article history:

Received 6 May 2016

Received in revised form 21 September 2016

Accepted 22 September 2016

Available online 24 September 2016

Keywords:

Insect

Tsetse fly

Glossina morsitans morsitans

Neuropeptide

Neuropeptide F

G protein-coupled receptor

ABSTRACT

Neuropeptides related to mammalian neuropeptide Y (NPY) and insect neuropeptide F (NPF) are conserved throughout Metazoa and intimately involved in a wide range of biological processes. In insects NPF is involved in regulating feeding, learning, stress and reproductive behavior. Here we identified and characterized an NPF receptor of the tsetse fly, *Glossina morsitans morsitans*, the sole transmitter of *Trypanosoma* parasites causing sleeping sickness. We isolated cDNA sequences encoding tsetse NPF (Glomo-NPF) and its receptor (Glomo-NPFR), and examined their spatial and temporal expression patterns using quantitative PCR. In tsetse flies, *npfr* transcripts are expressed throughout development and most abundantly in the central nervous system, whereas low expression is found in the flight muscles and posterior midgut. Expression of *npf*, by contrast, shows low transcript levels during development but is strongly expressed in the posterior midgut and brain of adult flies. Expression of *Glomo-npf* and its receptor in the brain and digestive system suggests that NPF may have conserved neuromodulatory or hormonal functions in tsetse flies, such as in the regulation of feeding behavior. Cell-based activity studies of the Glomo-NPFR showed that Glomo-NPF activates the receptor up to nanomolar concentrations. The molecular data of Glomo-NPF and Glomo-NPFR paves the way for further investigation of its functions in tsetse flies.

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1. Introduction

The insect neuropeptide F (NPF) is well known for its role in the regulation of feeding and shares this function with the evolutionarily related neuropeptide Y (NPY) family of vertebrates (Holzer et al., 2012). Functional insights in insect NPF signaling mainly come from studies in the fruit fly, where NPF is shown to be a pleiotropic factor. It regulates processes as diverse as feeding behavior (Beshel and Zhong, 2013; Nässel and Wegener, 2011; Rohwedder et al., 2015; Wang et al., 2013), ethanol sensitivity

(Shohat-Ophir et al., 2012; Wen et al., 2005), sexual and male-specific courtship behavior (Kim et al., 2013; Lee et al., 2006), sleep homeostasis (He et al., 2013a,b; Hermann et al., 2012), and aggression (Dierick and Greenspan, 2007). The role of NPF in other insects has been much less explored and studies primarily focused on feeding-related processes. For example, NPF signaling is thought to regulate feeding and growth in the silk moth *Bombyx mori* (Ni et al., 2015; Deng et al., 2014). In the blowfly *Protophormia terraenovae*, NPF affects feeding and cardiovascular activity (Setzu et al., 2012), while in the desert locust *Schistocerca gregaria* NPF signaling is involved in reproduction and ecdysteroidogenesis as well as feeding behavior (Van Wielendaele et al., 2013a,b,c).

Tsetse flies are obligate blood feeders with both males and females on average needing a blood meal once every three days. During consumption of a blood meal, infected flies can transmit *Trypanosoma* parasites, pathogenic protozoans that cause sleeping sickness in humans (human African trypanosomiasis) or nagana in cattle (animal African trypanosomiasis). So far, little is known about signaling pathways regulating the tsetse fly's feeding

Abbreviations: NPF, neuropeptide F; NPFR, neuropeptide F receptor; GPCR, G protein-coupled receptor; CHO, Chinese hamster ovary; sNPF, short neuropeptide F.
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behavior. Nevertheless, studying these systems should help to better understand tsetse biology and may lead to new vector control strategies (Verlinden et al., 2014; Gäde and Goldsworthy, 2003; Scherkenbeck and Zdobinsky, 2009; Van Hiel et al., 2010).

With the publication of the *Glossina morsitans morsitans* genome, several neuropeptide signaling systems have been predicted, including a putative NPF and NPF receptor (NPFR) (IGGI, 2014). Whereas a duplication of the *npf* gene has occurred in several taxa, a single *npf* gene is predicted in *G. m. morsitans* encoding one NPF (Glomo-NPF) of 36 amino acids (IGGI, 2014). The *G. m. morsitans* NPF receptor (Glomo-NPFR) belongs to the family of G protein-coupled receptors (GPCRs). Related NPFRs have been characterized in *Drosophila* and *Anopheles* (Garczynski et al., 2002, 2005), and are predicted in various other insects (Caers et al., 2012). Here we characterized the NPFR of the tsetse fly *G. m. morsitans* and found it is activated by Glomo-NPF with potential pleiotropic effects in the regulation of feeding behavior, neurotransmission and neuro-modulation, based on the expression patterns of Glomo-NPF and its receptor.

2. Materials and methods

2.1. Animal rearing conditions

A colony of *G. m. morsitans* is maintained at the Institute of Tropical Medicine Antwerp (Elsen et al., 1993). The flies are fed three times a week with defibrinated and gamma-irradiated bovine blood through an artificial membrane system and kept at 26 °C and 65% relative humidity.

2.2. Cloning of Glomo-NPFR and Glomo-NPF cDNAs

The open reading frames (ORFs) coding for the predicted NPFR (Glomo-npfr: VectorBase ID: GMOY006767) and NPF precursor (Glomo-npf: VectorBase ID: GMOY012182) of tsetse fly were amplified starting from female *G. m. morsitans* cDNA. Primers (Sigma-Aldrich) used for *Glomo-npfr* were: Fw 5'-CACCCTCGCAGAT TATGACCAC-3' and Rv 5'-TTACCGTAACACGATCTCTG-3'. A partial Kozak consensus sequence (CACC) was included at the 5' end of the Fw primer to facilitate initiation of translation in mammalian cells used in the Ca²⁺ luminescence assay (Kozak, 1987). Primers used for *Glomo-npf* were: Fw 5'-GAAGTCACTTCCTGTCTG-3' and Rv 5'-GATTAAACCGCTTGAAAGAG-3'. PCR was performed with the Advantage 2 Polymerase PCR Kit (Clontech). Purified PCR products were extended with a single 3' A-overhang for T/A cloning into the pcDNA3.1/V5-His-TOPO®TA expression vector (*Glomo-npfr*) or the pCR4-TOPO® TA sequencing vector (*Glomo-npf*) (Invitrogen). These plasmids were transformed into One Shot TOP10 chemically competent *Escherichia coli* cells (Invitrogen). Plasmids were isolated with the GenElute™ Plasmid Miniprep Kit (Sigma-Aldrich) and their sequences verified on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

2.3. Sequence analyses of Glomo-NPFR and Glomo-NPF precursor

Different software programs were applied to analyze the protein sequence of the cloned ORF for Glomo-NPFR. PSIPRED and MEMSAT3 were used to predict transmembrane regions (bioinf.cs.ucl.ac.uk/psipred) (Jones, 2007). Phosphorylation sites were predicted with the NetPhos 2.0 software (www.cbs.dtu.dk/services/NetPhos) (Blom et al., 1999). Glycosylation and palmitoylation sites were predicted with NetNGlyc 1.0 and GSS-PALM 4.0, respectively (www.cbs.dtu.dk/services/NetNGlyc) and csspalm.biocuckoo.org/online.php) (Gupta and Brunak, 2002; Ren et al., 2008). Clustal

Omega (www.clustal.org) Sievers et al., 2011 was used to align Glomo-NPFR with other dipteran NPFRs including *D. melanogaster* NPFR (Drome-NPFR: CG1147) (Garczynski et al., 2002), *Musca domestica* NPFR (Musdo-NPFR: MDOA001172), *Anopheles gambiae* NPFR (Anoga-NPFR: AAT81602) (Garczynski et al., 2005), *Aedes aegypti* NPFR (Aedae-NPFR: XP_001660966) and *Culex quinquefasciatus* NPFR (Culqu-NPFR: XM_001848696). The protein sequence of the cloned NPF precursor was analyzed with SignalP 4.1 (www.cbs.dtu.dk/services/SignalP/) to determine the presence of an N-terminal signal peptide (Petersen et al., 2011).

2.4. Spatial and temporal transcript profiles of Glomo-npf and Glomo-npfr

Quantitative real-time PCR (qRT-PCR) was applied to determine the expression profiles of *Glomo-npfr* and *Glomo-npf* in different tissues and developmental stages of tsetse flies (no sex could be distinguished for the larval stages). Tissues were dissected under a binocular microscope in ice-cold phosphate buffered saline (PBS) (NaCl 137 mM, KCl 2.7 mM, Na₂HPO₄ 10 mM, KH₂PO₄ 1.76 mM; pH 7.4) and immediately frozen in liquid nitrogen. The following tissues were dissected and transferred to MagNA Lyser Green Beads tubes (Roche): a single head, thorax and abdomen of both males and females; an 18 day old pupal head, thorax and abdomen; a complete 18-day-old pupa; an 11-day-old pupa; and first, second and third larval instars. Additional tissues were dissected from female adult flies: five tissues were pooled in samples of the female reproductive system (including the uterus and ovaries), flight muscles, fat body (including the milk gland), anterior midgut, posterior midgut, Malpighian tubules and entire brain (including the optical lobes, antennal lobes and suboesophageal ganglion); ten tissues were pooled in samples of the thoracic ganglion, corpora cardiaca – corpora allata (CC_CA), salivary glands and hindgut. Three biological repeats were prepared for each sample. The tissues were homogenized with the MagNA Lyser® (Roche) and total RNA was isolated using the RNeasy® Lipid Tissue Mini Kit (Qiagen), in combination with a DNase digestion (RNase-free DNase Set, Qiagen). cDNA was synthesized with the PrimeScript™ RT Reagent Kit (Perfect Real Time; TaKaRa Bio Inc.) and diluted 10-fold. Negative control samples, without reverse transcriptase added to the reactions, were prepared for every sample. To normalize qPCR results, suitable reference genes were determined with the geNorm applet of qbase⁺ 2.4 (Biogazelle; see Supplementary data 1 for primer sequences) (Hellemans et al., 2007; Vandesompele et al., 2002). *cyp33*, *actin C* and *β-tub* genes were found to be most stably expressed in the total sample set (which also contains *sdha*, *gapdh*, *EF1α* and *rpl32*) and the geometric mean of these expression levels was used to normalize the *npf* and *npfr* samples. All samples were analyzed in triplicate and a no template control was included for every primer set to indicate potential master mix contamination. qRT-PCR reactions were set up in 96 well plates using the SYBR Green PCR Master Mix and run on a StepOnePlus Real-Time PCR System (Applied Biosystems). Data analysis was performed with qbase⁺ according to the $\Delta\Delta C_T$ method (Livak and Schmittgen, 2001). P-values were calculated using an ANOVA post-hoc Tukey test in R.

2.5. Cell culture and transient transfections

Chinese hamster ovary cells (CHO-K1), stably overexpressing the mitochondrially targeted apoaequorin (mtAeq), the promiscuous human Gα₁₆ subunit and a zeocin resistance gene (CHO/mtAeq/Gα₁₆) were used to measure *in vitro* Ca²⁺ responses of Glomo-NPFR (Stables et al., 1997). The Gα₁₆ subunit couples with most agonist-induced GPCRs and redirects the intracellular signaling pathway to phospholipase C activation and subsequent

release of Ca^{2+} from intracellular stores (Offermanns and Simon, 1995). The CHO/mtAeq/ $G\alpha_{16}$ cell line was grown in monolayer in Dulbecco's Modified Eagles Medium nutrient mixture F12-Ham (DMEM/F12; Sigma-Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS, Invitrogen), 100 IU/ml of penicillin/streptomycin (P/S, Invitrogen), 250 $\mu\text{g}/\text{ml}$ zeocin (Invitrogen) and 2.5 $\mu\text{g}/\text{ml}$ fungizone (Amphotericin B; Invitrogen). The CHO/mtAeq cell line, not expressing the $G\alpha_{16}$ subunit, was used to study the involvement of Ca^{2+} in the downstream signaling pathway of Glomo-NPFR. DMEM/F12 supplemented with 10% FBS, 100 IU/ml P/S, 2.5 $\mu\text{g}/\text{ml}$ fungizone and 5 $\mu\text{g}/\text{ml}$ puromycin (Invitrogen) was used to culture CHO/mtAeq cells. Both cell types were passaged every three days (1:10) and incubated at 37 °C and 5% CO_2 in a humidified atmosphere. Cells (60–80% confluent) were transfected with pcDNA3.1-Glomo-npfr or empty pcDNA3.1 vector using the Lipofectamine LTX Kit (Invitrogen). After transfection, cells were incubated overnight (37 °C, 5% CO_2), supplemented with fresh cell medium and incubated another 24 h (37 °C, 5% CO_2) before measuring Ca^{2+} responses.

2.6. Ca^{2+} luminescence assay

An aequorin-based Ca^{2+} mobilization assay was used to measure Ca^{2+} responses of CHO-K1 cells transfected with Glomo-npfr and pcDNA3.1 as described in Caers et al. (2015a). To detect calcium fluxes, cells stably expressed a mitochondrially targeted apoaquorin (mtAeq) and the promiscuous $G\alpha_{16}$ protein. To investigate downstream signaling messengers, we also measured Ca^{2+} responses of Glomo-NPFR in CHO/mtAeq cells lacking $G\alpha_{16}$. Briefly, cells were detached and loaded for 4 h with coelenterazine h (Invitrogen) to reconstitute the aequorin Ca^{2+} indicator. Cells were injected into 96-well plates (25,000 cells/well) containing different concentrations of Glomo-NPFR. Four *G. m. morsitans* short neuropeptide F (Glomo-sNPFF) sequences, which share a C-terminal RxRFamide motif with Glomo-NPFR (see Section 2.7), were tested as controls to determine if the presence of this motif is sufficient for Glomo-NPFR activation. Wells containing assay medium without any peptide were used as negative control; ATP (1 μM), which induces activation of an endogenous CHO cell receptor, was included as positive control. The response of ATP is taken as a maximum response level of 100%. Cells transfected with an empty pcDNA3.1 construct were tested to assure that Glomo-NPFR does not evoke activation of endogenous receptors. Luminescent signals upon addition of the cells to the compound plate were measured for 30 s on a Mithras LB940 luminometer (Berthold Technologies)

at a wavelength of 469 nm. After 30 s, 0.1% triton X-100 was added to cause cell lysis and induce a maximal Ca^{2+} response monitored for 10 s. Three independent experiments were performed, each containing three replicates of the tested ligand concentration series. GraphPad Prism 5 was used for data analysis and the construction of concentration-response curves (nonlinear regression analysis with a sigmoidal concentration-response equation).

2.7. Peptides

Glomo-NPFR (NTRPTRNDEIANIEEALQYLQELNFYDGKARIRFamide), Glomo-sNPFR-1 (AQRSPSLRLRFamide), Glomo-sNPFR-2 (SPSLRLRFamide), Glomo-sNPFR-3 (PQRLRFamide) and Glomo-sNPFR-4 (PARLRFamide) (IGGI, 2014; Caers et al., 2015b), were synthesized by GL Biochem Ltd. (Shanghai). All peptides were purified by reversed-phase high performance liquid chromatography (HPLC) and verified with a matrix-assisted laser desorption/ionization tandem time-of-flight (MALDI TOF/TOF, Ultraflex II Bruker Daltonics) mass spectrometer.

3. Results

3.1. Cloning and sequence analyses of *G. m. morsitans* npfr and npfr cDNAs

Analysis of the *G. m. morsitans* genome predicts the presence of a single Glomo-npfr gene (IGGI, 2014). Using primers specific for the predicted ORF, we isolated the cDNA encoding Glomo-NPFR from tsetse flies. The cloned Glomo-npfr cDNA sequence suggested a polymorphic site in comparison with the predicted sequence. The cloned Glomo-npfr sequences were identical to the predicted cDNA and protein sequences (IGGI, 2014) in 50% of all the receptor clones. The remaining half of the clones contained a guanine residue at position 502 instead of the predicted thymine. The cDNA sequence was confirmed by two independent PCR experiments with independent cDNA samples from different tsetse flies. This nucleotide substitution induces an amino acid change in the protein sequence at position 168 (predicted TTA coding for leucine to GTA coding for valine) (Fig. 1).

The protein sequence of the cloned Glomo-npfr cDNA displays typical hallmarks of the rhodopsin-like GPCR family, and harbors various phosphorylation, glycosylation and palmitoylation sites (Fig. 1). Aligning Glomo-NPFR with other dipteran NPFRs revealed high sequence identity and similarity, with highest conservation in the seven transmembrane domains. For example, Glomo-NPFR shares 61% sequence identity with Drome-NPFR, 66% with

Fig. 1. Sequence analysis of the *G. m. morsitans* NPFR. The transmembrane (TM) α -helices are underlined and typical rhodopsin-like GPCR motifs are indicated in bold: helix 1: Gx₃N or GN, helix 2: N(S,H)LX₂DX_{7,8,9}P, helix 3: SX₂LX₂LX₂D(E,H)RY, helix 4: WX_{8,9}P, helix 5: FX₂PX₂Y, helix 6: FX₂CW(Y,F)XP, and helix 7: LX₂NSX₂N(D)PX₂YX_{5,6}F. Green: putative phosphorylation sites in the intracellular loops or the C-terminal tail; red: putative palmitoylation site in the C-terminal tail; yellow: predicted glycosylation sites in the extracellular loops or the N-terminal tail; black: amino acid change compared to the predicted sequence (valine instead of lysine). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

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1  gaagtcacttccttgttctgcccgttataatattatcaaccaacgtgtaattaggtgaaat
61  attatcggtatttctcacaataatttatttcataatggcaactactctacgtcggttctc
1  M A T T L R R V L
121 gtcctaatagacaattctgtctctgtttaacgcgaagtattcggaataatacaagacca
10 V L M T I L S L F N A K V F G K N T R P
181 acgcgtaacgatgaaattgccaatattgaggaagctctccaatacctgcaagaacttgaa
30 T N D E I A N I E E A L Q Y I Q E I E
241 aatttctacgacggcaggccagaattcgttttgaaaaacgtagtacttgatgcaaagt
50 N F Y D G K A I R F G K R S Y L M Q M
301 cttaaaaatcgcttagcgaggcaggagaaatgaaccacgctcaggactcgggagactct
70 L K N R L G E A G E M N H A Q D S G D S
361 ttccaagcgggttaatc
90 F Q A V -

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Fig. 2. Cloned *Glomo-npf* cDNA sequence with corresponding prepropeptide sequence. The signal peptide is indicated in bold and italics in the precursor sequence. The *Glomo*-NPF sequence is indicated in black, and a downstream dibasic cleaving site is shown in gray. Purple shading indicates conserved residues of the insect NPF consensus sequence: $x_nPxRx_nYLx_2Lx_2YYx_nRPRFamide$ (Nässel and Wegener, 2011). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

Fig. 3. Relative expression profiles of (A) *Glomo-npfr* and (B) *Glomo-npf* transcripts. All adult tissues were collected from female flies. Larval stages are mixed (no differentiation in sex could be made for the instar stages). Expression is shown relative to the transcript levels in the female head (He). The data represent the mean of three biological replicates measured in triplicate. Error bars indicate SEM. Expression levels in male and female tissues were compared by one-way analysis of variance (ANOVA) and Tukey post-hoc comparison (all P values >0.05). In 1: first instar; In 2: second instar; In 3: third instar; Pu-11: 11-day-old pupa; Pu-18: 18-day old pupa; PH-18: heads of 18-day old pupa; He: heads; PT-18: thoraces of 18-day old pupa; Th: thoracic ganglia; PA-18: abdomen of 18-day old pupa; Ab: abdomen; CC-CA: corpora cardiaca – corpora allata; TG: thoracic ganglion; SG: salivary gland; MT: Malpighian tubules; AM: anterior midgut; PM: posterior midgut; HG: hindgut; FB: fat body; RS: reproductive system; Mu: flight muscles; Br: brain. n.s.: not significant.

Fig. 4. Concentration-response curves for Ca²⁺ responses of Glomo-NPFR expressing (A) CHO/mtAeq/Gα₁₆ and (B) CHO/mtAeq cells evoked by Glomo-NPFR. Data points represent the mean ± SEM of three independent measurements, each containing three replicates of the concentration series. All values were corrected for the negative control (BSA). The EC₅₀ value is depicted with its 95% confidence interval. (C) Ca²⁺ responses of CHO/mtAeq/Gα₁₆ cells expressing Glomo-NPFR or transfected with an empty pcDNA3.1 vector (negative control) after addition of 10 μM Glomo-NPFR or Glomo-sNPFR peptides. Assay medium without peptide (BSA) was used as negative control, while 1 μM ATP was used to activate endogenous CHO receptors as a positive control. Mean responses of peptides in Glomo-NPFR expressing cells (n ≥ 2) are depicted relative to the Ca²⁺ response evoked by ATP.

Musdo-NPFR, 58% with Anoga-NPFR, 62% with Aedae-NPFR and 62% with Culqu-NPFR (Supplementary data 2).

We also cloned the *Glomo-npf* cDNA, which encodes a protein sequence identical to the NPF precursor annotated in the *G. m. morsitans* genome (IGGI, 2014). The precursor harbors an N-terminal signal peptide of 24 amino acids, followed by the 36 amino acids

long NPF with a C-terminal glycine residue and a dibasic cleaving site (Fig. 2).

3.2. Spatial and temporal expression profiles of *Glomo-npf* and *Glomo-npr*

We investigated the spatial and temporal distribution of *Glomo-npf* and *Glomo-npr* transcripts by RT-qPCR. Highest relative transcript levels of *Glomo-npf* were found in the brain. We also detected notable expression in the thoracic ganglion, posterior midgut and muscles of female flies (these tissues were not dissected from males), and in the head and thorax of flies of both sexes. No significant difference was observed between male and female tissues. Low expression was found in all other examined tissues. *Glomo-npr* transcripts are expressed throughout tsetse development, but displayed varying transcript levels in different larval stages (Fig. 3A).

Glomo-npf transcripts are predominantly expressed in the posterior midgut of adult female flies. Expression was also found in the brain and head, and at lower levels in the anterior midgut, thorax and abdomen. Expression of *Glomo-npf* was low during the different developmental stages, but clearly present in the head and abdomen of 18-day-old pupa and the posterior midgut of adult female flies (Fig. 3B).

3.3. Activation of Glomo-NPFR by Glomo-NPF

To determine whether Glomo-NPFR can activate Glomo-NPFR, we performed a cell-based receptor-activation assay. We heterologously expressed Glomo-NPFR using the Val¹⁶⁸-NPFR construct in CHO cells co-expressing the promiscuous Gα₁₆ subunit and mitochondrially targeted apoaequorin (CHO/mtAeq/Gα₁₆), which enable monitoring of intracellular Ca²⁺ responses to ligands. We then assayed receptor activation after addition of various concentrations of Glomo-NPFR, as well as Glomo-sNPFRs, which share the C-terminal RxRFamide motif of Glomo-NPFR (IGGI, 2014; Caers et al., 2015b), as controls. Glomo-NPFR elicited a concentration-dependent Ca²⁺ response in CHO/mtAeq/Gα₁₆ cells expressing Glomo-NPFR, with an EC₅₀ value of 7.67 ± 2.74 nM (95% confidence interval) (Fig. 4A). In contrast, no response was observed in cells transfected with an empty vector or when Glomo-NPFR was challenged with Glomo-sNPFRs (Fig. 4C), suggesting that the presence of the RxRFamide motif is not sufficient to activate the receptor and Glomo-NPFR does not activate endogenous receptors in CHO cells.

To investigate downstream signaling messengers, we measured Ca²⁺ responses of Glomo-NPFR in CHO/mtAeq cells lacking the promiscuous Gα₁₆ subunit. Glomo-NPFR induced concentration-dependent Ca²⁺ responses independent of Gα₁₆ with an EC₅₀ value of 12.59 ± 7.81 nM, similar to that of CHO/mtAeq cells expressing both Glomo-NPFR and Gα₁₆ (Fig. 4B). This result suggests that Glomo-NPFR can couple to endogenous Gα_q-type subunits in the CHO/mtAeq cells.

4. Discussion

The annotation of the genome sequence of the tsetse fly *G. m. morsitans* predicted the conservation of over 40 neuropeptidergic pathways that are bound to govern key aspects of tsetse fly biology (IGGI, 2014). In this study we characterized a neuropeptide pathway related to NPF signaling, encoded by a single *Glomo-npf* and *Glomo-npr* gene. The cDNA sequence of *G. m. morsitans npf* confirms the presence of a prepropeptide of 93 amino acids with a composition typical of the NPF precursor family: an N-terminal signal peptide followed by the mature NPF peptide, a C-terminal glycine residue for amidation and a dibasic cleavage site

(Nässel and Wegener, 2011). Cloning of the *npfr* cDNA sequence revealed a single nucleotide polymorphism in the open reading frame, causing a substitution in the protein sequence at position 168 (leucine to valine). This substitution preserves the presence of a hydrophobic residue at position 168, located in the third transmembrane domain, suggesting minor effects on the receptor conformation, ligand binding site or interaction with G proteins.

The presence of several characteristic residues and patterns in the seven transmembrane domains (GN in helix 1, NLX₃DX₈P in helix 2, SX₆IX₂DRY in helix 3, WX₈P in helix 4, PX₇Y in helix 5, FX₃WXP in helix 6 and NPX₂YX₆F in helix 7) indicates that the NPFR belongs to the rhodopsin-like GPCRs (Costanzi, 2012). Potential positions for post-translational modifications include sites for phosphorylation (S298, T303, T404, Y410, T426 and T450), glycosylation (N25, N53 and N59) and palmitoylation (C384, C385 and C386). Dipteran NPFRs show strong sequence conservation for their α -helices and intra- and extracellular loops, but are more loosely aligned at their N- and C-terminus. A notable difference is a longer third intracellular loop in the mosquito species (*A. gambiae*, *A. aegypti* and *C. quinquefasciatus*) in comparison with the fly species (*G. m. morsitans*, *D. melanogaster* and *M. domestica*). The length of this loop was also found to be inconsistent among other insect orders and different metazoan phyla (Deng et al., 2014). Although it remains unclear how the variable loop length affects the functioning of the NPFR, it may alter G protein coupling or affect post-transcriptional regulation through the presence of a varying number of sites for modifications.

Glomo-NPF activates Glomo-NPFR in a concentration-dependent manner and with a nanomolar EC₅₀ value, indicating this is likely the cognate ligand of the tsetse fly NPFR. On the other hand, Glomo-sNPFs that share the same C-terminal motif (-RxRFamide) were not able to activate the receptor. Similar to previous reports, this result implies that additional amino acids of the NPF consensus sequence x_nPxRx_nYLx₂Lx₂YYx₄RxRFamide are required for receptor activation and corroborates an only distant evolutionary relationship between the NPF and sNPF neuropeptide families (Nässel and Wegener, 2011). Glomo-NPF and Glomo-sNPF activation were only tested for one of the two Glomo-NPFR isoforms. We hypothesize that the conservative mutation in the second isoform does not influence ligand binding. The amino acid substitution is situated in a transmembrane position unlikely to be involved in ligand binding.

Previous work denotes that insect NPFR signaling can induce IP₃-dependent release of intracellular calcium, which was shown for *Bombyx mori* NPFR *in vitro* (Deng et al., 2014). The downstream coupling to this secondary messenger pathway seems to be conserved in *G. m. morsitans*. In our *in vitro* assay, Glomo-NPF activated Glomo-NPFR even in absence of the promiscuous G α_{16} subunit with a nanomolar EC₅₀ value. This finding indicates that the receptor can couple with an endogenous G α_q -type protein in the CHO/mtAeq cells. Evidence from other insects suggests that additional downstream signaling pathways might be involved in Glomo-NPF signaling. For example, *B. mori* NPFR activation was found to not only increase intracellular Ca²⁺, but also to inhibit adenylyl cyclase activity, suggesting that the receptor can act via a G α_i - and/or G α_q -mediated signaling cascade (Deng et al., 2014). Activation of the *D. melanogaster* NPFR by NPF also inhibited adenylyl cyclase (Garczynski et al., 2002).

A role for NPF in feeding behavior and other physiological processes has been described for various insects (Nässel and Wegener, 2011). These functions are linked to the presence of the NPF signaling system in the central nervous system and in the midgut of all insects studied so far (Nässel and Wegener, 2011; Rohwedder et al., 2015; Deng et al., 2014; Van Wielendaele et al., 2013b; Veenstra and Ida, 2014). For example, the activation of NPF neurons inhibits appetitive olfactory learning (Rohwedder et al.,

2015). In addition, NPF overexpression in *Drosophila* induces continuous feeding and tolerance to noxious food, while injection of NPF in locusts increases food uptake (Van Wielendaele et al., 2013b; Wu et al., 2003; Wen et al., 2005). This is in consonance with the observation that silencing of NPF or NPFR neurons abolishes food-odor attractiveness (Beshel and Zhong, 2013). In *G. m. morsitans*, *npfr* and *npf* transcripts are abundantly present in the brain and are also found in the posterior midgut, suggesting similar functions of NPF signaling in the control of feeding. Mass spectrometry analysis of tsetse brain extracts confirmed the expression of the NPF precursor by the detection of a spacer peptide from the Glomo-NPF precursor (Caers et al., 2015b). Besides the brain and midgut, transcripts of *Glomo-npfr* are also abundantly present in the thorax and more specifically in the thoracic ganglion and the flight muscles. To our knowledge, expression of *npfr* in muscle tissue has not been reported before, but these data suggest a putative role for NPF signaling in locomotion or the control of energy metabolism. As odor attractiveness involves moving away from or towards a food source, NPF signaling could induce, directly or indirectly, a locomotory program. Additionally we observed expression of the *npfr* transcript in the salivary glands. While speculative, NPF might increase salivation upon encountering favorable odors through its olfactory function. Contrary to the expression of *D. melanogaster npfr* in Malpighian tubules (<http://flyatlas.gla.ac.uk/>), we did not observe any expression of *Glomo-npfr* in Malpighian tubules of tsetse flies. In fruit flies males show a higher expression of NPF in brains, possibly due to the presence of male-specific NPF neurons, (Chintapalli et al., 2012; Lee et al., 2006). In contrast to fruit flies we did not observe any difference in *npf* expression between male and female heads and bodies. For *Glomo-npf*, low transcript levels are noticed in the anterior midgut and thorax. Whether this involves putative production of NPF by neuroendocrine midgut cells remains to be investigated, but may be linked to a role in the digestive process. *Glomo-npfr* is expressed throughout all life stages of tsetse flies, while expression of *npf* transcripts is strongly upregulated in the adult stage. Overall, our results suggest a role for NPF signaling in the regulation of feeding behavior, among other physiological processes, in tsetse flies. The pleiotropic character of NPF in other insects including its role in feeding behavior points out a potential as target for new insecticides. Our molecular data provide a basis to further characterize and validate this signaling system in tsetse flies *in vivo*.

Acknowledgments

The research was funded by the Research Foundation Flanders (FWO-Flanders, G069713N), the European Research Council (ERC grant 282312), the InterUniversity Attraction Pole program P7/41 and the ITM-SOFI funding. Isabel Beets (postdoctoral fellowship) and Katleen Peymen (doctoral fellowship) are supported by the FWO.

Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.jinsphys.2016.09.013>.

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