Identification of the short neuropeptide F precursor in the desert locust: Evidence for an inhibitory role of sNPF in the control of feeding

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ABSTRACT

Peptides of the short neuropeptide F (sNPF) family have been shown to modulate feeding behavior in a wide variety of insect species. While these peptides stimulate feeding and food-searching behavior in Drosophila melanogaster and Apis mellifera, an opposite effect has recently been demonstrated in the desert locust, Schistocerca gregaria. In this study, we elaborate on these observations with the identification of the nucleotide sequence encoding the Schgr-sNPF precursor and the study of its role in the regulation of locust feeding behavior. We confirm that both Schgr-sNPF-like peptides, previously identified in mass spectrometric studies, are genuine precursor-encoded peptides. RNA interference mediated silencing of the Schgr-sNPF precursor transcript generates novel evidence for an inhibitory role of Schgr-sNPF in the regulation of feeding in S. gregaria. Furthermore, we show that starvation reduces the Schgr-sNPF precursor transcript level in the optic lobes, the primary visual centers of the locust brain. Our data indicate that Schgr-sNPF exerts an inhibitory effect on food uptake in the desert locust, which contrasts with effects of sNPF reported for several other insect species.

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1. Introduction

Peptides of the short neuropeptide F (sNPF) family, characterized by a carboxyterminal xPxLRLRFamide sequence, have a widespread occurrence throughout the arthropod phylum and have been found in all arthropod species of which ample sequence data are available [22]. Interestingly, no sNPF-like peptides have been discovered in non-arthropod species, making the sNPF signaling pathway a prime candidate for further research exploring the use of neuropeptidergic signaling in the control of insect pests [11].

The sNPF peptides are cleaved from a larger prepropeptide precursor, containing an aminoterminal signal peptide which allows for transport of the precursor peptide to the endoplasmatic reticulum for further processing and for secretion to the extracellular environment. These sNPF prepropeptides typically yield multiple sNPF isoforms. In Drosophila melanogaster, for example, the sNPF precursor (sNPFp) encodes four Drome-sNPFs [31], while the sNPF prepropeptide of Apis mellifera yields two Apime-sNPF isoforms [12]. While these sNPF peptide precursors typically yield multiple sNPF isoforms, this is not the case in all insect species. In the kissing bug, Rhodnius prolixus, for example, only a single sNPF is cleaved from the Rhopr-sNPF prepropeptide [24].

In the desert locust, Schistocerca gregaria, the presence of two sNPF-like peptides has previously been confirmed by mass spectrometry. These peptides display a large overlap in amino acid sequence and at the same time their discovery, it could not be excluded that the smaller Schgr-sNPF4-11 (SPSLRLRFamide) was only a degradation product of the larger Schgr-sNPF (SNRSPRLRLR-Famide) [8]. Recent in vivo experiments, however, pointed out that both Schgr-sNPF and Schgr-sNPF4-11 display physiological activity. Furthermore, both Schgr-sNPF peptides are potent ligands of the Schgr-sNPF receptor (sNPFR), capable of inducing both a rise of intracellular calcium ion levels and a decrease of intracellular cyclic AMP [10].

Mass spectrometric analysis showed that the Schgr-sNPF peptides are abundant throughout the central nervous system and their presence has been confirmed in the brain, corpora cardiaca, corpora allata, the recurrent and suboesophageal nerves and a variety of ganglia [9].

Although peptides of the sNPF family were demonstrated to play a role in a wide variety of physiological processes including molting [18], sleep homeostasis [7] and reproduction [5], their main function seems to lie in the regulation of feeding and food-searching behavior.

A first indication of these effects on feeding was obtained when overexpression of sNPF in D. melanogaster promoted food uptake...
and yielded larger and heavier flies. Conversely, flies in which the Drome-sNPFp transcript was knocked down, displayed the opposite phenotype. It should be noted, however, that these stimulatory effects on feeding were only observed in larvae in the feeding stage of development and that the feeding responses of larvae in the wandering stage did not differ from those observed in control animals [15]. Similar to the results obtained for Drome-sNPF, overexpression of the Drosophila sNPFR, Drome-sNPFR1, led to an increase in body size while RNA interference (RNAi) mediated knockdown of the receptor transcript yielded smaller animals when compared to wild-type controls. Interestingly, this reduction in Drome-sNPF signaling activity also led to an increase in lifespan, an effect that is likely mediated by the Drosophila insulin-like peptides [14].

A positive correlation between sNPF signaling and feeding, as demonstrated in Drosophila, was also observed in several other insect species. When sNPF and sNPFR transcript levels were studied in the honey bee, A. mellifera, both were found to be higher in foraging animals compared to animals providing brood care [2]. Furthermore, sNPF reduced the latency to feeding in the silk moth, Bombyx mori, rendering the animals more prone to feed [21]. sNPF and sNPFR expression levels were also higher in starved fruit flies [25] and cockroaches [19], suggesting a correlation with an increase in food-searching behavior. In D. melanogaster, this starvation-dependent upregulation of sNPF signaling was mapped to certain olfactory receptor neurons in the antennal lobes of the brain. Upon starvation, sNPF expression in these neurons increased and flies demonstrated augmented food-searching behavior. When sNPF signaling in these neurons was inhibited however, this starvation-dependent increase in food-searching behavior was abolished [25].

Although there seems to be a positive correlation between sNPF signaling and feeding in the fruit fly and the honey bee, this does not seem to be the case in all insect species. Early studies on the Aedes aegypti sNPF-like peptide, Head Peptide 1 (HP-1), showed that the levels of circulating peptide increased fivefold after a replete blood meal. Furthermore, injection of this Aedes-HP-1 potentely inhibited host-seeking behavior in adult female mosquitoes [4]. In the red imported fire ant, Solenopsis invicta, transcript levels of the sNPF receptor were also shown to be higher in starved animals compared to non-starved controls [6].

Further indications were acquired in the silk moth, Bombyx mori. While injection of Bommo-sNPF-2 reduced the latency to feeding in silk moth larvae [21], Nagata et al. also showed that the expression levels of Bommo-sNPF-1 and -2 decreased upon starvation, a state in which the animals are more likely to engage in food-searching behavior [20].

Arguably the strongest indications for an inhibitory role of sNPF in the regulation of feeding were recently obtained in the desert locust, S. gregaria. In these studies, starvation resulted in a decrease of the Schgr-sNPF receptor transcript levels, while feeding led to a transient peak in the receptor transcript level. Furthermore, injecting either of the two Schgr-sNPF isoforms resulted in an augmentation of food-uptake. When sNPF signaling activity was reduced by RNAi-induced knockdown of the Schgr-sNPFR transcript, the opposite (stimulatory) effect was observed [10].

In this paper, we describe the identification of the Schgr-sNPF peptide precursor and discuss its role in the regulation of feeding behavior. We show that both Schgr-sNPF and Schgr-sNPF4-11 are genuine Schgr-sNPF precursor-derived isoforms. Furthermore, we present more evidence for an inhibitory role of sNPF in the regulation of feeding. Silencing the Schgr-sNPF precursor mRNA, significantly increases food uptake, while starvation leads to a decrease in sNPF precursor transcript levels in the optic lobes, the primary visual centers of the locust brain.

2. Materials and methods

2.1. Identification and cloning of the Schgr-sNPF precursor cDNA

From a recently generated whole-body transcriptome database of S. gregaria (Verdonck et al., in preparation), a cDNA sequence encoding the two Schgr-sNPF isoforms was retrieved. This nucleotide sequence was amplified from brain-derived cDNA, using the following nucleotide primers: Schgr-sNPF-Fw: 5′-GTCGCGCTGTGCAAGGCT-3′; Schgr-sNPF-Rv: 5′-GTTGAGCTCGACCGTATTTC-3′ (Sigma–Aldrich) and the following program: 95 °C for 120 s, followed by 30 cycles of 95 °C for 30 s, 55 °C for 30 s and 72 °C for 120 s. After these cycles, the program was completed with a final elongation step of 72 °C for 120 s and ended at 10 °C. The PCR product was run on a 1% agarose gel, from which it was purified using the GenElute™ Gel Extraction Kit (Sigma–Aldrich). Amplicons were then cloned in a pCR®-2.1-TOPO® vector (Invitrogen), which was used to transform One Shot TOP10 chemically competent Escherichia coli cells (Invitrogen). These were plated on LB agar plates (35 g/l, Sigma–Aldrich) with ampicillin (10 mg/ml, Invitrogen) and grown overnight at 37 °C. Colonies were collected and transferred to LB medium (25 g/l, Sigma–Aldrich) with ampicillin (10 mg/ml, Invitrogen) and again grown overnight at 37 °C. Plasmids were extracted using the GenElute™ HP Plasmid Miniprep kit (Sigma–Aldrich) and the sequences of the insert fragments were verified on an ABI PRISM 3130 Genetic Analyzer (Applied Biosystems) using the ABI PRISM BigDye Terminator Ready Reaction Cycle Sequencing Kit (Applied Biosystems).

2.2. Animal rearing conditions

All animals used in this study were gregarious adult locusts. They were reared under crowded conditions (over 200 locusts per cage) at constant temperature (32 ± 1 °C) and photoperiod (LD: 14 h/10 h). During rearing, they were fed daily with cabbage and dry oat flakes. The locusts used in the feeding experiment were placed in separate cages and placed on a controlled dietary regimen one week prior to the assay in order to normalize feeding responses. In this controlled regimen animals were allowed to feed ad libitum for 2 h each day (9–10 h, 16–17 h). The animals used in starvation studies were placed in separate cages and deprived of food for a period of five days. Non-starved control animals were also placed in these cages but were allowed to feed ad libitum.

2.3. Schgr-sNPF precursor transcript distribution

Locust tissues were dissected under a binocular microscope and collected in MagNa Lyser Green Beads (Roche). The dissected tissues were immediately frozen in liquid nitrogen and stored at –80 °C. Prior to RNA extraction, the tissues were homogenized using the MagNa Lyser instrument. RNA extraction was performed using the RNeasy® Lipid Tissue Mini Kit (Qiagen), in combination with a DNase digestion of the purified nucleic acids (RNaase-free DNase Set, Qiagen), or the RNaqueous®-Micro Kit (Ambion). The resulting total RNA was reverse transcribed to cDNA using the PrimeScript™ RT Reagent Kit (Takara). The resulting cDNA was diluted twentyfold and used as template in quantitative (realtime) reverse transcription PCR (qRT–PCR).

The primers used in the qRT–PCR analysis were designed by means of the Primer Express® software (Applied Biosystems, Table 1) and subjected to melting curve analysis to verify their specificity and efficiency of amplification. Amplification products were also analyzed by means of electrophoresis on a 1% agarose gel. Visual inspection of the PCR products showed a single band of the expected size. Additionally, the PCR products were sequenced
to further verify the specificity of the qRT-PCR amplification. The reference genes used in the study were analyzed using the geNorm applet [32], revealing ribosomal protein 49 (RP49) and Elongation Factor 1α (EF1α) to be the most stably expressed pair over the sample set. For qRT-PCR, we used Fast SYBR® Green Master Mix (Applied Biosystems), as per manufacturer’s instruction, and the StepOnePlus™ Real-Time PCR system (Applied Biosystems). The Fast SYBR® Green Master Mix contains the fluorescent ROX™, which is used as a passive reference. All samples were measured in duplicate and all plates contained a no template control for all three primer pairs to check for possible contaminations in the master mix. The following program was used: 95 °C for 10 min, followed by 40 cycles of 95 °C for 3 s and 60 °C for 30 s. Data were analyzed using the ΔΔCt method, in which the cycle threshold (Ct) value for the gene of interest (GOI) is normalized to the Ct1 of one or more reference genes (ΔCt) and to the Ct2 GOI of a calibrator sample (ΔΔCt), included on each plate [16].

2.4. Schgr-sNPF precursor transcript knockdown

T7 promoter sites were incorporated on either side of a 256 bp fragment of the Schgr-sNPF precursor cdNA. This was done by performing PCR, using the following primers: Schgr-sNPF-T7-Fw 5'-TAATACGACTCACTATAGGGAGATTTCTCCACT-GAATTCGAG-3' (Sigma–Aldrich) and Schgr-sNPF-T7-Rv 5'-TAATAGCCTACTATAGGGAGATTTCTCCACT-GAATTCGAG-3' (Sigma–Aldrich) and the following program: 95 °C for 120 s, followed by 10 cycles of 95 °C for 30 s, 60 °C for 30 s and 72 °C for 60 s, and 15 cycles of 95 °C for 30 s, 65 °C for 30 s and 72 °C for 60 s. After these cycles, the program was completed with a final elongation step of 72 °C for 120 s and ended at 10 °C. Amplions were cloned into a pCR®2.1-TOPO® vector (Invitrogen), used in the transformation of One Shot TOP10 chemically competent Escherichia coli cells (Invitrogen), and sequenced, as described above for the Schgr-sNPF precursor cdNA.

Double stranded RNA (dsRNA) was generated using the MEGAscript® RNAi Kit (Ambion) and its length was checked on a 1% agarose gel. The efficiency of the knockdown was assayed by injecting adult locusts with 2 μl of a 100 ng/μl dsRNA solution, the dsRNA corresponding to either Schgr-sNPF (experimental) or GFP (control). Animals were injected dorsally, between the first and second abdominal segment, using a 710 RN injector (Hamilton). Five and eight days after injection, locust brains (including the optic lobes) were dissected. Since S. gregaria shows a highly potent and systemic RNAi response, a knockdown of the Schgr-sNPF transcript in this tissue will also be indicative of effects on other tissues. The high efficiency of RNAi in multiple tissues of the desert locust has previously been demonstrated in a number of studies and has been studied in detail by Wynant et al. [17,27,28,34]. Total RNA was extracted from the dissected tissues and was reverse transcribed into cDNA and used as template in qRT-PCR analysis, as described previously for the Schgr-sNPF precursor transcript distribution.

2.5. Feeding assay

Two groups of age- and sex–matched locusts were injected with 2 μl of a 100 ng/μl solution of dsRNA, corresponding to either Schgr-sNPF or GFP. The feeding assay was performed five days after injection, at the time of the first daily feeding session. Pieces of cabbage leaf were weighed in advance and presented to the locusts for 1 h, after which all animals had stopped feeding. The pieces of cabbage were then weighed again and the total food uptake was determined. In parallel, similar pieces of cabbage leaf were put in empty cages during the course of the experiment, allowing for a correction for weight-loss due to evaporation. The results were analyzed and visualized using GraphPad Prism 5 (GraphPad Software).

3. Results

3.1. Identification of the Schgr-sNPF precursor

Using the peptide sequences of the two Schgr-sNPF isoforms as a query, we were able to identify the sNPF precursor sequence in a recently developed whole-body transcriptome database of S. gregaria. The precursor’s nucleotide sequence was cloned and the confirmed sequence was uploaded to Genbank [GenBank: KF360057]. The region encoding the two sNPF isoforms is flanked by a start- and stop-codon, indicating that the complete coding sequence is represented (Fig. 1). At the aminoterminal end of the precursor peptide, the presence of a secretary signal peptide is predicted (InterProScan, EMBL European Bioinformatics Institute). The amino acid sequences of Schgr-sNPF and Schgr-sNPF-11 are both present in the precursor sequence and each of these is flanked by mono- or dibasic cleavage sites and followed by an amidation signal.

3.2. Schgr-sNPF precursor transcript distribution

Quantitative reverse transcription PCR was used to study Schgr-sNPF transcript levels in a wide variety of tissues. The transcript was primarily detected in the central nervous system, with only a very limited expression in the flight muscles and the male and female gonads. The brain, optic lobes, and corpora cardiaca proved to be the major sites of Schgr-sNPF mRNA expression. Lower transcript levels were detected in the corpora allata and the suboesophageal, thoracic, and abdominal ganglia (Fig. 2). The effect of starvation on sNPF transcript levels was studied in the brain, the optic lobes, the corpora cardiaca and the suboesophageal ganglion. Starvation was shown to reduce Schgr-sNPF transcript levels in the optic lobes by 35%, while no significant differences were detected in the other three tissues that were analyzed (Fig. 3).

3.3. Knockdown of Schgr-sNPF transcript levels stimulates food uptake

To further study the functional role of sNPFs in the desert locust, we knocked down the Schgr-sNPF transcript by means of RNA interference. This procedure yielded a potent reduction of the abundance of Schgr-sNPF transcripts. Five days after injection, Schgr-sNPF transcript levels in the brain showed a decrease of over 90% when compared to control animals injected with dsRNA corresponding to the mRNA corresponding to GFP. This 90%
reduction in Schgr-sNPFp transcript levels was shown to persist until at least eight days after injection (Fig. 4). When the animals were subjected to a feeding assay five days after dsRNA injection, locusts in which the Schgr-sNPFp specific mRNA was silenced, showed a highly significant (approximately fourfold) increase in food-uptake compared to control animals (Fig. 5).

4. Discussion

The identified sequence displays all characteristics that are typical for a neuropeptide precursor [31]. All necessary elements for the formation of mature Schgr-sNPF peptides are therefore present (Fig. 1). Since arthropod genomes typically contain only a single sNPF-encoding gene, these results indicate that no other Schgr-sNPF isomers are likely to be present, apart from the two peptides previously identified in mass spectrometric studies [8,22]. At present, it is not clear whether, in addition to sNPFs, other bioactive peptides might be encoded in this precursor. It is interesting, however, to notice some conserved amino acid sequence pattern (starting with SDP) in the adjacent sequences following the amidation signal of the sNPFs. Although the function of these SDP-motifs remains unclear, they can also be observed in the sNPF precursors of Tribolium castaneum [GenBank: DAA34847.1], Aedes aegypti [GenBank: ABE72968.1], Anopheles gambiae [UniProt: A0SIX6.2], and D. melanogaster [RefSeq: NP_724239.1].

Based on the structure of the Schgr-sNPFp sequence, an update of nomenclature seems appropriate. At the time of discovery of the Schgr-sNPF peptides, Clynen et al. were unable to ascertain whether the observed peptides were genuine sNPF isoforms or whether the shorter Schgr-sNPF4-11 (SPSRLRLFA) was derived from the larger Schgr-sNPF (SNRSPLRLRIFa). These peptides were therefore named emphasizing their overlap in amino acid sequence: Schgr-sNPF and Schgr-sNPF4-11 [8]. The identification of the Schgr-sNPF precursor, however, has shown that both Schgr-sNPF and Schgr-sNPF4-11 are separately encoded in the precursor and that both peptides are genuine Schgr-sNPF isoforms. Therefore, we recommend adjusting the names of the peptides from Schgr-sNPF and Schgr-sNPF4-11 to Schgr-sNPF-1 and -2 respectively, according to their order of appearance in the precursor sequence. This way, the structure of the peptide precursor is taken into account and nomenclature is adjusted to correspond to that used in other insect species, such as D. melanogaster (Drome-sNPF-1 to -4) and A. mellifera (Apime-sNPF-1 and -2) [12,31].

![Graph showing relative transcript level sNPFp upon starvation](image)

**Fig. 3.** Relative Schgr-sNPF precursor transcript levels upon starvation. Expression levels of the Schgr-sNPFp transcript in the optic lobes were significantly lower after five days of starvation. Br: brain; OL: optic lobes; CC: corpora cardiac; and SOG: suboesophageal ganglion. Data represent mean values ± SEM (n = 9). Results were analyzed using Student’s t-test. **p < 0.005.

![Graph showing relative transcript level sNPFp in brain](image)

**Fig. 4.** Relative Schgr-sNPF precursor transcript levels in the brain upon Schgr-sNPFp knockdown. Locusts were injected with 200 ng dsRNA corresponding to either GFP or sNPFp. Injection of sNPFp dsRNA resulted in a 90% reduction in transcript levels five days after injection. Similar transcript levels were maintained until at least eight days after injection. Data represent mean values ± SEM (n = 6).

![Graph showing food uptake and body weight](image)

**Fig. 5.** RNAi mediated knockdown of the Schgr-sNPF precursor transcript and its effect on food uptake. Silencing of the Schgr-sNPFp mRNA yields a fourfold augmentation of food uptake in non-starved adult locusts. Data represent mean values ± SEM (n = 15). Results were analyzed using Student’s t-test. ***p < 0.0001.
The main function of sNPF in arthropod physiology appears to be the regulation of feeding, a highly complex process regulated by numerous peptides, as discussed in recent studies [1,2,9,30,33] and reviewed by Spit et al. [26]. Although sNPF’s effects on feeding are evident in the vast majority of studies, the direction of the effect appears to differ between insect species. In D. melanogaster, Bombyx mori, A. mellifera, Periplaneta americana, and Lepinotarsa decemlineata, sNPF signaling either stimulates feeding and food-search, or is correlated with a physiological state in which the animals more actively engage in food-search behavior [13,15,19,21]. In Aedes aegypti and S. Invicta, however, sNPF does not seem to follow this positive correlation with feeding [4,6]. More conclusive evidence of sNPF’s inhibitory role in feeding came when the nucleotide sequence encoding the sNPF receptor was identified in S. gregaria [3]. RNAi mediated knockdown of this receptor significantly stimulated food uptake, while injection of either of the two Schgr-sNPF isoforms resulted in a potent reduction of feeding [10]. In the present study, we elaborate on these results with the identification of the nucleotide sequence encoding the Schgr-sNPF precursor and with the finding that silencing this precursor mRNA strongly stimulates food uptake in this locust species. Schgr-sNPFp transcript levels in the brain significantly declined by injecting a fragment of double stranded RNA, corresponding to a 256 bp section of the Schgr-sNPFp cDNA. This procedure generated a potent transcript knockdown of over 90% (Fig. 4). When food uptake was analyzed in these animals, we observed an increase of the ingested amount of food of more than 300% (Fig. 5). These results provide new evidence for an inhibitory role of Schgr-sNPF in the regulation of feeding behavior in S. gregaria, although it remains mostly unclear how these effects are established exactly.

The distribution of the Schgr-sNPF transcript appeared to be limited to tissues of neural origin, while its expression was very low or nearly undetectable in male and female gonads and flight muscles (Fig. 2). In this aspect, the Schgr-sNPF transcript distribution resembles that of the sNPF peptides in D. melanogaster [15] and Reticulitermes flavipes [23], and of the Schgr-sNPF receptor [10]. In several insect species, such as Bombyx mori and Aedes aegypti, sNPF expression levels have been shown to decline in starved animals compared to non-starved controls [4,20]. Similar results have previously also been obtained for the sNPF receptors of S. invicta and S. gregaria [6,10]. While no effects of starvation were observed in the central mass of the locust brain, the suboesophageal ganglion or the corpora cardiaca, starvation during a period of five days significantly reduced Schgr-sNPF transcript levels in the optic lobes, the primary visual centers of the brain (Fig. 3). Interestingly, in D. melanogaster, starvation was shown to induce Drome-sNPF expression in sensory receptor neurons in the antennal lobes of the brain, which are responsible for the processing and propagation of olfactory stimuli. It was shown that sNPF signaling in these neurons plays a key role in starvation-dependent behavior and the authors argued that sNPF expression might modulate the organism’s receptivity to external olfactory cues [25]. Analogous to these results, but bearing in mind that in S. gregaria sNPF has inhibitory rather than stimulatory effects on feeding, the observed starvation-dependent decrease of sNPFp transcript levels in the optic lobes may also be indicative for a transition to a physiological state more receptive to sensory (i.e. visual) stimuli, thereby facilitating food-search and feeding behaviors.

In conclusion, we have shown that both Schgr-sNPF and Schgr-sNPF4–11 are genuine, precursor-encoded Schgr-sNPF isoforms and therefore suggest an update of their nomenclature to Schgr-sNFP–1 and –2 respectively. Furthermore, we have generated more evidence supporting an inhibitory role of sNPF in the control of feeding in S. gregaria. Although the exact mode of action remains largely unknown, the observation that sNPFp transcript levels in the optic lobes decrease upon starvation may suggest that Schgr-sNFP can modulate the animal’s receptivity to visual cues, thereby affecting food searching behavior.

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