



Effects of starvation on brain short neuropeptide F-1, -2, and -3 levels and short neuropeptide F receptor expression levels of the silkworm, *Bombyx mori*

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In our previous report, we demonstrated the possibility that various regulatory neuropeptides influence feeding behavior in the silkworm, *Bombyx mori*. Among these feeding-related neuropeptides, short neuropeptide F (sNPF) exhibited feeding-accelerating activity when injected into *B. mori* larvae. Like other insect sNPFs, the deduced amino acid sequence of the cDNA encoding the sNPF precursor appears to produce multiple sNPF and sNPF-related peptides in *B. mori*. The presence of three sNPFs, sNPF-1, sNPF-2, and sNPF-3, in the brain of *B. mori* larvae was confirmed by direct MALDI-TOF mass spectrometric profiling. In addition, all three sNPFs are present in other larval ganglia. The presence of sNPF mRNA in the central nervous system (CNS) was also confirmed by Reverse transcription-polymerase chain reaction. Semi-quantitative analyses of sNPFs in the larval brain using matrix-assisted laser desorption ionization time-of-flight mass spectrometry further revealed that brain sNPF levels decrease in response to starvation, and that they recover with the resumption of feeding. These data suggest that sNPFs were depleted by the starvation process. Furthermore, food deprivation decreased the transcriptional levels of the sNPF receptor (BNGR-A10) in the brain and CNS, suggesting that the sNPF system is dependent on the feeding state of the insect and that the sNPF system may be linked to locomotor activity associated with foraging behavior. Since the injection of sNPFs accelerated the onset of feeding in *B. mori* larvae, we concluded that sNPFs are strongly related to feeding behavior. In addition, semi-quantitative MS analyses revealed that allatostatin, which is present in the larval brain, is also reduced in response to starvation, whereas the peptide level of Bommyosuppressin was not affected by different feeding states.

Keywords: *Bombyx mori*, feeding behavior, peptide, short neuropeptide F

INTRODUCTION

Although phytophagous insects feed on their preferred host plants, feeding is not continuous. A number of biochemical and behavioral studies have found that chemical components extracted from host plants serve as attractants or deterrents for plant feeders (Harborne, 1993; Hanson, 2003), and thus contribute to host selection. Therefore, it is believed that feeding behavior in phytophagous insects may be initiated by the chemical components present in their preferred host plants.

The biology and physiology of the mechanisms that regulate insect feeding behavior have been extensively investigated (Simpson, 1995). To date, sequential behaviors in regularly occurring feeding patterns have been monitored using a number of phytophagous insects, including orthopteran (Blaney et al., 1973; Simpson, 1982; Simpson and Ludlow, 1986) and lepidopteran species (Reynolds et al., 1986; Bowdan, 1988a,b; Timmins et al., 1988; Bernays and Woods, 2000; Nagata and Nagasawa, 2006). In the case of the silkworm, *Bombyx mori*, larvae exhibit characteristic feeding behavioral patterns that are generated independent of

circadian rhythms at regular intervals of approximately every 2 h (Nagata and Nagasawa, 2006). Since these characteristic behaviors are primarily timed by the mode-changing process from quiescent status to feeding status, we assumed that some process might be involved in the decision to initiate feeding activity.

Based on previous physiological reports, the feeding state of an insect can be defined according to the following two indicators: (1) duration elapsed from the end of the previous meal and (2) head-swaying behavior immediately before the onset of eating or under feeding-motivated states (Nagata et al., 2011a). These two physiologically defined feeding states, hunger and satiety, support the evaluation of feeding behavior in *B. mori* larvae by measuring the latency period prior to the initiation of feeding (Nagata et al., 2011b). We were previously able to identify several biologically active peptides as feeding modulators by measuring the length of the latency period prior to the initiation of feeding in *B. mori* larvae (Nagata et al., 2009, 2011a,b). Among these peptides, two classes of peptides with different biological activities on feeding behavior were characterized: (1) inhibitory peptides, which prolong the

latency to the initiation of feeding, and (2) acceleratory peptides, which shorten the latency to feeding initiation. Consistent with a previous proposal regarding control of invertebrate feeding behavior (Simpson, 1995), we found that FMRFamide-related peptides (FaRPs) and myosuppressin have inhibitory effects on the initiation of feeding behavior by peptide administration in *B. mori* larvae (Nagata et al., 2011b). In contrast, the administration of short neuropeptide F (sNPF) was shown to shorten the latency period, possibly resulting from the activation of locomotor activity. Quantitative analysis of sNPF levels during different feeding states, however, has not been fully examined.

The compiled genomic information from a number of insect species has allowed us to identify *in silico* various conserved factors, including the identification of sNPFs from a number of insect species. sNPFs belong to a very diverse neuropeptide group sharing a C-terminal RF-amide or RY-amide. In vertebrates, a representative RY-amide peptide, neuropeptide Y (NPY), has been characterized as a neuromodulator controlling feeding behaviors (Tatemoto et al., 1982). In invertebrates, a NPY homologous peptide has been identified in the tapeworm, *Moniezia expansa*, as a factor that regulates myo-stimulatory effects (Maule et al., 1991). By using an antibody against NPF [antiserum against Arg-X-Arg-Phe/Tyr-NH₂; serum code 792(3)], shorter peptides that share the C-terminal RF-amide sequence, subsequently designated as short NPF, have been identified in the Colorado potato beetle (Spittaels et al., 1996). However, the detailed function of sNPF *in vivo* remains to be elucidated.

A cDNA encoding sNPF was first identified in the fruit fly, *Drosophila melanogaster* (Vanden Broeck, 2001). The deduced amino acid sequence contained four sNPFs, which were predicted to be produced after the post-translational modification of a dibasic amino acid cleavage site and C-terminal amidation (Vanden Broeck, 2001). In *D. melanogaster*, sNPF has been shown to be pleiotropic including regulation of feeding behavior (Lee et al., 2004, 2008; Nassel and Wegener, 2011). sNPFs are conserved across arthropod species with the number of sNPF isoforms encoded by a single cDNA varying depending on the insect species (Nassel and Wegener, 2011). In *B. mori*, sNPF has been identified as a juvenile hormone (JH) regulating peptide that functions in tandem with allatotropin to regulate the secretion of JH from the corpora allata (Yamanaka et al., 2008). Identification of a cDNA encoding sNPFs revealed the presence of three sNPFs in *B. mori* (Mita et al., 2003; Roller et al., 2008; Yamanaka et al., 2008), which is consistent with reports in other insects of multiple sNPFs.

In *D. melanogaster*, the presence of sNPFs isoforms has been characterized by direct matrix-assisted laser desorption ionization time-of-flight mass spectrometry (MALDI-TOF MS) profiling (Predel et al., 2004; Baggerman et al., 2005; Yew et al., 2009; Carlsson et al., 2010; Wegener et al., 2011). Although sNPF-2 and a partial fragment of sNPF-3 were purified from *B. mori* brain extracts (Yamanaka et al., 2008), there is currently no experimental evidence for the presence of all three sNPF isoforms at the peptide level in *B. mori*. We consequently performed direct mass spectrometric profiling of larval brains to reassess the presence of sNPFs. In addition, to further address the mechanisms underlying sNPF-mediated feeding behaviors, we semi-quantitatively measured the levels sNPFs during different feeding states in *B. mori*.

MATERIALS AND METHODS

CHEMICALS AND REAGENTS

Chemicals and reagents, α -cyano-4-hydroxycinnamic acid (CCA), ethidium bromide, agarose for electrophoresis and Trizma-base, and EDTA used in the present study were purchased from Nacal-lab (Osaka, Japan). Methanol, diethylether, formic acid, trifluoroacetic acid (TFA), and acetonitrile for reversed-phase HPLC (RP-HPLC) were purchased from Kanto Chemicals (Tokyo, Japan). Fmoc derivatives of amino acids were purchased from Watanabe Chemical Industries (Hiroshima, Japan).

INSECTS

Silkworm eggs from the hybrid *B. mori* strain (Kinshu \times Showa) were purchased from UEDA SANSU Ltd. (Ueda, Japan). Larvae were reared in plastic containers at $26 \pm 1^\circ\text{C}$ with $70 \pm 10\%$ relative humidity under long-day lighting conditions (16L: 8D) using SILKMATE 2S artificial diet purchased from NIPPON NOSHAN Co. Ltd. (Yokohama, Japan). Larvae were provided with fresh diet on a daily basis. Only larvae whose growth was synchronized were utilized for the present experiments.

PREPARATION OF STARVED AND RE-FED *B. mori* LARVAE

Bombyx mori larvae were starved by food deprivation with feces frequently removed from the containers throughout starvation. Food deprivation was initiated in synchronously growing fifth instar, day-2 larvae (2.7 ± 0.3 g). Resumption of feeding was initiated with addition of an artificial diet block. One hour after resumption of feeding, the re-fed-larvae were anesthetized on ice. For mass spectrometric analyses, brains were dissected out from the prepared larvae.

PREPARATION OF SYNTHETIC PEPTIDES

Short neuropeptide Fs were chemically synthesized based on the Fmoc method using an automated peptide synthesizer (APEX-SC, apex396, Advanced ChemTech, Louisville, KY, USA) and deprotection as previously reported (Nagata et al., 2011b). In brief, after deprotection and cleavage from resins, the synthetic peptides in diethylether were collected by centrifugation. The resulting crude synthetic peptides were partially purified using Sep-Pak C18. The adsorbed sample was eluted with 60% acetonitrile/0.1% TFA. The eluate was subjected to RP-HPLC (Jasco SC 802, PU-880, UV-875, Jasco; Tokyo, Japan) on a Senshu Pak Pegasil-300 ODS column (4.6 mm i.d. \times 250 mm, Shenshu Kagaku, Tokyo, Japan) with a 25-min linear gradient of 10–60% acetonitrile containing 0.05% TFA at a flow rate of 1.0 ml/min. The purity of the synthetic peptide was confirmed by MALDI-TOF MS using a Voyager-DETMSTR (Applied Biosystems, CA, USA) in the positive ion mode and CCA as the matrix. A highly purified byproduct (m/z 983) of sNPF synthesis was used for semi-quantitative analyses in the present study.

REVERSE TRANSCRIPTION-POLYMERASE CHAIN REACTION

Total RNA was extracted from fat body, silk glands, foregut, midgut, hindgut, Malpighian tubules, hemocytes, central nervous ganglia, brain, ovary, and testis using TRIzol reagent (Invitrogen, Carlsbad, CA, USA) according to the manufacturer's protocol. After treatment with DNase

I (TaKaRaBio, Shiga, Japan), cDNA was synthesized using a reverse transcriptase, Superscript III (Invitrogen), and an oligo-dT₃₀ primer. Gene specific primers were sNPF-Fw (5'-GATGCTCCTAAAGAAGACAAT-3') and sNPF-Rv (5'-TCAGGCTTTCGGTGCTTCCG-3'). Primers for sNPF (BNGR-A10) were sNPF-Fw (5'-GCTACAATCCATTCCTCTATGCTT-3') and sNPF-Rv (5'-AGGTTCCCTGTGCTGAAGAAATCAC-3'). Ribosomal protein 49 (*rp49*) was used as an experimental control with the following gene specific primer: Rp-Fw (5'-GACCTGTTTACAGGCCGACAATCG-3') and Rp-Rv (5'-TTATATTTATTTCATTCTCCTGGGAGCGGAG-3'). Amplified fragments were electrophoresed on a 1.2% agarose gel and detected by staining with ethidium bromide. The saturation of PCR products were observed after 35 cycles of amplification. We consequently performed our analyses at 30 cycles. To quantitate the sNPF expression levels (data not shown), gel images were analyzed using the public domain NIH Image program (the U.S. National Institutes of Health at <http://rsb.info.nih.gov/nih-image/>). The analyzed PCR product bands were normalized to bands derived from the PCR experimental control, *rp49*.

QUANTITATIVE RT-PCR

Extraction of total RNA and RT reaction were performed as described above. The primers for Quantitative RT-PCR (qRT-PCR) for sNPF were: forward 5'-TGCTTGGCTCAACGAAAAC-3'; reverse 5'-GAAAGCACCCAAACATGGAA-3'. As a control, the following primers for *rp49* were used: forward 5'-TGGGAGTTTCCCCATT-3'; reverse 5'-CCATGCAGCAACCCTTGAT-3'. PCR was performed using a 7300 Real-Time PCR system (Applied Biosystems). Thermal cycling conditions were as follows: after initial denaturation at 95°C for 10 min, 40 cycles of 95°C, 15 s; 60°C, 1 min. The singly targeted qRT-PCR product was confirmed by constructing a melt curve to all reactions. The reaction was performed using Power SYBR® Green PCR Master Mix (Applied Biosystems) and 50 nM

of each primer. Data were analyzed using the 7300 Real-Time PCR system software v1.3 (Applied Biosystems). Results are represented as a ratio of sNPF and RpL3 by Ct method.

DIRECT MALDI-TOF MS ANALYSIS

Brain and other ganglia were isolated from *B. mori* larvae and placed in a drop of distilled water. After removal of excess water, the matrix solution was covered with the dried brain or ganglia prior to mass spectrometry analysis. Mass spectra were measured on a MALDI-TOF mass spectrometer with CCA as matrix in the positive ion mode. The matrix solution was prepared by saturating CCA in 60% acetonitrile containing 0.1% TFA.

SEMI-QUANTITATIVE ANALYSES USING MALDI-TOF MS

For semi-quantitative analyses, the intensities of the mass spectra ion peaks were evaluated by comparing with an exogenously added internal standard peptide as previously reported (Jimenez et al., 1997). The internal standard peptide, which is not observed in the spectrum of brain extracts, has a *m/z* 983 and is the byproduct of Fmoc chemical synthesis of sNPF-2 with Fmoc at the N-terminus of sNPF-2. The internal standard was added in extraction solution (90% methanol in 0.1% formic acid). The relative intensities of each ion peak were compared with the intensity of the ion peak corresponding to the internal standard peptide. Five samples were measured for analyses of ion peak intensities. At least three measurements were carried out per sample. Single measurement of MALDI-TOF MS was carried out by accumulation of more than 50 shots using the same laser desorption settings.

STATISTICAL ANALYSIS

The intensities of the semi-quantitative analyses of sNPF were statistically compared with control samples derived from brains of larvae fed *ad libitum* using one-way ANOVA. Significances were determined with Dunnett's *post hoc* test (data from larvae fed *ad libitum* were used as experimental controls for comparison).

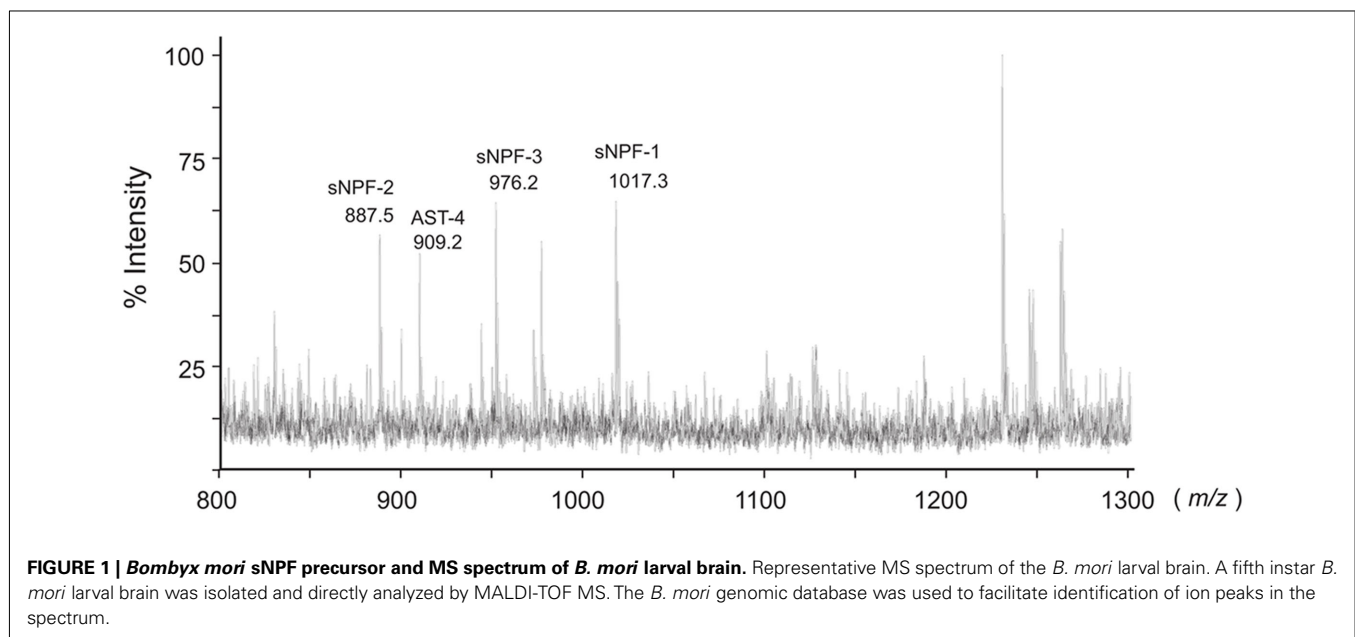


FIGURE 1 | *Bombyx mori* sNPF precursor and MS spectrum of *B. mori* larval brain. Representative MS spectrum of the *B. mori* larval brain. A fifth instar *B. mori* larval brain was isolated and directly analyzed by MALDI-TOF MS. The *B. mori* genomic database was used to facilitate identification of ion peaks in the spectrum.

RESULTS

sNPF IN THE SILKWORM, *BOMBYX MORI*

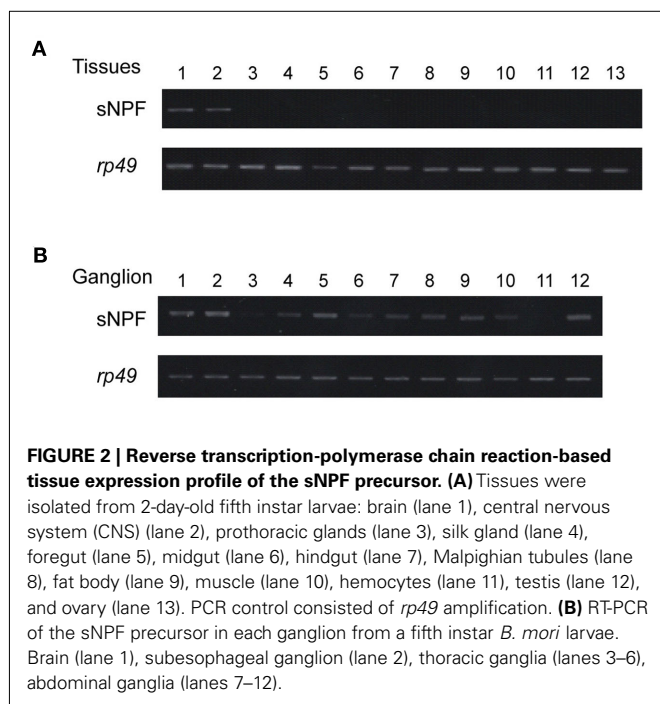
In *B. mori*, three sNPFs, sNPF-1, sNPF-2, and sNPF-3, are encoded by a single cDNA sequence (Yamanaka et al., 2008). Of these three sNPFs, sNPF-2 and a partial fragment of sNPF-3 were purified from brain extracts (Yamanaka et al., 2008). To confirm the presence of sNPFs in the central nervous system (CNS), we performed direct MALDI-TOF mass spectrometric analyses using the isolated brain of a last instar larva (Figure 1). In total, more than 15 detectable ion peaks were observed. As deduced from the cDNA sequence encoding the sNPF precursor, ion peaks at m/z 1017.2, 887.2, and 976.1 corresponding to sNPF-1, sNPF-2, and sNPF-3, respectively, were observed. These data suggest the presence of all three forms of sNPF in the brain. A database search revealed that these ion peaks coincided with those of known neuropeptides, including sNPFs (Figure 1).

RT-PCR ANALYSES OF *B. mori* sNPFs

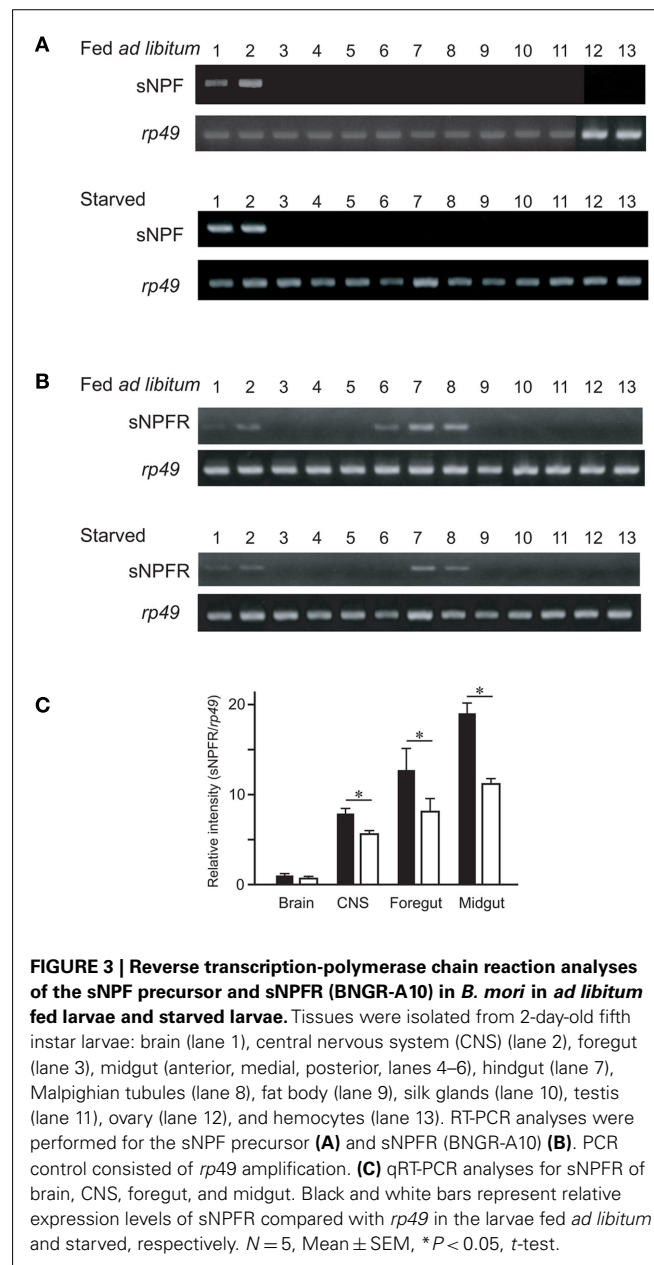
We subsequently confirmed the expression sites of the sNPF precursor transcript in *B. mori* larvae. Reverse transcription-polymerase chain reaction (RT-PCR) using specific primers for the sNPF precursor cDNA sequence revealed that the expression of sNPFs was observed in the brain and CNS (Figure 2A). To analyze the detailed location of sNPF expression, we examined the expression profile in each ganglion. We found that all ganglia expressed the sNPF precursor mRNA, even though biased cDNA amplification was observed (Figure 2B). These RT-PCR data are consistent with our MS data.

sNPF EXPRESSION DURING DIFFERENT FEEDING STATES

While determining the sNPF precursor expression profile, we observed clear differences in expression levels. The resulting RT-PCR products were compared between the tissues of *ad libitum*



fed larvae and starved larvae. These results showed similar levels for the sNPF precursor transcript in the brain and CNS in the different feeding states (Figure 3A). Furthermore, the transcriptional expression level of the sNPF receptor (sNPF-R, BNGR-A10) was also analyzed by RT-PCR. The widely distributed expression sites of the sNPF-R were reconfirmed, supporting previous reports (Yamanaka et al., 2008). Comparison of the transcriptional levels of the sNPF-R between RT-PCR products derived from tissues of *ad libitum* fed larvae and starved larvae revealed that starvation reduces sNPF-R transcript levels (Figure 3B). The reduced levels of sNPF-R transcript by starvation were confirmed using several sNPF-R expressing tissues, brain, CNS, midgut, and hindgut by qRT-PCR (Figure 3C). We observed a significant reduction in sNPF-R expression levels following starvation in all tissues



examined except for the brain, which likewise showed a slight albeit not significant reduction in sNPF.

SEMI-QUANTITATIVE ANALYSES OF sNPFs IN THE BRAIN

We next examined the peptide levels of sNPFs in the brain during different feeding states. The amount of brain sNPFs were semi-quantitatively analyzed by comparing the relative levels of sNPFs in crude larval brain extracts with an exogenous internal standard peptide. A byproduct of sNPF-2 chemical synthesis was used as an exogenous standard peptide after separation from sNPF-2 by RP-HPLC. In the resulting spectra, we observed ion peaks at m/z 1017.2, 887.2, and 976.1 corresponded to sNPF-1, sNPF-2, and sNPF-3, respectively. Reduced levels of sNPF-1 and sNPF-2 were observed in starved larval brains. The levels of sNPF-1 and sNPF-2 further decreased with an increase in the starvation period (Figure 4). A resumption of feeding returned the levels of brain sNPF-1 and sNPF-2 back to basal levels. It is noteworthy that sNPF-1 and sNPF-2 were significantly reduced by starvation, whereas sNPF-3 was not as clearly affected. However, there did appear to be a similar trend, albeit not statistically significant, in the reduction sNPF-3 levels, most noticeably after 2 days of starvation. The less pronounced reduction in sNPF-3 levels could be due to a lower ionization efficiency.

The mass spectral profiles (Table 1) obtained while performing the semi-quantitative analyses of sNPFs allowed us to examine the effects of starvation two other significant ion peaks. These ion peaks correspond to Bommyosuppressin (BMS) at m/z 1229.1 and allatostatin-4 (AST-4) at m/z 909.1, which have been suggested to be feeding-related peptides (Audsley and Weaver, 2009; Nagata et al., 2011b). We found that, similar to sNPF-1 and sNPF-2, AST-4 levels were reduced by starvation and were recovered following a resumption of feeding (Figure 5). In contrast, the levels of brain of BMS did not change significantly.

DISCUSSION

In *B. mori*, post-translational cleavage of the sNPF precursor yields three sNPF forms. In the current study, we examined the presence and expression profile of these three cleavage products and have shown that sNPF mRNA is expressed throughout the CNS with all three sNPF forms in each *B. mori* larval ganglion. Since sNPFs have been linked to the regulation of feeding behavior (Nagata et al., 2011b; Nassel and Wegener, 2011), the presence of sNPFs in all ganglia is consistent with a putative role in regulating feeding behavior in *B. mori*.

In other insect species, multiple sNPF forms (from one to five) are present (Nassel and Wegener, 2011). This is consistent with evidence showing the presence of three sNPFs in *B. mori*. For example, based on the deduced amino acid sequence of the sNPF precursor, the *D. melanogaster* sNPF gene appears to encode four separate sNPF forms (Vanden Broeck, 2001). The presence of these four sNPFs at the peptide level has been confirmed by direct mass spectrometry analyses (Predel et al., 2004; Baggerman et al., 2005; Yew et al., 2009; Carlsson et al., 2010). In the mass spectrum profiling, trypsin-like enzymatic processing of sNPFs appeared to generate shorter sNPF-like products (Baggerman et al., 2005; Wegener et al., 2011), which would suggest that at least six sNPF isoforms are present in the *D. melanogaster* larval CNS. In contrast, as demonstrated in Figure 1, no ion peaks corresponding to sNPF precursor-derived peptides were observed in the crude *B. mori* larval brain extract.

Both our direct mass spectrometry measurements and RT-PCR analyses of the *B. mori* CNS indicated that sNPF expression is localized, if not restricted to, the nervous tissues. However, it has been reported that, in addition to the CNS, *D. melanogaster* sNPFs are also expressed in the midgut (Reiher et al., 2011). In the case of *B. mori*, we were unable to detect any ion signals for sNPFs in the crude extract of the larval midgut nor could we detect sNPF

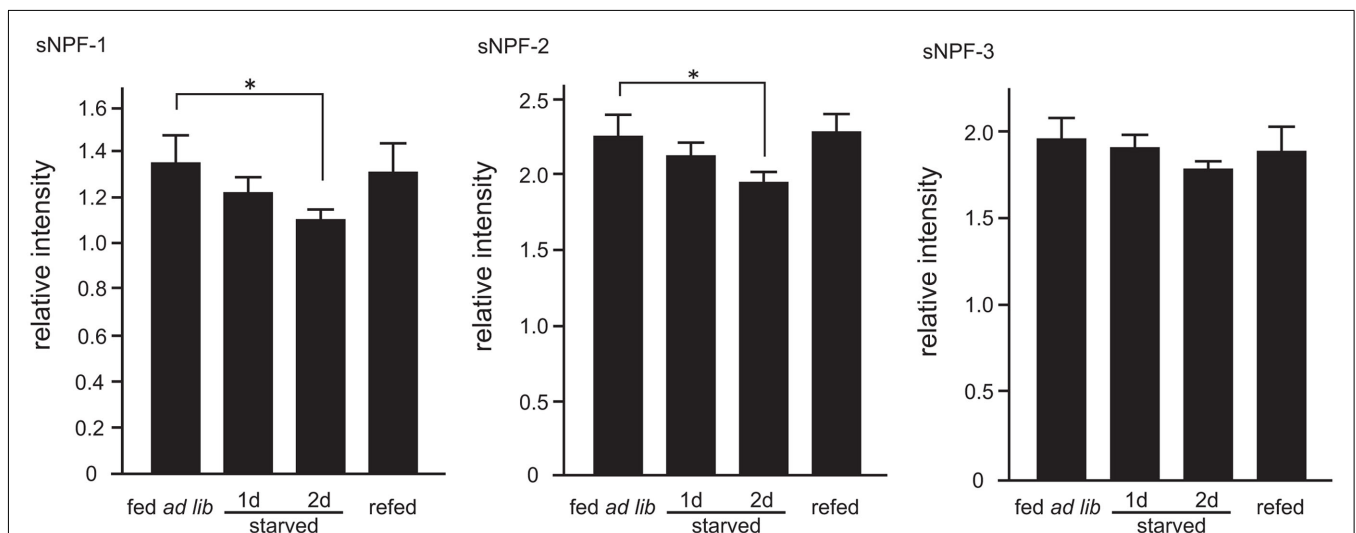


FIGURE 4 | Semi-quantitative mass spectrometry analyses of sNPFs. The sNPFs levels were measured by comparing with an exogenous nonsense peptide. Samples were prepared from 2-day-old fifth instar larvae. Larvae were starved for 1 and 2 days. Re-fed-larvae

were prepared as described in the Materials and Methods. $N = 5$, Mean \pm SE. Data were statistically analyzed using a one-way ANOVA with significant differences determined with a *post hoc* Dunnett's test, $*P < 0.05$.

precursor mRNA in the midgut via RT-PCR. As sNPFs exert a number of biological events, including regulating feeding behavior, sNPFs in the midgut might function as enterogastric peptides in *D. melanogaster*. Although sNPF expression was not observed in the *B. mori* larval midgut, sNPFs secreted from the individual ganglia could potentially function in all tissues because receptor expression has been reported to be widespread in *B. mori* larvae (Yamanaka et al., 2008). Additionally, antiserum against RF-amide, which can recognize sNPFs in addition to other RF-amide peptides, indicated that RF-amide peptides are preset in many neurons (Walker et al., 2009; Nassel and Wegener, 2011). Consequently, even though sNPFs are not expressed in the midgut of *B. mori*, it is possible that they function in gastric control.

Even though sNPFs were initially identified based on cross-reactivity with an anti-neuropeptide F (NPF) antibody, our knowledge of their functionality remains limited. A number of studies, however, have proposed possible functions for sNPFs (Nassel and Wegener, 2011). For example, in *D. melanogaster*, sNPFs are produced in numerous small neurons in the brain that are thought to be associated with locomotor modulation and regulation as well as other various functions (Hanesch et al., 1989; Strauss, 2002; Kahsai and Winther, 2011). This possible function supports our previous data, which showed that the injection of sNPFs into *B. mori* larvae influenced the initiation of feeding behavior (Nagata et al., 2011b). sNPFs sometimes co-express with other peptide hormones in *D. melanogaster*, such as tachykinin and ion transport peptides, resulting in a proposal that sNPFs may regulate hormone release (De Lange et al., 1997; de Jong-Brink et al., 2001; Kahsai et al., 2010). In *B. mori*, sNPFs also appear to negatively act on the allatotropin producing organ, the corpora allata, such that JH secretion and biosynthesis are decreased (Yamanaka et al., 2008).

To have a better understanding of how sNPF may function in *B. mori*, we estimated the amount of sNPFs at both the transcriptional and peptide levels. The present data indicate that starvation has no effect on the transcriptional level of sNPFs (data not shown)

but did reduce the amount of mature sNPF peptides in the brain. Although the sNPF content of the remaining ganglia was not measured in this study, we found that the brain sNPF levels changed significantly in response to starvation (Figure 4). We speculate that a reduction in brain sNPFs, which likely results from functional consumption of the peptides, would trigger several biologically important events in *B. mori* larvae such as the activation of locomotor activity accompanied with other functions. This would be consistent with the observation that sNPFs drive locomotor activity in *D. melanogaster* (Kahsai et al., 2010).

Table 1 | Ion peaks detected by direct MALDI-TOF MS analyses of fifth instar *B. mori* larvae brain.

Observed ion peak	Peptide
830.1	n.d
887.2	sNPF-2
899.1	AST-6
909.1	AST-4
928.0	n.d
943.1	AST-5
951.2	AST-3
976.1	sNPF-3
1017.2	sNPF-1
1031.0	n.d
1100.9	PTSP
1127.0	n.d
1229.1	Bommysuppressin
1246.1	n.d
1267.0	n.d
1295.1	n.d

sNPF, short neuropeptide F; AST, allatostatin; PTSP, prothoracicostatic hormone; n.d, not determined.

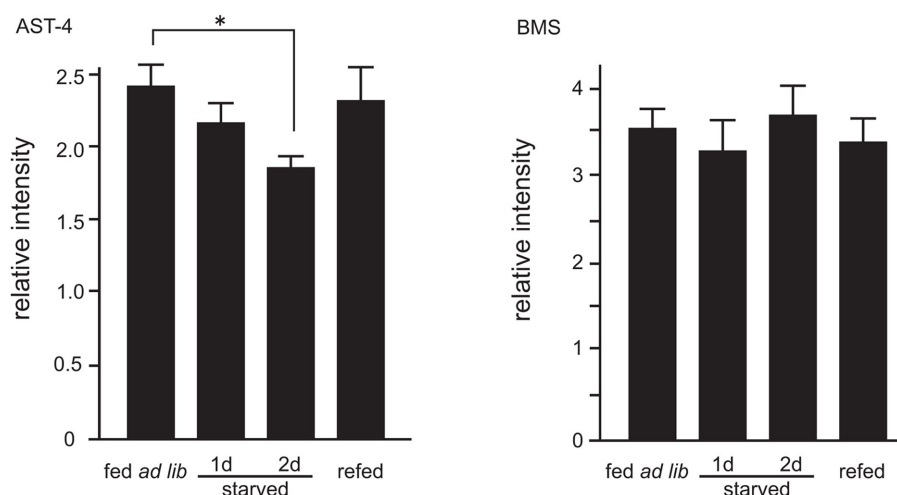


FIGURE 5 | Semi-quantitative analyses of allatostatin (AST)-4 and Bommyosuppressin (BMS). Ion peaks corresponding to AST-4 at m/z 909.1 and BMS at m/z 1229.1 were analyzed. Analytical conditions were the same

as those for sNPFs analyses. $N = 5$, Mean \pm SE. Data was statistically analyzed by one-way ANOVA. Significant differences were determined by the *post hoc* Dunnett's test, $*P < 0.05$.

Intriguingly, the sNPF receptor transcript levels were reduced in response to starvation (Figures 3B,C). Furthermore, different behavioral states were observed after short versus long starvation, with the latter showing much lower levels of locomotor activity (Wolfgang and Riddiford, 1987; Nagata and Nagasawa, 2006). As sNPFs are possible locomotor modulators (Kahsai et al., 2010), the reduction in sNPF levels is consistent with the fact that larvae starved for longer periods would exhibit decreased locomotor activity as a means of conserving crucial energy stores.

Database-assisted transcriptomic analyses revealed that several GPCRs might be allocated to receptors for RF-amide peptides in most insect species, including *D. melanogaster* (Mertens et al., 2002) and *Anopheles gambiae* (Hill et al., 2002). In *B. mori*, there are three sNPF receptors, BNGR-A10, BNGR-A11, and BNGR-A7 (data not shown, manuscript in preparation). Interestingly, these three receptors are expressed in almost all tissues of the larval body, but exhibit biases in distribution (Yamanaka et al., 2008; unpublished data by our group). This observation suggests that each receptor has different ligands or different functions *in vivo*. Therefore, careful analyses of sNPF expression levels, in addition to that of the associated receptors, are required to further develop our understanding of the mechanisms underlying sNPF-modulated feeding behavior.

REFERENCES

- Audley, N., and Weaver, R. J. (2009). Neuropeptides associated with the regulation of feeding in insects. *Gen. Comp. Endocrinol.* 162, 93–104.
- Baggerman, G., Boonen, K., Verleyen, P., De Loof, A., and Schoofs, L. (2005). Peptidomic analysis of the larval *Drosophila melanogaster* central nervous system by two-dimensional capillary liquid chromatography quadrupole time-of-flight mass spectrometry. *J. Mass Spectrom.* 40, 250–260.
- Bernays, E. A., and Woods, H. A. (2000). Foraging in nature by larvae of *Manduca sexta* influenced by an endogenous oscillation. *J. Insect Physiol.* 46, 825–836.
- Blaney, W. M., Chapman, R. F., and Wilson, A. (1973). The pattern of feeding of *Locusta migratoria* (L.) (Orthoptera, Acrididae). *Acrida* 2, 119–137.
- Bowdan, E. (1988a). The effect of deprivation on the microstructure of feeding by the tobacco hornworm caterpillar. *J. Insect Behav.* 1, 31–50.
- Bowdan, E. (1988b). Microstructure of feeding by tobacco hornworm caterpillar, *Manduca sexta*. *Entomol. Exper. Appl.* 47, 127–136.
- Carlsson, M. A., Diesner, M., Schachtner, J., and Nassel, D. R. (2010). Multiple neuropeptides in the *Drosophila* antennal lobe suggest complex modulatory circuits. *J. Comp. Neurol.* 518, 3359–3380.
- De Lange, R., van Golen, F. A., and can Minnen, J. (1997). Diversity in cell specific co-expression of four neuropeptide genes involved in control of male copulation behaviour in *Lymnaea stagnalis*. *Neuroscience* 78, 289–299.
- de Jong-Brink, M., ter Maat, A., and Tensen, C. P. (2001). NPY in invertebrates: molecular answers to altered functions during evolution. *Peptides* 22, 309–315.
- Hanesch, U., Fischbach, K. F., and Heisenberg, M. (1989). Neuronal architecture of the central complex in *Drosophila melanogaster*. *Cell Tissue Res.* 257, 343–366.
- Hanson, J. B. (2003). “Phenolics,” in *Natural Products, Their Chemistry and Biological Significance*, eds J. Mann, J. B. Davidson, J. B. Hobbs, D. V. Banthorpe, and J. B. Harborne (Upper Saddle River, NJ: Prentice Hall), 362–388.
- Harborne, J. B. (1993). *Introduction to Ecological Biochemistry*, 4th Edn. London: Academic Press.
- Hill, C. A., Fox, A. N., Pitts, R. J., Kent, L. B., Tan, P. L., Chrystal, M. A., Cravchik, A., Collins, F. H., Robertson, H. M., and Zwiebel, L. J. (2002). G protein-coupled receptors in *Anopheles gambiae*. *Science* 298, 176–178.
- Jimenez, C. R., Li, K. W., Dreisewerd, K., Mansvelter, H. D., Brussaard, A. B., Reinhold, B. B., Van der Schors, R. C., Karas, M., Hillenkamp, F., Burbach, J. P. H., Costello, C. E., and Geraerts, W. P. M. (1997). Pattern changes of pituitary peptides in rat after salt-loading as detected by means of direct, semi-quantitative mass spectrometric profiling. *Proc. Natl. Acad. Sci. U.S.A.* 94, 9481–9486.
- Kahsai, L., Martin, J. R., and Winther, A. M. (2010). Neuropeptides in the *Drosophila* central complex in modulation of locomotor behavior. *J. Exp. Biol.* 213, 2256–2265.
- Kahsai, L., and Winther, A. M. (2011). Chemical neuroanatomy of the *Drosophila* central complex: distribution of multiple neuropeptides in relation to neurotransmitters. *J. Comp. Neurol.* 519, 290–315.
- Lee, K. S., Kwon, O. Y., Lee, J. H., Kwon, K., Min, K. J., Jung, S. A., Kim, A. K., You, K. H., Tatar, M., and Yu, K. (2008). *Drosophila* short neuropeptide F signalling regulates growth by ERK-mediated insulin signalling. *Nat. Cell Biol.* 10, 468–475.
- Lee, K. S., You, K. H., Choo, J. K., Han, Y. M., and Yu, K. (2004). *Drosophila* short neuropeptide F regulates food intake and body size. *J. Biol. Chem.* 279, 50781–50789.
- Maule, A. G., Shaw, C., Halton, D. W., Thim, L., Johnston, C. F., Fairweather, I., and Buchanana, K. D. (1991). Neuropeptide F: a novel parasitic flatworm regulatory peptide from *Moniezia expansa* (Cestoda: Cyclophyllidae). *Parasitology* 102, 309–316.
- Mertens, I., Meusen, T., Huybrechts, R., De Loof, A., and Schoofs, L. (2002). Characterization of the short neuropeptide F receptor from *Drosophila melanogaster*. *Biochem. Biophys. Res. Commun.* 297, 1140–1148.
- Mita, K., Morimyo, M., Okano, K., Koike, Y., Nohata, J., Kawasaki, H., Kadono-Okuda, K., Yamamoto, K., Suzuki, M. G., Shimada, T., Goldsmith, M. R., and Maeda, S. (2003). The construction of an EST database for *Bombyx mori* and its application. *Proc. Natl. Acad. Sci. U.S.A.* 100, 14121–14126.
- Nagata, S., Morooka, N., Asaoka, K., and Nagasawa, H. (2011a). Identification of a novel hemolymph peptide that modulates silkworm feeding motivation. *J. Biol. Chem.* 286, 7161–7170.
- Nagata, S., Morooka, N., Matsumoto, S., Kawai, T., and Nagasawa, H. (2011b). Effects of neuropeptides on feeding initiation in larvae of the silkworm, *Bombyx mori*. *Gen. Comp. Endocrinol.* 172, 90–95.
- Nagata, S., Morooka, N., Matsumoto, S., and Nagasawa, H. (2009). Characterization of feeding-delaying factors from the silkworm *Bombyx mori*. *Ann. N. Y. Acad. Sci.* 1163, 481–483.
- Nagata, S., and Nagasawa, H. (2006). Effects of diet-deprivation and physical stimulation on the feeding behaviour of the larvae of the silkworm, *Bombyx mori*. *J. Insect Physiol.* 52, 807–815.

CONCLUSION

In the present study, we monitored sNPF levels in the CNS and brain of *B. mori* larvae. The level of sNPFs in the *B. mori* brain was influenced by the feeding state of the specimens examined. As previously proposed, sNPFs may function as possible neuromodulators of locomotion in *B. mori* larvae. We suggest that one of the pleiotropic functions of sNPFs is neuromodulatory in nature in that they potentially drive feeding behavior associated locomotor activity in response to the animal's feeding state.

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- Nassel, D. R., and Wegener, C. (2011). A comparative review of short and long neuropeptide F signaling in invertebrates: any similarities to vertebrate neuropeptide Y signaling? *Peptides* 32, 1335–1355.
- Preedel, R., Wegener, C., Russell, W. K., Tichy, S. E., Russell, D. H., and Nachman, R. J. (2004). Peptidomics of CNS-associated neurohemal systems of adult *Drosophila melanogaster*: a mass spectrometric survey of peptides from individual flies. *J. Comp. Neurol.* 474, 379–392.
- Reynolds, S. E., Yeomans, M. R., Timmins, W. A. (1986). The feeding behaviour of caterpillars (*Manduca sexta*) on tobacco and on artificial diet. *Physiol. Entomol.* 11, 39–51.
- Reiher, W., Shirras, C., Kahnt, J., Baumeister, S., Isaac, R. E., Wegener, C. (2011). Peptidomics and peptide hormone processing in the *Drosophila* midgut. *J. Proteome Res.* 10, 1881–1892.
- Roller, L., Yamanaka, N., Watanabe, K., Daubnerova, I., Zitnan, D., Kataoka, H., and Tanaka, Y. (2008). The unique evolution of neuropeptide genes in the silkworm *Bombyx mori*. *Insect Biochem. Mol. Biol.* 38, 1147–1157.
- Secher, T., Lenz, C., Cazzamali, G., Sorensen, G., Williamson, M., Hansen, G. N., Svane, P., and Grimmelikhuijzen, C. J. (2001). Molecular cloning of a functional allatostatin gut/brain receptor and an allatostatin preprohormone from the silkworm *Bombyx mori*. *J. Biol. Chem.* 276, 47052–47060.
- Simpson, S. J. (1982). Patterns in feeding: a behavioural analysis using *Locusta migratoria* nymphs. *Physiol. Entomol.* 7, 325–336.
- Simpson, S. J. (1995). “The control of meals in chewing insects,” in *Regulatory Mechanisms of Insect Feeding*, eds R. F. Chapman and J. de Boer (New York: Chapman & Hall), 137–156.
- Simpson, S. J., and Ludlow, A. R. (1986). Why locusts start to feed? A comparison of causal factors. *Anim. Behav.* 34, 480–496.
- Spittaels, K., Verhaert, P., Shaw, C., Johnston, R. N., Devreese, B., and Van Beeumen, J. (1996). Insect neuropeptide F (NPF)-related peptides: isolation from Colorado potato beetle (*Leptinotarsa decemlineata*) brain. *Insect Biochem. Mol. Biol.* 26, 375–382.
- Strauss, R. (2002). The central complex and the genetic dissection of locomotor behaviour. *Curr. Opin. Neurobiol.* 12, 633–638.
- Tatemoto, K., Carlquist, M., and Mutt, V. (1982). Neuropeptide Y-a novel brain peptide with structural similarities to peptide YY and pancreatic polypeptide. *Nature* 296, 659–660.
- Timmins, W. A., Bellward, K., Stamp, A. J., and Reynolds, S. E. (1988). Food intake, conversion efficiency and feeding behaviour of tobacco hornworm caterpillar given artificial diet of carrying nutrient and water content. *Physiol. Entomol.* 13, 303–314.
- Vanden Broeck, J. (2001). Neuropeptides and their precursors in the fruitfly, *Drosophila melanogaster*. *Peptides* 22, 241–254.
- Walker, R. J., Papioannou, S., and Holden-Dye, L. (2009). A review of FMRFamide- and RFamide-like peptides in metazoan. *Invert. Neurosci.* 9, 111–153.
- Wegener, C., Hervert, H., Kahnt, J., Bender, M., and Rhea, J. M. (2011). Deficiency of prohormone convertase dPC2 (AMONTILLADO) results in impaired production of bioactive neuropeptide hormones in *Drosophila*. *J. Neurochem.* 118, 581–595.
- Wolfgang, W. J., and Riddiford, L. M. (1987). Cuticular mechanics during larval development of the tobacco hornworm, *Manduca sexta*. *J. Exp. Biol.* 128, 19–33.
- Yamanaka, N., Yamamoto, S., Zitnan, D., Watanabe, K., Kawada, T., Satake, H., Kaneko, Y., Hiruma, K., Tanaka, Y., Shinoda, T., and Kataoka, H. (2008). Neuropeptide receptor transcriptome reveals unidentified neuroendocrine pathways. *PLoS ONE* 3, e3048. doi:10.1371/journal.pone.0003048
- Yew, J. Y., Wang, Y., Barteneva, N., Dikler, S., Kutz-Naber, K. K., Li, L., and Kravitz, E. A. (2009). Analysis of neuropeptide expression and localization in adult *Drosophila melanogaster* central nervous system by affinity cell-capture mass spectrometry. *J. Proteome Res.* 8, 1271–1284.

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