Drosophila short neuropeptide F regulates food intake and body size

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Abbreviations: sNPF, short neuropeptide F; dNPF, Drosophila neuropeptide F; NPY, neuropeptide Y; CNS, central nervous system; PNS, Peripheral nervous system

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SUMMARY

Neuropeptides regulate a wide range of animal behavior including food consumption, circadian rhythms, and anxiety. Recently, Drosophila neuropeptide F (dNPF), which is the homologue of the vertebrate neuropeptide Y, was cloned and the function of dNPF in feeding behaviors was well characterized. However, the function of the structurally related short neuropeptide F (sNPF) was unknown. Here, we report the cloning, RNA and peptide localizations, and functional characterizations of the Drosophila sNPF gene. The sNPF gene encodes the pre-protein containing putative RLRFamide peptides and was expressed in the nervous system of late stage embryos and larvae. The embryonic and larval localization of the sNPF peptide in the nervous systems revealed the larval CNS neural circuit from the neurons in the brain to thoracic axons and to connective axons in the ventral ganglion. In the adult brain, the sNPF peptide was localized in the medulla and the mushroom body. However, the sNPF peptide was not detected in the gut. The sNPF mRNA and the peptide were expressed during all developmental stages from embryo to adult. From the feeding assay, the gain-of-function sNPF mutants expressed in nervous systems promoted food intake, whereas the loss-of-function mutants suppressed food intake. Also, sNPF over-expression in nervous systems produced bigger and heavier flies. These findings indicate that the sNPF is expressed in the nervous systems to control food intake and regulate body size in Drosophila melanogaster.
INTRODUCTION

Neuropeptides regulate a wide range of animal behavior. In vertebrates, neuropeptide Y (NPY) regulates food consumption, circadian rhythms, anxiety, and other physiological processes (1). NPY, a 36 amino acids neuromodulator, is expressed abundantly in the mammalian brain and controls feeding (2). The NPY injection into the hypothalamus of rat’s brain resulted in hyperphagia and obesity, whereas the NPY deficient mouse in the leptin mutant background (NPY−/−; ob/ob) showed the less obese phenotype (3).

In invertebrates, neuropeptide F (NPF) peptides share structural similarity with the vertebrate NPY (4). NPF peptides isolated from various invertebrate animals have the conserved (A/L)R(P/L)RFamide sequence at their C terminal ends (5, 6). From a completely sequenced Drosophila melanogaster genome, short NPF (sNPF; CG13968) and Drosophila NPF (dNPF; CG10342) were found. The dNPF peptide was isolated by the radioimmunoassay. The pre-protein is processed to the 36-amino acids peptide containing RVRFamide in the C terminus. dNPF is considered the homologue of the vertebrate NPY. dNPF mRNA and peptide are expressed in the brain and midgut of Drosophila larvae and adults (7). The dNPF neural network in the larval central nervous system (CNS) is changed by the gustatory stimulation of sugar, indicating that dNPF is an integral part of the chemosensory system that regulates eating behavior (8). Drosophila larvae eat continuously and grow in a relatively short period. About 5 days after egg laying (AEL), they stop feeding, leave the food, and enter the wandering stage to prepare for pupation (9). Recently, Wu et al. (10) showed that dNPF is expressed in the larval brain during the feeding stage but not in the older larval brain during the
wandering stage. Loss-of-function \textit{dNPF} mutation in the larval feeding stage leads to the premature behavioral phenotypes similar to wandering larvae, whereas over-expression of \textit{dNPF} in the wandering larval stage shows the continuous feeding phenotype. These indicate that the Neuropeptide F system, which is consisted of \textit{dNPF} and its receptor, regulates larval feeding behavior (10).

The DmNPFR, the receptor for dNPF, was cloned and showed the homology with the vertebrate NPY Receptor (NPYR) family which are the seven transmembrane G-protein coupled receptors (11). \textit{DmNPFR1} mRNA is expressed in larval CNS and midgut. In the binding assay with CHO cells, the dNPF binds to the DmNPFR1 receptor (12). NPFR76F, the receptor for sNPF, was also cloned and showed the similarity with NPYR2. It is expressed in the CNS of embryos and larvae. In the electrophysiological assay using \textit{Xenopus} oocytes and in the bioluminescence assay using CHO cells, the receptor was maximally activated by the putative sNPF peptides (13, 14). Although the functions of \textit{dNPF} and \textit{DmNPFR1} genes in the dNPF system are well characterized in feeding behavior (10), the function of the \textit{sNPF} gene is unknown.

In this report, we present the cloning, RNA and peptide localizations, and functional characterization of the \textit{sNPF} gene from \textit{Drosophila}. We demonstrated that the \textit{sNPF} gene encodes the pre-protein containing putative RLRFamide peptides and \textit{sNPF} mRNA and peptide were expressed in the nervous system of late stage embryos, larvae, and adult brain. From the \textit{sNPF} antibody staining, we found that the neural circuit from neurons in the brain to thoracic axons and to connective axons of the ventral ganglion in the larval CNS. Using the feeding assay, we showed that the \textit{sNPF} over-expressing mutants in nervous systems promoted food intake, whereas the \textit{sNPF}
loss-of-function mutants suppressed food intake. We also showed that sNPF over-expressing flies seemed to increase appetite and produced bigger and heavier flies.
**EXPERIMENTAL PROCEDURES**

*Cloning of the sNPF gene and sequencing* - The forward (5‘GAATTCATGTTTTCTTGAAGCGGGGA3’) and reverse (5’TCTAGATTAGTTCTGTGTCTTTGGTG3’) primers were used to clone the coding region of the sNPF gene. The sequences of primers came from the predicted sNPF mRNA in the *Drosophila* genome database. To perform PCR-based cloning, total RNA from wild type (Oregon-R) adult heads was isolated with RNeasy Midi Kit (Qiagen), and mRNA was purified with Oligotex mRNA Midi Kit (Qiagen). From purified 0.5µg mRNA, 1st strand cDNA was synthesized with the AMV Reverse Transcriptase Kit (Roche Biochemical) according to the manufacturer’s instruction. To isolate the coding region of the sNPF gene, the PCR reaction containing 100pM of the primers, 1ul of 1st strand cDNA, and 25ul of High Fidelity Master Mix (Roche Biochemical) was performed in thirty cycles of 94°C for 1min, 55°C for 1min, and 72°C for 1min followed by additional 72°C for 10min. The 0.85kb PCR product was subcloned into the pGEM-T easy vector (Promega) and was sequenced using ABI automated sequencer (Applied Biosystem).

*Northern blot analysis* - The antisense sNPF Digoxigenin (Dig) labeled probe was generated with linearized pGEM-sNPF, SP6 RNA polymerase, and NTP/Dig-UTP mixture (Roche Biochemical). The sense sNPF Dig-labeled probe for the negative control was generated with T7 RNA polymerase. The Northern blot analysis was performed as previously described (14) with the Dig-labeled antisense sNPF RNA probe. The rp49 were used as the control. After post-hybridization washes, the signal was detected with the anti-Dig antibody conjugated with alkaline phosphatase.
and visualized by chemiluminescent reagent (Roche Biochemical).

**In situ hybridization -** In situ hybridization in whole mount embryos or larval CNS with the Dig-labeled antisense *sNPF* RNA probe was performed as previously described (15). The embryos and larval CNS were mounted on the Gary’s Magic mounting media.

**Generation of the sNPF antiserum and Western blot analysis -** The sNPF antiserum was produced by the immunization of a rabbit with the synthetic peptide (WFGDVNQKPIRSLRLRF) corresponding to the amino acid residues of the sNPF2 peptide. Western blot analysis was performed as previously described (16) with the 1000 times diluted polyclonal sNPF2 antibody.

**Immunohistochemistry -** Immunohistochemical staining in whole mount tissue was performed according to Yu et al. (17). The embryos, the larval CNS, and sectioned adult brain were mounted in the Gary’s Magic mounting media.

**Vector constructions and transgenic fly generations -** The coding sequence of the *sNPF* gene was subcloned into the EcoRI-XbaI sites of the *pUAST* vector and produced the *pUAST-sNPF* construct. The EcoRI-5′sNPF3′-NdeI-3′sNPF5′-XbaI construct was generated and subcloned into the *pUAST* vector to produce the *pUAST-sNPF RNAi* construct (Fig. 6A). The germ-line mediated transformation was performed according to Rubin and Spreadling (18). Three independent *pUAST-sNPF* and three independent *pUAST-sNPF RNAi* transgenic lines were generated.
Feeding assay and Measuring adult body weight and size - 3 days old wild-type (Oregon-R) female flies or synchronized wild-type feeding larvae (before AEL 96 hr) were cultured in the fresh corn meal agar medium for 2 hr, transferred into the vial containing the yeast paste with blue color (0.05% Bromophenol Blue), and kept for 10 min (19). The ones with blue color in their abdomens were counted for the statistical analysis and homogenized to measure the amount of blue color with a spectrophotometer at 595 nm. For weight measuring, the 3 day-old female flies were measured with the balancer (METTLER AJ100). For body size measuring, the length from the anterior end of a head to the posterior end of abdomen in the 3 day-old female fly was measured. At least three experiments were performed in each assay and 50 individuals were used in each experiment.

Statistics - The data was presented as the mean and error bar (±S.E.M.). ANOVA (one-way F-test) program was used for the statistical analyses and P<0.05 was accepted as statistically significant.
RESULTS

Cloning, the Genomic Organization, and the Sequence Comparison - We have cloned the 846 base pairs cDNA which represent the coding region of the sNPF gene from the PCR-based cloning method. This encodes the 281 amino acids protein which contains the 30 amino acids signal peptide in the N-terminus (the dotted underline) and two putative RLRF peptides flanked by pairs of basic residues (Fig. 1A, sNPF1, 2). In the Drosophila genome database (http://flybase.bio.indiana.edu), there are two predicted transcripts for the sNPF gene: one is CG13968-PA and the other is CG13968-PB. The amino acid sequences of the sNPF pre-protein have 98% identity with CG13968-PB (280/281). However, the sNPF gene does not have a significant vertebrate homolog.

The PCR amplifications with the pair of primers (Fig. 1A, underlined DNA sequences) produced the 0.85Kb fragment from adult cDNA and the 1.1Kb fragment from adult genomic DNA (Fig. 1B). The sequence comparison between the 1.1Kb genomic DNA from the Drosophila genome database and the sNPF cDNA indicates that the coding region of the sNPF transcript has four exons and the transcript was produced by splicing of typical GU/AG intron/exon boundaries (Fig. 1C).

Since the sNPF pre-protein contained the putative 11 amino acid sNPF1 and 19 amino acids sNPF2 peptides, we compared peptide sequences among sNPF1, sNPF2, dNPF, and NPY peptides (Table 1). The amino acid sequences of sNPF1 and 2 peptides are quite different with the vertebrate NPY other than C-terminal RXRF/Y sequences, whereas dNPF has not only the same number of amino acids (36/36) but also 31% homology with NPY. It suggests that the dNPF peptide, not sNPF peptides, is considered as the fly homologue of the vertebrate NPY.
Expression of sNPF during development and in the nervous systems of embryos and larvae - To study temporal expression of sNPF mRNA, we performed the developmental Northern blot analysis with the Dig-labeled sNPF antisense RNA probe. The similar amounts of the 2.5Kb sNPF transcripts were expressed throughout all developmental stages (Fig. 2A).

To exam the spatial expression of the sNPF mRNA, we performed the in situ hybridization in the whole mount embryos and the CNS of the feeding larva with the Dig-labeled sNPF antisense RNA probe. Expression of the sNPF transcript was observed in the nervous system from stage 14 embryos (data not shown). In stage 17 embryos, it was clearly seen in the CNS composed of two brain hemispheres and connected ventral ganglion, and PNS (Fig. 2B, C, & D). In the brain, the sNPF was strongly expressed in neural cells located in the dorsal posterior region (Fig. 2C, arrow). In the ventral ganglion, the sNPF was expressed in the pairs of neural cells along the ventral midline (Fig. 2D, arrow) and cells with a bilaterally symmetrical pattern in both edges of the ventral ganglion (Fig. 2D, arrowheads). Interestingly, the expression pattern of the sNPF in the brain and ventral nerve cord is very similar with that of the sNPF receptor, NPFR76F (14). In the PNS, the sNPF was expressed in the antennal-maxillary sensory cells (Fig. 2B, C, arrowheads), which is not overlapped with the expression pattern of NPFR76F (14).

In the brain hemispheres from the feeding third instar larva, the sNPF was expressed in neural cells located in the dorsal-anterior region of the protocerebrum (Fig. 2G, labeled a). In the larval ventral ganglion, the sNPF was expressed in neural cells located in the subesophageal region (Fig. 2G, labeled b), along the ventral midline (Fig. 2G, labeled c), and in thoracic (Fig. 2G, labeled d) and abdominal segments (Fig. 2G,
labeled e). sNPF expression in various neural cells suggests that the sNPF gene may be involved in regulating a wide range of neural activities. Comparing the expression pattern of the sNPF transcript with the dNPFergic neuron expression in the subesophagial ganglion (8) suggests that one of the sNPF functions is regulating feeding behavior.

*sNPF peptide production during development* - We have generated the sNPF antibody against WFGDVNQKPIRSPSLRLRF peptide (sNPF2 peptide sequences) to investigate the sNPF peptide production during development. In the Developmental Western blot analysis with the sNPF antibody, the processed 2kD sNPF peptide bands were found during all developmental stages (Fig. 3). The presence of the sNPF mRNA and the peptide during all developmental stages suggests that sNPF may play various roles during different developmental stages like other neuropeptides.

*sNPF peptide localization in the nervous systems of embryos, larval CNS, and the adult brain* - The localization of the sNPF peptide was examined by the immunohistochemistry using the sNPF antiserum in embryos, larval CNS, and the adult brain. In stage from 14 to 16 embryos, the localization of the sNPF peptide was observed both in CNS and PNS. In the stage 14 embryonic CNS, the sNPF peptide was localized along the connectives (Fig. 4A, arrows), two commissures in each segment of the ventral nerve cord (Fig 4B, arrowheads). In the stage 16 embryonic CNS, the sNPF peptide was localized in the connectives axons (Fig. 4F, arrows) and neural cells in the midline (Fig. 4C, arrowheads). In the embryonic PNS, the sNPF peptide was localized in the subset of sensilla (Fig. 4D, G, arrowheads).
In the larval CNS, the sNPF peptide was localized symmetrically in the brain hemispheres and the ventral ganglion (Fig. 5A). In the brain hemispheres, the sNPF peptide was localized in cell bodies located in the dorsal-anterior region of the protocerebrum and the connected axons (Fig. 5A, arrows). In the ventral ganglion, the sNPF peptide was localized in the connective axons, six thoracic axons (Fig. 5A, upper box), three neural hemal organs which are specialized neuroendocrine glands, (Fig. 5B, dorsally focused and enlarged from the upper box in Fig. 5A), and the descending axons which are located in the outside of connective axons (Fig. 5C, arrows). The neural circuits of the sNPF from thoracic axons to the brain indicate that the sNPF function may be related with chemosensing. Also, the localization of the sNPF in the neural hemal organs suggests that the sNPF peptide functions in the neuroendocrine system. The localization of the sNPF in eye discs, wing discs, and leg discs was not observed (data not shown).

In the adult brain, the sNPF peptide was found in the medulla (Fig. 5D, E) and the mushroom body calyx (Fig. 5D, F). The mushroom body calyxes is the main center for sensory processing after receiving chemosense responses (20, 21, 22). These localizations in larval CNS and adult brain suggest that the function of sNPF is related with the detection of chemosenses in thoracic neurons and sensory processing in brain. In the adult gut, the localization of the sNPF peptide was not observed (data not shown).

The sNPF gene regulates food intake - To study the function of the sNPF gene in feeding behavior, we prepared the gain-of-function and loss-of-function constructs (Fig. 6A) (23) to use the UAS/Gal4 system (24) and generated transgenic mutants (18).
feeding assays, *MJ94-Gal4*, which is expressed in CNS and PNS during larval to adult stages (25), and *HS-Gal4* drivers were used (17). First, we tested the production of the sNPF peptide in the *MJ>sNPF* and *HS>sNPF* gain-of-function mutants, and in the *MJ>sNPF-RNAi* loss-of-function mutant. *MJ>sNPF* and *HS>sNPF* mutants produced more sNPF peptide than wild-type flies, whereas *MJ>sNPF-RNAi* mutants produced less sNPF peptide (Fig. 6B). In the feeding assay, we fed blue colored food for 10min to the 3 day-old female adults and feeding larvae (Fig. 7A & 7B). In the adults, over-expression of sNPF significantly increased the number of fed flies, whereas expression of the loss-of-function mutants (*sNPF-RNAi*) significantly decreased the number of fed flies compared with controls (Fig. 7C). In feeding larvae, the feeding assays showed the same results which are over-expression of sNPF significantly increased the number of fed larvae whereas expression of the loss-of-function mutants decreased the number of fed larvae compared with controls (Fig. 7D). However, in wandering larvae, over-expression of the gain-of-function or the loss-of-function sNPF mutants did not change the number of fed larvae compared with the control (data not shown). This is different with the function of the dNPF peptide because the dNPF over-expression in wandering larvae prolonged feeding (10), suggesting that sNPF and dNPF are involved in the different aspects of feeding behavior during the larval stage.

The amount of feeding measured by the colored food that has been fed with a spectrophotometer showed the proportional increase of the absorbance by increasing the sNPF transgene from one copy to two copies (Fig. 7E).

In *Drosophila* the appetite signal promotes the rate of movement toward food in larvae and the proboscis extension response in adults (19, 26). We assayed the prolonged food intake to test whether the increased number of fed flies by over-
expression of the sNPF gene in the first 10 min reflected the increased appetite or not. The number of fed flies reached nearly 100% after 40 min in the wild-type control while over-expression of sNPF flies reached nearly 100% after 15 min (Fig. 7F), suggesting that the sNPF gene may regulate appetite and controls food intake.

The sNPF gene controls body size - To test the effect of the sNPF gene in the adult phenotype, we measured the size and weight of the sNPF gain-of-function and loss-of-function mutant flies. Over-expression of MJ>2XsNPF showed heavier (32%) and bigger (25%) flies than MJ>sNPF flies or wild-type flies (Fig. 8B, C), suggesting that the over-expressing sNPF gene may induce hyperphagia and produce the obese-like phenotype (Fig. 8A). However, MJ>sNPF-RNAi flies did not decrease the size and weight compared with wild-type flies (Fig. 8B, C), suggesting that there is another functionally redundant gene(s) in Drosophila.
DISCUSSION

The *Drosophila short NPF* (*sNPF*) gene, which encodes the pre-protein containing putative sNPF peptides, was expressed in the nervous system of late stage embryos and larvae. The localization of the sNPF peptide in embryonic and larval nervous systems disclosed the neural circuit from the brain to thoracic axons and to connective axons in the larval CNS. In the adult brain, the sNPF peptide was localized in the medulla and mushroom body. From the feeding assays with the *sNPF* mutants, the gain-of-function sNPF mutants promoted food intake, whereas expression of loss-of-function sNPF-*RNAi* mutants suppressed food intake. Also, the *sNPF* over-expression produced bigger and heavier flies than wild-type flies. These findings indicate that the *sNPF* is expressed in the nervous systems to regulate food intake and controls body size in *Drosophila melanogaster*.

The sNPF pre-protein is predicted to produce two putative RLRF peptides and two putative RLRW peptides by cleaving dibasic amino acids. Those are sNPF1 (AQRSPSLRLRFamide), sNPF2 (WFGDVNKPIRSPSLRLRFamide), sNPF3 (PQRLRWamide), and sNPF4 (PMRLRWamide) (27). In the electrophysiological assay using *Xenopus* oocytes expressing the NPFR76F receptor, the application of sNPF1, sNPF2, or short sNPF peptide (PIRSPSLRLRFamide) showed high inward current responses, whereas the application of sNPF3 or sNPF4 showed low inward current responses (14). On the contrary to the electrophysiological data, all four sNPF peptides elicited clear calcium responses in the bioluminescence assay using CHO cells expressing the NPFR76F receptor (13). However, the presence of all four putative
sNPF peptides \textit{in vivo} was not known. Recently, the peptidomic analysis with the \textit{Drosophila} larval CNS using the liquid chromatography followed by the mass spectrometry identified the sNPF2\textsuperscript{1-9} peptide (WFGDVNKPI) and the sNPF-AP (Associated Peptide: SDPDMLNSIVE-OH). The sNPF-AP was considered as the oxidized product during sample preparation. sNPF2\textsuperscript{1-9} peptide was the product after the monobasic cleavage of the arginene located in 10\textsuperscript{th} position of sNPF2 (28). Most recently, the peptidomic analysis with adult CNS using mass spectrometry detected sNPF1 (AQRSPSLRLRFamide), sNPF1\textsuperscript{4-11} (SPSLRLRFamide), and the sNPF-AP. sNPF1\textsuperscript{4-11} peptide was the product after the monobasic cleavage of the arginene located in 3rd position of sNPF1 (29). These peptidomic analyses indicate that sNPF3 and 4 peptides were not detected in larval and adult CNS and possible active sNPF peptides were the cleaved products in monobasic arginine such as sNPF1\textsuperscript{4-11} and sNPF2\textsuperscript{1-9}.

The neural circuit of the sNPF from the brain to thoracic axons in the larval CNS suggests that one of the sNPF functions is related with chemosensing because thoracic axons send a signal for chemosensing which is detected by chemosensory neurons located in the legs (30). The additional neural circuits from the brain to the neuropile in the ventral ganglion suggest that the \textit{sNPF} gene has different function(s) other than chemosensing. The neural circuits from the six thoracic axons to the three neural hemal organs (Fig. 5A, B), which are a neuroendocrine system in \textit{Drosophila}, suggest that the sNPF functions as a neurohormone. The same neural circuits were detected by the staining of the antibody against the \textit{Drosophila} FMRFamide neuropeptide (31). Interestingly, one of known functions in the FMRF related peptide is regulating feeding (32). In the adult brain, the sNPF peptide was found in the
medulla and the mushroom body calyx (Fig. 5D). The adult brain of Drosophila is composed of approximately 200,000 neurons that are organized into three discrete substructures. The optic lobe, which consists of the lamina, medulla, lobula, and lobula plate, is primarily involved in the processing of visual sensing in the brain (33, 34, 35). The antennal lobes are mainly responsible for the processing of olfactory sensing (36). The mushroom bodies are involved in learning and memory and other complex behaviors (20, 37, 38, 39). The mushroom body is also the main center for sensory processing, and the mushroom body calyces receive chemosensing response input (21, 22). The neural circuit from six thoracic axons to brain through connective axons in the larval CNS and the mushroom body staining in the adult brain suggest that the function of the sNPF peptide is related to detect chemosense in thoracic neurons and send the information to brain to process chemosensing input.

The localization of the sNPF peptide in subesophagus ganglion in the larval CNS (Fig. 5A) suggests that the function of sNPF may be related with feeding. To test this hypothesis, the loss-of-function sNPF transgenic flies were also generated using the 5′sNPF3′-3′sNPF5′ (sNPF-Ri) construct. The similar 5′coding sequence3′-3′coding sequence 5′construct were demonstrated to have RNA interference effects in Drosophila and C. elegans (23). As the result of more food intake, the phenotype of MJ>2XsNPF showed bigger and heavier flies (Fig. 8) compared with the wild-type controls. However, the noticeable size increase was not detected in MJ>sNPF although MJ>sNPF flies also showed the hyperphagic phenotype (Fig. 7E), suggesting that more than one copy of the sNPF gene is required to show body size increase. Since over-expression of sNPF in the fly eyes showed the increased eye size due to the
increased number of ommatidia (data not shown), it remains to be tested whether the increased body size of \( MJ>2XsNPF \) flies is the result of the cell number increase in the body.

Various evidences suggest that sNPF and dNPF peptides have different functions. The sNPF peptide was found only in nervous systems while the dNPF was found as the \( Drosophila \) brain-gut peptide (7). The expression patterns of \( sNPF \) and dNPF differ in the larval brain. For example, the sNPF expression was found in the anterior dorsal neurons of the brain (Fig. 2G), whereas the dNPF expression was detected in the four neurons of larval brain (7). In the feeding behavior analysis, over-expression of \( sNPF \) in wandering larvae did not extended feeding period (data not shown) on the contrary to the extension of feeding period in the wandering larval stage by over-expression of \( dNPF \) (10). At the receptor level, each peptide works in different receptors such as the NPFR76F receptor is for sNPF peptides (13, 14) and the DmNPFR1 receptor is for the dNPF (12). These differences indicate that the dNPF and sNPF peptides function in different neurons and may regulate different aspects of feeding behaviors in \( Drosophila \).

Like other neuropeptides, the sNPF peptide may be involved in regulating various physiological processes other than regulating food intake because the sNPF peptide and transcript were expressed during all developmental stages (Fig. 2A, 3) and the sNPF is localized in the mushroom body calyx and medulla of the adult brain (Fig. 5D, E, F). The mushroom body is involved in learning and memory (20). These unknown multi-functions of the sNPF peptide in various biological processes are the
subjects of future studies.
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Fig. 1. The sNPF cDNA sequence and encoded amino acids, and the genomic organization of the sNPF gene. (A) The sequences of 843 bp sNPF cDNA and encoded 280 amino acids are shown. The pair of primer sequences used in the PCR-based cloning is underlined and the signal peptide by the Signal P program is underlined with a dotted line. The predicted sNPF1 and 2 peptides are boxed and flanked with underlined dibasic amino acids. (B) The 1.1Kb genomic sNPF DNA was amplified by PCR with the same pair of primers used in Fig. 1A. (C) The comparison between the coding sequence of the sNPF cDNA and the 1.1Kb genomic DNA sequences revealed that there are four exons and three introns spliced by CU/AG intron/exon boundaries.

Fig. 2. The sNPF transcript was expressed during all developmental stages and localized in the nervous systems of embryos and larvae. (A) In the developmental Northern blot analysis, single 2.5 kb sNPF mRNA bands were detected throughout all developmental stages. The transcript of rpl49 was used as the control. (B-F) The antisense Dig-labeled sNPF RNA probe was used in the whole mount embryos and larval CNS in situ hybridization. (B) In the lateral view of the stage 17 embryo, the sNPF mRNA was expressed in the brain hemispheres, the fused ventral ganglion, and in the antennal-maxillary sense organ (AMSO; arrowhead). (C) In the dorsal view of the stage 17 embryo, the sNPF was expressed in the cells located in the posterior of the brain hemispheres (arrow) and in the AMSO (arrowhead). (D) In the ventral view of the stage 17 embryo, the sNPF was expressed in the pairs of cells along the midline (arrow) and cells with a bilaterally symmetrical pattern in both edges of the ventral
ganglion (arrowheads).  (E) The ventrally focused sNPF mRNA expression in the CNS of the third instar feeding larva.  (F) The dorsally focused sNPF mRNA expression in the CNS of the third instar feeding larva.  (G) Composite diagram from (E) and (F). In the brain hemispheres, the sNPF was expressed in cells located in the dorsal-anterior region of the protocerebrum (a).  In the ventral ganglion, the sNPF was expressed in cells located in the subesophagus region (b), along the ventral midline (c), and in thoracic (d) and abdominal segments (e).

**Fig. 3. The sNPF peptide was produced during all developmental stages.** In the developmental Western blot analysis, 2kD sNPF bands were present throughout all developmental stages.  The β-actin was used as the control.

**Fig. 4. The sNPF peptide was localized in the nervous systems of embryos.** Immunohistochemical staining with the sNPF peptide antibody was performed in the whole mount embryos.  (A, B) In the stage 14 embryo, the sNPF peptide was localized along the connectives (A, arrows), two commissures axons (B, arrowheads), and descending segmental nerves in the ventral ganglion.  (C) In the stage 16 embryo, the sNPF peptide was localized in the neural cells in the midline (arrowheads).  (D) In the PNS of the stage 14 embryo, the sNPF peptide was localized in the subset of sensillae (arrowheads).  (E) In the lateral view of the stage 16 embryo, the sNPF peptide was detected from the brain to the end of ventral ganglion of CNS.  (F) In the stage 16 embryo, the sNPF peptide was localized in the connectives axons (arrows) and neural cells in the midline (C, arrowheads).  (G) In the PNS of the stage 16 embryo, the sNPF peptide was localized in the subset of sensilla (arrowheads).
Fig. 5.  The sNPF peptide was localized in the nervous systems of larvae and adults.  Immunohistochemical staining with the sNPF peptide antibody was performed in the whole mount larval CNS from the feeding stage and the sectioned adult head.  

(A) The sNPF antibody staining revealed the neural network from the protocerebrum (arrows) to six thoracic axons through connective axons.  (B) sNPF was localized in the dorsal neurohemal organs (NHO).  (C) In the abdominal portion of the ventral nerve cord, sNPF was localized in the axons of descending neurons located around connective axon fibers (arrows).  (D, E, F) The immunohistochemical staining with the sNPF peptide antibody in the sectioned adult brain are shown.  Strong stained neural cells were distributed in the medullar (E) and the mushroom body calyx (F).

Fig. 6.  The gain-of-function and loss-of-function sNPF constructs, and sNPF productions in the transgenic flies.  (A) Schematic diagrams of the gain-of-function UAS-sNPF construct and the loss-of-function UAS-sNPF-RNAi construct.  (B) In the Western blot analysis, MJ94>sNPF and HS>sNPF mutants produced more sNPF peptide than wild-type flies, whereas Hs>sNPF-RNAi mutants produced less sNPF.

Fig. 7.  The sNPF regulates food intake.  (A) The fed colored food was detected in the abdomen of the female adult.  (B) The fed colored food was detected in the gut of the third instar feeding larva.  (C) After 10 min of feeding, 93% of MJ>sNPF flies were fed colored food compared to 32% of the wild-type flies, whereas only 3% of MJ>sNPF-RNAi flies were fed colored food.  (D) After 10 min of feeding, 30% of HS>sNPF and 85% of HS>2XsNPF larvae were fed colored food compared to 21% of the wild-type larvae, whereas 8% of HS>sNPF-RNAi larvae were fed colored food.
(E) The fed blue color was measured in absorbance at 595nm with a spectrometer. After 10 min of feeding, 0.38 absorbance in MJ>sNPF and 0.58 absorbance in MJ>2XsNPF flies were measured compared to 0.21 absorbance in the wild-type flies, whereas 0.05 absorbance in MJ>sNPF-RNAi flies were measured. (F) In the prolong feeding assays, MJ>sNPF flies saturated feeding to nearly 100% after 15 min, while wild-type flies saturated to nearly 100% after 40 min.

**Fig. 8. The sNPF controls body size.** (A) MJ>2X sNPF fly was larger than the wild-type fly. (B) MJ>2XsNPF flies were 20% bigger sizes than MJ>sNPF or the wild-type flies, whereas MJ>sNPF-RNAi flies were similar sizes as the wild-type. (C) MJ>2XsNPF flies were 25% heavier than MJ>sNPF or wild-type flies, whereas MJ>sNPF-RNAi flies were similar weight as the wild-type.
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Table 1. *Drosophila* sNPF1, 2, dNPF, and vertebrate NPY amino acid sequences.
Fig. 1

A

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MFHLKRLSLQSQRCLALICLPSIQMQQQPAQA

GAGCTGCG TGCGCTGCGA CGCGCTGCG CCGCTGCG AGCCCTGCG CGCGCTGCG TGGCTGCG CGTCGTCG

EYSASAQGTPLSNLYDNAOLLQREYAGPYVFPN

CAAGTCGCG AGCGCGCGA CGCGCGCG ACGCGCGCG CGCGCGCG CGCGCGCG CGCGCGCG CGCGCGCG

HQVERKARQPSLRLRFGRSQPDMLIYI

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GACTCGGT CGCGCGCGA CGCGCGCG CGCGCGCG CGCGCGCG CGCGCGCG CGCGCGCG CGCGCGCG

DGLDARYDIGLYERYVRKPRQRLRGRSYPQFE

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FQREYVPRKMRRLRGRGSTKAPSEQKHTPEE

ACGTCATG ACTCGGCGA GAAATGACG AACTAA

TSSIPPKTQMN

B

M cDNA Genomic DNA

1.1 kb 0.9 kb

C

1.1 kb

1 109 167 624 767 1808 1064 1111

ATG TAA
Fig. 5
**Fig. 8**

A) Images of wild-type (WT) and MJ>2XsNPF flies.

B) Bar graph showing body weight of adults (mg) for different genotypes: WT, MJ>1XsNPF, MJ>2XsNPF, and MJ>3NPFRi.

C) Bar graph showing body size of adults (mm) for different genotypes: WT, MJ>1XsNPF, MJ>2XsNPF, and MJ>3NPFRi.
Drosophila short neuropeptide F regulates food intake and body size
Kyu-Sun Lee, Kwan-Hee You, Jong-Kil Choo, Yong-Mahn Han and Kweon Yu

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