Processed short neuropeptide F peptides regulate growth through the ERK-insulin pathway in Drosophila melanogaster

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ABSTRACT

The Drosophila sNPF gene regulates growth through the ERK-insulin pathway. sNPF encodes a precursor protein that is processed and produces biologically active sNPF peptides. However, the functions of these peptides are not known. In Drosophila neuronal cells in culture and in flies in vivo, sNPF1 and sNPF2 activated the ERK-insulin pathway and regulated body growth. In addition, the sNPF precursor and the processed sNPF peptide were co-localized in the neurons of the central nervous system. These results indicate that sNPF1 and sNPF2 peptides processed from the sNPF precursor are sufficient for regulating body growth through the ERK-insulin pathway in Drosophila.

1. Introduction

The short neuropeptide F (sNPF) gene has been found in diverse insect families which include fruit flies, locusts, fire ants, potato beetles, and mosquitoes [1–5]. sNPF regulates various physiological processes like the reproduction in locusts [1], feeding behavior in fire ants [2], and adult diapause in the Colorado potato beetles [3].

In Drosophila melanogaster, sNPF regulates body size and food intake [4]. In order to regulate body size, sNPF produced in the fly brain binds to the sNPF receptor (sNPFR1) and activates extracellular signal-related-kinase (ERK), which turns on the Drosophila insulin-like peptide (Dilp) genes in the insulin producing cells of the brain. The up-regulated Dilp peptides are secreted into the system and bind to the insulin receptor in target tissues like the fat body, which activates the insulin receptor signaling through the Akt/FOXO/4E-BP pathway resulting in body growth [6].

The sNPF gene encodes the 280 amino acids precursor protein which contains the 30 amino acid signal peptide in the N-terminus. It may produce sNPF1 (AQRSPSLRLRFa), sNPF2 (WFGDVNQKPI), sNPF3 (PQRLRWa), and sNPF4 (PMRLRWa) peptides by cleaving flanking dibasic amino acids and amidating in their C-termini [4] (Fig. 2A). However, in previous mass spectrometry studies using larval and adult tissues, AQRSPSLRLRFa (sNPF1), SPSLRLRFa (sNPF2), SDPDSLNSIVE (sNPF-AP-1), WFGDVNQKPI (sNPF2), SPSLRLRFa (sNPF2), and PQRLRWa (sNPF3) and PMRLRWa (sNPF4) are also identified in adult body extract [10,11].

In the mammalian CHO cell based bioluminescence assay for sNPFR1, sNPF1, sNPF2, and sNPF3, and sNPF4 peptides show high calcium responses [12]. However, in the Xenopus oocytes electrophysiological assay for sNPFR1, sNPF1, sNPF2, and sNPF3 or sNPF4 [13]. In the mammalian CHO-K1 cell based radioreceptor assay, sNPF1, sNPF2, and sNPF3 or sNPF4 [10] (Supplementary Table S1).

Since the sNPF peptides show different biological activities for sNPFR1 in vitro and heterologous assays and its functions are unknown, we examined whether sNPF peptides regulate growth through the ERK-insulin pathway. We found that sNPF1 and sNPF2 peptides activated ERK and up-regulated Dilps mRNA expression in Drosophila neuronal cells and D. melanogaster. In flies, sNPF1 and sNPF2 peptides regulated body size and weight. In addition, we investigated expression patterns of the sNPF precursor protein and the processed sNPF peptide. They were co-localized in the neurons of the larval central nervous system (CNS).
2. Materials and methods

2.1. Drosophila culture and stocks

*D. melanogaster* were cultured and kept at 25 °C using the standard method. Wild-type Oregon-R, *w-*, and UAS-2XEGFP were obtained from the Bloomington stock center (Bloomington, USA). UAS-2xNSPF and UAS-NSPF-Ri transgenic flies were described in Lee et al. [4]. To express these UAS lines, UAS/Gal4 system was used.

2.2. UAS-sNSPF1, UAS-sNSPF2, sNSPF-Gal4 constructions and transgenic fly generations

sNSPF1 276 bp DNA fragment containing the signal peptide and sNSPF1 coding sequences was subcloned into the pUAS vector to generate pUAS-sNSPF1 construct (Fig. 2B). sNSPF2 coding sequences was amplified and ligated into the PstI–XbaI sites of the pUAS-sNSPF1 construct to generate pUAS-sNSPF2 (Fig. 2C). 2.4 kb genomic DNA fragment of 5'-untranslated region of the sNSPF gene was isolated and subcloned into the pGal4 vector to generate sNSPF-Gal4 (Supplementary Fig. S1A). UAS-sNSPF1, UAS-sNSPF2, and sNSPF-Gal4 transgenic flies were obtained by the P-element-mediated germ line transformation [14].

2.3. Cell culture

*Drosophila* third instar central nervous system (CNS) derived BG2-c6 cell line [15], purchased from the Drosophila Genomics Resource Center (DGRC, Indiana University, Bloomington, IN, USA), was maintained at 26 °C in a 60 mm culture dish containing Schneider medium (Gibco/Invitrogen, Burlington, USA) supplemented with 10% bovine calf serum. The BG2-c6 cell line expresses snPF1 mRNA and is positively stained with the snPF1 antibody [6]. Before peptide treatments, cells were starved for 8 h in the serum-free medium and treated with 100 nM synthetic snPF1, snPF2, snPF3, snPF4, and snPF21-10 peptides, respectively.

2.4. Western blots

Total proteins from snPF peptides treated BG2-c6 cells or snPF mutant flies were isolated using the PROPREP protein extraction buffer (iNtRON Biotechnology, Korea). Western blot analyses were performed as described previously [4,6]. Diphospho-ERK (Sigma, St. Louis, USA) and β-actin (Abcam, Cambridge, USA) primary antibodies were used.

2.5. Quantitative RT–PCR

Total RNA from snPF peptide treated BG2-c6 cells or snPF mutant flies were isolated. Quantitative RT-PCR method and primers were described previously [4,6].

2.6. Immunostaining

Immunostaining in the larval CNS was performed as described previously [4,6]. snPF precursor (snPFp) (1:5000, a gift from Dr. D.R. Nässel, Stockholm University, Sweden) and snPF1 antibody were used. snPFp antibody was raised against DPSLPQMRRTAYDDLLEREL of the snPF precursor (green color in Fig. 2A) in rabbits [16,17] and snPF1 antibody was raised against the amidated snPF1 (AQRSPSLRLRFa) in guinea pigs.

2.7. Measurement of body size and weight

To avoid overcrowding and lack of nutrition, the eggs laid by five female flies for 6 h at 25 °C were cultured. Body sizes of the
50 3–5-day-old female flies were measured from the anterior end of the head to the posterior end of the abdomen. For weight, 3-day-old female flies were measured with the balancer (METTLER AJ100).

3. Results

3.1. sNPF1 and sNPF2 peptides activated ERK and regulated Dilp expression in Drosophila neuronal cells

Since sNPF2 peptide activated ERK-mediated Dilp1 and Dilp2 mRNA expression in Drosophila neuronal BG2-c6 cells [6], we tested whether other sNPF peptides also activate the ERK-Dilps pathway in BG2-c6 cells. After treatments of various sNPF peptides in BG2-c6 cells, we performed the Western blot analysis with the antibody against the phospho-ERK (pERK) which detects the active form of ERK (Fig. 1A). sNPF1 and sNPF2 peptide treatments increased ERK activation compared to those of the non-treated and sNPF21–10 (WFGDVNKPI) peptide treated controls. However, sNPF3 and sNPF4 peptide treatments did not increase ERK activation compared to the controls.

Next, we tested Dilps mRNA expression by the treatments of various sNPF peptides in the BG2-c6 cells using the quantitative RT-PCR analysis (Fig. 1B). sNPF1 and sNPF2 peptide treatments up-regulated Dilp1 and Dilp2 mRNA expression by more than 5-folds and 10-folds respectively, compared to those of the non-treated and sNPF21–10 treated controls. However, sNPF3 and sNPF4 peptide treatments did not up-regulate Dilp1 and Dilp2 mRNA expression. These results indicate that sNPF1 and sNPF2 activated ERK and up-regulated Dilp1 and Dilp2 mRNA expression in Drosophila neuronal cells.

3.2. sNPF1 and sNPF2 activated ERK and regulated Dilp expression in Drosophila

Since sNPF1 and sNPF2 peptides activated the ERK-insulin pathway in Drosophila neuronal cells, we examined whether sNPF1 and sNPF2 flies activate the same pathway in vivo. We generated UAS-sNPF1 and UAS-sNPF2 transgenic flies containing the signal peptide, the processed sNPF1 or sNPF2 peptide, and flanking dibasic amino acids (Fig. 2B and C).

After over-expression of UAS-sNPF1 and UAS-sNPF2 with sNPF-Gal4 (sNPF > sNPF1 and sNPF > sNPF2), we performed Western blot analysis with the pERK antibody (Fig. 3A). sNPF > sNPF1 and sNPF > sNPF2 flies increased ERK activation compared to the sNPF-Gal4 control. sNPF > 2XsNPF, which is the over-expression of two copies of the sNPF gene, increased ERK activation whereas sNPF > sNPF-Ri flies, the inhibition of the sNPF gene, decreased ERK activation.

In the quantitative RT-PCR analysis, sNPF > sNPF1 and sNPF > sNPF2 over-expression flies up-regulated Dilp1 and Dilp2 mRNA expression by more than six-folds compared to the sNPF-Gal4 control flies. sNPF > 2XsNPF over-expressing flies also up-regulated Dilp1 and Dilp2 mRNA expression. These results indicate that sNPF1 and sNPF2 activated ERK and up-regulated Dilp1 and Dilp2 mRNA expression in Drosophila neuronal cells.

Fig. 3. sNPF1 and sNPF2 activated ERK and regulated Dilp expression in Drosophila. (A) Over-expression of UAS-sNPF1 and UAS-sNPF2 with sNPF-Gal4 (sNPF > sNPF1 and sNPF > sNPF2) increased ERK activation, like over-expression of two copies of the sNPF gene (sNPF > 2XsNPF), compared to the sNPF-Gal4 control. sNPF inhibition (sNPF > sNPF-Ri) decreased ERK activation. β-Actin was the loading control. (B) sNPF > sNPF1 and sNPF > sNPF2 up-regulated expression of Dilp1 and Dilp2 mRNA like sNPF > 2XsNPF, compared to the control. sNPF > sNPF-Ri down-regulated expression of Dilp1 and Dilp2 mRNA. Data are expressed as mean ± S.E.M. from three independent experiments (*P < 0.05, **P < 0.001, Student's t-test).

Fig. 4. sNPF1 and sNPF2 controlled body growth. (A and B) sNPF > sNPF1 and sNPF > sNPF2 increased body size by 16–18% and body weight by 20–22% like sNPF > 2XsNPF compared to the wild-type (WT) and sNPF-Gal4 control flies. sNPF > sNPF-Ri inhibiting flies decreased body size and weight. Data are expressed as mean ± S.E.M. from three independent experiments (n = 50 per each experiments, *P < 0.05, **P < 0.001, Student's t-test).
ulated Dilp1 and Dilp2 mRNA expression whereas sNPF > sNPF-Ri inhibiting flies down-regulated Dilp1 and Dilp2 mRNA expression (Fig. 3B). These findings indicate that over-expression of sNPF1 and sNPF2 activated ERK and up-regulated Dilps mRNA expression in Drosophila.

3.3. sNPF1 and sNPF2 controlled body growth

sNPF regulated body weight and size: sNPF > 2XsNPF over-expressing flies increased body size and weight by 20% compared to the wild-type (WT) and sNPF-Gal4 control flies whereas sNPF > sNPF-Ri inhibiting flies decreased body weight and size by 12–18% (Fig. 4A and B), sNPF > sNPF1 and sNPF > sNPF2 over-expressing flies also increased body size by 16–18% and body weight by 20–22%. These results indicate that sNPF1 and sNPF2 peptides are sufficient for regulating body growth.

3.4. sNPFp and sNPF1 were co-localized in neurons of the larval CNS

To compare expression pattern of the processed sNPF1 with the sNPF precursor (sNPFp), we performed immunostaining in the third instar larval CNS with the antibodies against the sNPFp and sNPF1. Large numbers of neuronal cell bodies and axons in the brain, ventral ganglion, and ring gland were stained with the antibodies against the sNPFp and sNPF1 (Fig. 5A and B). When they were merged, almost all of staining was overlapped (Fig. 5C).

In the brain, the sNPFp and sNPF1 were detected in the neurons, Kenyon cells, and mushroom body (Fig. 5A–F, MB). In the ring gland, corpora cardiaca region (Fig. 5D–F dot line, CC) had strongly stained axonal projections from neurons adjacent to insulin producing cells (Fig. 5D–Fasterisks). These results indicate that sNPF precursor and the processed sNPF peptide were co-localized in the same neurons. However, sNPFp signal was relatively strong in cell bodies whereas sNPF1 signal was relatively strong in the axons of sNPF expressing neurons.

4. Discussion

In silico analysis suggests that the sNPF precursor may produce sNPF1, sNPF2, sNPF3, and sNPF4 peptides. Flanking dibasic amino acids of these peptides in the precursor are proteolytic sites cleaved by the evolutionary conserved proprotein convertase (PC). Three PCs are found in D. melanogaster [18]. PCs sometimes cleave a monobasic amino acid, which suggests that sNPF2 may be cleaved further and produce sNPF21–10 (WFGDVNQKPI) and sNPF212–19 (SPSLRLRFa) found in the larval and adult tissues. sNPF21–10 is a biologically inactive peptide confirmed in the Western blot analysis with Drosophila neuronal cells (Fig. 1A). Alpha-amidation in the C-terminal of a neuropeptide by the PHM enzyme is required to produce active neuropeptides. In Drosophila, more than 90% of neuropeptides are amidated [19].

sNPF > sNPF-Ri inhibiting flies decreased body size and weight (Fig. 4A and B). On the contrary, sNPF-Ri inhibition with MJ94-Gal4 (MJ94 > sNPF-Ri) did not decrease body size and weight [4,6]. sNPF-Gal4 expression, shown by sNPF-Gal4 > UAS-GFP, is very similar with the endogenous sNPF mRNA expression pattern in the larval CNS (Supplementary Fig. S1B). However, MJ94-Gal4 expression, shown by MJ94-Gal4 > UAS-GFP, is in the entire sensory neurons and sensory structures of the larval CNS (Supplementary Fig. S1C) [20]. These expression differences explain why sNPF inhibition in the sNPFnergic neurons (sNPF > sNPF-Ri) decreased body size and weight while sNPF inhibition in the non-sNPFnergic neurons (MJ94 > sNPF-Ri) did not.

In the brain, sNPFp and sNPF1 immunostained in the mushroom body which is involved in learning and memory and other complex behaviors like circadian regulations [16,21]. The mushroom body calyxes receive chemosensory input and Kenyon cells of the mushroom body calyx are the main center for olfactory processing [22]. It suggests that a part of functions of sNPF peptides may be related to processing chemosensory and olfactory information.

Fig. 5. sNPFp and sNPF1 were co-localized in the neurons of the larval CNS. (A and B) Immunostaining with antibodies against sNPF precursor (sNPFp) and sNPF1 in the whole mount larval CNS. (C) Almost all of the stained neurons were overlapped. (D–F) Higher magnification of the upper region of brain hemispheres including ring glands (dot lines) and mushroom body revealed that sNPFp and sNPF1 were in the lobes of the mushroom bodies (MB), axonal projections to the corpora cardiaca (CC) of the ring gland, and axonal terminals adjacent to insulin producing cells (asterisks). Scale bar = 100 μm in A–C; 50 μm in D–F.
We found that sNPF1 and sNPF2 peptides processed from the sNPF precursor are sufficient for regulating body growth through the ERK-insulin pathway in Drosophila. However, sNPF3 and sNPF4, which are PXRLRW (Fig. 2A) and found in the adult brain [11], did not activate ERK-insulin pathway in Drosophila neuronal cells. Since the sNPF gene may have multiple functions as a neurohormone and neuromodulator [17], it is possible that sNPF3 and sNPF4 peptides control different function(s) other than ERK-insulin mediated growth regulation.

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Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at doi:10.1016/j.febslet.2009.07.024.

References