Minibrain/Dyrk1a Regulates Food Intake through the Sir2-FOXO-sNPF/NPY Pathway in Drosophila and Mammals

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Abstract

Feeding behavior is one of the most essential activities in animals, which is tightly regulated by neuroendocrine factors. Drosophila melanogaster short neuropeptide F (sNPF) and the mammalian functional homolog neuropeptide Y (NPY) regulate food intake. Understanding the molecular mechanism of sNPF and NPY signaling is critical to elucidate feeding regulation. Here, we found that minibrain (mnb) and the mammalian ortholog Dyrk1a target genes of sNPF and NPY signaling and regulate food intake in Drosophila melanogaster and mice. In Drosophila melanogaster neuronal cells and mouse hypothalamic cells, sNPF and NPY modulated the mnb and Dyrk1a expression through the PKA-CREB pathway. Increased Dyrk1a activated Sirt1 to regulate the deacetylation of FOXO, which potentiated FOXO-induced sNPF/NPY expression and in turn promoted food intake. Conversely, AKT-mediated insulin signaling suppressed FOXO-mediated sNPF/NPY expression, which resulted in decreasing food intake. Furthermore, human Dyrk1a transgenic mice exhibited decreased FOXO acetylation and increased NPY expression in the hypothalamus, as well as increased food intake. Our findings demonstrate that Mnb/Dyrk1a regulates food intake through the evolutionary conserved Sir2-FOXO-sNPF/NPY pathway in Drosophila melanogaster and mammals.

Introduction

Neuropeptides regulate a wide range of physiological processes in animals. In mammals, NPY is widely distributed in the brain and involved in various physiological functions including food intake. In the mammalian brain, the hypothalamus is the center for controlling food intake. The hypothalamic injection of NPY in the rat brain induces hyperphagia and obesity. In the hypothalamus, the arcuate nucleus (ARC) that contains orexigenic NPY and AgRP expressing neurons and anorexigenic POMC neurons senses hormonal levels of insulin and leptin and regulates food intake [1]. In Drosophila, sNPF, a functional homolog of NPY produced in sNPFergic neurons of the fly brain, regulates food intake and growth [2]. Recently, we reported that sNPF and sNPF receptor (sNPFR1) regulate body growth through evolutionary conserved ERK-mediated insulin signaling in Drosophila and rat insulinoma cells [3].

Drosophila Minibrain (Mnb) and its mammalian ortholog Dual specificity tyrosine-phosphorylation-regulated kinase 1a (Dyrk1a) are highly expressed in the neural tissues [4,5,6]. The Dyrk1a gene has been implicated in Down Syndrome (DS) [5,7] and the expression level of Dyrk1a is increased in DS patients and Ts65Dn mice, a mouse model of Down syndrome [4,8]. Mutations of mnb and Dyrk1a in Drosophila and mammals show neural phenotypes like defects in neuroblasts proliferation and brain development [6,9]. Human patients with truncated mutations in the Dyrk1a gene also show microcephaly [10,11]. To date, however, the effects of mnb and Dyrk1a upon food intake have not been described.

FoxO1 modulates food intake by regulation of orexigenic Argp and anorexigenic Pomc genes in the hypothalamus of mice. In the ARC of hypothalamic neurons, FoxO1 is localized in the nuclei during fasting and in the cytoplasm by feeding [12]. Sirti1 (Sir1), the mammalian ortholog of Drosophila Silent information regulator 2 (Sir2), in the ARC also regulates food intake [13]. The Sir1 protein level increases during fasting. Sir1 inhibition by the hypothalamic knock-out in the AgRP neurons decreases food intake [14]. In N43 hypothalamic cells, pharmacological inhibition of Sir1 increases anorexigenic Pomc expression but co-treatment with Sir1 inhibitor and FoxO1 siRNA does not [15], suggesting
Author Summary

Feeding behavior is one of the most essential activities in animals. Abnormal feeding behaviors cause metabolic syndromes including obesity and diabetes. Neuropeptides regulate feeding behavior in animals from nematode to human. Here, we presented molecular genetic evidences of how neuropeptides regulate food intake using fruit fly and mouse model systems. Drosophila short neuropeptide F (sNPF) and the mammalian functional homolog neuropeptide Y (NPY) are produced from neurons in the brain of fruit fly and mouse, respectively. These neuropeptides turned on the minibrain, in mammals also called Dyrk1a, a target gene through the PKA-CREB pathway. Then, this Mnb/Dyrk1a enzyme activated Sir2/Sirt1 enzyme, which activated FOXO transcriptional factor, turning on the expression of a sNPF/NPY target gene. The increased sNPF/NPY increased food intake in fruit flies and mice. On the contrary, increased food intake induced insulin and activated insulin signaling. When insulin signaling is activated, FOXO transcriptional factor acts as a gatekeeper for fasting–feeding transition by regulating sNPF/NPY expression in Drosophila and mammals.

that Sirt1-mediated FoxO1 deacetylation is involved in the regulation of POMC mRNA and food intake.

In this study, we identified mnb and Dyrk1a as target genes of sNPF and NPY signaling, respectively, and describe a molecular mechanism of how Mnb and Dyrk1a regulate food intake in Drosophila and mice.

Results

sNPF Targets mnb to Regulate Food Intake in Drosophila

To find genes affected by sNPF signaling, we performed a DNA microarray analysis using the Affymetrix Drosophila Genome 2.0 Array GeneChip with mRNA extracted from Drosophila neuronal BG2-c6 cells treated with sNPF peptide. Among the 159 genes with at least a two-fold change, mRNA of mnb increased 34-fold compared to the control (Table S1). To test whether the expression of mnb is dependent on sNPF signaling in vivo, we examined the expression levels of mnb in sNPF and sNPFR1 mutants. When sNPF was overexpressed in sNPFergic neurons with the sNPF-Gal4 driver [16] (sNPF> sNPF, sNPF>2XsNPF), mnb mRNA increased 4 to 5-fold compared with the sNPF-Gal4 control (sNPF> sNPF-Ri) or by an sNPF mutant (sNPF<0.00448) (Figure 1A and Figure S1A). When sNPFR1 was overexpressed via a sNPFR1-Gal4 driver (Figure S2) (sNPFR1>sNPFR1), mnb mRNA was increased 3-fold compared with the sNPFR1-Gal4 control. When sNPFR1 was inhibited (sNPFR1<sNPFR1-Ri) or suppressed (sNPFR1<sNPFR1-DN), mnb mRNA was decreased by more than 50% (Figure 1A and Figure S1A). Like mnb mRNA, Mnb proteins were also increased in sNPF or sNPFR1 overexpression with the sNPF-Gal4 or sNPFR1-Gal4 driver, (sNPF>2XsNPF, sNPFR1>sNPFR1) while reduced in an sNPF mutant (sNPF<0.00448) or sNPFR1 inhibition (sNPFR1<sNPFR1-Ri) compared with the sNPF-Gal4 or sNPFR1-Gal4 control (Figure S3A). However, the numbers of Mnb expression neurons (asterisks) are consistent in the sNPFR1-Gal4 control, sNPFR1 overexpression (sNPFR1>sNPFR1), sNPFR1 inhibition (sNPFR1<sNPFR1-Ri), and an sNPF<0.00448 mutant (Figure S3B–S3F). These results indicate that sNPF-sNPFR1 signaling regulates mnb mRNA and protein expression in Drosophila.

To understand how Mnb protein may interact with the sNPFR1 receptor, we immunostained fly adult brains with Mnb and sNPFR1 antibodies. The Mnb antibody produced strong and weak staining in neuronal cells (Figure 1H, 1K, red) while the sNPFR1 receptor antibody stained many neurons (Figure 1L, green). Among the strongly stained Mnb neurons, cell bodies of symmetrically localized median neurons behind the antennal lobe show overlap with the antibody against sNPFR1 (Figure 1J, IM, arrows). At least ten neuronal cell bodies in median neurons were stained with the both antibodies. This coincidence suggests that at least part of Mnb function may be regulated by sNPF-sNPFR1 signaling.

Since sNPF signaling regulates food intake and growth, and growth is regulated by ERK-mediated insulin signaling [3], we hypothesized that sNPF may regulate food intake through the mnb gene. To assess this hypothesis, we used the CAFE† assay [17] to measure feeding in mnb mutant adults. Because homozygous mnb deletion mutants (mnb<0.0057 and mnb<0.00447) generated by the imprecise excisions of the P-element (Figure S4A) are lethal (as are homozygous Dyrk1a mutant mice) we analyzed mnb overexpression and hypomorphs generated by RNAi. mnb overexpression in sNPFR1 neurons (∆NPFR1>2Xmnb) increased cumulative food consumption compared to the sNPFR1-Gal4 control whereas inhibiting mnb (∆NPFR1>mnb-Ri) decreased cumulative food consumption (Figure 1C), indicating that mnb expression in sNPFR1 neurons can regulate food intake. Likewise, we measured the amount of food intake by the amount of digested dye from colored food. Overexpression of mnb in sNPFR1 neurons (∆NPFR1>2Xmnb) increased consumed dye up to 57% compared with that of the sNPFR1-Gal4 control whereas mnb inhibition (∆NPFR1>mnb-Ri) or the mnb mutant (mnb<0.0057) decreased this intake by 30% (Figure 1B and Figure S1B). As expected, levels of mnb mRNA and protein were markedly reduced by mnb inhibition and by the mnb<0.0057 mutant relative to the sNPFR1-Gal4 and w- controls (Figure S4B, S4C). Since sNPFR1 signaling in the insulin producing cells (IPC) regulates body growth through insulin signaling [3], we examined the effect of mnb in IPCs upon food intake. However, food intake was not affected by mnb overexpression in IPCs driven via Dilp2-Gal4 (Dilp2>2Xmnb) while mnb inhibition in IPCs (Dilp2>2Xmnb) decreased food intake by about 30% (Figure 1B), expression of mnb in sNPFR1 neurons but not in IPCs (Figure 1D–1G) is sufficient to regulate food intake.

To determine the consequences of mnb control upon food intake we measured the body weight of young adults from mutant and control. Overexpression of mnb in sNPFR1 neurons (∆NPFR1>2Xmnb) increased body weight relative to that of the sNPFR1-Gal4 controls, similar to the effect seen when sNPFR1 is overexpressed (∆NPFR1>sNPFR1). On the contrary, body weight is decreased when mnb is repressed in sNPFR1 neurons (∆NPFR1>mnb-Ri) and mnb<0.0057 mutant (Figure S4D). The amounts of food intake in the mutants were similar when they were normalized to body mass or to the number of flies (Figure S4E).

Since mnb is involved in neural development [6,9], we restricted mnb expression in the adult stage using the tub-Gal80ts inducible system [18] and tested food intake. mnb overexpression (∆NPFR1-Gal4>2Xmnb) and mnb inhibition (∆NPFR1-Gal4>2Xmnb) flies were cultured in the 32°C permissive temperature until adulthood to suppress sNPFR1-Gal4 expression by the tub-Gal80ts. Then, these adult flies were shifted to the 30°C restrictive temperature in which the tubGal80ts cannot suppress sNPFR1-Gal4. In the permissive
Figure 1. Expression and distribution of *Drosophila mnb* in adults in relation to *sNPf, sNPFR1*, and feeding. (A) *mnb* mRNA prepared from fly heads was measured by RT-qPCR. *mnb* mRNA was increased relative to *sNPFGal4* and *sNPFR1-Gal4* controls when *sNPf* and *sNPFR1* was overexpressed in sNPFnergic neurons (*sNPF>*sNPf, *sNPF>*2XsNPf) and in sNPFR1 neurons (*sNPFR1>*sNPFR1). *mnb* mRNA was decreased when *sNPf* and *sNPFR1* were inhibited (*sNPf>*sNPFRi, *sNPf>c00448, *sNPFR1>*sNPFR1-Ri, *sNPFR1>*sNPFR1-DN). (B) Food consumption measured by the colorimetric assay. Relative to *sNPFR1-Gal4* control, *mnb* overexpression in sNPFR1 neurons (*sNPFR1>*mnb, *sNPFR1>*2Xmnb) increased feeding whereas *mnb* suppression (*sNPFR1>*mnb-Ri, *mnbG1767*) decreased feeding. Overexpression or inhibition of *mnb* in the insulin producing cells with the *Dilp2-Gal4* driver (*Dilp2>*mnb, *Dilp2>*2Xmnb, *Dilp2>*mnb-Ri) did not change the feeding. (C) Food consumption measured by CAFÉ assay. Relative to the *sNPFR1-Gal4* (open triangle) control, *sNPFR1>*mnb (closed triangle) increased while *sNPFR1>*mnb-Ri (closed circle) decreased cumulative food consumption. Data are presented as means ± s.e.m. from three independent experiments. *P*<0.05, **P*<0.001 (One-way ANOVA analysis). (D-G)
condition, the mnb overexpression and mnb inhibition flies did not change the amount of food intake compared with the control flies (sNPFR1-Gal4; tub-Gal80ts) (Figure S3A). However, in the restrictive condition, the mnb overexpression increased food intake compared with the control and the mnb inhibition suppressed food intake (Figure S5B). These results indicate that the food intake phenotype of mnb mutants is not due to developmental effects.

**sNPF-PKA-CREB-mnb Signaling in Drosophila Neuronal BG2-c6 Cells**

To study how sNPFR1 regulates mnb expression, we treated *Drosophila* central nervous system-derived BG2-c6 cells [19] with synthetic sNPF peptide, which changed sNPF and sNPFR1 expression slightly (Figure S6A). Consistent with our initial observations and with patterns in genetically manipulated flies, sNPF treatment increased mnb mRNA more than 5-fold compared to the control when measured by quantitative PCR (Figure 2A). Then, we tested whether the induction of this mnb mRNA is mediated by ERK, as we have previously observed for the induction of *Drosophila* insulin like peptides (Dilps) by sNPF [3]. However, ERK inhibitor PD98059 treatment of the sNPF peptide-treated cells did not suppress the mnb expression. On the other hand, sNPFR1 is a G-protein coupled receptor (GPCR), and the second messenger of GPCRs is cAMP or Ca**²** which respectively activates PKA or PKC [20]. Thus, we treated BG2-c6 cells with the protein kinase A (PKA) inhibitor H89 or with protein kinase C (PKC) inhibitor Chelerythrine Chloride (CC). H89 decreased both basal and sNPF-induced mnb expression level but the PKC inhibitor CC showed no effect (Figure 2A). sNPF signaling appears to control mnb expression through PKA, not through ERK or PKC. Consistent with this interpretation, BG2-c6 cells treated with sNPF showed increased levels of cAMP in a time-dependent manner, peaking at 15 min (Figure S6B).

To find the G**α** subunit of the sNPFR1 G-protein heterotrimer, we examined G**α** and G**β** of both of which modulate cAMP [20]. When transfected into BG2-c6 cells G**α** sIRNA inhibited sNPF-induced cAMP whereas transfection with G**β** sIRNA did not (Figure 2C), suggesting that G**α** is a G**α** subunit of sNPFR1 that can modulate the cAMP-PKA pathway in *Drosophila* neuronal cells. Next, we examined the activation of the cAMP responding element binding protein (CREB), which is a PKA down-stream transcription factor [21]. sNPF stimulated the phosphorylation of CREB in control cells whereas G**α** sIRNA transfection suppressed this sNPF dependent activation of CREB (Figure 2E). In addition, G**α** sIRNA transfection completely blocked the induction of mnb by sNPF, but G**β** sIRNA transfection did not (Figure 2G). These data indicate that G**α** is a key G**α** subunit of the sNPFR1 G-protein as it regulates mnb expression. Taken together, these findings demonstrate that sNPF signaling effectively regulates mnb expression through the G**α**-cAMP-PKA-CREB pathway in *Drosophila* neuronal cells.

**NPY-PKA-CREB-Dyrk1A Signaling in Mouse Hypothalamic GT1-7 Cells**

To compare the functional conservation of sNPF-sNPFR1-PKA-CREB-mnb signaling with the signaling of mammalian NPY, we conducted similar experiments with mouse GT1-7 hypothalamic cells [22]. NPY treatment increased Dyrk1a mRNA while the PKA inhibitor H89 strongly suppressed NPY-induced Dyrk1a expression (Figure 2B). NPY signaling activates Dyrk1a expression through PKA, much like the PKA mediated mnb expression by sNPF in fly neuronal cells. Next, we measured the cAMP level in the NPY treated GT1-7 cells. As expected, cAMP level increased time-dependently and peaked at 15 min (Figure S6C). Five NPY receptors (NPYR1, 2, 4, 5, and 6) mediate the NPY signal [23]. Among them, NPYR1, 2, and 5 receptors are broadly expressed in the mouse nervous system and mediate NPY-induced food intake [24]. We treated GT1-7 cells with chemical inhibitors against these receptors BIBO3304 for NPYR1, BIIE0246 for NPYR2, and CGP71683 for NPYR5. The NPYR1 inhibitor BIBO3304 substantially decreased the NPY-induced cAMP level; little effect was seen for the inhibitors of NPYR2 and NPYR5 (Figure 2D). Thus, NPY appears to activate the cAMP-PKA pathway mainly through NPYR1 in GT1-7 hypothalamic cells. Next, we measured the CREB activation. As expected, inhibiting PKA or NPYR1 suppressed the NPY-induced activation of CREB (Figure 2F), confirming that NPY signal is mediated through NPYR1-cAMP-PKA-CREB. In addition, the NPYR1 inhibitor strongly suppressed NPY-induced Dyrk1a expression; this was not seen with the inhibitors of NPYR2 and NPYR5 (Figure 2H). Taken together, these findings indicate that NPY signaling regulates Dyrk1a expression mainly through the NPYR1-cAMP-PKA-CREB pathway in mouse hypothalamic cells. Importantly, this signal transduction pathway is conserved between fly neuronal cells and mammalian hypothalamic cells.

**Genetic Interactions among sNPFR1, G**α**, P**KA**, C**REB**, and mnb Genes, and CREB ChIP Analysis**

To study genetic interactions among sNPFR1, G**α**, P**KA**, C**REB**, and mnb genes, we suppressed G**α**s, P**KA**, C**REB**, and mnb by RNAi and Dominant Negative (DN) forms in neurons that simultaneously overexpressed sNPFR1. Each of these suppression genotypes reduced the level of mnb mRNA compared with sNPFR1-Gal4 and UAS controls (Figure 3A and Figure S7A). In contrast to the strong induction of mnb produced by sNPFR1 overexpression alone (sNPFR1> sNPFR1), mnb induction was inhibited in genotypes where sNPFR1 overexpression occurred with each of the suppression constructs (sNPFR1> sNPFR1+ G**α**-Ri, sNPFR1> sNPFR1+ P**KA**-DN, sNPFR1> sNPFR1+ C**REB**-DN, sNPFR1> sNPFR1+mnb-Ri) (Figure 3B). In sNPFR1 neurons of flies, as in isolated cells, G**α**, P**KA**, and C**REB** may work downstream of sNPFR1 to regulate mnb expression. The consequences of these interactions are also seen in terms of food intake. G**α**, P**KA**, C**REB**, and mnb suppression mutant flies have reduced food intake compared to those of the sNPFR1-Gal4 and UAS controls (Figure 3C and Figures S1C, S7B). Furthermore, increased food intake of sNPFR1 overexpression was suppressed by co-inhibition of G**α**, P**KA**, and C**REB**, respectively (Figure 3D). These results suggest that the sNPFR1 may regulate food intake through G**α**, P**KA**, C**REB**, and mnb.

Based on promoter analysis of mnb genes from twelve *Drosophila* species, we found a conserved cAMP responding element (CRE) site (Figure S8). Interestingly, the promoters of human *Dyrk1a* and mouse *Dyrk1a* genes contain CRE [25]. To test whether CRE binds to the promoter of the mnb gene, we performed the chromatin immunoprecipitation (ChIP)-PCR analysis with the CREB antibody in sNPF treated *Drosophila* neuronal BG2-c6 cells.
Figure 2. sNPF/NPY-sNPFR1/NPYR1-PKA-CREB-mnb/Dyrk1a signaling in Drosophila neuronal BG2-c6 cells and mouse hypothalamic GT1-7 cells. (A) mnb mRNA in Drosophila neuronal BG2-c6 cells increased in response to treatment with sNPF peptide, but not when co-treated with H89 PKA inhibitor. The ERK inhibitor PD98059 and PKC inhibitor CC did not suppress sNPF-induced mnb expression. (B) Dyrk1a mRNA in mouse hypothalamic GT1-7 cells increased in response to treatment with NPY peptide, but not when co-treated with the PKA inhibitor H89. (C) In Drosophila BG2-c6 cells, sNPF peptide induced cAMP, while transfection of cells with Gαs siRNA but not Gαi siRNA repressed this effect. (D) In mouse GT1-7 cells, NPY peptide induced cAMP, while co-treatment with NPYR1 inhibitor BIBO3304 but not NPYR2 and NPYR5 inhibitors strongly decreased this effect. (E) Western blot to detect activated CREB (pCREB) in Drosophila BG2-c6 cells. sNPF peptide increased pCREB but not in cells transfected with Gαs siRNA.
CREB binding was enriched at the sNPF treated promoter region of the mnb gene by 3-fold compared to the Act5C and sNPF non-treated controls (Figure 3E). Together these in vivo and in vitro findings indicate that sNPF-sNPFR1-Gαs-PKA-CREB pathway controls expression of the mnb target gene and regulates food intake in Drosophila.

Positive Regulation of sNPF/NPY by the Mnb/Dyrk1a-Sir2-FOXO Pathway

A possible avenue through which Mnb regulates food intake could involve Sirt1/Sir2. Notably, Dyrk1a kinase phosphorylates Sir2 in HEK293T cells [26], and activated Sirt1 deacetylates FoxO1 to modulate the activity of this transcription factor in the rat hypothalamus [15]. Accordingly we determined if these interactions were present and associated in mouse hypothalamic GT1-7 cells. In cells transfected with Dyrk1a or treated with NPY, phosphorylation of Sirt1 was increased as detected by immunoprecipitation with Sirt1 antibody, followed by immunoblotting with phospho-threonine (pThr) antibody. Sirt1 phosphorylation was reduced by Dyrk1a siRNA or Dyrk1a siRNA with NPY (Figure 4A). In addition, FoxO1 acetylation was reduced in cells transfected by Dyrk1a or treated with NPY, while FoxO1 acetylation was increased by Dyrk1a siRNA, Dyrk1a siRNA with NPY, or Dyrk1a transfection coupled with the Sirt1 inhibitor EX527 (Figure 4C).

Importantly, NPY mRNA itself was increased in cells transfected with Dyrk1a or treated with NPY peptide, and NPY mRNA was decreased by Dyrk1a siRNA, Dyrk1a siRNA with NPY, or Dyrk1a overexpression in the presence of Sirt1 inhibitor (Figure 4B). In mouse hypothalamic GT1-7 cells, Dyrk1a phosphorylates Sirt1 and this activated Sirt1 appears to deacetylate FoxO1 which in turn positively regulates expression of NPY.

To study genetic interactions among mnb, Sir2, and dFOXO in an animal model, we manipulated Sir2 and dFOXO in the Drosophila mnb overexpression genotype. When mnb, Sir2, and dFOXO were overexpressed in sNPFR1-Gal4 neurons (sNPFR1>mnb, sNPFR1>Sir2, sNPFR1>dFOXO) (Figure S9A), sNPF mRNA and food intake were increased compared to sNPFR1-Gal4 and UAS controls (Figure 4D and 4E, Figure S7B and S7C). Conversely, when mnb, Sir2, and dFOXO were inhibited in sNPFR1 expressing neurons (sNPFR1>mnb-Ri, sNPFR1>Sir2-Ri, sNPFR1>dFOXO-Ri) (Figure S9B), the expression levels of sNPF and food intake were decreased or similar to those of sNPFR1-Gal4 and UAS controls (Figure 4D and 4E, Figure S7B and S7C). Finally the level of sNPF mRNA and food intake were reduced in adults when Sir2 or dFOXO were inhibited in sNPFR1 neurons that overexpressed mnb (sNPFR1>mnb+Sir2-Ri, sNPFR1>mnb+dFOXO-Ri) compared with flies only overexpressing mnb (sNPFR1>mnb). These data suggest that mnb may regulate sNPF expression and food intake through Sir2 and dFOXO.

Since fasting can stimulate food intake, we tested whether an acute period of food deprivation affected the expression of mnb and sNPF of adult flies. Levels of mnb and sNPF mRNA increased 2-fold after 12 h starvation (Figure 4F). We propose that dFOXO contributes to this expression of sNPF in starved flies. We identified a common dFOXO consensus binding site (RWVAAAGA) in the sNPF promoter from twelve Drosophila species (Figure S10) and performed a chromatin immunoprecipitation (ChIP)-tiled gene array analysis with dFOXO antibody in fed and starved adult flies. dFOXO binding was enriched at the promoter region of sNPF more than 3-fold in the starved flies compared to the Act5C and fed controls (Figure 4G). These results suggest that the dFOXO transcriptional factor regulates sNPF mRNA expression by direct binding to its promoter in Drosophila, as seen for FoxO1 regulation of NPY expression in mice [27].

Overall, these results from mouse hypothalamic GT1-7 cells and Drosophila indicate that the Mnb/Dyrk1a-Sir2-FOXO pathway positively regulates sNPF/NPY expression and food intake.

Negative Regulation of sNPF/NPY by Insulin Signaling

The positive feedback regulation of sNPF signaling we have described to this point must occur alongside a system to negatively regulate sNPF signaling. Insulin, one of several anorexic hormones, inhibits food intake through AKT-mediated FoxO1 inactivation in the hypothalamus [27]. In Drosophila, neuronal overexpression of Dilps negatively regulates larval food intake [29]. To understand the inhibitory mechanism of insulin on food intake, we analyzed the phosphorylation of FOXO and the expression of NPY and NPT. In the mouse hypothalamic GT1-7 cells, insulin treatment increased FoxO1 phosphorylation and decreased NPT mRNA while insulin combined with AKT inhibitor co-treatment slightly decreased FoxO1 phosphorylation and increased NPT expression (Figure 5A, 5B). Likewise, in fly neuronal BG2-c6 cells, insulin with AKT inhibitor co-treatment increased sNPF mRNA (Figure 5C). Thus, in both models AKT-mediated insulin signaling increased FOXP phosphorylation and suppressed NPY or sNPF expression.

We extended these results with analyses of Drosophila with insulin and insulin receptor transgenes. Compared to Dilp2-Gal4 and sNPFR1-Gal4 controls, sNPF mRNA and food intake were decreased when Dilp2 was overexpressed in insulin producing cells (Dilp2>Dilp2) and when insulin receptor (InR) was overexpressed in sNPFR1 expressing neurons (sNPFR1>hInR) (Figure 5D, 5E). On the other hand, sNPF expression and food intake were increased when InR was suppressed by a dominant negative construct expressed in sNPFR1 neurons (sNPFR1>hInRΔ) (Figure 5D, 5E). Fasting may contribute to sNPF expression and the propensity for food intake because fasting in the adult reduces the expression of several Dilps (Figure 5F), as previously observed to occur in Drosophila larvae [29].

Taken together, the results from mouse and Drosophila neuronal cells and from adult flies indicate that the insulin signaling negatively regulates sNPF/NPY expression and food intake.

Dyrk1a TG Mice Regulate Food Intake through the FOXO-NPY Pathway

To evaluate these Mnb/Dyrk1a-Sir2-FOXO-NPY interactions and consequences in a mammalian animal model, we examined FoxO1 acetylation and NPY expression in the hypothalamus of transgenic mice containing the human Dyrk1a and BAC clone (hDyrk1a TG). As expected, in the Western blot, Dyrk1a in the hypothalamus was increased in hDyrk1a TG mice compared to controls (Figure 6A). On the other hand, FoxO1 in the hypothalamus was less acetylated in hDyrk1a TG mice compared to controls (Figure 6C). Hypothalamic NPY mRNA and serum NPY were increased in hDyrk1a TG mice compared to controls (Figure 6B, 6D). These results indicate that Dyrk1a regulatesFoxO1 expression and food intake through the FOXO-NPY pathway.
levels were elevated in hDyrlk1a TG mice compared to controls (Figure 6B). Thus, mammalian Dyrlk1a appears to regulate FoxO1 acetylation and NPY expression in the mouse hypothalamus, as we have observed for this system in Drosophila sNPFR1 neurons.

To assess whether mammalian Dyrlk1a also regulates food intake as seen for the homolog mnb of Drosophila, we monitored food intake in seven-week-old hDyrlk1a TG mice. Daily food consumption was increased in the transgenic mice compared to littermate controls (Figure 6D) and the average food intake of hDyrlk1a transgenic mice was elevated by 15% (Figure 6E). Correspondingly, the hDyrlk1a transgenic mice presented slightly increased mass (Figure S11). Dyrlk1a thus appears to regulate food intake through the expression of NPY mediated by FOXO in a molecular pathway that is evolutionarily conserved in Drosophila.

Discussion

The production of sNPF and NPY in sNPFergic and hypothalamic neurons of flies and mammals respectively, is increased during fasting. These neuropeptides are secreted to produce paracrine and endocrine effects [24] but also feedback upon their synthesizing neurons where they respectively induce mnb and Dyrlk1a gene expression through the PKA-CREB pathway (Figure 6F). This Mnb/Dyrlk1a kinase phosphorylates and activates the Sirt2/Sirt1 deacetylase, which in turn deacetylates and activates the FOXO transcription factor. Among its many potential targets, FOXO then increases sNPF/NPY mRNA expression. Negative controls modulate the positive feedback of sNPF/NPY. Feeding activates the insulin receptor-PI3K-AKT pathway. FOXO becomes phosphorylated and transcriptionally inactivated by translocation to the cytoplasm [30]. In this state the induction of sNPF/NPY by FOXO is decreased. Because sNPF and NPY are orexogenic, their positive feedback during fasting should reinforce the propensity for food intake whereas the negative regulation of sNPF and NPY mRNA during feeding condition would then contribute to satiety (Figure 6F).

FOXO family transcriptional factors are involved in metabolism, longevity, and cell proliferation [31]. FOXO is in part regulated in these processes by post-transcriptional modifications including phosphorylation and acetylation [30]. In many model systems, the ligand activated Insulin-PI3K-AKT pathway phosphorylates FOXO to inactivate this transcription factor by moving it to the cytoplasm. The cytoplasmic localization of FOXO is mediated by 14-3-3 chaperone proteins in Drosophila and mammals [32,33]. FOXO may also be acetylated, as in FoxO1 of mice, by the CREB-binding protein (CBP)/p300 acetylase and this inhibits FOXO transcriptional function by suppressing its DNA-binding affinity. Such FoxO1 acetylation can be reversed by SirT1 to help activate the FoxO1 transcription factor [34]. Here we describe for Drosophila how dFOXO in sNPFR1 neurons regulates the expression of sNPF and food intake (Figure 4D, 4E). This mechanism parallels how hypothalamic FoxO1 regulates food intake through its control of orexigenic NPY and Agp in rodents [12,27]. Post-transcriptional modification of FOXO is central to these controls in both animals. sNPF and NPY expression is increased when FOXO is deacetylated by Sir2/Sirt1, while sNPF and NPY are decreased when FOXO is phosphorylated via the insulin-PI3K-AKT pathway. Post-transcriptional modifications of FOXO proteins play a critical role for controlling food intake through the sNPF and NPY expression in flies and rodents.

Mnb/Dyrlk1a has been described to participate in olfactory learning, circadian rhythm, and the development of the nervous system and brain [6]. Mnb and Dyrlk1a proteins contain a nuclear targeting signal sequence, a protein kinase domain, a PEST domain, and a serine/threonine rich domain. The kinase domains are evolutionarily well-conserved from flies to humans [35]. In Down syndrome (DS), chromosome 21 trisomy gives patients three copies of a critical region that includes the Mnb/Dyrlk1a; trisomy of this region is associated with anomalies of both the nervous and endocrine systems [36]. DS patients often show high Body Mass Index due to the increased fat mass. Children with DS have elevated serum leptin coupled with leptin resistance, both of which contribute to the obesity risk common to DS patients [37,38]. We now observe a novel function of Mnb/Dyrlk1a that may underlay this metabolic condition of DS patients. Mnb/Dyrlk1a regulates food intake in flies and mice. This is controlled by sNPF/NPY-PKA-CREB up-stream signaling and thus produces down-stream affects upon Sirt2/Sirt1-FOXO-sNPF/NPY. Fasting not only increases the expression of mnb, but also of sNPF, suggesting that Mnb kinase activates a positive feedback loop where Sirt2-dFOXO induces sNPF gene expression. Notably, fasting increases Sirt1 deacetylase activity and localizes FoxO1 to the nucleus in the orexogenic AgRP neurons of the mouse hypothalamus [15]. Increased dosage of Dyrlk1a in DS patients may reinforce the positive feedback by NPY and disrupt the balance between hunger and satiety required to maintain a healthy body mass.

Insulin produced in the pancreas affects the hypothalamus to regulate feeding in mammals [1]. Insulin injected into the intracerebroventral of the hypothalamus reduces food intake while inhibiting insulin receptors of the hypothalamic ARC nucleus causes hyperphasia and obesity in rodent models [39,40]. Here we saw a similar pattern for Drosophila where overexpression of insulin-like peptide (Dilp2) at insulin producing neurons decreased food intake while food intake was increased by inhibiting the insulin receptor in sNPFR1 expressing neurons (Figure 5E). Likewise, during fasting, serum insulin and leptin levels are decreased in mammals [1], as is mRNA for insulin-like peptides of Drosophila [29,41] (Figure 5F). Thus, the mechanism by which insulin and insulin receptor signaling suppresses food intake is conserved from fly to mammals in at least some important ways.

Previously, we reported how sNPF signaling regulates Dilp expression through ERK in IPCs and controls growth in Drosophila [3]. Here, we show that sNPF signaling regulates mnb expression through the PKA-CREB pathway in non-IPC neurons and controls food intake (Figure 1, 2, 4). Since sNPF works through the sNPFR1 receptor, sNPFR1 in IPCs and non-IPC neurons might transduce different signals and thereby modulate different phenotypes. Four Dilps (Dilp1, 2, 3, and 5) are expressed in the IPCs of the brain [42]. Interestingly, levels of Dilp1 and 2 mRNA are reduced in the sNPF mutant, which has small body size [3], but we find only Dilp3 and 5 mRNA levels are reduced upon 24 h fasting. Likewise, only Dilp3 is reduced when adult flies are maintained on yeast-limited diets [43]. In addition, Dilp1 and 2...
null mutants show slight reduced body weights but Dlp3 and Dlp5 null mutants do not [44]. These results suggest that Dlp1 and 2 behave like a mammalian insulin growth factor for size regulation while Dlp3 and 5 act like a mammalian insulin for the regulation of metabolism. However, in the long term starvation, Dlp2 and Dlp5 mRNA levels are reduced and Dlp3 mRNA expression is increased [45].

During fasting, sNPF but not sNPER1 mRNA expression was increased in samples prepared from fly heads (Figure 4F and Figure S9C), which increases food intake. On the other hand, in feeding, the high level of insulin signaling reduced sNPF but not sNPER1 mRNA expression and suppressed food intake (Figure 5D and 5E, Figure S9D). Interestingly, in the antenna of starved flies, sNPER1 but not sNPF mRNA expression is increased and induces presynaptic facilitation, which resulted in effective odor-driven food search. However, high insulin signaling suppresses sNPER1 mRNA expression and prevents presynaptic facilitation in DM1 glomerulus [46]. These results indicate that starvation-mediated or insulin-signaling-mediated sNPF-sNPER1 signaling plays a critical role in Drosophila feeding behavior including food intake and food search even though the fine tuning is different.

In this study, we present a molecular mechanism for how sNPF and NPY regulate food intake in Drosophila and mice. We describe a system of positive feedback regulation for sNPF and NPY signaling that increases food intake and a mode of negative regulation for sNPF and NPY by the insulin signaling that suppresses food intake. Modifications of the FOXO protein play a critical role for regulating sNPF and NPY expression, resulting in the control of food intake.

Materials and Methods

Drosophila Culture and Stocks

Drosophila melanogaster were cultured and at 25°C on standard cornmeal, yeast, sugar, agar diet. Wild-type Canton-S, w+, and UAS-CREB-DBN were obtained from the Bloomington stock center. sNPF [64-68] was obtained from the Harvard stock center (Exelixis stock collection). UAS-sNPF, UAS-2XsNPF, UAS-sNPF-Ri, UAS-sNPER1, UAS-sNPER1-DBN and UAS-NeuroGal4 transgenic flies were described in our previous reports [2,3,16]. The sNPF-Ri-Gal4 construct was generated from a 2.5 kb genomic DNA fragment of the 5’-untranslated region of the sNPF-Ri gene. The full-length cDNA sequence of Drosophila minibrain (mnb, CG 42273) was subcloned into the µUAS vector to generate the µUAS-mnb construct. sNPF-Ri-Gal4 and UAS-mnb transgenic flies were generated by the P-element-mediated germ line transformation [47]. mnb [27,72-74], an EP line for minibrain, was purchased from the GenExel, Inc. (KAIST, Korea). UAS-sNPER1-Ri (VDRG9379), UAS-mnb-Ri (VDRG28628), UAS-Sir2-Ri (VDRC23201) and UAS-FOXO-Ri (VDRC106097) were obtained from the Vienna Drosophila RNAi Center (VDRC). Dlp2-Gal4, UAS-Gzx-Ri, UAS-PAK-DBN (a dominant-negative form of PAK), UAS-Sir2 transgenic flies were described previously [42,48,49,50,51]. To express these US lines, UAS-Gal4 system was used [52]. For minimizing the genetic background effect among tested Drosophila lines, all stocks were crossed with w+ and then crossed to the second (w+; Be, Elp/ CyO) or third (w+; D/TM3, Sb) chromosome balancers, respectively. For making double mutants, w+; T(2;3) Apb+/CyO; TM3. Then, w+; +/CyO; UAS-Y/TM3 flies generated by the similar way were crossed with w+; UAS-X/CyO; +/-TM3; then, w+; +/CyO; UAS-Y/TM3 flies were obtained from the Bloomington stock center.

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Drosophila Food Intake Assay

We measured food intake of Drosophila in two ways. The CAFE assay [17] was performed with 3 day-old adult male flies. Twelve hours before the assay, ten flies were placed in the CAFE device [17] containing 5% sucrose solution in calibrated glass micropipettes (WVR, West Chester, PA). At time zero, the micropipettes with 5% sucrose solution were replaced and the amount of liquid consumed was measured every 6 h. A colorimetric food intake
Figure 5. Negative regulation of sNPF/NPY by insulin signaling. (A, B) Insulin treatment increased FoxO1 phosphorylation and decreased NPY expression in mouse hypothalamic GT1-7 cells while insulin with AKT inhibitor co-treatment slightly decreased FoxO1 phosphorylation and increased NPY expression. (C) Insulin with AKT inhibitor co-treatment increased sNPF expression in fly neuronal BG2-c6 cells. (D, E) sNPF expression (D) and food intake (E) were decreased in adult flies overexpressing Dilp2 in IPCs (Dilp2>\text{Dilp2}) and overexpressing insulin receptor (InR) in sNPFR1 neurons (sNPFR1>\text{InR}^\text{WT}), while sNPF mRNA and food intake increased when InR was suppressed in sNPFR1 neurons (sNPFR1>\text{InR}^\text{DN}). (F) Fasting (at 24 h) reduces Dilp3, and Dilp 5 mRNA but not Dilp2 mRNA. Data are presented as means ± s.e.m. from three independent experiments. *P<0.05 (One-way ANOVA analysis).

[Data and figures from the paper]
Figure 6. hDyrk1a transgenic mice regulate food intake through the FOXO-NPY pathway. (A, C) In the hypothalamus of hDyrk1a transgenic mice, Dyrk1a was increased and FoxO1 acetylation was reduced compared with those of the littermate control mice. (B) NPY mRNA from hypothalamus and serum NPY were increased in hDyrk1a transgenic mice. (D) Daily food intake was increased in hDyrk1a transgenic mice compared with the littermate controls. (E) Average food intake of hDyrk1a transgenic mice increased by 15%. Data are presented as means ± s.e.m. from three independent experiments. *P<0.05 (One-way ANOVA analysis). (F) The model of this study.

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Quantitative RT–PCR Analysis

Drosophila Starvation

Twenty 3-week-old male Drosophila flies were starved overnight and fed for 2 h for the physiological synchronization. Then, starvation for the experiment was started. The heads from starved flies were collected for the Quantitative RT-PCR analysis. The experiments were repeated three times.

Measurement of Drosophila Body Weight

Eggs laid by five female flies for 6 h at 25°C were cultured to avoid overcrowding and lack of nutrition. For weight of individual fly, over 50 three-day-old adult male flies were measured with the balancer (METTLER Aj100) and divided with the number of flies. At least three experiments were performed in each assay.

Quantitative RT–PCR Analysis

Adult heads from 20 flies were collected for RNA preparation. Total RNA was extracted using the easy-BLUE (TM) reagent (iNtRON biotechnology). All RNA samples were treated with RNase-free DNase (Promega). cDNA was synthesized using a SuperScript III First-Strand Synthesis System (Invitrogen). For quantitative RT-PCR analysis, ABI Prism 7900 Sequence Detection System (Applied Biosystems) and SyberGreen PCR Core reagents (Applied Biosystems) were used. mRNA levels were expressed as the relative fold change against the normalized rp49 mRNA. The comparative cycle threshold (Ct) method (User Bulletin 2, Applied Biosystems) was used to analyze the data. All experiments were repeated at least three times. The statistical significance was tested by Microsoft Excel-based application for the student t-test statistical analysis. Primers used in the RT-PCR analyses were listed in Table S2.

Generation of the Minibrain and sNPFR1 Antiserum and Immunostaining in the Adult Brain of Drosophila

Minibrain antiserum was generated by the immunization of rabbits with the synthetic peptide (CQHRVRFNVPTNGNQ) corresponding to the N-terminal sequence (75–88) of the Minibrain-H protein. Antiserum against sNPFR1 was generated by the immunization of rat with the synthetic peptide (GEAI-GAGGAEGLRRIN) corresponding to the C-terminal sequence (585–600) of the sNPFR1 protein. For immunostaining, adult brain from newly eclosed flies (3 day old) was dissected in PBS, fixed in 4% paraformaldehyde, and blocked in 5% BSA and 5% normal goat serum. Primary antibodies were incubated two days at 4°C and secondary antibodies were incubated for 2 h at room temperature. The tissues were mounted in the DABCO solution (70% glycerol, 2.5% DABCO, Sigma, St. Louis, MO) and fluorescence images were acquired by Fluoview confocal microscope (Olympus). sNPF (1:200), sNPFR1 (1:200), and Minibrain (1:200) primary antibodies, and anti-rat IgG Alexa 488, anti-rabbit IgG Alexa 488, or Alexa 594 (1:200, Molecular Probes) and anti-guinea pig Cy5 (1:200, Jackson ImmunoResearch) secondary antibodies were used.

Western Blot Analysis

The cells were lysed by the Lysis buffer (Cell signaling) containing NaF, PMSF and Na3VO4. Total cell lysates were immunoprecipitated with Sirt1 antibody (Cell signaling) and protein A-agarose (Pierce). The immunoprecipitates were washed three times with Lysis buffer and solubilized in the SDS sample buffer (63.5 mM Tris-HCl; pH 6.8, 2% w/v SDS, 10% glycerol, 50 mM DTT, 0.01% w/v bromphenol blue). Western blot analyses were performed as described previously [2]. Phospho-CREB, phospho-Threonine, FoxO1 (1:1000, Cell signaling), Ac-FKHR (1:1000, Santa Cruz), β-actin (1:3000, Abcam) primary antibodies, and horseradish peroxidase-conjugated anti-rabbit IgG (1:5000, Santa Cruz) and anti-mouse IgG secondary antibody (1:5000, Sigma) were used.

cAMP Assay

Intracellular cAMP was measured with the cAMP Biotrak Enzyme Immunoassay Kit (GE Healthcare) by the manufacturer’s instruction. Briefly, samples were incubated with anti-cAMP antibody, which was immobilized in the secondary antibody coated micro-plates. Following enzyme substrate conversion, an optical density was measured at 450 nm with microplate reader (Fluostar Optima, BMG labtechn). cAMP concentration was expressed as the cAMP pM per mg of protein and converted to the fold change relative to the basal control value.

ChIP-on-chip and ChIP–PCR Analysis

About 250 of 3-day-old W[Dahi] female flies were collected after 12 h starvation. Then, flies were homogenized and cross-linked in 1X PBS containing 1% formaldehyde. The ChIP protocol was performed as described in Telem et al. [56]. Immunoprecipitation was performed using Dynal protein G beads (Invitrogen) and anti-dFOXO antibody (a gift from Heather Broihier). Purified DNA was amplified and labeled following Affymetrix ChIP Assay Protocol. Drosophila Tiling 2.0R Array was used to detect dFOXO binding enrichment. For ChIP-PCR analysis, about 10^6 of BG2-c6 cells were treated with sNPFP2 peptide as described above. sNPFE-treated and untreated cells were cross-linked with 1% formaldehyde. After immunoprecipitation with the CREB antibody (Cell signaling) and Protein A Sepharose CL-4B (GE Healthcare), quantitative RT-PCR analysis was performed using input DNA and immunoprecipitated DNA for the CREB binding site in the mnb promoter region and the 3rd axon of Actin5C.

Statistics

Values in the paper are presented as means ± s.e.m. Statistical significant of all data were evaluated by the One-way ANOVA test.
(GraphPad Prism software). P<0.05 was accepted as statistically significant.

Supporting Information

Figure S1  (A) mnb mRNA expression levels of Figure 1A and UAS controls. (B) Relative food consumption of Figure 1B and UAS controls. (C) Relative food consumption of Figure 3C, Figure 4E, and UAS controls.

Figure S2  sNPFR1-Gal4 expression was detected by sNPFR1-Gal4>UAS-DsRed (sNPFR1>DsRed) in the fly adult brain. (A-D) In the anterior focal planes, sNPFR1>DsRed was detected in the optic lobes (OL, A), insulin producing cells (IPCs, C), mushroom body (MB, B), and subesophageal ganglia (SOG, D). (E, F) In the posterior focal planes, sNPFR1>DsRed was detected in the median neurons above esophagus (E, dot box; F, asterisk). Scale bars are 100 μm (A, E), 50 μm (B, D, F) and 30 μm (C).

Figure S3  (A) Western blots with the Mnb antibody in the sNPFR1-Gal4 control, sNPFR1 overexpression (sNPFR1>2xUAS), sNPFR1G1767 mutant, sNPFR1-Gal4 control, sNPFR1 overexpression (sNPFR1>2xUAS), and sNPFR1 inhibition (sNPFR1>2xUAS). (B-F) Numbers of strong Mnb expression neurons (asterisks) are similar in the sNPFR1-Gal4 control, sNPFR1 overexpression (sNPFR1>2xUAS), sNPFR1 inhibition (sNPFR1>2xUAS), and sNPFR1G1767 mutant. Scale bars are 100 μm.

Figure S4  (A) The mnb genomic organization. Open boxes represent exons, the triangle shows the p-element insertion site in mnb and an arrow indicates the transcriptional initiation of the mnb isoform containing the longest coding sequence among mnb isoforms. mnb deletion mutants (mnb and mnbH) were generated by imprecise excisions of the inserted p-element. (B) mnb mRNA expression levels in the sNPFR1-Gal4 control, sNPFR1 overexpression (sNPFR1>2xUAS), sNPFR1 inhibition (sNPFR1>2xUAS), and mnbG1767 mutant. (C) Western blot with the Mnb antibody in the mnb control and mnbG1767 mutant. (D) mnb overexpression (sNPFR1>2xUAS) increased the body weight compared with the sNPFR1-Gal4 control whereas mnb suppression (sNPFR1>2xUAS, mnbG1767) decreased the body weight. (E) Amount of food intake by the normalized to body mass and to the number of flies. Data are presented as means ± s.e.m. from three independent experiments. *P<0.05.

Figure S5  Adult specific food intake assay using the tubGal80ts inducible system. (A) In the 22°C permissive temperature condition in which tubGal80ts suppress sNPFR1-Gal4 expression, mnb overexpression (sNPFR1-Gal4+tubGal80ts>mnb, sNPFR1-Gal4+tubGal80ts>2Xmnb) and mnb inhibition (sNPFR1-Gal4+tubGal80ts>mnb-Ri) flies did not change the amount of food intake compared with the control flies (sNPFR1-Gal4+tubGal80ts). (B) In the 30°C restrictive temperature in which tubGal80ts cannot suppress sNPFR1-Gal4, the mnb overexpression increased food intake compared with the control and the mnb inhibition suppressed food intake. Data are presented as means ± s.e.m. from three independent experiments. *P<0.05.

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Author Contributions

Conceived and designed the experiments: S-HH K-SL S-JK MT KY. Performed the experiments: S-HH K-SL S-JK A-KK HB M-SJ. Analyzed the data: S-HH K-SL S-JK O-YK W-JS KY. Wrote the paper: S-HH K-SL MT KY.
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