Hypoxia-Inducible Factor Directs POMC Gene to Mediate Hypothalamic Nutrient Sensing and Energy Balance Regulation

By

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Hypoxia-inducible factor (HIF) is a transcription factor that responds to environmental and pathological hypoxia to induce metabolic adaptation, vascular growth, and cell survival. Here we found that HIF subunits and HIF2α in particular were normally expressed in the mediobasal hypothalamus of mice. Hypothalamic HIF was up-regulated by glucose to mediate the feeding control of hypothalamic glucose sensing. Two underlying molecular pathways were identified, including suppression of PHDs by glucose metabolites to prevent HIF2α degradation and the recruitment of AMPK and mTOR/S6K to regulate HIF2α protein synthesis. Amino acid leucine and hormone insulin also activate HIF2α on protein levels through up-regulating mTOR/S6K pathway.

HIF activation was found to directly control the transcription of POMC gene. Genetic approach was then employed to develop conditional knockout mice with HIF deletion in POMC neurons. HIF loss-of-function in POMC neurons impaired hypothalamic glucose sensing, leucine sensing and insulin sensing and in turn caused energy imbalance to promote development of obesity development. The metabolic effects of HIF in hypothalamic POMC neurons were independent of leptin signaling or pituitary ACTH pathway. Hypothalamic gene delivery of HIF counteracted overeating and obesity under conditions of nutritional excess. In conclusion, HIF controls hypothalamic POMC gene to direct the central nutrient sensing in regulation of energy and body weight balance.
CHAPTER 1. BACKGROUND AND SIGNIFICANCE

1. Energy homeostasis regulation by hypothalamus

1.1 Overview of Energy homeostasis regulation by hypothalamus

The energy homeostasis of mammals relies on the precise balance of food intake and energy expenditure. A series of experiments showed that hypothalamus was a key component which was revealed by ablation studies that targeted different regions of the hypothalamus (1). For example, ventromedial ablation of hypothalamus caused hyperphagia and obesity while lesion of the lateral hypothalamus resulted in hypophagia and weight loss. Parabiosis experiments (connecting of the circulation system of two animals by surgery) showed that circulating substances can regulate food intake and hypothalamus senses the cues (2;3). However, only recently, research has provided insights onto the molecules that are involved in energy homeostasis in the circulation. The mechanism can be divided into two categories, hormone- and nutrient-sensing pathways.

1.2 Hormonal regulation of energy homeostasis.

The hormonal regulators come from at least three parts of the peripheral organs, white adipocytes, the gastrointestinal tract and the pancreas. The most well studied hormone for hypothalamic regulation of energy balance is leptin. Leptin was secreted by white adipose tissue and its amount is in proportion of the fat mass, thus it serves perfectly as a indicator of the energy level of the body (4). The leptin receptors are mainly expressed in the hypothalamus and immune cells (5). It has a strong anorexic effect through its target neurons in the hypothalamus and loss of function mutant in
mice which showed severe obesity (6). Leptin responsive neurons are mainly located in the arcuate nucleus (ARC) in the medial basal region of the hypothalamus (7). There are two major types of leptin-responsive neurons in ARC, POMC neurons and AgRP neurons (see Fig. 1-1). The POMC neurons express a precursor peptide pro-opiomelanocortin (POMC) and the peptide cocaine and amphetamine-related transcript (CART) and both of the peptides are anorexigenic (appetite inhibiting). The AgRP neurons express Agouti-related peptide (AgRP) and Neuropeptide Y (NPY), which are both orexigenic (appetite stimulating) (8). Leptin excites POMC neurons and up-regulates the expression of POMC mRNA by JAK2/STAT3 pathway (9). Meanwhile, leptin inhibits AgRP neurons and down-regulates the expression of AgRP mRNA (10). Therefore, leptin has anorexigenic effects through both POMC and AgRP neurons. Another key related hormone in hypothalamic control of energy balance is insulin. Similar to leptin, insulin inhibits AgRP neurons but activates POMC neurons, leading to food intake reduction and body weight control (11;12).
Fig. 1-1. Anatomical and cellular structure of the arcuate nucleus.

In the mouse brain, arcuate nucleus (ARC) is located in the medial basal region of the hypothalamus. The leptin/insulin-sensitive populations of cells in the ARC are POMC and AgRP neurons. Both neural types respond to leptin and insulin in synergistical manner to control food intake and energy expenditure and maintain body weight balance.

1.3 Nutritional regulation of energy homeostasis.

In the ARC, both POMC and AgRP neurons sense changing levels of glucose (13). Glucose excites POMC neurons but inhibits AgRP neurons (14). One model of how POMC and AgRP neurons sense glucose is that these neurons employ the glucose-sensing mechanism of pancreatic β cells. Indeed, the ARC expresses the same form of glucokinase as pancreatic β cells do (15;16). Thus, just like in the β cells of the pancreas, glucokinase may play a major role in the glucose-sensing process of
these neurons. Another hypothalamic glucose sensor is AMP-activated protein kinase (AMPK). In general, AMPK is known as a sensor of energy insufficiency. When the energy levels in cells drop, the AMP/ATP ratio increases and thus activates AMPK (17). In the mediobasal hypothalamus, activation of AMPK increases food intake and body weight, conversely, inhibition of AMPK has the opposite effects (18).

Unlike glucose, amino acids seems to employ a different pathway – the mTOR pathway, to modulate hypothalamic control of energy balance (19). Among various amino acids, L-leucine has been shown to exert the strongest effects in activating mTOR pathway and therefore inhibiting food intake (20;21). For example, immediate hypothalamic administration of L-leucine can instantly decrease food intake and this effect can be abolished by intra-brain injection of mTOR inhibitors. Interestingly, mTOR was reported to be inhibited by AMPK(22), thus interaction between hypothalamic mTOR and AMPK may exist.

The mechanism of fatty acid-sensing process in the hypothalamus may be particularly related to the fatty acid metabolites in target cells. When the lipid levels in the blood stream are elevated, the long-chain fatty acid (LCFA) concentration in the hypothalamic cells increases. As a result, though the metabolic process, the levels of LCFA-CoA increase, which can inhibit food intake and glucose production(23).

Altogether the hypothalamus can use multiple signaling pathways to sense different nutrients. One major question is whether there is a universal sensor of nutrients that can sense all or most nutrients. Some evidence suggests that it might be possible. For example, one group recently proposed that mitochondrial reactive
oxygen species (ROS) might be necessary for both glucose and lipid sensing in the hypothalamus (24;25). This indicates that ROS could serve as a common readout of various nutrients and a pathway that can react to ROS could be a universal sensor of all the nutrients in the hypothalamus.

2. Hypoxia-inducible factor (HIF)

2.1. Functions of HIF

HIF genes were first discovered in research of studying how the EPO (erythropoietin) gene is up-regulated by hypoxia (26;27). In this endeavor, a transcription factor hypoxia-inducible factor 1 (HIF1) was identified to bind to a region in the 3' untranslated portion of the Epo gene (26;28;29).

There are three HIFα subunits, HIF1α, HIF2α and HIF3α. All of these isoforms have the property of sensing O₂ concentration (30-36). In structure, HIF2α and HIF1α are most similar. They also share the same binding sequence on DNA and mechanism of degradation regulation (34;35;37-39). HIF3α was recently discovered to act as a transcription inhibitor (40;41) but because its biological function and target genes are unclear, our research interest will focus on HIF1α and HIF2α. The classic target genes of HIF are hypoxia-responsive genes which have been shown to participate in the biological processes, such as angiogenesis, cell invasion and metastasis (42). More recent research has shown that HIF also regulates genes in other aspects including metabolism, cell proliferation and differentiation (43-48). For example, HIF regulates the transcription of many glycolytic genes, such as the genes that encode phosphoglycerate kinase (PDK) and lactate dehydrogenase A (LGHA) in the
peripheral tissues. This research advance suggests that HIF may also play a role in the hypothalamic regulation of energy homeostasis.

2.2. Regulations of HIF

HIF is a heterodimeric complex consisting of an α subunit (HIFα) and a β subunit (HIFβ, also called aryl hydrocarbon receptor nuclear translocator (ARNT)) (49;50). The activity of HIF-1 is mainly regulated by posttranslational modification(51). The stability of HIF1α is controlled by a region that is known as the oxygen-dependent degradation domain (ODD)(52). At normal oxygen levels (normoxia), HIF1α is hydroxylated at two proline residues, pro402 and pro564, by a family of prolyl hydroxylase domain (PhD) proteins(53;54). The hydroxylation causes the conformation change of HIF1α and the binding of von Hippel-Lindau tumor suppressor (VHL), which is a component of an ubiquitin ligase complex. The binding of VHL leads to the ubiquitinylation and degradation of HIF1α. Therefore, under normoxia condition, HIF1α undergoes constant degradation and the activity of HIF1 remains low(55-57). The activation of prolyl hydroxylase relies on the concentration of O2. Under the condition of hypoxia, the prolyl hydroxylases are inhibited and HIF1α is, thus stabilized (see Fig. 2)(58;59). Recently, more and more evidences demonstrate that other molecules can also activate HIF by inhibiting the prolyl hydroxylases. Among them are ROS and a number of metabolites - succinate, fumarate, citrate, isocitrate, malate, oxaloacetate, and pyruvate(60-66). Considering that ROS is generated by the electron transport chain during aerobic respiration and those metabolites are mainly the members of the Krebs cycle, nutrients including
glucose, fatty acid and amino acid could generate these chemicals during their metabolic processes. This indicates that HIF has the potential to be a common sensor of multiple nutrients.

**Fig. 1-2. Regulation of HIFα through its protein levels**

Under normal conditions, HIFα is constantly hydroxylated and degraded. Hypoxia, metabolites and ROS activate HIFα by inhibiting its hydroxylase leading to protein stabilization of HIFα. mTOR pathway, on the other hand, directly enhances the translation of HIFα mRNA and activates HIF by increasing its protein production.

**2.3. HIF and nutrient sensing**

Besides the regulation of ROS and metabolites which suggests that HIF may serve as a multinutrients-sensor, HIF also was regulated directly and indirectly by classic nutrient sensing pathways. Several lines of research in the cancer systems showed that mTOR pathway directly regulates HIF though upregulating its protein
synthesis at translational level (22;67;68). mTOR is well known to play an important role in general nutrient and energy sensing and in turn function (69) as a master regulator of protein translation by its downstream S6 kinase and eIF-4E signals (69-71). More specifically mTOR pathway is strongly activated directly by one of the amino acids – leucine. Therefore mTOR is often considered one of the main pathways for amino acid sensing (72). However, it is also activated by other energy sources. For example, glucose can activate mTOR pathway at least through inhibiting AMPK signals (71). Therefore, HIF could in turn response to amino acid and glucose availability through regulation by mTOR signals.

Compared to mTOR pathway, AMPK is even better known as the energy gauge. When cellular energy level is low, intracellular AMP:ATP ratio increases which leads to allosteric activation of AMPK. Glucose replenish ATP levels through its catabolic metabolism, decreases AMP:ATP ratio and inhibit AMPK (73). AMPK also negatively regulate HIFα through mTOR (68;74). Therefore, both major nutrient sensing pathways – mTOR and AMPK regulate HIF activity. Considering metabolic byproducts – metabolites and ROS can activate HIF as well, HIF may serve as a multiple nutrient sensor.
CHAPTER 2. MATERIALS AND METHODS

2.1 Animal models and metabolic phenotyping

HIFβ\textsuperscript{lox/lox} mice (75) and POMC-Cre mice (48) were maintained on the C57BL/6 strain background. ROSA-flox-STOP-flox-YFP mice were from Jackson Laboratory. All the mice were housed in standard conditions. High-fat diet (58.5 Kcal\% fat) was purchased from Research Diets, Inc. Mice were measured for body weight and food intake on either a daily or regular basis. DEXA scanning was performed using the DEXA scanner at the Primate Center at the University of Wisconsin. The physiological markers of energy expenditure, including \( \text{O}_2 \) consumption and \( \text{CO}_2 \) production, were measured using the metabolic chambers (Columbus Instrument, Inc.) at the DRTC core at Albert Einstein College of Medicine. The Institutional Animal Care and Use Committee approved all the procedures.

2.2 Third ventricle cannulation, chemical infusion, and injection

As previously described (76), an ultra-precise small animal stereotactic apparatus (10-mm resolution, David Kopf Instrument) was used to implant a guide cannula into the third ventricle of anesthetized mice at the midline coordinates of 1.8 mm posterior to the bregma and 5.0 mm below the bregma. The mice were allowed 2 wk for surgical recovery. Angiotensin II-stimulated drink response was used to verify success of implantation. Individual mice were restrained in a mouse restrainer, and infused with an indicated reagent over the indicated time period a 26-gauge guide cannula and a 33-gauge injector (Plastics One) connected to a Hamilton Syringe and
an infusion pump (Harvard Apparatus). Glucose, leucine, diethyl fumarate ester, and diethyl succinate ester were from Sigma. Nutrient/chemical infusion experiments for terminal molecular assays: glucose (10 nmol/min), diethyl fumarate (50 nmol/min), diethyl succinate (50 nmol/min) and leucine (10 nmol/min) were infused over 2 to 8 h. Rapamycin, AICAR and insulin injections were, respectively, 50 ng, 15 mg and 50 mU per injection (at hour 0, 2, and 4 during the 5-h experimental period). The injection experiments for acute physiological tests used a single injection of glucose (40 nmol), diethyl fumarate ester (0.2 mmol), diethyl succinate ester (0.2 mmol), AICAR (3 mg), leucine (10 nmol) and insulin (4 mU) in a 2 ml vehicle over 5 min.

2.3 Chemicals, plasmids, and recombinant lentiviral vectors

Glucose, leucine and rapamycin were from Sigma, and AICAR from Toronto Research Chemicals. Artificial cerebrospinal fluid (aCSF) was used as the vehicle for glucose, leucine, insulin, leptin, and AICAR. Full-length cDNAs for HIF1α, HIF2α, and HIFβ, provided by D. Peet, were sub-cloned into pcDNA3.1 plasmids (Invitrogen). DNA for POMC promoter (provided by S. Melmed) was sub-cloned to pGL3 (Promega). Mutant POMC promoter was generated by deleting 59-GCGTG-39 in the WT version of POMC promoter. For the lentivirus that co-expresses HIF subunits, we introduced the encoding cDNA of HIF1α or HIF2α and HIFβ into the lentiviral vector Lox-Syn-Syn (provided by G. Francisco) in which two synapsin promoters control neuron-specific coexpression of two inserts. For lentivirus that directs expression of single gene, we sub-cloned PCR fragment of dominant-negative
HIF2α (HIF2α amino acids 1–485) (77;78) or constitutively active Rheb (CARheb) (provided by J. Avruch) into pLenti6/V5 vector (Invitrogen).

2.4 Viruses, injection, and verification

The lentiviruses were produced from HEK293T cells through co-transfecting the target plasmid with two package plasmids (VSVg and delta 8.9) using Ca3(PO4)2. Lentiviruses were purified by ultracentrifugation. Ultracentrifuge purified lentivirus in 0.2 μl aCSF was injected over 10 min through a 26-gauge guide cannula and a 33-gauge injector (Plastics One) connected to a Hamilton Syringe and an infusion pump (WPI Instruments). As previously described (79), bilateral injections to the mediobasal hypothalamus were directed using an ultra-precise stereotax with 10-mm resolution (Kopf Instruments) to the coordinates of 1.5 mm posterior to the bregma, 5.8 mm below the bregma, and 0.3 mm lateral to midline.

2.5 Western blots and histology

As we previously described (79), the hypothalamus was dissected along the anterior border of the optic chiasm, posterior border of the mammillary body, upper border of the anterior commissure, and lateral border halfway from the lateral sulcus in the ventral side of brain. Animal tissues were homogenized, the proteins dissolved in a lysis buffer, and Western blot was performed as previously described (79-81). Proteins dissolved in a lysis buffer were separated by SDS/PAGE and identified by immunoblotting or immunoprecipitation followed by immunoblotting. Primary
antibodies included anti-HIF1α, anti-HIF2α, and anti-PHD2 (Novus Biologicals); anti-p300 (Santa Cruz); and anti-VHL, anti-pS6K, anti-S6K, anti-pAMPKa, anti-AMPKa, anti-pAkt, anti-Akt, and anti-β-actin (Cell Signaling). Secondary antibodies included HRP-conjugated anti-rabbit and anti-mouse antibodies (Pierce). Western blots were quantified using NIH Image J software. Tissue histology: Various tissues were removed from mice and fixed in Bouin’s solution (Sigma). Parafilm sections were prepared, stained with H&E, and examined under a bright field microscope.

2.6 GHO assay and biochemical measurements

PHD activity was determined using the GHO assay as established in the literature (82). Briefly, a wheat germ in vitro transcription-translation (IVTT) system (Promega) was used to produce unhydroxylated HA-tagged GHO protein (substrate of PHDs). Hydroxylation of GHO protein was performed by incubation with hypothalamic protein lysates (dissolved in HEB buffer) at 37°C for 15 min in the presence of 1 mM ascorbate and 100 mM FeSO₄ (Sigma). The reaction was terminated by adding SDS loading buffer, and hydroxylated versus unhydroxylated GHO protein was separated by PAGE gels and detected by Western blot analysis of HA tag. Fumarate and succinate were measured using Fumarate Assay Kit (BioVison) and Succinic Acid Kit (Megazyme).

2.7 Cell culture and luciferase assay
HEK 293 and HEK 293T (ATCC) were maintained in DMEM with 5%–10% FBS, glutamate, antibiotics, and in 5%–10% CO₂ at 37°C. Transfection of cultured cells with luciferase plasmids and expression plasmids was performed through Lipofectamine 2000 (Invitrogen). Co-transfection of pRL-TK (Promega) was used to internally control firefly activity. Empty plasmids pGL3 and pcDNA3.1 were used as negative controls.

2.8 Quantitative RT-PCR

We extracted total RNA from the homogenized hypothalamus using TRIzol (Invitrogen). Complementary DNA was synthesized using the M-MLV RT system (Promega). PCR amplification was quantified using SYBRHGreen PCR Master Mix (Applied Biosystems). Results were normalized against the expression of house-keeping genes including TATA box-binding protein (TBP) and GAPDH.

2.9 Heart perfusion, immunostaining, and imaging

Mice under anesthesia were trans-heart perfused with 4% PFA, and the brains were removed, post-fixed in 4% PFA for 4 h, and infiltrated in 20%–30% sucrose. Brain sections (20-mm thickness) were made using a cryostat at -20°C. For cell culture, cells were cultured in coverslips and fixed using 4% PFA. Fixed tissues or cells were blocked with serum of appropriate species, penetrated with 0.2% Triton-X 100, treated with primary antibodies including rabbit anti-HIF1α, anti-HIF2α (Novus Biologicals), anti-HIFβ (Cell Signaling), mouse anti-HuCD (Molecular Probes), and
subsequently followed by a fluorescent reaction with Alexa Fluor 488 or 555 secondary antibody (Invitrogen). Naive IgGs of the appropriate species were used as negative controls. DAPI staining was used to reveal all the cells in the slides. A confocal microscope was used to image fluorescence.

2.10 Behavioral tests and treatments

Fasting induced food intake. The individually housed mice were measured for food intake. One hour before the designated “night time”, a reagent (nutrient species) dissolved in 1–2 ml of vehicle or the empty vehicle (control) was injected to fasted mice through third ventricle cannula that were pre-implanted 1 to 2 wk prior to the commencing of experiments. Food was then returned to the mice immediately before the designated “night time”. Food intake for each mouse was measured for the following periods as indicated. Hypoxia treatment: Animal was maintained in a hypoxia chamber supplied with 8% O₂ and 92% nitrogen for 6 h as established in the literature (83). Taste aversion test: C57BL/6 mice were habituated to experimental protocol for several days and then presented to 0.2% saccharine for 60 min after removing the drinking water for 23 h. After habitation, mice were ICV injected with an indicated drug and the vehicle via preimplanted third ventricle cannula, and subsequently had access to 0.2% saccharine for 60 min (Test 1). Intraperitoneal (IP) injection of LiCl and saline was used as a positive and negative control, respectively. After 4 d, mice were presented with 0.2% saccharine, and saccharine intake of mice during 60 min was measured (Test 2)
2.11 Statistical analyses

ANOVA and Turkey’s post hoc analyses were used for comparisons, which involved more than two groups. Kolmogorov-Smirnov (KS) test was applied to each dataset to determine the appropriate statistical test (e.g., parametric or nonparametric) for analysis of each set of data. Two-tailed Student’s t tests were used for comparisons, which involved only two groups. Software for performing statistics included Excel, GraphPad Instat 3, and Prism. Data were presented as mean 6 SEM. p,0.05 was considered significant.
3.1 Abstract

Hypoxia-inducible factor (HIF) is transcription factor in the nuclear that responds to hypoxia to induce metabolic changes, vascular growth, and cell survival. Here we found that HIF subunits, HIF2α in particular, were normally expressed in the mediobasal hypothalamus of mice. Hypothalamic HIFα was up-regulated by glucose to mediate the control of feeding by hypothalamic glucose sensing. Two underlying molecular pathways were identified, including inhibition of PHDs by glucose metabolites to prevent HIF2α degradation and the recruitment of AMPK and mTOR/S6K to regulate HIF2α protein synthesis. HIF activation directly controls the transcription of POMC gene. Genetic approach was employed to develop conditional knockout mice with HIF deletion in POMC neurons, revealing that HIF loss-of-function in POMC neurons impaired glucose sensing in hypothalamus and caused energy imbalance to promote obesity development. The metabolic effects of HIF in hypothalamic POMC neurons were independent of leptin signaling or pituitary ACTH pathway. Hypothalamic delivery of the gene of HIF counteracted overeating and obesity under conditions of overnutritional. In conclusion, HIF controls hypothalamic POMC gene to direct the central glucose sensing in regulation of energy and body weight balance.

3.2 Introduction

Hypoxia-inducible factor (HIF) is the central transcription factor that is induced under environmental (e.g., high altitude) and pathological (e.g., cancer) hypoxia
HIF exists as the heterodimer of an α subunit and a β subunit; HIFα protein levels are regulated by tissue oxygen availability, while HIFβ (also called ARNT, aryl hydrocarbon receptor nuclear translocator) is constitutively present (86-89). Under normoxia, HIFα undergoes protein hydroxylation, and ubiquitination-dependent degradation, and this process is mediated by prolyl hydroxylases (PHDs) and E3 ligase pVHL coded by von Hippel-Lindau (VHL) gene (90-93). Under hypoxia, PHDs are suppressed, leading to stabilization of HIFα proteins and thus the transcriptional action of HIFα/β to induce genes that classically regulate metabolic adaptation, vascular growth, and cell survival (94-97). Among three HIFα isoforms (HIF1α, HIF2α and HIF3α), HIF1α and HIF2α have been most extensively studied in the literature (98-101). While both HIF1α and HIF2α mediate hypoxia adaptation, HIF2α can control a distinct set of genes (102;103). Consistently, the biological consequences of HIF2α versus HIF1α deletion in mice are different (104-106), suggesting that HIF2α and HIF1α may have divergent physiological functions.

Recent research has shown that regulation of HIF1/2α by hypoxia involves metabolic mediators, such as ROS that can modulate mitochondrial complex III (107-113). HIF is also subjected to normoxic regulation, and the underlying mechanism has been related to several metabolic signaling pathways including the PI3K-mTOR cascade (114-116) and SIRT1 (117). The biochemical regulation of HIF by metabolic signals has been implicated to underlie the metabolic programming of tumorigenesis (118-121). It remained unexplored, however, whether a reverse relationship might exist, i.e. if HIF could be a primary regulator of metabolic
physiology at an organism level and hence a target for controlling metabolic disease, and if so, what could be the responsible tissue/cell types and the underlying molecular basis.

The hypothalamus is the master regulator of food intake, energy expenditure, and body weight balance (122-126). The responsible neuronal regulation involves not only hormonal sensing by molecules such as insulin and leptin (124;127-130) but also nutrient sensing by molecules such as glucose, amino acid, and fatty acids (131-141). Compared to the long-term homeostatic regulation of body weight by hypothalamic hormonal signaling, hypothalamic glucose sensing can be rapid and predicted to provide an acute and real-time regulation on metabolic homeostasis.

Proopiomelanocortin (POMC)-expressing neurons, termed POMC neurons, have been identified to be account for hypothalamic glucose sensing (142;143). In this chapter, we demonstrate that hypothalamic glucose sensing is mediated by HIF activation and resulting up-regulation of $POMC$ gene and that loss-of-function of HIF in POMC neurons causes glucose desensitization and promotes energy imbalance and obesity development.

3.3 Results

3.3.1 HIF Controls POMC Gene Transcription

In order to screen for transcription factors that control hypothalamic neuropeptide genes, we analyzed the DNA sequence of $POMC$ promoter and identified a HIF-binding element at the proximal promoter region across species from rodents to
humans. POMC peptide is the precursor of hypothalamic neuropeptide, 
\( \alpha \)-melanocyte-stimulating hormone \((\alpha\text{-MSH})\), which is an important hypothalamic regulator of feeding and energy balance, and mutation of \( POMC \) gene is sufficient to cause severe obesity and diabetes in both rodents \((144)\) and humans \((145;146)\). Notably, the HIF-binding element \((5发出-GCGTG-3发出)\) is located immediately upstream of the transcriptional initiation site \((\text{TATA box})\) in the \( POMC \) promoter \((\text{Figure 3-1A})\). In contrast, the binding elements for STAT3, the most established nuclear transcription factor for \( POMC \) gene in leptin signaling \((147-149)\), are located further upstream. Using a reporter system, we found that overexpression of HIF\(1\alpha\) and HIF\(2\alpha\) increased the activities of \( POMC \) promoter by 12 folds and 26 folds, respectively \((\text{Figure 3-1B})\). When HIF\(1\alpha\) or HIF\(2\alpha\) was co-overexpressed with HIF\(\beta\), the heterodimeric complex activated \( POMC \) promoter by \(362-466\)-folds \((\text{Figure 3-1B})\). On the other hand, deletion of the 5-bp HIF-binding element \((5发出-GCGTG-3发出)\) substantially prevented HIF from activating the mutant \( POMC \) promoter \((\text{Figure 3-1B})\). All these data suggest that \( POMC \) represents a HIF target gene.
Figure 3-1. Regulation of HIF on POMC gene and HIF profile in the hypothalamus. (A) Analysis of the proximal region of rat POMC promoter. Putative HIF-binding DNA element, STAT3-binding DNA element, TATA box, and initial site of mRNA transcription are indicated. (B) HEK 293 cells were transfected
with firefly luciferase pGL3 controlled by either wildtype (WT) or mutant (Mu) rat POMC promoter, and control renilla luciferase pRL-TK, together with the indicated combination of pcDNA-HIF1α, pcDNA-HIF2α, pcDNA-HIFβ, and pcDNA. Mutant POMC promoter was generated by deleting 5’-GCGTG-3’ in the WT version of POMC promoter. ** p < 0.01, *** p < 0.001; n = 4 per group. Error bars reflect mean ± SEM. (C) HIF2α and HIF1α expression levels in different brain areas of C57BL/6 wildtype mice were analyzed using Western blotting. β-actin (β-act) was used as loading control. PN, pons; CB, cerebellum; OF, olfactory bulb; CR, cortex; TH, thalamus; HP, hypothalamus. (D) Brain sections of the mediobasal hypothalamus were prepared from regular C57BL/6 wildtype mice and immunostained with HIF2α antibody (upper panel) or HIF1α antibody (lower panel). HIF2α or HIF1α (green) was co-immunostained with neuronal marker HuCD (red). Merged images (yellow/orange) revealed neuronal expression of HIF2α or HIF1α protein. 3V, third ventricle; ARC, arcuate nucleus. Bar = 50 µm.

### 3.3.2 Differential Patterns of HIF1α and HIF2α in the Hypothalamus

To explore whether there was an anatomic basis of supporting a metabolic role of hypothalamic HIF, we then profiled HIF isoform distribution in the hypothalamus as well as other brain regions. Western blot analysis of HIF2α showed high levels of protein in the hypothalamus but low levels in many other brain regions, including cortex, thalamus, olfactory bulb, pons, and cerebellum (Figure 3-1C). Unlike HIF2α, HIF1α protein expression was generally weak throughout the brain (Figure 3-1C). We
then performed immunostaining of HIF2α versus HIF1α in the brain to map HIF2α versus HIF1α of normal C57BL/6 mice. Immunostaining revealed that HIF2α was abundant in neurons of the mediobasal hypothalamus that comprises the arcuate nucleus (Figure 3-1D), but less abundant in many other brain regions (data not shown). Compared to HIF2α, HIF1α was weakly expressed in the hypothalamus (Figure 3-1D). Altogether, these data suggest that HIFα, in particular HIF2α, might be involved in hypothalamic regulation of whole-body physiology.

3.3.3 HIF Inhibition in POMC Neurons Abrogates Feeding Regulation of Glucose Sensing

POMC neurons have been known as a major hypothalamic neuronal type that mediates glucose sensing of the hypothalamus (150;151). Hence, we investigated whether HIF inactivation in POMC neurons could affect hypothalamic glucose sensing in the regulation of feeding. To test this question, we chose to delete HIFβ gene, since HIFβ is required for the DNA binding and activation of both HIF1 and HIF2 complexes (152-155). By crossing POMC-Cre mice (156) with HIFβlox/lox mice (157), we created a knockout mouse model with HIFβ gene ablated in hypothalamic POMC neurons, termed POMC/HIFβlox/lox mice. To evaluate the efficiency of HIFβ ablation in the knockout mice, we further crossed POMC/HIFβlox/lox mice with ROSA-flox-STOP-flox-YFP mice in order to visualize POMC neurons in brain sections. Using this tool, we revealed that HIFβ protein expression was disrupted in the majority (~90%) of POMC neurons in POMC/HIFβlox/lox mice (Figure 3-2A&B). The total number and morphology of hypothalamic POMC neurons were not affected
by HIFβ gene ablation (Figure 3-2C), suggesting that HIF inactivation did not impair the development and survival of POMC neurons.

POMC/HIFβ<sup>lox/lox</sup> mice were developmentally indistinguishable from littermate control HIFβ<sup>lox/lox</sup> mice. POMC/HIFβ<sup>lox/lox</sup> mice and controls at young ages (1~3 months old) had similar body weight. This knockout mouse model was then used to test whether HIFβ ablation could compromise nutrient-induced hypothalamic POMC mRNA expression. Two experimental paradigms were employed: supply of general nutrients through re-feeding after fasting and direct glucose administration through third-ventricle infusion. First, it was found that re-feeding after fasting significantly increased hypothalamic levels of POMC mRNA in control mice, but failed to do so in POMC/HIFβ<sup>lox/lox</sup> mice (Figure 3-2D). HIFβ ablation did not alter mRNA levels of other hypothalamic neuropeptides CART, AGRP, and NPY (Figure 3-2D) or hindbrain neuropeptide nesfatin-1 (Figure 3-3A). We also examined a few other transcription factors including BSX, FoxO1, and CREB, which also control neuropeptide expression. The expression levels of these genes were unchanged in the hypothalamus of POMC/HIFβ<sup>lox/lox</sup> mice compared to controls (Figure 3-3B). Second, we performed the experiment using third-ventricle glucose infusion. Similar to re-feeding, glucose infusion up-regulated hypothalamic POMC mRNA levels in control mice; however, this up-regulation was not induced in POMC/HIFβ<sup>lox/lox</sup> mice (Figure 3-2E). In summary, the data suggested that HIF mediates glucose-dependent hypothalamic POMC gene expression.

Subsequently, we investigated whether the HIFβ ablation could compromise the
feeding-suppression effect of hypothalamic glucose sensing. Following a prolonged fasting (24 h), $POMC/HIF\beta^{lox/lox}$ mice and their littermate controls received third-ventricle injection of glucose via pre-implanted cannula. Glucose suppressed fasting-induced feeding in control mice. This anorexic effect occurred rapidly within 6 h post-injection (Figure 3-2F) and lasted throughout 24h follow-up period (Figure 3-2G). In contrast, glucose-induced appetite suppression was substantially abolished in $POMC/HIF\beta^{lox/lox}$ mice (Figure 3-2F&G). Hence, HIF in POMC neurons is required for glucose-dependent hypothalamic regulation of feeding behavior.
Fig. 3-2. HIF inactivation in POMC neurons impairs glucose regulation of feeding.

(A&B) Hypothalamic sections from POMC/HIFβ<sup>lox/lox</sup> mice and control HIFβ<sup>lox/lox</sup> mice were stained with antibody against HIFβ (red). YFP (green) was introduced to these mice (via crossing these mice with ROSA-flox-STOP-flox-YFP mice) to visualize POMC neurons. DAPI staining shows nuclei of all cells in the sections. Bar
= 25 µm. Graphs: percentage of HIFβ-positive POMC neurons. *** p < 0.001; n = 3–4 per group. Error bars reflect mean ± SEM. (C) Counting analysis for the total numbers of POMC neurons across anterior, medial, and posterior arcuate nucleus of POMC/HIFβ\textsuperscript{lox/lox} mice versus control HIFβ\textsuperscript{lox/lox} mice. n = 4 per group. Error bars reflect mean ± SEM. (D) 24-h fasted young POMC/HIFβ\textsuperscript{lox/lox} mouse (P/HIFβ\textsuperscript{l/l}) and control littermate HIFβ\textsuperscript{lox/lox} mice (HIFβ\textsuperscript{l/l}) received 6h re-feeding (R) versus continued 6h fasting (F). Hypothalami were subsequently harvested for the measurement of neuropeptide mRNA levels. (E) 24h-fasted young POMC/HIFβ\textsuperscript{lox/lox} mice (P/HIFβ\textsuperscript{l/l}) and control littermate HIFβ\textsuperscript{lox/lox} mice (HIFβ\textsuperscript{l/l}) received injection of glucose (Glu) versus vehicle (Veh) via cannula pre-implanted into the third ventricle. Hypothalami were then harvested for the measurement of POMC mRNA. (F&G) Young, male POMC/HIFβ\textsuperscript{lox/lox} mice (P/HIFβ\textsuperscript{lox/lox} mice) versus littermate control HIFβ\textsuperscript{lox/lox} mice were fasted for 24 h and received third-ventricle injection of glucose or vehicle. Food was placed in cages, and mice were subsequently monitored for food intake for 6 h (F) and 24 h (G). (D–G) * p < 0.05, ** p < 0.01, NS, non-significant; n = 6–14 per group. Error bars reflect mean ± SEM.
Figure 3-3  Gene expression profiles in POMC/HIFβ^{lox/lox} mice.
Following 24h fasting, POMC/HIF\textsuperscript{β\textasciitilde}lox/lox mice (P/HIF\textsuperscript{β\textasciitilde}) and control HIF\textsuperscript{β\textasciitilde}lox/lox mice (HIF\textsuperscript{β\textasciitilde}) received 6h re-feeding (R) versus continued 6h fasting (F). Hindbrain (A) and hypothalami (B) were subsequently harvested for the measurement of mRNA levels of indicated genes. \( n = 6–8 \) per group. Error bars reflect mean ± SEM.

Following 24h fasting, C57BL/6 mice received injection of glucose versus vehicle via cannula pre-implanted into the third ventricle. Hypothalami were harvested for the measurement of HIF1\textalpha versus HIF2\textalpha mRNA levels. \( n = 5–8 \) per group. Error bars reflect mean ± SEM.

3.3.4 Absence of Pituitary Changes in POMC/HIF\textsuperscript{β\textasciitilde}lox/lox Mice

POMC cells are present in the hypothalamus as well as in the pituitary; thus, both places were targeted by the Cre-loxp technique for HIF\textbeta ablation in the knockout mice. Following the above observations in POMC/HIF\textsuperscript{β\textasciitilde}lox/lox mice, we examined whether the pituitary POMC cells were affected by HIF\textbeta ablation in the knockout mice. Because pituitary POMC is the precursor of adrenocorticotropic hormone (ACTH), we evaluated the pituitary ACTH expression via ACTH immunostaining. Data revealed that the numbers of pituitary ACTH-positive cells and ACTH expression levels were similar between POMC/HIF\textsuperscript{β\textasciitilde}lox/lox mice and littermate controls (Figure 3-4A&B). Consistently, pituitary morphology (Figure 3-4A), ACTH release (Figure 3-4C), and pituitary mass (Figure 3-4D) in the POMC/HIF\textsuperscript{β\textasciitilde}lox/lox mice were normal. Also, since the main function of ACTH is to control adrenal growth and corticosterone release, we analyzed the histology of adrenal glands from
POMC/HIFβ<sup>lox/lox</sup> mice and matched controls. The adrenal morphology and mass were comparable between POMC/HIFβ<sup>lox/lox</sup> mice and the controls (Figure 3-4E&F). In line with this profile, blood corticosterone concentrations in POMC/HIFβ<sup>lox/lox</sup> mice and controls were similar (Figure 3-4G). Altogether, these data indicated that the pituitary POMC-ACTH system was not involved in glucose-related feeding dysregulation of POMC/HIFβ<sup>lox/lox</sup> mice.
Fig. 3-4. Profile of pituitary POMC-ATCH system in POMC/HIFβ<sup>lox/lox</sup> mice.
(A–D) POMC/HIFβ<sup>lox/lox</sup> (P/HIFβ<sup>lox/lox</sup>) mice and littermate control HIFβ<sup>lox/lox</sup> mice were analyzed for pituitary ACTH immunostaining (A&B), blood ACTH concentration (C), and pituitary weight (D). (A) Pituitary sections contained anterior pituitary (AP) and posterior pituitary (PP) that had no ACTH cells and was outlined by broken lines. (B) ACTH cells were counted based on pituitary cross-sections that were cut at midline point, and data presented represent the analysis of no less than 3 mice per group. Error bars reflect mean ± SEM. (E&F) POMC/HIFβ<sup>lox/lox</sup> (P/HIFβ<sup>lox/lox</sup>) mice and littermate controls (HIFβ<sup>lox/lox</sup> mice) were analyzed for adrenal gland morphology via H&E staining (E) and adrenal gland weight (F). (G) POMC/HIFβ<sup>lox/lox</sup> (P/HIFβ<sup>lox/lox</sup>) mice and control littersmates (HIFβ<sup>lox/lox</sup> mice) mice were psychosocially stressed or left intact(158). Serum samples were collected from these mice and measured for corticosterone concentrations. n = 5–6 per group. Error bars reflect mean ± SEM.

3.3.5 Glucose Suppresses PHDs to Stabilize Hypothalamic HIF2α

The next question was: how could glucose activate hypothalamic HIF? Because mRNA levels of hypothalamic HIF2α and HIF1α were not affected by third-ventricle glucose infusion (Figure 3-3C), glucose regulation of hypothalamic HIF was not mediated via HIF mRNA levels. In contrast, third-ventricle glucose infusion significantly increased HIF2α protein levels in the hypothalamus (Figure 3-5A&B, Figure 3-6A). This effect was not significant in peripheral tissues of mice that were i.p. injected with glucose (Figure 3-6B&C). To understand the molecular basis for
glucose-induced hypothalamic HIF up-regulation, we examined if it involved PHDs-dependent HIFα hydroxylation and degradation—which is the classical molecular cascade in regulation of HIF activity (159;160). Using the GHO assay, which was established in the literature (161), we found that third-ventricle glucose delivery suppressed the hydroxylation activities of PHDs in the hypothalamus (Figure 3-5C). We also examined pVHL, an E3 ligase that mediates ubiquitination and degradation of hydroxylated HIFα. Glucose did not change protein levels of pVHL in the hypothalamus (Figure 3-5A), suggesting that pVHL was not a primary factor for glucose-induced HIFα up-regulation. Therefore, glucose employs the PHD-pVHL system to induce hypothalamic HIF up-regulation, although the magnitude of this effect was much smaller than that of hypoxia (Figure 3-5B&C). We also analyzed the binding of HIF2α to p300, since the complex functions in the nuclear to exert the transcriptional activity. As shown in Figure 3-5D, increased hypothalamic HIF2α protein levels were associated in proportion with the increased binding of HIF2α to p300, suggesting that up-regulation of hypothalamic HIF2α by glucose is transcriptionally functional.
**Fig. 3-5. Regulation of hypothalamic HIF by glucose.**

(A) 24h fasted adult C57BL/6 mice received 5-h third-ventricle infusion of glucose (Glu) or vehicle (Veh), and hypothalami were analyzed by Western blots. Bar graphs: Western blot results were normalized by β-actin (β-act) and analyzed statistically. *** $p < 0.001$, $n = 6$ per group. (B) 24h fasted regular C57BL/6 mice received third-ventricle infusion of glucose (Glu) versus vehicle (Veh). Positive controls included 6h hypoxia (Hpx) treatment. Hypothalami were then harvested for Western blot analysis of HIF2α protein levels. β-actin (β-act) was used as an internal control. Bar graphs: HIF2α protein levels were normalized by β-actin and analyzed statistically. ** $p < 0.01$; $n = 5–6$ per group. Error bars reflect mean ± SEM. (C) 24h fasted adult C57BL/6 mice received 5-h third-ventricle infusion of glucose (Glu) versus vehicle (Veh). Positive controls included matched mice that received 6h hypoxia (Hpx) treatment. Hypothalami were dissected and lysed for the measurement of PHD activity using the GHO assay. PHD activity is expressed by the ratio of hydroxylated GHO substrate ($^{\text{OH}}$Sub) to intact GHO substrate (Sub). PHD2 and β-actin levels in the lysates were also measured using Western blots. Bar graphs: quantitative analysis of PHD activities. * $p < 0.05$, ** $p < 0.01$; $n = 6$ per group. Error bars reflect mean ± SEM. (D) 24-h fasted regular C57BL/6 mice received third-ventricle infusion of glucose (Glu) versus vehicle (Veh). Hypothalami were isolated, lysed, and analyzed for co-immunoprecipitation of endogenous HIF2α and p300 using HIF2α immunoprecipitation (IP) followed by HIF2α and p300 immunoblotting (IB) detection. Protein lysates were examined by Western blots for
β-actin (β-act). Bar graphs: immunoprecipitated HIF2α and p300 were normalized by β-actin and analyzed statistically. * $p < 0.05$, ** $p < 0.01$; $n = 5–6$ per group. Error bars reflect mean ± SEM. (A–D) All mice were male C57BL/6 under normal chow feeding.
Fig. 3-6. Profiles of hypothalamic versus peripheral glucose-HIF connection.

(A) Following 24h fasting, C57BL/6 mice received third-ventricle injection of
glucose at the indicated doses. HIF2α protein levels in the hypothalamus were examined by Western blots. β-actin was used as an internal control. (B&C) Following 24h fasting, C57BL/6 mice received intraperitoneal injection of glucose (Glu) (2 g/kg body weight) or vehicle. HIF2α protein levels in the liver (B) and lung (C) tissues were examined by Western blots. β-actin was used as an internal control.

### 3.3.6 HIF Mediates Hypothalamic Glucose Sensing Without Involving Leptin Signaling

We further asked whether HIF2α might be involved in the action of leptin, a well-studied hormone that employs nuclear transcription factor STAT3 to mediate hypothalamic regulation of feeding (124;162;163). First, in contrast with the effect of glucose, leptin administration via the third ventricle did not alter HIF2α protein levels in the hypothalamus (Figure 3-7A). Then, we investigated whether HIF was required for the signaling and function of leptin in the hypothalamus. To test this question, we employed a loss-of-function strategy by analyzing hypothalamic leptin signaling and leptin-dependent feeding regulation in POMC/HIFβlox/lox mice. Compared to the control mice, POMC/HIFβlox/lox mice showed similar leptin-induced STAT3 phosphorylation in the hypothalamus including the comprised POMC neurons (Figure 3-7C&D). Thus, hypothalamic HIF was responsive to glucose but not leptin, which agrees with the observation that hypothalamic glucose sensing did not involve the induction of leptin signaling (Figure 3-7B). To evaluate the physiological relevance of this finding, experiments were performed to compare leptin-dependent feeding
regulation in \( POMC/HIF\beta^{lox/lox} \) mice versus control mice. Data showed that food intake in \( POMC/HIF\beta^{lox/lox} \) mice and the controls were suppressed by leptin in a similar manner (Figure 3-7E). Altogether, while STAT3 is a critical transcription factor in leptin signaling, HIF represents a transcription factor that crucially mediates the glucose-sensing process of the hypothalamus.
Fig. 3-7. Relationship between leptin signaling and hypothalamic HIF pathway.

(A) C57BL/6 wildtype mice received third ventricle injection of leptin or vehicle (Veh). The hypothalami were harvested for Western blot analysis HIF2α protein levels. β-actin was used as an internal control. (B) C57BL/6 wildtype mice received third
ventricle infusion of glucose (Glu), leucine (Leu), or vehicle (Veh). For comparison, a subset of mice received third ventricle injection of leptin (Lep). Hypothalami were harvested for Western blot analysis of STAT3 phosphorylation (pSTAT3) and STAT3 protein levels. Western blots of β-actin were performed as an internal control. (C&D) POMC/HIF$^{β^{lox/lox}}$ mice (P/HIF$^{β^{lox/lox}}$) and control littermate HIF$^{β^{lox/lox}}$ mice received third ventricle injection of leptin or control vehicle. Brain sections of mediobasal hypothalamus were prepared and immunostained for phosphorylated STAT3 (pSTAT3) (green) and α-MSH (red). DAPI nuclear staining (blue) revealed all cells in the sections. ARC, arcuate nucleus. Bar = 50 µm. (D) POMC neurons (α-MSH-immunoreactive) positive for pSTAT3 in multiple sections were counted and analyzed statistically. Data represent the observations from at least 3 mice per group. Error bars reflect mean ± SEM. (E) Young male POMC/HIF$^{β^{lox/lox}}$ mice (P/HIF$^{β^{lox/lox}}$ mice) versus littermate control HIF$^{β^{lox/lox}}$ mice were fasted 24 h and received third-ventricle injection of leptin or vehicle. Food was placed in cages, and mice were subsequently monitored for food intake. * $p < 0.05$; $n = 6–8$ per group. Error bars reflect mean ± SEM.

3.3.7. Glucose Up-regulates Hypothalamic HIF2α via Fumarate and Succinate

It was recently demonstrated that glucose metabolites fumarate and succinate can inhibit PHDs to activate HIF in cancer cells to promote tumorigenesis (164). Based on this information, we questioned if fumarate and succinate could mediate glucose-dependent HIF activation in the hypothalamus. To test this idea, we first
confirmed the prediction that glucose delivery via the third ventricle increased the production of fumarate and succinate in the hypothalamus (Figure 3-8A&B). Then after having established the appropriate dose- and time-course conditions (Figure 3-9A–D), we revealed that HIF2α protein levels in the hypothalamus of normal C57LB/6 mice significantly increased with a third-ventricle delivery of either fumarate or succinate (Figure 3-8C, Figure 3-9E&F). Furthermore, fumarate and succinate were both found to suppress the PHD hydroxylation activities in the hypothalamus (Figure 3-8D). These data indicated that these two glucose metabolites activated hypothalamic HIF via the PHD-pVHL pathway.

Subsequently, we tested whether manipulation of hypothalamic fumarate or succinate could affect feeding in mice. As established in the literature (165;166), levels of succinate and fumarate can be increased by using inhibitors of either a succinate dehydrogenase, thenoyltrifluoroacetone (TTFA), or a fumarate hydratase inhibitor, trans-aconitate, or 3-nitropropionic acid (3-NPA). We found that individual delivery of these chemicals via third-ventricle injection inhibited fasting-induced food intake of mice (Figure S9A) without evident toxic effects (Figure 3-10B). Then, we tested if succinate or furmarate administration into the third ventricle of mice could affect their feeding behavior. Indeed, we found that a third-ventricle injection of either fumarate or succinate suppressed food intake in the control mice. In contrast, such effects were significantly reduced in POMC/HIFβ<sup>lox/lox</sup> mice (Figure 3-8E&F). Hence, fumarate and succinate are two metabolites that can mediate glucose up-regulation of hypothalamic HIF.
Fig. 3-8. Glucose metabolites mediate glucose regulation of hypothalamic HIF.

(A&B) 24h fasted C57BL/6 mice received third-ventricle infusion of glucose versus vehicle. Hypothalami were harvested and measured for tissue contents of fumarate (A) and succinate (B). * p < 0.05; n = 5–6 per group. Error bars reflect mean ± SEM.

(C&D) 24h fasted C57BL/6 mice received third-ventricle infusion of diethyl fumarate.
(Fum) or diethyl succinate (Suc) or control vehicle (Veh). Hypothalami were harvested for Western blot analysis of HIF2α protein (C) and GHO assay for PHD activities (D). Bar graphs: HIF2α protein levels (C) and PHD activities (D) were quantitated and analyzed statistically. ** $p < 0.01$; $n = 5–6$ per group. Error bars reflect mean ± SEM. (E&F) 24h fasted POMC/HIF$\beta^{lox/lox}$ mice (P/HIF$\beta^{l/l}$) and control littermates HIF$\beta^{lox/lox}$ mice (HIF$\beta^{l/l}$) received third-ventricle injection of diethyl fumarate (Fum) (E), diethyl succinate (Suc) (F), or vehicle (Veh) (E&F), and subsequently monitored for food intake for 6h.  * $p < 0.05$; ** $p < 0.01$; $n = 7–10$ per group. Error bars reflect mean ± SEM. (A–F) All mice were adult males and normal chow-fed.
Fig. 3-9. Dose-course and time-course actions of fumarate or succinate.

Following 24h fasting, C57BL/6 mice received third-ventricle infusion of diethyl fumarate or diethyl succinate at the dose of 0, 0.6, 3.0, or 15 µmol/h for 5 h (A&C) or at the dose of 3.0 µmol/h for 0, 2, 5, or 8 h (B&D). Hypothalami were harvested and measured for the tissue contents of fumarate and succinate (A–D) and HIF2α protein (E&F). Bar graphs: * $p < 0.05$; $n = 6–8$ per group. Error bars reflect mean ± SEM.
Fig. 3-10. Food intake and taste aversion effects of pharmacologic chemicals.

(A) Following 24h fasting, C57BL/6 mice received third-ventricle injection of trans-aconitate (ACON), thenoyltrifluoroacetone (TTFA), 3-nitropropionic acid (3NPA), or vehicle (Veh). Food was provided to mice and food intake of mice was recorded. *p < 0.05; n = 7–10 per group. Error bars reflect mean ± SEM. (B) C57BL/6 wildtype mice were habituated to experimental protocol for several days and
then presented to 0.2% saccharine for 60 min after removing the drinking water for 23 h. After habitation, mice were ICV injected with an indicated drug and the vehicle (Veh) via pre-implanted third ventricle cannula, and subsequently had access to 0.2% saccharine for 60 min (Test 1). Intraperitoneal (IP) injection of LiCl and saline was used as a positive and negative control, respectively. After 4 d, mice were presented with 0.2% saccharine and saccharine intake of mice during 60 min was measured (Test 2). Data represent 60-min saccharine intake in Test 2. ** p < 0.01, ns, non-significant; n = 6–7 per group. Error bars reflect mean ± SEM. ACON, trans-aconitate; TTFA, thenoyltrifluoroacetone; 3NPA, 3-nitropropionic acid.

3.3.8. AMPK Down-Regulation by Glucose Mediates Hypothalamic HIF Activation

Recent research has revealed that AMPK is an “energy gauge” in hypothalamic regulation of energy balance (167-169). In this context, we asked whether AMPK could be involved in hypothalamic HIF signaling and function. Using phosphorylation of AMPKα to reflect AMPK activity, we found that an intra-third ventricle injection of glucose inhibited hypothalamic AMPK (Figure 3-11A&B). Then, we performed intra-third-ventricle injection of glucose with or without AICAR, an established AMPK activator. Data revealed that AICAR markedly reduced the effects of glucose on hypothalamic HIF up-regulation (Figure 3-11A&B). This result indicated that AMPK might work as an inhibitory regulator for glucose-dependent HIF activation in the hypothalamus.

Also, it has recently been reported that activation of hypothalamic AMPK with
AICAR can promote food intake in mice (170;171). Having confirmed this effect in our experiment, we tested whether hypothalamic HIF could prevent the feeding-promoting effects of AICAR. To achieve this, we delivered HIF2α/HIFβ complex into the neurons in the mediobasal hypothalamus, since POMC neurons are predominantly localized in this region. Through a neuron-specific lentiviral vector in which dual synapsin promoters were used to co-express two genes (Figure 3-12A), HIF2α and HIFβ were co-delivered into the mediobasal hypothalamus of standard C57BL/6 mice (Figure 3-12B&C). Mice receiving lentiviral expressing GFP were used as controls (Figure 3-12B). As shown in Figure 3-11C&D, a third-ventricle administration of AICAR promoted food intake in control mice as expected; however, this effect of AICAR was eliminated by the exogenous expression of HIF2α/HIFβ. Thus, AMPK suppression by glucose is mechanistically involved in glucose sensing of the hypothalamic HIF pathway.
Figure 3-11. Effect of AMPK in glucose-mediated activation of hypothalamic HIF.

(A&B) Western blot analyses of AMPK signaling were performed for the hypothalami harvested from C57BL/6 wildtype mice that received a 5-h intra-third ventricle infusion of glucose (Glu) versus ventricle (Veh) in the presence or absence of a prior third-ventricle injection of AICAR. β-act, β-actin. Bar graphs: Western blots were quantitated and analyzed statistically. (C&D) C57BL/6 wildtype mice received mediobasal hypothalamic injection of neuron-specific lentiviruses expressing HIF2α/HIFβ (HIF2α/β) or GFP, and simultaneously received cannula implantation
into the third ventricles. After 2-wk post-surgical recovery, mice received 6-h fasting and then were injected with AICAR or vehicle (Veh) via the cannula. Mice subsequently had free access to food and were measured for food intake during 1h (C) and 3h (D) periods. (B–D) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$; $n = 6$ per group. Error bars reflect mean ± SEM.
Fig. 3-12. Lentivirus-directed hypothalamic HIF delivery up-regulates POMC gene.

(A) Schematic map of lentivirus that co-expressed myc-conjugated HIFα (either HIF2α or HIF1α) and HA-conjugated HIFβ (Lenti-HIF2α/HIFβ) under the control of neuron-specific synapsin (Syn) promoter. Matched GFP-expressing lentiviral vector (Lenti-GFP) was used as a control. (B&C) C57BL/6 mice received intra-mediobasal hypothalamic injection of Lenti-HIFα/HIFβ or control Lenti-GFP. (B) Site-specific gene delivery was verified by GFP (green) and HA staining (red). DAPI nuclear staining (blue) reveals all cells in the sections. Right panels: GFP (green) and neuronal marker HuCD staining (red) are merged to indicate neuron-specific gene delivery (yellow). (C) Site-specific gene delivery was verified by Western blot analysis of myc and HA expression. Data shown in (B&C) were obtained from the mice injected with Lenti-HIF2α/HIFβ versus Lenti-GFP, but also represented similar patterns in mice injected with Lenti-HIF2α/HIFβ versus Lenti-GFP. ARC, arcuate nucleus; 3V, third ventricle; β-act, β-actin. Bar = 50 µm. (D) Chow-fed regular C57BL/6 mice that received bilateral MBH injections of Lenti-HIF2α/HIFβ or Lenti-GFP. At 2 wk post-injection, hypothalami were harvested for the measurement of POMC mRNA levels. * p < 0.05; n = 5 per group. Error bars reflect mean ± SEM.

3.3.9. Up-regulation of mTOR/S6K by Glucose Mediates Hypothalamic HIF Activation
We also examined the potential relevance of mTOR and its downstream component S6K, since mTOR/S6K can promote HIF1/2α protein synthesis in various models (172;173), and also AMPK can inhibit mTOR/S6K (174;175). We found that glucose-dependent hypothalamic HIF2α up-regulation was associated with increased S6K activities (Figure 3-13A&B). Conversely, glucose induction of hypothalamic HIF2α was significantly reversed by third-ventricle injection of mTOR inhibitor, rapamycin (Figure 3-13A&B). Supported by recent findings that has shown that hypothalamic mTOR (176;177) and S6K (178) restrict feeding and weight gain, we further evaluated whether mTOR/S6K might participate in the action of hypothalamic HIF in regulation of feeding. Using the site-specific gene delivery approach described above, we delivered lentiviruses expressing constitutively active Rheb (CA Rheb) to directly activate mTOR in the mediobasal hypothalamus of POMC/HIFβ<sup>lox/lox</sup> mice versus littermate controls (Figure 3-13C–F). As revealed in Figure 3-13F, while CA Rheb decreased food intake in control mice, this effect was reduced in POMC/HIFβ<sup>lox/lox</sup> mice. In sum, glucose sensing of HIF in POMC neurons critically involves mTOR/S6K signaling.
Fig. 3-13. Regulation of mTOR pathway on hypothalamic HIF.

(A&B) 24h fasted adult C57BL/6 mice received 5h third-ventricle infusion of glucose (Glu) versus vehicle (Veh) in the presence or absence a prior third-ventricle injection of rapamycin (Rap). Hypothalami were harvested and analyzed by Western blotting for phosphorylated S6K (pS6K) and HIF2α. β-act, β-actin. Bar graphs: Western blots were quantitated and analyzed statistically. * p < 0.05, ** p < 0.01; n = 4 per group.
Error bars reflect mean ± SEM. AU, arbitrary unit. (C–F) POMC/HIFβ\textsuperscript{lox/lox} mice (P/HIFβ\textsuperscript{β/β}) and control littermate HIFβ\textsuperscript{lox/lox} mice (HIFβ\textsuperscript{β/β}) received MBH injection of lentiviruses expressing the constitutive-active form of Rheb tagged with Flag (Lenti-\textsuperscript{CA}Rheb/Flag) or matched GFP-expressing control lentiviruses (Lenti-GFP). Site-specific gene delivery was verified by immunostaining (C) and Western blots of Rheb/mTOR signaling proteins (D) as well as the quantitation analysis of Western blots (E). Food intake of these mice was monitored on a daily basis for 2 weeks (F). * \( p < 0.05 \), ** \( p < 0.01 \); \( n = 5–6 \) per group. Error bars reflect mean ± SEM. AU, arbitrary unit. (A–F) All mice were adult males and normal chow-fed.

3.3.10. HIF Inhibition in POMC Neurons Causes Overfeeding and Energy Imbalance

Following the studies addressing glucose sensing by hypothalamic HIF (Figures 3-2~3–16), we examined whether HIF inhibition in POMC neurons could be sufficient to affect the steady-state levels of feeding and energy homeostasis. First, energy (food) intake and expenditure were profiled in POMC/HIFβ\textsuperscript{lox/lox} mice and littermate controls under normal chow feeding. Compared to the controls, POMC/HIFβ\textsuperscript{lox/lox} mice were found hyperphagic (Figure 3-14A) with impaired energy expenditure (Figure 3-14B&C), but resulting in only a mild overweight condition (unpublished data). Despite the lack of dramatic body weight effect, DEXA scanning revealed that fat mass of POMC/HIFβ\textsuperscript{lox/lox} mice increased evidently (Figure 3-14D). Morphological examination of various fat tissues further confirmed that the size of fat...
cells isolated from the knockout mice increased (Figure 3-14E). These physiological changes in the knockout mice were associated with impaired thermogenic response of brown fat tissue to re-feeding (Figure 3-15). We also tested if the obesogenic effect of hypothalamic HIF loss-of-function could result from leptin resistance. To do this, ob/ob mice received mediobasal hypothalamic injection of lentiviruses expressing dominant-negative HIFα, which has been established to inhibit both HIF1α and HIF2α (179;180). The results revealed that the obesity-promoting effect of HIF loss-of-function remained in ob/ob mice (Figure 3-16), indicating that leptin signaling was not involved in the metabolic action of hypothalamic HIF. Taken together, HIF loss-of-function in POMC neurons causes positive energy balance in favor of obesity development.
Fig. 3-14. Inactivation of HIF in POMC neurons causes energy and metabolic imbalance.

(A–C) Chow-fed POMC/HIFβlox/lox mice (P/HIFβlox/lox) and littermate control HIFβlox/lox were analyzed for food intake (A) and O₂ consumption (B&C). * p < 0.05; n = 10 per group (A) and n = 4 per group (B&C). Statistics in (A) delineate the difference between two mice groups at the indicated time points. Error bars reflect
mean ± SEM. (D) DEXA scanning of fat mass (versus lean mass) in chow-fed
POMC/HIFβ
lox/lox
(P/HIFβ
lox/lox
) mice and littermate control HIFβ
lox/lox
(HIFβ
l/l
) mice. * p < 0.05; n = 6 per group. (E) H&E staining of epididymal fat from normal
chow-fed POMC/HIFβ
lox/lox
(P/HIFβ
lox/lox
) mice and littermate control HIFβ
lox/lox
mice. Bar = 100 µm. (F&G) HFD-fed POMC/HIFβ
lox/lox
mice (POMC/HIFβ
lox/lox
) and
littermate control HIFβ
lox/lox
mice were monitored for food intake (F) and weight gain
(G). * p < 0.05; n = 9–13 per group. Statistics display the difference between two
mice groups at the indicated time points. Error bars reflect mean ± SEM. (A–G) Data
were male mice but similarly observed in females.

**Fig. 3-15. Thermogenic activities in POMC/HIFβ
lox/lox
mice.**

Following 24-h fasting, POMC/HIFβ
lox/lox
mice (P/HIFβ
l/l
) and control HIFβ
lox/lox
mice (HIFβ
l/l
) received 6-h re-feeding versus continued 6-h fasting. Hypothalami were
collected and analyzed for mRNA levels of indicated genes. * p < 0.05, ** p < 0.01,
ns, non-significant; n = 5–8 per group. Error bars reflect mean ± SEM.
Fig. 3-16. Effects of HIF on the metabolic phenotype in ob/ob mice.

(A&B) Body weight-matched ob/ob mice (8 wk old) received intra-mediobasal hypothalamic injection of neuron-specific lentiviruses expressing dominant-negative HIF1/2α (DNHIF1/2α) or control GFP. Mice were monitored for body weight (BW) gain during 2-wk follow-up (A) and daily food intake during this follow-up period (B). * $p < 0.05$; $n = 5–6$ per group. Error bars reflect mean ± SEM.

### 3.3.11. HIF Inhibition in POMC Neurons Exacerbates Dietary Obesity

To further elucidate the significance of hypothalamic HIF inhibition in obesity development, we maintained $POMC/HIFβ^{lox/lox}$ mice and the littermate controls under high-fat diet (HFD) feeding since weaning. Despite caloric abundance (58.5 Kcal% fat) in the HFD, $POMC/HIFβ^{lox/lox}$ mice continued to display an overeating behavior (Figure 3-14F). Thus, the knockout mice were insensitive to the enriched levels of calories from HFD, again supporting the notion that HIF inactivation in POMC neurons reduced the nutrient-sensing function of the hypothalamus. We monitored the
longitudinal course of body weight gain and obesity development in HFD-fed
\(\text{POMC/HIF}^{\beta\text{lox/lox}}\) mice versus controls. Compared to HFD-fed control mice, HFD-fed
\(\text{POMC/HIF}^{\beta\text{lox/lox}}\) mice gained body weight more rapidly and displayed obesity in an
exacerbated manner (Figure 3-14G). Thus, when challenged with obesity-prone
conditions (such as HFD feeding), the susceptibility of disease development is highly
increased by HIF dysfunction in hypothalamic POMC neurons, supporting the
importance of HIF in the pathogenesis of obesity-diabetes syndrome.

3.3.12. Treatment of Obesity by Targeting HIF in the Hypothalamus

Finally, we performed animal studies to evaluate whether hypothalamic HIF could be
targeted to generate strong therapeutic effects against obesity. Although HIF2\(\alpha\) is the
major subunit in the hypothalamus (Figure 3-1C&D), we designed experiments to
evaluate the potential use of both HIF2\(\alpha\) and HIF1\(\alpha\). Using the lentiviral
cो-expression system shown in Figure 3-12A&B, we delivered HIF2\(\alpha\)/HIF\(\beta\) versus
HIF1\(\alpha\)/HIF\(\beta\) into the mediobasal hypothalamus of normal C57BL/6 mice. Matched
mice with gene delivery of GFP were used as the controls. Following viral injection,
all mice were maintained on a HFD and longitudinally followed up for feeding and
weight gain. As shown in Figure 3-17A&B, the control mice gained body weight,
rapidly leading to overt obesity over a 3-mo period. In contrast, delivery of either
HIF2\(\alpha\)/HIF\(\beta\) or HIF1\(\alpha\)/HIF\(\beta\) markedly reduced obesity development (Figure
3-17A&B). The anti-obesity effect of HIF gain-of-function was clearly attributed to
feeding restriction (Figure 3-17C&D) presumably resulting from POMC gene
expression up-regulation (Figure 3-12D). In conclusion, although hypothalamic HIF2α/HIFβ is more physiologically relevant to metabolic regulation, both HIF2α and HIF1α hold significant therapeutic potentials and can be targeted, individually or in combination, in order to counteract obesity and related metabolic diseases.

**Fig. 3-17. Anti-obesity effect of delivering HIF to the hypothalamus.**

Age- and weight-matched C57BL/6 mice received mediobasal hypothalamic injection of neuron-specific lentiviruses expressing HIF2α/HIFβ (Lenti-HIF2α/HIFβ) (A&C), HIF1α/HIFβ (Lenti-HIF1α/HIFβ) (B&D), or GFP (Lenti-Control) (A–D). Mice were subsequently maintained under HFD feeding and monitored for body weight gain (A&B) and HFD intake (C&D). The control mice were shared by both groups but repeatedly presented in separate figures. Statistics show the difference between two
mice groups at the time points indicated by lines. * $p < 0.05$; $n = 5–8$ per group. Error bars reflect mean ± SEM.

3.4. Discussion

The study in this chapter demonstrates that HIF is present in the hypothalamus and sensitively up-regulated by the local availability of glucose and its metabolites. Glucose up-regulation of hypothalamic HIF is mediated by PHD/pVHL-dependent HIFα degradation and AMPK/mTOR-dependent HIFα synthesis. POMC neurons in the hypothalamus are critical for the metabolic role of hypothalamic HIF through its direct transcriptional regulation on POMC gene. HIF loss-of-function in POMC neurons can cause overeating and weight gain to promote obesity development, while HIF gain-of-function can provide strong therapeutic benefits against obesity (and related diseases) (Figure 3-18). Taken together, all these unexpected findings reveal an unappreciated role for neuronal HIF in the brain regulation of energy, body weight, and metabolic balance.

Energy homeostasis is fundamental for living, and this physiological process relies on the complex property of the life’s regulatory system in coordinately sensing and transducing the metabolic dynamics in the body. The hypothalamus is known as the “headquarters” for the regulation of energy balance (181-184). This work discovered that the HIF complex plays a critical role in linking hypothalamic glucose sensing to glucose-dependent hypothalamic control of feeding and energy balance. Known as a nuclear transcription factor that mediates hypoxia adaptation, the
physiological and disease significance of HIF has traditionally been explored for the pathological roles in diseases, particularly cancers (185-187). Recently, research in cancer biology has begun to appreciate that tumorigenesis involves the connection between HIF and metabolism at cellular levels (188;189). Here, we have demonstrated an important but unappreciated role of HIF in the hypothalamus at an organism level from the perspective of feeding and body weight regulation as well as metabolic disease, especially obesity and related health problems. Indicated by our findings, further lines of research can be expected to explore possible roles of neuronal HIF in other physiological functions and disease relevance of the hypothalamus.

For almost a decade, the hypothalamus has been established as a lipostatic feeding regulator through hormonal action of leptin, which involves activation of POMC by STAT3 (190-193). Recently, the role of the hypothalamus in nutrient sensing has become a major interest in the research field, leading to the elucidation of hypothalamic glucose sensing involving AMPK (194-196) and KATP channels (197) and hypothalamic amino acid-sensing process involving mTOR (198). In this study, we discovered that HIF in the hypothalamus acts as a nutrient sensor. In alignment with the fast turnover rate of HIF proteins (half life: 5~10 min) (199;200), the HIF pathway in hypothalamic POMC neurons likely directs the real-time homeostasis of feeding and energy homeostasis. This regulation complements STAT3-dependent

\[ POMC \] gene expression in leptin signaling and its long-term regulation on feeding and body weight (201-203). Additional research seems needed to address if HIF pathway
can work in other neuronal types in addition to POMC neurons in mediating hypothalamic control of metabolic physiology. Overall, this work has specifically focused on the glucose-sensing process of the hypothalamus, and along this line, it deserves further endeavors to study whether HIF pathway can function in the central sensing process of other nutrient species (such as amino acids and fatty acids) and their metabolites.

The hypothalamus has been well appreciated as the master regulator of body weight and metabolic balance and a pathogenic culprit for obesity and related disease (124;204-207). Recent research attentions have been extensively directed to hypothalamic hormonal dysregulations and most notably the development of leptin resistance and insulin resistance (208-212). However, it remains poorly understood how hypothalamic nutrient sensing is mediated. More importantly, it is still unclear if a molecular pathway in central nutrient sensing could be targeted to effectively counteract obesity and related disease. In this work, we found that enhancing hypothalamic glucose sensing through HIF induction per se is effective in treating obesity. Our findings are supported by a recent work showing an anti-obesity effect of HIF activation through the ablation of a HIF inhibitor FIH throughout the brain (213). We recognize that there is a great deal of research efforts aimed to develop HIF inhibitors for cancer therapeutics, but concerns were recently raised about some serious problems that can arise from HIF inhibition in certain tissues and cells (214). Here our research further points out that HIF inhibition in the hypothalamus can result in adverse metabolic outcomes and needs to be avoided in drug designs. On the other
hand, selective activation of neuronal HIF especially in the hypothalamus could be developed to provide a new therapeutic avenue against obesity and related metabolic disease. Neuron-specific HIF activation might not have critical concerns in terms of oncogenesis since neurons are non-replicable, but the potential application of this strategy in treating metabolic disease will certainly require future technology development and safety assessment.
Fig. 3-18. Glucose sensing of HIF in hypothalamic control energy and metabolic balance. Proposed model of hypothalamic HIF in neural control of energy balance.
Glucose activates hypothalamic HIF through metabolite-induced suppression of HIF protein degradation and AMPK/mTOR-related promotion of HIF protein synthesis. Activation of HIF up-regulates POMC gene, linking hypothalamic glucose sensing to the hypothalamic control of feeding, energy expenditure, body weight, and metabolic homeostasis. In conclusion, HIF in the hypothalamus represents a molecular target of protecting hypothalamic nutrient sensing in order to improve whole-body metabolic regulation of the hypothalamus and prevent against obesity and related diseases.
CHAPTER 4. HYPOXIA-INDUCIBLE FACTOR MEDIATES HYPOTHALAMIC AMINO ACID SENSING AND INSULIN SENSING

4.1 Abstract

Hypothalamus is the center that senses nutritional (glucose, amino acid etc.) and hormonal (leptin, insulin etc.) signals from periphery and regulates whole-body energy balance. Our previous work showed that hypoxia-inducible factor (HIF) responses to glucose in the hypothalamus and hence control energy balance by directly mediating the transcription of neuropeptide pro-opiomelanocortin (POMC), while HIF is not a part of leptin signals. In this study, we examined whether hypothalamic HIF is involved in amino acid and insulin sensing. First, intracerebroventricular (i.c.v.) injection of leucine and insulin in mice increased protein levels of HIF2α by 2 folds and 1.8 folds as we detected by western blots. However, the mRNA levels of HIF2α and other HIF subunits remained the same during leucine and insulin treatment, indicating that leucine and insulin upregulate HIF on the post-transcriptional level. Both leucine and insulin activate mTOR pathway and mTOR is known to upregulate translation of HIFα mRNAs. Therefore we combined leucine and insulin injection with rapamycin injection to block mTOR activity. mTOR inhibition reversed the upregulation effects of leucine and insulin on HIF2α protein, proving that leucine and insulin activate HIF through mTOR pathway in the hypothalamus. To further study whether the sensing of leucine and insulin by HIF plays physiological roles in the feeding regulation by hypothalamus, we used
CRE-lox system in mice to delete HIFβ in hypothalamic POMC neurons so that HIF activity will be eliminated. In wild type control mice, i.c.v. injection of leucine or insulin inhibited fasting-induced food intake at 6 hr and 24 hr by 30-50%. However, in the POMC/HIF knockout mice, the feeding-inhibiting effects were mostly blocked, suggesting that HIF is required for the feeding response to leucine and insulin. In conclusion, hypothalamic HIF works downstream of mTOR pathway and is responsible for amino acid and insulin sensing and control of energy balance.

4.2 Introduction

Hypoxia-inducible factor is a transcription factor whose usual function is to activate cellular and molecular responses to hypoxia. It consists of an α subunit and a β subunit and they form a heterodimer. There are 3 kinds of HIFαs, HIF1α, HIF2α and HIF3α. 1α and 2α are most well-studied while 3α is an inhibitory subunit. HIFβ is also called ARNT (aryl hydrocarbon receptor nuclear translocator) and it’s considered stable (215;216). HIFα subunits constantly undergo degradation under normoxia conditions unless its degradation is suppressed by low oxygen levels (217). Besides classic hypoxic response, HIF also regulates metabolism such as glycolysis and mitochondria functions (215;216;218). Also, several lines of researches have reported that peripheral loss of function of HIF is related to metabolism diseases, such as diabetes and obesity (219;220). Our recent research also showed that HIF in the central nervous system regulate global energy balance by controlling profoundly the neuropeptide POMC that controls feeding and energy expenditure. HIF is also a novel
glucose sensor in the hypothalamus. Glucose inhibits AMPK and further activates mTOR pathway to activate HIF. HIF is not involved in leptin signals in the hypothalamus. Another group also reported that knock out a HIF inhibitor FIH in the brain regulates energy balance in line with our research. This may be because of the function of HIF in the hypothalamus.

Hypothalamus also senses other nutrients and hormones to regulate energy balance. mTOR pathway has been revealed to be a key negative mediator of food intake and it’s responsible for amino acid sensing in hypothalamus(19;221). Activated mTOR, we know, can activate HIFα through upregulation its translation(67;72). We also found that glucose activates HIF through mTOR pathway. HIF might also utilize this pathway to sense amino acid. Also insulin is known to inhibit food intake by regulating expression of neuropeptide including POMC (222;223). The classic pathway is that insulin activates Akt and Akt phosphorylates FoxO and inhibits its translocation into the nuclear. FoxO is a transcriptional inhibitor of POMC. On the other hand, insulin also mediates mTOR pathway through inhibiting TSC1/2 which suppresses mTOR. In this research we set out to study the role of HIF in hypothalamic amino acid sensing and insulin sensing and the function of mTOR pathway involved. Also, we examined the physiological functions of HIF in this context.

4.3 Results

4.3.1 Leucine and insulin activate hypothalamic HIF2α on protein levels

To dissect the role of HIF in hypothalamic leucine sensing or insulin sensing,
we first tested whether HIF responses to leucine and insulin. We used i.c.v. injection to deliver leucine and insulin into the ventral third ventricle proximal to the hypothalamus. Our previous work showed that HIF2α is the major form of HIFα in hypothalamus (224). Western blots showed that leucine and insulin increased the protein level of HIF2α by 2.1 folds and 2.4 folds respectively (Fig. 4-1A-D). Since the activity of HIF primarily relies on the protein level of its α subunits, our results suggested that leucine and insulin can activate HIF in the hypothalamus. To tease out whether at which level did the activation happen, we further measured the mRNA levels of HIF subunits with quantitative real-time PCR (Fig. 4-1E, F). Leucine or insulin injection didn’t change the mRNA levels of HIF1α, HIF2α or HIFβ significantly, indicating the activation occurs on the protein level.

Fig. 4-1 Activation of hypothalamic HIF by central administration of leucine and
insulin

(A&B) C57BL/6 mice received third ventricle injection of leucine (Leu) or vehicle control (Con). The hypothalami were harvested for Western blot analysis for HIF2α protein levels. β-actin was used as an internal control. Bar graphs: Western blots were quantitated and analyzed statistically. (C&D) C57BL/6 mice received third ventricle injection of insulin (Ins) or vehicle control (Con). The hypothalami were harvested for Western blot analysis for HIF2α protein levels. β-actin was used as an internal control. Bar graphs: Western blots were quantitated and analyzed statistically. (E) C57BL/6 mice received the same treatment as in A&B. The hypothalami were harvested for real-time PCR analysis of HIF1α, HIF2α and HIFβ mRNA levels. (F) C57BL/6 mice received the same treatment as in C&D. The hypothalami were harvested for real-time PCR analysis of HIF1α, HIF2α and HIFβ mRNA levels. (E&F) AU: artificial unit (A-D) ***p < 0.001; n = 4 per group. Error bars reflect mean ± SEM. (E&F) n = 6 per group. Error bars reflect mean ± SEM.

4.3.2 Leucine activates hypothalamic HIF though mTOR pathway

Many groups have reported that mTOR pathway upregulates HIFα protein levels by enhancing the translation of its mRNA(67;225). Leucine is a direct and a major activator of mTOR pathway although the mechanism is not completely clear yet (226). Meanwhile, insulin also activates mTOR pathway (227). Therefore, we set out to explore if leucine and insulin activate HIF in an mTOR-dependent manner. First we examined mTOR signals under leucine injection conditions by measuring
phosphorylation of S6K, a main marker of mTOR activation. Leucine increased phosphorylation of S6K in the hypothalamus while increasing protein levels of HIF2α (Fig. 4-2A&B). To further dissect whether mTOR activation is required for activation of HIF, we used rapamycin, a potent and specific inhibitor of mTOR to block mTOR activity. As shown in Fig. 4-2(C-E), rapamycin completely blocked both the basal and leucine-induced phosphorylation of S6K and inhibited the effects of leucine on HIF2α. This proved that leucine activates HIF2α through activating mTOR pathway.
Fig. 4-2 Rapamycin blocks the activation of HIF2α by leucine

(A&B) C57BL/6 mice received third ventricle injection of leucine (Leu) or vehicle (Veh). The hypothalami were harvested for Western blot analysis for HIF2α, phospha-S6K and total S6K protein levels. β-actin was used as an internal control. Bar graphs: Western blots were quantitated and analyzed statistically. (C-E) C57BL/6 mice received third ventricle injection of leucine (Leu) or vehicle control (Con) combined with injection of rapamycin (Rap) and its matching control (Con). The hypothalami were harvested for Western blot analysis for HIF2α, phospha-S6K and total S6K protein levels. β-actin was used as an internal control. Bar graphs: Western blots were quantitated and analyzed statistically. (A-E) * p < 0.05, ** p < 0.01, NS, non-significant; n = 3–4 per group. Error bars reflect mean ± SEM.

4.3.3 Insulin activates hypothalamic HIF though mTOR pathway

We then used rapamycin to inhibit the effects on mTOR. In Fig. 4-3. A-C, insulin i.c.v. injection increased HIF2α protein by 2.6 folds. However, combination with i.c.v. injection of rapamycin almost completely blocked this effects. Meanwhile, rapamycin didn’t affect the upstream insulin signals as characterized by phosphorylation of AKT, further supporting that rapamycin only blocked the signal at mTOR level. Fig. 4-2 and Fig. 4-3 proved that both leucine and insulin activate HIF in an mTOR dependent manner.
Fig. 4-3 Rapamycin blocks the activation of HIF2α by insulin

(A-C) C57BL/6 mice received third ventricle injection of insulin (Ins) or control vehicle (Con). The hypothalami were harvested for Western blot analysis for HIF2α, phospho-AKT and total AKT protein levels. β-actin was used as an internal control.

Bar graphs: Western blots were quantitated and analyzed statistically. (B&C) * p < 0.05, ** p < 0.01, *** p < 0.001, NS, non-significant; n = 4 per group. Error bars reflect mean ± SEM.

4.3.4 HIF in POMC neurons is required for hypothalamic leucine sensing

After understanding the mechanism how leucine and insulin activate HIF, we further asked the question that whether the leucine/insulin – HIF axis in the hypothalamus has any physiology significance. As we reported previously, HIF in hypothalamus directly regulates the transcription of neuropeptide POMC and thus
exerts important role in whole body energy balance (224). We therefore utilize the conditional knockout mice with HIFβ deleted in POMC neurons to study the effects of loss-of-function of HIF on hypothalamic leucine and insulin sensing. We measured fasting-induced feeding after i.c.v. injection of leucine or the empty vehicle – aCSF. Leucine inhibits 6hr food intake by up to 48%. However, in POMC-HIFβ knockout mice leucine can only decrease food intake by less than 20% (Fig. 4-4A). This suggests that hypothalamic HIF is required for leucine sensing. Then we measured the mRNA levels of POMC in hypothalamus with or without leucine i.c.v. (Fig. 4-4B). Leucine upregulated POMC mRNA to 2 folds, while this effect is completed gone when HIF is knocked out in POMC neurons, suggesting that leucine regulates POMC mRNA directly though HIF and thus regulates food intake.
Fig. 4-4 Knocking out of HIFβ in POMC neurons compromise hypothalamic leucine sensing.

(A) Young, male \(POMC/HIF^{\beta_{\text{lox/lox}}}\) mice (\(P/HIF^{\beta_{\text{lox/lox}}}\) mice) versus littermate control \(HIF^{\beta_{\text{lox/lox}}}\) mice were fasted 24 h and received third-ventricle injection of leucine (Leu) or vehicle (aCSF). Food was placed in cages, and mice were subsequently monitored for food intake for 6 h. (B) 24-h fasted young \(POMC/HIF^{\beta_{\text{lox/lox}}}\) mice (\(P/HIF^{\beta_{\beta}}\)) and control littermate \(HIF^{\beta_{\text{lox/lox}}}\) mice (\(HIF^{\beta_{\beta}}\)) received injection of leucine (Leu) versus
vehicle (Veh) via cannula pre-implanted into the third ventricle. Hypothalami were then harvested for the measurement of POMC mRNA. (A&B) * \( p < 0.05 \), ** \( p < 0.01 \), NS, non-significant; \( n = 6–10 \) per group. Error bars reflect mean ± SEM.

**4.3.5 HIF in POMC neurons is required for hypothalamic insulin sensing**

We got similar results from insulin (Fig. 4-5). First, insulin in the hypothalamus decreased 6 hour food intake by about 60%. In the HIF knock-out mice, this effects are largely reduced, leaving only 25% inhibition on food intake (Fig. 4-5A). Meanwhile, insulin injection in the brain elevated mRNA levels of POMC by 1.5 times (Fig. 4-5B). Insulin had significant but much less effects on POMC mRNA in POMC/HIFβ mice. The fact that losing HIF does not completely block insulin sensing suggests that other insulin signals were not altered such as the insulin-FOXO pathway.
Fig. 4-5 Knocking out of HIF$\beta$ in POMC neurons compromises hypothalamic insulin sensing.

(A) Young, male $POMC/HIF^{\beta_{\text{lox/lox}}}$ mice ($P/HIF^{\beta_{\text{lox/lox}}}$ mice) versus littermate control $HIF^{\beta_{\text{lox/lox}}}$ mice were fasted 8 h and received third-ventricle injection of insulin (Ins) or vehicle (aCSF). Food was placed in cages, and mice were subsequently monitored for food intake for 6 h. (B) 24-h fasted young $POMC/HIF^{\beta_{\text{lox/lox}}}$ mice ($P/HIF^{\beta_{\text{lox/lox}}}$) and control littermate $HIF^{\beta_{\text{lox/lox}}}$ mice ($HIF^{\beta_{\text{lox/lox}}}$) received injection of insulin (Ins) versus
vehicle (Veh) via cannula pre-implanted into the third ventricle. Hypothalami were then harvested for the measurement of POMC mRNA. (A&B) * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$, NS, non-significant; $n = 6–10$ per group. Error bars reflect mean ± SEM.

4.5 Discussion

To summarize, this study is the first to show that both leucine and insulin activate HIF2 in the hypothalamus on the protein level. This activation is dependent on the activity of mTOR pathway (Fig. 4-6). On the physiological level, hypothalamic leucine and insulin sensing and its function on energy balance is at least partially depend on the activity of HIF in POMC neurons. This finding completed our previous study on the glucose-sensing function of HIF and expanded the scope into amino acid and hormone sensing of HIF.
Fig. 4-6. Leucine and insulin sensing of HIF in hypothalamic control energy and metabolic balance.

Proposed model of hypothalamic HIF senses leucine and insulin and in turn control energy balance. Leucine and insulin activate hypothalamic HIF through mTOR pathways to promote HIF protein synthesis. Activation of HIF up-regulates POMC gene, linking hypothalamic leucine and insulin sensing to the control of food intake.

In conclusion, HIF in the hypothalamus represents a multi-functional target in nutrient
sensing in order to improve whole-body energy balance of the hypothalamus and prevention of obesity and related diseases.

The ability of living organisms to be able to adapt nutrient fluctuation in the environment and maintain energy homeostasis is fundamental for their survival. In modern society, the human body faces the challenge more from the overnutrition direction, also testing the energy balancing capacity of our systems. The hypothalamus is well-recognized as the “headquarter” of whole body energy regulation (7;228-230). It gathers energy signals from all over the body including hormones and nutrients. Hormone sensing is well studied in hypothalamus including leptin, insulin and some other hormones. On the other hand, nutrient sensing is less explored. My previous work showed that HIF present in hypothalamus and mediate whole-body energy homeostasis through direct transcriptional regulation of neuropeptide POMC (224). Also, HIF is a key pathway in hypothalamic glucose sensing, linking nutrients all the way to nuclear programs. Now, we established the function of HIF in amino acid sensing and insulin sensing setting its role as a multi-nutrient sensor and nutrient/hormone sensor. HIF also serves as a hub of different energy signals that converges everything onto the nuclear level. This is the first time we found an energy sensor working on the nuclear level connecting extracellular signal molecules directly to the expression of neuropeptide that controls energy balance. Considering HIF being a multifaceted transcription modulator that has been shown to have more and more target in metabolism, there may be more
targets of HIF that are involved in central regulation of metabolism.

Before the discovery of HIF in hypothalamic energy control, people found the role of AMP/ATP ratio-dependent AMP kinase in central glucose sensing (18); they also found the hypothalamic amino acid sensing pathway mTOR/S6K (19). However, what the downstream signals of these pathways are were unknown. HIF filled up this gap. Now there is a complete pathway in nutrient sensing from nutritional molecules to gene products. HIF even converged insulin signals on itself, connecting classic hormonal signals to nutrient pathways. This indicates that in the history of evolution, organisms may have borrowed pathways from different categories to set up the network of nutrient sensing.

Because of the special position of HIF in energy sensing, it represent a unique opportunity in treating metabolic diseases. Targeting HIF could potentially solve problems in several energy pathways all in once. With the difficulties the field faces for finding a solution for central leptin resistance. Utilizing the nutrient sensing signals might be an alternative solution for curing diet-induced obesity.
CHAPTER 5. DISCUSSION AND CONCLUSIONS

5.1 Other factors that regulate HIF in hypothalamus

In the previous chapters, I have showed that both nutritional molecules like glucose and leucine and hormone like insulin can activate HIF2 in the hypothalamus. I dissected that at least two pathways are involved in the mechanism – metabolites-PHD pathway and AMPK/mTOR pathway. HIF can response to a variety of signals or molecules that’s related to metabolism (217). Also, as a transcription factor HIF may function as partners of other transcriptional factors or co-factors that are involved in nutrient sensing and metabolism regulation. I will discuss all these possibilities.

We have discussed that succinate and fumarate can activate HIF in the hypothalamus by directly inhibiting PHD. Several other intermediates in Kreb cycle can regulate the activity of PHD as well. Citrate, isocitrate, malate, oxaloacetate, and pyruvate all activate HIF through similar mechanisms(63;63;63;65;231). Nutrient flux elevates the availability of these metabolites. They might as well play a role in how glucose or other nutrients activate HIF.

Besides metabolic intermediates, reactive oxidative species (ROS) also can modulate HIF activity(217). Although the mechanism is not fully understood, it’s clear that ROS can slow down the rate of HIFα hydroxylation, hence keep it from degradation. The activation of HIF by ROS may have physiological relevance. Several papers reports that ROS is required for central glucose and lipid sensing(24;25). Central administration of glucose stimulates ROS generation in
hypothalamus. Elimination of ROS in the hypothalamus blunt glucose and hyperlipid sensing in the brain. Evidence showed that ROS can directly modulate melanocortin tone and feeding(232). ROS may also mediate the effects of some feeding control hormones. For example, ROS scavengers remove 5-HT-induced hypothalamic ROS and inhibit the anorexigenic action of 5-HT(233). It would be interesting to test in HIF knock-out mice if inhibiting HIF would block the effects of hypothalamic ROS on feeding.

Other than signaling molecules, HIF may also be regulated or co-regulated by other transcriptional factors and co-factors. Most of the energy or nutritional signals eventually converge to nuclear programs. In hypothalamus, leptin utilizes JAK2 kinase to activate transcriptional factor Stat3. Insulin, in a similarly manner, passes signal to downstream PI3K and Akt pathway and regulate FoxO1 activity. Stat3 and FoxO1 in turn directly control the expression of neuropeptide POMC and AgRP(234). Recent data in adipose tissue showed that HIF1α regulates a SOCS3-Stat3-adiponectin signal(235). In pancreatic and prostate carcinomas, HIF1α and Stat3 co-exist in the same transcription complex(236). In endothelial cells, both FoxO1 and FoxO3a proteins mediate certain aspect of hypoxia response(237). Preliminary data from my lab also suggests that, at least in cell culture system, HIF may regulate the activity of FoxOs. Therefore, it would be interesting to look at the co-regulatory relationship of HIF and classic transcriptional factors that regulate energy balance in hypothalamus.

As a transcription factor, HIF is supposed and known to bind co-activators to
modify local chromatin structure and facilitate the transcription activation of HIF target genes. For example, HIFα binds to the transcriptional co-activator and integrator p300 and CBP(238-240). p300/CBP either acts as molecular scaffolds to link various transcription factors or remodel chromatin structure and acetylate transcription factors. In fact, several other histone/protein acetylase (HAT) and deacetylase (HDAC) bind to HIF and modulate its activity. HAT PCAF acetylates HIF-1α at Lys674, while HDAC SIRT1 removes that modification. SIRT1 blocks the interaction of HIF-1α with p300 and inhibit HIF(241). Another group showed that SIRT1 selectively augments HIF-2 rather than HIF-1 signaling during hypoxia. HIF-2 also promote SIRT1 expression under hypoxia conditions(242). Another sirtuin, SIRT6 also binds to the promoter of HIF1 target genes and deacetylates histone H3K9 (243). In this way, SIRT6 functions as a co-repressor of HIF1 and downregulate the transcription of glycolytic genes. Sirtuins, SIRT1-7, are all NAD dependent deacetylases and their activities are influenced by NAD/NADH ratio(244). Due to involvement of NAD in electron transport chain and many other enzymatic reactions, Sirtuins reponse to metabolic states and are known to be energy sensors(245;246). Therefore, it would be interesting to invest the roles of sirtuins in HIF-mediated hypothalamic energy sensing.

5.2 Other functions of HIF in hypothalamic energy regulation

We already showed that glucose activates HIF in hypothalamus and HIF in turn regulate the transcription of POMC gene and mediate food intake and energy balance.
As a multi-functional transcription factor, HIF has many target genes that are involved in metabolism, primarily genes involved in glycolysis and mitochondrial functions (247). Therefore, we further examined whether there are other HIF target genes that might be involved in hypothalamic nutrient sensing. First, we measured the changes of known HIF target genes after glucose injection in the hypothalamus (left column, Table 5-1.). 2 genes in glucose sensing were upregulated by glucose injection, Glut3 and GK; PGK1 and Enolase1 in glycolysis were upregulated; Bnip3 and Complex4-2 in mitochondrial function were upregulated.
Table 5-1. Hypothalamic expression profiles of HIF target genes in response to ICV delivery of glucose vs. vehicle or POMC-specific HIFβ ablation vs. WT controls.

All groups of mice were fasted for 24 hours. Some mice were ICV injected with 7.2μg glucose in 1μL aCSF. The control group was injected with 1μL aCSF only. It was done in both C57BL/6 wildtype mice and POMC-HIFβ<sup>lox/lox</sup> knockout mice. The
middle column presents the data from HIFβlox/lox vs. POMC-HIFβlox/lox mice without icv injection. Hypothalami were then harvested for the measurement of mRNA of HIF target genes. Quantitative RT-PCR data represent mean±SEM; *p<0.05, **p<10^-2, ***p<10^-3, n=5-8/group. Abbreviations: Glut: glucose transporter; GK: glucokinase; HK: hexokinase; PGK: phosphoglycerate kinase 1; PKLR: pyruvate kinase liver and red blood cell; PKM: pyruvate kinase, muscle; LDHA: lactate dehydrogenase A; MCT: monocarboxylate transporter; CA: carbonic anhydrase; UCP: uncoupling protein; Bnip: BCL2/adenovirus E1B interacting protein; PDK: pyruvate dehydrogenase kinase; LON: lon peptidase; iNOS: inducible nitric oxide synthase; eNOS: endothelia nitric oxide synthase; TF: Transferrin; Tie: tyrosine kinase with immunoglobulin-like and EGF-like domains 1; PDGF: platelet-derived growth factor; Ang: angiogenin; CCD1: DIX domain containing 1; IGF: insulin-like growth factor; IGFBP: insulin-like growth factor binding protein; CCND: cyclin D1; CXCR: chemokine (C-X-C motif) receptor 4; MMP: matrix metallopeptidase; LOX: lysyl oxidase; PAI: plasminogen activator inhibitor type 1; Oct: octamer-binding protein.

These changes may be though activation of HIF by glucose or may not, so we did the same treatment in POMC-HIFβ knockout mice (right column, Table 5-1.). Most of the upregulated were unaltered except PGK1 and Bnip3. There is no significant elevation of PGK1 and Bnip3 mRNA after glucose injection in POMC-HIFβ knockout mice. Also, both PGK1 and Bnip3 mRNA were reduced by a
half in POMC-HIFβ knockout mice compared to wildtype littermates. This suggests that HIF regulates the expression of these two genes in hypothalamus in a glucose dependent manner. \textit{PGK1} encodes phosphoglycerate kinase 1, which is part of the glycolysis process. \textit{Bnip3} encodes BCL2/adenovirus E1B 19 kDa protein-interacting protein 3, which functions in mitophagy and mitochondrial dynamics and induces apoptosis. Bnip3 is also found regulating lipid metabolism in liver (248).

So far there is no evidence of whether these two genes play a role in hypothalamic energy sensing and balance. To study their functions, first we can look at their expression patterns in hypothalamus. Then we can use cell/tissue specific knock out or delivery of virus to gain or lose their activity in hypothalamus and test if they have altered feeding or body weight phenotypes. On another note, since HIF is not only expressed in POMC neurons but other neurons as well, we may have missed some genes that are controlled by HIF in other cell types. A screening in pan-neuron knockout model of HIF would be more suitable to address this possibility. For example, we can use Nestin-CRE to delete HIF in all neurons in the brain and screen for genes that stop responding to glucose. In summary, HIF may regulate genes other than POMC that also contribute to hypothalamic nutrient sensing and energy balance regulation.

5.3 Hypothalamic HIF as a therapeutic target of metabolic diseases

We showed that HIF is essential for physiological central nutrient sensing and energy balance control. Under pathological conditions, such as high-fat diet feeding,
artificial enhancing HIF activity in hypothalamus have anti-obesity effects against overnutrition-induced overeating and overweight (Fig. 3-17). It would be plausible to use HIF as a target to treat obesity and related metabolic syndromes.

Compared to virus-based gene delivery, pharmacological methods of HIF activation will be less intrusive and suitable for the non-life-threatening nature of metabolic diseases. There are already preclinical studies of HIF activators. For example, HIF activators may be effective in treating tissue ischemia, other ischemia/hypoxia-related disorders, tissue regenerations and healing(249;250). Many HIF activators have been found or developed and tested at least in vitro. Besides gene therapy, approaches to activate HIF include small molecules that inhibit the HIF hydroxylases (251-254), and peptides that block the degradation of HIFα protein (255;256). Among them, many molecules are natural product and that will decrease the risk of toxicity (257). Caution needs to be taken in developing HIF activators for treating metabolic disease. HIF activation may contribute to tumor development by providing anti-hypoxia capacity and enhancing angiogensis(258;259). HIF overexpression has been linked to majority of common cancers(260). We need to screen for HIF activators that doesn’t significantly increase the incidences of cancer occurrence. Also, drug delivery of HIF activators by nasal spray may narrow the effects of these compounds in the central nervous system(261). Nonetheless, it would be interesting to tests these compounds and peptides on hypothalamic HIF activation featured by POMC expression and improvement in energy balance by reducing food intake and increase energy expenditure.
5.4 Conclusions

In summary, my work presented in the previous chapters achieved

1) The expression of HIF$\alpha$ is enriched in the hypothalamic neuron populations. Between HIF1$\alpha$ and HIF2$\alpha$, HIF2$\alpha$ is the predominant subtype, which represents the majority of HIF activities. Through different assays, we further found that HIF directly binds on the promoter of neuropeptide gene POMC and regulates the expression of POMC transcriptionally.

2) Since POMC is an important hypothalamic neuropeptide that controls energy balance by regulating energy intake and energy expenditure, we examined the physiological function of HIF in POMC neurons. We first used the knock-out (KO) mouse model with HIF$\beta$ deleted in the POMC neurons. The KO animals showed increased food intake and body weight and decreased energy expenditure under normal diet feeding. This proved that HIF is required for maintaining whole-body energy balance. We further overexpressed HIF in arcuate nucleus in hypothalamus with virus delivery and HIF can protect the effects of diet-induced obesity. All these phenotypes are associated with alteration of POMC mRNA levels, suggesting that POMC is at least a main effector of HIF.

3) HIF serves as a glucose sensor in hypothalamus. Deletion of HIF$\beta$ in POMC neurons impairs the glucose regulation of feeding and the glucose-induced elevation of POMC mRNA. Glucose activates HIF activity in the hypothalamus though increasing HIF2$\alpha$ protein levels. We further found that at least two pathways are involved in this process. First, glucose metabolism generates fumarate and succinate
which inhibits the hydroxylase of HIFα – PHD, preventing HIFα from degradation. On the other hand, glucose can upregulate HIF protein levels though the AMPK-mTOR pathway. Both hypothalamic succinate/fumarate and AMPK-mTOR signals regulate feeding as well and their effects depend on HIF activity in POMC neurons. Meanwhile, HIF doesn’t seem to be involved in hypothalamic leptin functions.

4) Not only HIF senses glucose in the hypothalamus, it also is an amino acid sensor and insulin sensor. We found that both leucine and insulin can activate HIF2 by increase the protein levels of its α subunit. Further experiments showed that leucine and insulin activate HIF in an mTOR pathway dependent manner. The HIFβ knock-out model showed that HIF in POMC neurons is required or partially required for hypothalamic leucine and insulin sensing.
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