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Energy balance pathways converging on the Nhlh2 transcription factor

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1. ABSTRACT

Multiple regulatory pathways exist to control the expression levels of neuropeptides in response to body weight and energy availability changes. Since many neuropeptides are first synthesized in a pro-neuropeptide form, the availability of processing enzymes in a neuron can control the amount of active mature neuropeptide produced at any given time. In this review, we will focus on the regulation of prohormone convertase 1 (PC1) and prohormone convertase 2 (PC2), as well as downstream neuropeptide genes. Evidence from our laboratory suggests that Nescient helix-loop-helix 2 (Nhlh2) regulates the transcription of PC1 and PC2, possibly in conjunction with the leptin-stimulated transcription factor, STAT3. Furthermore, Nhlh2 itself is a target of leptin and other energy availability signals, with high levels of expression during energy surplus, and low levels of expression in conditions of reduced energy availability such as food deprivation or cold exposure. Overall, coordinate regulation of Nhlh2, PC1, PC2 and downstream hypothalamic neuropeptides such as thyrotropin releasing hormone (TRH) and pro-opiomelanocortin (POMC) does lead to energy balance modulation and ensuing long-term changes in body weight.

2. INTRODUCTION

Billions of dollars in research money are spent annually to understand the cellular and molecular mechanisms governing the regulation of body weight. As a result, numerous gene products have been identified that play a role in regulating weight using signals from the gut to the brain. The term "energy balance" describes the amount of available energy for daily metabolic requirements and physical activity balanced against our daily food consumption (for a review see (1)). If more food is consumed than is needed for physical and metabolic expenditure, the excess is stored as fat. If less food is consumed, then fat stores are utilized. The control of energy balance involves complex signaling and nutrient sensing pathways between the central nervous system (mainly the hypothalamus and hindbrain), the gut and the fat stores. Neurons in the hypothalamus play a key role in sensing and regulating energy balance in the body. Specifically, the hypothalamus regulates feeding, physical activity, and metabolism through the neuroendocrine expression of neuropeptides and the secretion of hormones. To accomplish these coordinated expression levels, groups of genes must be differentially regulated in response to signals from the periphery. This regulation occurs both

transcriptionally (i.e. promoter-mediated gene regulation) and post-transcriptionally (i.e. post-transcriptional mRNA stability, protein translational control, protein modification and control of neuropeptide processing).

At least eight different transcription factors have been implicated in the regulation of body weight by hypothalamic neurons (2). Many of these, including SF-1 (3), STAT3 (4), AP-1 (5) and Nhlh2 (Vella *et al.*, submitted), are reported to be stimulated either transcriptionally or post-transcriptionally by leptin. STAT3 is one of the seven members of the signal transducers and activators of transcription (STAT) family of proteins. STAT3 is phosphorylated by a janus kinase (JAK), homodimerizes, and moves into the nucleus to stimulate transcription of target genes. There is little evidence to suggest that STAT3 expression itself is regulated transcriptionally. Rather, both up- and down-regulation of STAT3 protein action occurs post-translationally through either phosphorylation or sequestering mechanisms (6). Steroidogenic factor 1 (SF-1) is a member of the nuclear hormone receptor superfamily of transcription factors. Evidence suggests that SF-1 may also be regulated post-transcriptionally by phosphorylation which simulates ligand binding (7). The activator protein-1 (AP-1) complex, a member of the leucine zipper family of transcription factors, is composed of a jun subunit and a fos subunit. Transcriptional regulation of c-fos in response to leptin is well documented (2). In addition, junB, the major hypothalamic jun mRNA, has been shown to be transcriptionally up-regulated in response to energy availability (8). Identification of the specific gene targets for STAT3, AP-1 and SF-1 and the role of these transcription factors in body weight regulation are still under intense investigation by other laboratories. In this review, the function of the basic helix-loop-helix transcription factor Nhlh2 in regulating and coordinating energy balance signals received by the hypothalamus is analyzed in detail. The phenotype of the Nhlh2 knockout mice (N2KO) is described, putative hypothalamic gene regulatory targets of Nhlh2 are identified, and the mechanism by which Nhlh2 expression and protein activation are activated or repressed by changes in energy availability are investigated. All of this information will be placed in the context of gene regulatory pathways converging on hypothalamic neurons during energy balance cascades.

3. ENERGY BALANCE AND THE NHLH2 TRANSCRIPTION FACTOR

3.1. The Nhlh2 transcription factor knockout mice

Nhlh2 is a member of the basic helix-loop-helix (bHLH) transcription factor family. Members of the bHLH family form homo- or heterodimers with other bHLH family members and with members of the leucine zipper family of transcription factors, such as jun and fos, retinoblastoma protein, Ets transcription factors and LIM homeodomain proteins, to activate or repress transcription through E-box motifs (9-11). Nhlh2 was originally identified in a homology search using a probe to the related bHLH cDNA Nhlh1 (NSCL-1) to screen a murine 11.5-day embryo library (12). The murine Nhlh2 (also called NSCL-2 and Hen-2) is expressed as early as 9.5 days of gestation in mice and can be found in both the postnatal cerebellum (13) and the adult hypothalamus and pituitary (14, 15).

Like other bHLH transcription factors, the Nhlh2 protein most likely requires a dimerization partner to modulate the transcription of target genes. We have found that Nhlh2 can homodimerize and can form heterodimers with both the related bHLH protein Nhlh1 and with the class I bHLH factors E12 and E47 (Good, unpublished). A yeast two-hybrid assay found interaction of Nhlh2 with Id-1 bHLH factor (Ilan R. Kirsch, personal communication), but no other *in vivo* assays were done to confirm this or the other interactions. We have found that Nhlh2 can interact with leucine zipper transcription factors such as jun and fos using pull-down assays, but the role of these interactions in Nhlh2 function has yet to be established. Recently, Nhlh2 was shown to form a complex with Bex2 (formerly called Bex1) and LMO2 on the E-box motif CAGCTG. This complex activates transcription in COS-7 cells (16). Nhlh2 also forms a complex with LMO3 (17), but the promoter context of this complex and the genes regulated by either of the complexes are unknown at this time. On the necdin promoter, Nhlh2 binds to and regulates necdin gene expression through two different E-box motifs, CATGTC and CACATG (18).

Nhlh2's role in the brain was not clear until the phenotype of the Nhlh2 knockout mouse (N2KO) was examined (14). Hypogonadism is evident as early as birth in N2KO males and females, and fertility in these animals is impaired by a combination of both reduced gamete numbers and reduced sexual behavior (14, 19). Thus, at least one role of Nhlh2 and its target genes is in regulating fertility through gametogenesis and sexual behavior. Nhlh2 is also required for body weight control, and in particular, weight regulation in adults. A study designed to examine body weight changes and body fat content in normal, heterozygous and N2KO mice found that increased weight gain was not detectible until at least 12 weeks of age in male N2KO mice and 7 weeks of age in female N2KO mice. Both sexes continued to gain weight through the end of the study at 52 weeks, although age-matched WT mice maintained weight to a similar level ($\pm$ 5 grams) of that obtained at ~12 weeks of age. The increased body weight in both sexes can be attributed to increased body fat, as carcass fat content continues to increase linearly through 52 weeks of age (20).

In a long-term study of the phenotypic changes in N2KO mice, our laboratory showed normal food intake levels in pre-obese N2KO mice. Slight hyperphagia is evident only when the animals become obese and is proportional to body weight (20). We also showed that body temperature is normal in these animals, but that spontaneous voluntary exercise is decreased by more than 50% in both male and female N2KO mice given access to computerized running wheels. There was no difference in the circadian rhythm of running, as the little running done by N2KO mice was nocturnal. Likewise, N2KO mice do

not show motor deficiencies in standard rotarod apparatus tests indicating that lack of physical exercise is not due to motor or balance problems (20). Most importantly, our contention that alterations in physical activity levels causes increased body weight in N2KO mice is supported by data showing that reduced activity levels in these animals precedes the onset of obesity by at least 2 weeks (females) and up to 7 weeks (males). Male mice were tested for activity levels starting at 5 weeks of age when they are pre-obese and weigh the same or less than WT mice. Under these conditions, male N2KO mice are 5-fold less active than male WT mice (20). Female mice, tested at 7 weeks of age to account for wheel running behavior during estrous cycles showed a similar trend (20). Thus, reduced physical activity in N2KO mice is at least one of the main causes of adult-onset obesity in this animal model. This is a unique phenotype among other obese mouse models, as many show hyperphagia as a main component of their weight gain. To ensure that food intake is not involved in the weight gain in N2KO mice, we re-examined food intake in these animals using several measures. Cumulative food intake totals for mice between 12 to 25 weeks of age shows that N2KO mice eat significantly more cumulative food than WT males (Coyle and Good, unpublished). There are three points that need to be considered in relation to this data. First, the N2KO mice are obese during these ages, but an analysis of food intake per gram body weight shows that for all genotypes, animals consume the same amount of food for their body weight (Coyle and Good, unpublished). This suggests that the animals are eating more to account for the increased energy needs of their increased body weight. Second, at eight weeks of age, when the animals are pre-obese and physical activity is significantly reduced (20), twenty-four hour food intake of individually-housed animals is similar for both genotypes (Fox *et al.*, unpublished). Third, even during periods of increased energy requirement, such as 24 hour cold exposure, N2KO mice do not show increased food intake (Vella and Good, unpublished). Taken together, these points argue against food intake playing more than a peripheral part in maintaining the higher body weight of the obese animals. Reduced spontaneous activity, on the other hand, is the more likely culprit in body weight gain in these animals, as it is significantly reduced in N2KO mice tested during 5-10 weeks of age (20), when food intake is the same for all genotypes.

Leptin levels in mammals reflect their state of energy availability at the time of the assay (1). In young, pre-obese N2KO mice, leptin levels are similar to age-matched WT mice, while older obese N2KO mice display hyperleptinemia, reflective of their increased body fat (21). Interestingly, leptin levels in young pre-obese N2KO mice who have been food deprived for 24 hours are elevated compared to WT food deprived animals (Fox *et al.*, unpublished). In addition, N2KO mice can conserve body weight better during food deprivation, losing one gram of body weight less than WT mice over 24 hours (Fox *et al.*, unpublished). Weight loss during cold exposure is more pronounced in N2KO mice after a four hour exposure, although similar total weight loss in WT and N2KO mice occurs during the 24 hour exposure (Vella and Good, unpublished). This indicates that N2KO mice may be unable to respond well to the initial energy balance challenge, but can respond to a long-term depletion of energy reserves. Overall, N2KO mice appear to have a defect in leptin signaling that extends to include physiological differences when energy depletion is sensed.

Although these mutant animals display adult-onset obesity, including fatty deposits in their abdomen, around their gonads, and within their liver (14), they do not appear to be overtly diabetic. In fact, N2KO mice display normal glucose levels, and can clear glucose slightly faster than WT mice (Fox *et al.*, unpublished). One animal model with a similar phenotype to N2KO mice with respect to glucose tolerance are CD26 Knockout mice (22). CD26 is a serine protease that affects pain perception, T-cell function and cytokine secretion (23-25). There has been no report on the adult-body weight of CD26 animals and, thus, it is unclear whether Nhlh2 may function upstream of CD26 protease or if the similar glucose and insulin profiles are a coincidence.

Overall, N2KO mice are a unique model of adult-onset obesity—unique for their inactivation of a neuronal basic helix-loop-helix transcription factor. Also unique about these animals is the cause for their obesity—reduced spontaneous or motivation-induced physical activity. Finally, these animals are a model for obesity unlinked from diabetes, and can potentially be used to understand the mechanism of body weight gain in the absence of glucose intolerance.

3.2. Gene targets of Nhlh2 and their role in energy balance modulation

Nhlh2 is bHLH transcription factor family member. Based on the phenotype of the N2KO mice, at least some of the potential gene targets of this transcription factor should be involved in body weight control. bHLH transcription factors bind to a semi-palindromic site called the E-box, and represented as CANNTG (10). Gene expression analysis in N2KO animals has identified Prohormone convertase 1 (PC1) and Prohormone convertase 2 (PC2) as putative gene regulatory targets of Nhlh2 (15). Both PC1 and PC2 contain conserved E-box motifs in their proximal promoters (Figure 1). In the human PC1 promoter, one of the E-boxes (CACATG) is an exact match to that found to be necessary for necdin gene regulation by Nhlh2 (18) (Figure 1B). In the PC2 promoter, both of the E-boxes (CAGCTG) exactly match the Nhlh2 binding motif identified by Han and colleagues (Figure 1B) (16). In this section, the role of both of PC1 and PC2 in body weight regulation and their potential transcriptional regulation by Nhlh2 will be discussed.

3.2.1. PC1 and PC2

The obese phenotype of the N2KO mice led to the examination of levels for several hypothalamic neuropeptides, including POMC. In the original paper describing the N2KO mice, we reported that there were reduced levels of immunoreactive POMC peptide in these mutant animals (14). When POMC mRNA levels in N2KO mice were analyzed by *in situ* hybridization and found to be

normal, we realized that reduced levels of POMC peptide in N2KO mice resulted from reduced peptide processing of the pro-peptide. A 40-60% reduction in both PC1 and PC2 mRNA in several hypothalamic nuclei of the N2KO mice was reported by Jing and co-workers, making these two genes putative targets of Nhlh2 (15).

PC1 and PC2 were originally identified using a probe derived from the cDNA sequence from the active sites in the yeast Kex2 and human Furin1 gene. The proteins are found primarily in endocrine and neural tissues as demonstrated via *in situ* hybridization and Northern blots in both vertebrates and invertebrates (26). PC1 and PC2, along with the other members of the subtilisin/kexin-like pro-protein convertase family, act in the secretory pathway to cleave precursor proteins into their more mature forms usually after selected pairs of basic residues in the proneuropeptide amino acid sequence, mainly lysine-arginine or arginine-arginine residues (26). PC1 and PC2 transcripts and protein products are widely distributed in different areas of the brain including the cerebral cortex, hippocampus and hypothalamus (26, 27). However, their expression does not entirely overlap. The processing of many neuroendocrine precursors, including proinsulin, proglucagon, proopiomelanocortin (POMC), pro-cocaine and amphetamine-regulated transcript (pro-CART), proenkephalin, prodynorphin, prosomatostatin, pro-thyrotropin releasing hormone (proTRH), and pro-growth hormone releasing hormone (pro-GHRH) are mediated by PC1 and PC2 but are differentially dependent on these two processing enzymes (26). It is thought that the expression patterns and levels of PC1 and PC2 in different neuroendocrine cells dictate which mixtures of peptide products will be produced.

Although PC1 was thought to be responsible for processing POMC peptides in the hypothalamus, including alpha-melanocyte stimulating hormone (αMSH) which is involved in food intake and energy expenditure, the PC1 knockout mice are not obese. However, a new PC1 mutant animal identified using a forward genetics screen has both obesity and reduced αMSH production (28). The homozygous mutation in PC1 is thought to impair PC1 functionally because it does not entirely eliminate its expression. Targeted disruption of the PC1 gene in mice causes severe postnatal growth impairment and multiple endocrine peptide processing defects, including processing of pro-GHRH, pituitary POMC, islet proinsulin, and intestinal pro-glucagon (29, 30). Mice lacking PC1 appear normal at birth, but exhibit a growth defect in which they are 60% of normal size at 10 weeks, most likely due to the lack of mature GHRH. They also display hyperproinsulinemia but without impaired glucose tolerance (29, 30). Interestingly, the PC1 heterozygous mice are mildly obese starting at approximately 10 weeks of age, similar to the adult-onset obesity of N2KO mice. This is particularly interesting as PC1 heterozgyous animals have a ~50% reduction in PC1 expression, while N2KO mice have between a 40-60% reduction in PC1 expression (14, 15, 31). Surprisingly, functional inactivation of PC1 has a different effect in humans. To date, heterozygous inactivation of PC1 in humans has no phenotypic consequence and has not been associated with obesity. However, two human patients with inactivating mutations (compound heterozygotes) in both alleles of PC1 display severe obesity. This indicates that PC1 gene expression is necessary for human body weight regulation and suggests that a 50% reduction in PC1 heterozygous humans can be compensated for by other means (31, 32). The phenotypes of these human patients are similar to the PC1 mutant mouse, suggesting that impaired function of PC1 leads to obesity.

Disruption of the PC2 gene in mice causes reduced litter size and a reduced growth rate that is not as severe as in the PC1 null mice (33). PC2 knockouts fail to produce any mature αMSH and accumulate POMC processing intermediates (34). PC2 mice also have defects in proinsulin processing, as one can find mildly elevated circulating serum and pancreatic proinsulin levels in these animals (35). Similar to the PC1 null and heterozygous mice, this defective processing of proinsulin in the PC2 nulls does not lead to the development of diabetes PC2 null mice also display defects in glucagon processing resulting in hypoglycemia that can be corrected by glucagon replacement (36). PC2 null mice are not obese, and humans with a mutation in PC2 have yet to be identified.

3.2.2. Transcriptional regulation of PC1 and PC2 genes

Changes in the expression of the PC1 and PC2 genes are mediated by glucose, thyroid hormone, leptin, and gp130-related cytokines that are associated with the leptin JAK/STAT pathways (37-40). The promoter regions of both of these genes have been extensively studied with respect to thyroid hormone mediated regulation. While less well studied, both the human and mouse PC1 and PC2 promoters contain putative STAT3 and E-box motifs (Figure 1). The E-box motifs in the PC2 promoter are identical to the identified Nhlh2 E-box motif CAGCTG (16). The spacing and sequence of both the Ebox motifs, the STAT3 site are conserved between the mouse and human PC2 promoter sequences (accession numbers AF416733 and HSU73595 respectively) (Figure 1B). The human PC1 promoter has three E-box motifs while the mouse PC1 promoter has two (Figure 1A) (accession numbers HSU24128 and S74618, respectively). What is most striking about the mouse and human PC1 promoters is the close proximity of putative binding sites for the STAT family of transcription factors to the consensus E-box motifs (Figure 1A). Leucine zipper transcription factors, like STAT, form complexes with bHLH transcription factors (41). We have evidence that Nhlh2 can form a complex with both jun and fos, two other leucine zipper transcription factors (Good, unpublished), leaving open the possibility that Nhlh2 and STAT could form a heterodimeric complex to regulate PC1 gene expression. This possibility is further supported by the findings that STAT3, Nhlh2 and PC1 are all downstream targets of leptin stimulation of hypothalamic neurons (37, 42-44) (Vella *et al.*, submitted). A role for Nhlh2 in controlling PC1 and possibly PC2 expression via the leptin and Jak/STAT pathway is currently being investigated. Confirmation of this pathway would directly link the action of this bHLH transcription factor to the transcriptional

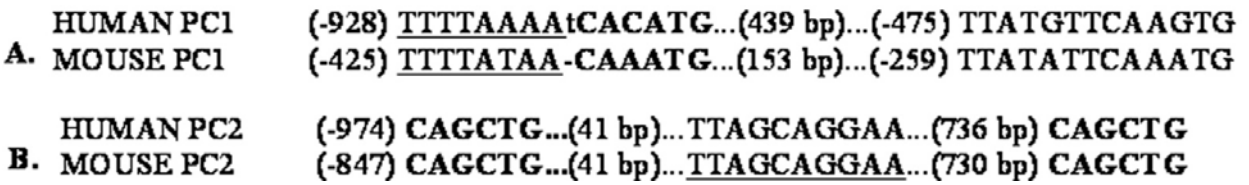

Figure 1. E-box and STAT motifs in the PC1 and PC2 promoters. The published mouse and human PC1 (A) and PC2 (B) promoters were scanned for the presence of consensus STAT binding motifs ($TT(N)_{4\text{-}6}AA$) (underlined) and consensus E-box motifs (CANNTG) (bolded).

control of neuropeptide processing enzymes and the regulation body weight.

3.2.3 .Identifying downstream effects of PC1 and PC2 reduction in N2KO mice

While to date we have reported only on PC1 and PC2 as possible transcriptional targets of Nhlh2, regulation of these two targets has many possible downstream effects. In the arcuate nucleus, Nhlh2 regulation of PC1 was shown to modulate levels of POMC-derived peptides, such as αMSH and ACTH (Figure 2) (15). New data from our laboratory supports our previous work showing that while Nhlh2 regulates αMSH levels through PC1 gene regulation, Nhlh2 does not directly affect POMC mRNA levels, even during leptin stimulation. We had previously shown that levels of POMC mRNA were normal to slightly elevated in N2KO mice (15). We now show that POMC mRNA levels in N2KO mice are also not adversely affected following leptin stimulation (Vella *et al.*, submitted). In other words, POMC mRNA is stimulated by leptin to similar levels in both N2KO and normal mice. . This suggests that Nhlh2 action does not affect POMC gene expression directly, but rather modulates POMC peptide expression through Nhlh2's direct action on the PC1 promoter.

Reduction of the POMC-derived peptide αMSH via Nhlh2 regulation of PC1 and PC2 could also have an indirect effect on TRH mRNA levels, as TRH neurons express melanocortin-4 receptor (MC4R) and respond to αMSH or melanocortin agonists with increased TRH expression (Figure 2) (45). In our paper by Jing *et al.*, (15), we showed that thyrotropin releasing hormone (TRH) mRNA and peptide levels were reduced in N2KO mice. Since PC1 levels were also reduced in the PVN where TRH is synthesized, we concluded that reduced TRH peptides were due to reduced processing of pro-TRH caused by reduced PC1 (15). While we hypothesized that reduced TRH mRNA could be due to reduced levels of processed αMSH, we also speculated that reduced TRH mRNA could be a direct effect of lack of the Nhlh2 transcription factor. Recent evidence from our laboratory supports this latter hypothesis, as TRH mRNA levels increase in N2KO mice in response to food deprivation (Vella *et al.*, submitted). These data suggest that Nhlh2 actually functions to regulate both the peptide level and mRNA level of TRH in the PVN during energy balance changes. The TRH promoter contains a STAT3 site, which functions to directly regulate TRH gene expression following leptin stimulation (45). The STAT3 site is just upstream from an SP1 site, and approximately 100 bp from one of 9 putative E-box motifs in the TRH promoter that we have identified by visual inspection of the sequence (data not shown). More work will need to be done to determine if the TRH promoter is a direct target of Nhlh2 or if Nhlh2 in conjunction with STAT3 mediates the response of this promoter to leptin stimulation.

A number of other possible indirect (via PC1 and PC2) and direct targets of Nhlh2 transcriptional regulation in the hypothalamus exist, but have not yet been identified. We are searching for these targets using the expected means of microarray analysis (for genes that may be directly regulated through their promoter and mRNA levels), proteomic analysis (for protein processing mediated indirect effects of Nhlh2 regulation) and by analyses based on Nhlh2 expression pattern and known functions of proteins in body weight regulation. Identification of these direct and indirect targets of Nhlh2 will allow us to complete the picture of the complex phenotype of the N2KO mouse with regards to proneuropeptide processing and the downstream effects on motivated exercise, body weight regulation and sexual behavior.

3.3. Regulation of the Nhlh2 gene in response to energy balance modulation

The phenotype of the N2KO mice and an analysis of putative Nhlh2 hypothalamic gene regulatory targets suggest that the Nhlh2 promoter may itself be a regulatory target of leptin or other energy availability signals. Below, we discuss how Nhlh2 mRNA expression is modulated when experimental perturbations to the energy balance equation are made. These data complete the pathway from leptin signaling to gene regulatory changes in the hypothalamus, indicating that at least some of the leptin-mediated changes in gene regulation occur through regulation of Nhlh2 expression patterns and activity.

3.3.1. Food intake

Nhlh2 is expressed in the ARC, PVN, LH, DMH and VMH of the hypothalamus (15, 18, 42), areas known to express genes responsive to changes in energy availability. This, coupled with the adult-onset obesity phenotype of N2KO mice, make Nhlh2 an excellent candidate gene target for energy balance signals. We and others have shown that hypothalamic Nhlh2 mRNA expression is reduced following 24 hour food deprivation in WT mice compared to ad libitum fed mice ((42), Vella *et al*, submitted). A 50% reduction of PC1 and PC2 protein occurs following 65-hour food deprivation in rats (37, 43), and our published data shows that reduction of Nhlh2

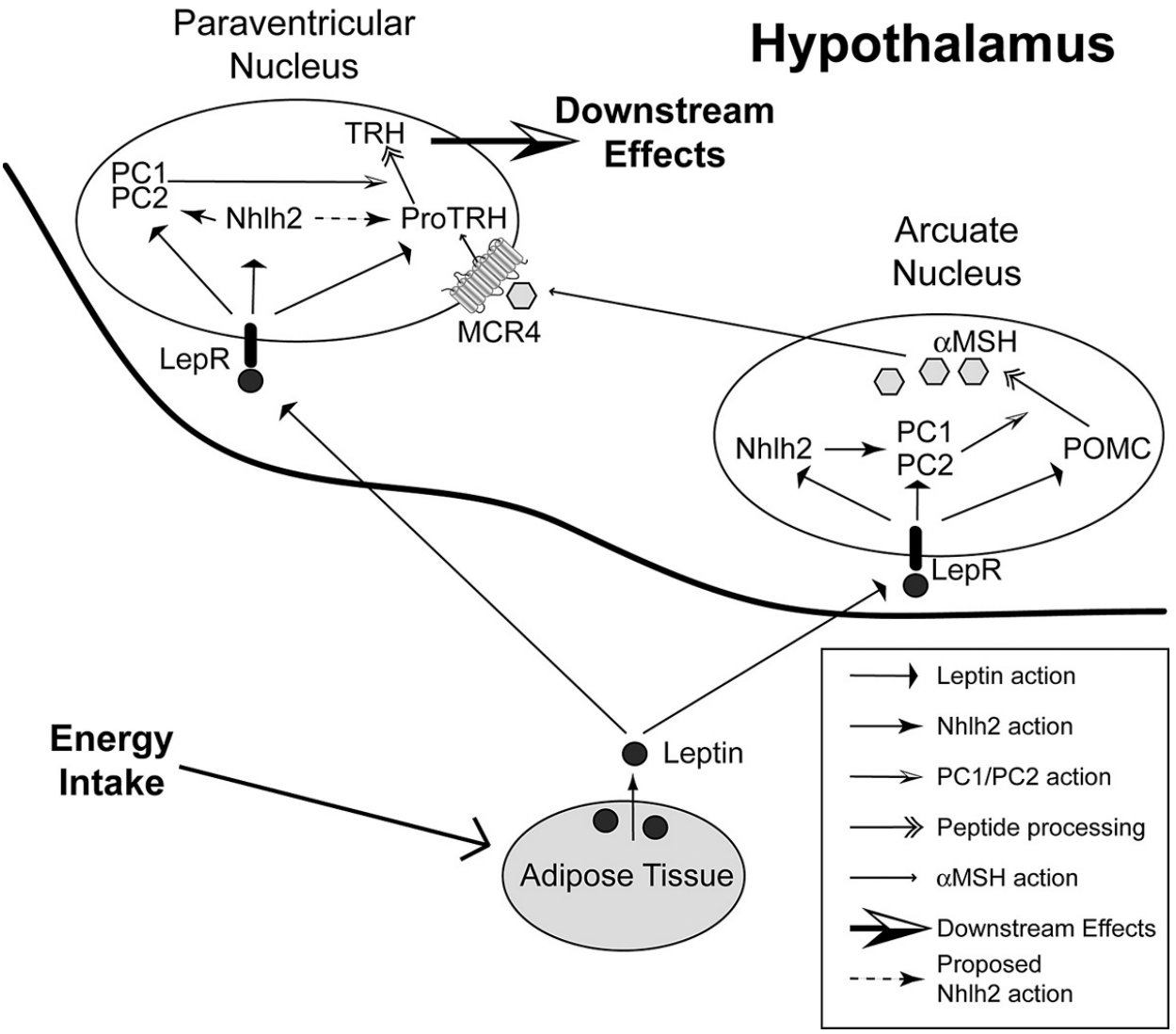

Figure 2. Proposed Nhlh2 signaling pathway following leptin stimulation of hypothalamic neurons. Diagram shows a section of the third ventricle of the hypothalamus (3rd ventricle) receiving peripheral leptin stimulation, which activates neurons in both the arcuate nucleus and paraventricular nucleus. These neurons communicate through αMSH production, such that paraventricular nucleus neurons receive both direct and indirect stimulation from leptin. Nhlh2 is expressed in neurons from both of these areas and controls expression of PC1 and PC2 in each of these neurons, leading to production of mature neuropeptides and downstream effects on body weight control. It is hypothesized that Nhlh2 may also control TRH gene expression, either directly or indirectly as indicated by the dashed arrow.

coincides with reduced levels of POMC-derived peptides in the ARC and TRH peptide in the PVN (15). Taken together, this suggests that Nhlh2-mediated signals are necessary for energy-balance mediated effects on PC1 expression and downstream proneuropeptide processing (Figure 2).

Nhlh2 expression returns to baseline when ad libitum food is given following deprivation (Vella *et al.*, submitted). In WT mice allowed ad libitum access to food for 2 hours following a 24 hour food deprivation period, levels of Nhlh2 mRNA expression returned to levels in ad libitum fed animals in the PVN and whole hypothalamus and to 55% *ad libitum* levels in the ARC. Nhlh2 expression in these hypothalamic areas was co-expressed with TRH mRNA in the PVN and with POMC mRNA in the ARC (Figure 2) (15) and this colocalization is reduced following 24-hour food deprivation (Vella *et al.*, submitted). Return of food to mice for two hours following food deprivation brought an increase in colocalization in the PVN and ARC, but not to the same levels as that of ad libitum fed mice. Overall, these data suggest that modulation of Nhlh2 expression may occur earlier, and may be upstream of changes in the levels of hypothalamic neuropeptides which serve as the downstream mediators of energy balance modulation. This is an area of active investigation.

3.3.2. Leptin

The effect of food return on Nhlh2 expression within that short 2 hour time span is indicative of signaling from the periphery to the hypothalamus. Leptin, the most-studied peripheral signal with respect to energy balance modulation, is a prime candidate in Nhlh2 stimulation as leptin levels can be modulated by changes in food intake (46). Recent data from our laboratory shows that changes in Nhlh2 mRNA levels occur following peripheral injection of leptin (Vella *et al.*, submitted). In experiments where food deprived mice were injected with 3mg / kg body weight mouse recombinant leptin and euthanized 2 hours post-injection, Nhlh2 mRNA expression, as measured by *in situ* hybridization increased to 1.5 fold greater than ad libitum fed mice in both the PVN and ARC. Quantitative RT-PCR analysis of whole hypothalamus showed a 100-fold increase in Nhlh2 mRNA expression over ad libitum controls. This increase coincides with colocalization studies with TRH in the PVN and POMC in the ARC. In both areas of the hypothalamus, colocalization of Nhlh2 with both of these peptides is increased over ad libitum controls (Vella *et al.*, submitted).

These studies all used 2 hours as an end-point for energy return. However, PC1 and TRH mRNA expression have been found to peak at 3 and 6 hours following food deprivation (37). In order to examine the time course of Nhlh2 expression following leptin stimulation, and to determine if Nhlh2 expression was increased prior to, co-incident with or following increases in POMC and TRH, we examined Nhlh2, TRH and POMC mRNA levels at various time points following peripheral leptin injection. Nhlh2 is increased as early as 15 minutes post-leptin injection and continues to rise with a peak at 2 hours (Vella *et al*, submitted). Thus, high level Nhlh2 expression is stimulated early following leptin injection, and remains high in the hypothalamus for at least eight hours. Relative to Nhlh2 expression, increases in POMC and TRH mRNA expression are delayed, and do not show the extended high level expression pattern that we found for Nhlh2. This pattern further supports a role for Nhlh2 as an upstream regulator in leptin-mediated signals involved in body weight regulation.

3.3.3. Transcriptional regulation of Nhlh2

The sequence of ~300 base pairs on the proximal promoter of the human NHLH2 gene and its closely homologous family member NHLH1 was reported in 1992 (47). While these genes most likely diverged from a common ancestral gene, the promoter regions of the two genes are quite different. Within the first 300 base pairs, the NHLH2 promoter contains an AP-1 site, several GATA3 sites and an E-box motif which could be autoregulatory. Interestingly, the NHLH2 promoter is quite highly conserved among mammals with approximately 90% conservation within the first 300 base pairs of the proximal promoter (48, 49). Within the first 1000 base pairs of the promoter a number of putative motifs are conserved between bovine, mouse and human NHLH2 promoter, including 3 AP-1 motifs, a progesterone receptor binding motif, an NF kappa B motif, a retinoic acid alpha 1 motif, a GATA site and the E-box motif (Burnside and Good, data not shown). The human and mouse NHLH2 promoters also contain five putative binding motifs for the leptin-stimulated transcription factor STAT3 (21). This finding supports work from our laboratory showing regulation of Nhlh2 by leptin (Vella *et al.*, submitted). The functionality of the STAT motifs has been further analyzed leading to a somewhat complicated story. Oligonucleotides to each of the five STAT3 motifs on the mouse and human NHLH2 promoters were analyzed for STAT3 binding using electrophoretic mobility shift analysis. Surprisingly, only one of the sites displays STAT3 binding, and this site is the only one that is 100% homologous for the human and mouse promoters (Burnside and Good, unpublished), as well as the bovine NHLH2 promoter sequence (Good, unpublished). The binding by STAT3 requires copious amounts of the transcription factor, and is still weak compared to the control reaction (Burnside and Good, unpublished). The site did not show a supershift using anti-STAT3 antibody, while control STAT3 binding site was supershifted (Burnside and Good, unpublished). In addition, using N29/2 cells transfected with STAT3, leptin receptor and an Nhlh2 promoter reporter construct we have been unable to demonstrate high levels of expression from the Nhlh2 promoter, although the 1.3-fold increase is significant over baseline (Burnside and Good, unpublished), similar to effects on PC1 promoter transactivation by cytokines (38) and similar to the 1.5-fold stimulation of Nhlh2 by leptin as measured by *in situ* hybridization. Further analysis of the role of STAT3 and possibly other interacting direct and indirect components of the leptin pathway in Nhlh2 transcriptional regulation is underway.

3.3.4. Post-transcriptional regulation of Nhlh2

With the exception of pro-neuropeptide processing and post-translational modification of STAT3 and other factors, post-transcriptional mechanisms of gene regulation are not well studied with respect to pathways of energy balance regulation. In particular, mechanisms of pre-translational gene regulation such as mRNA stability need more directed research in the obesity field. The 3' untranslated region (UTR) of Nhlh2 contains numerous AUUUA motifs (12, 47), long thought to be involved in mRNA stability. In addition, a recent analysis of Nhlh2 3' UTR using the Sanger microRNA Target Database (50) reveals that both human and mouse Nhlh2 has 16 putative microRNA (miRNA) homology regions overlapping some of these AUUUA motifs (data not shown). These small, evolutionarily conserved, non-protein coding RNA molecules have been shown to regulate mRNA translation or stability by interacting with conserved sequences in the 3' non-coding region of a gene (51). miRNAs could play an important role in body weight control, as it is thought that they represent ancient mechanisms for gene regulation (52). As pathways of energy conservation and utilization are present from Drosophila to mammals, it is quite plausible that miRNA will be present and active as one pathway of gene regulation following energy balance modulation in the mammalian hypothalamus. Much more work is needed in this area to determine if Nhlh2 is a target of mRNA stability signals in response to changes in energy availability.

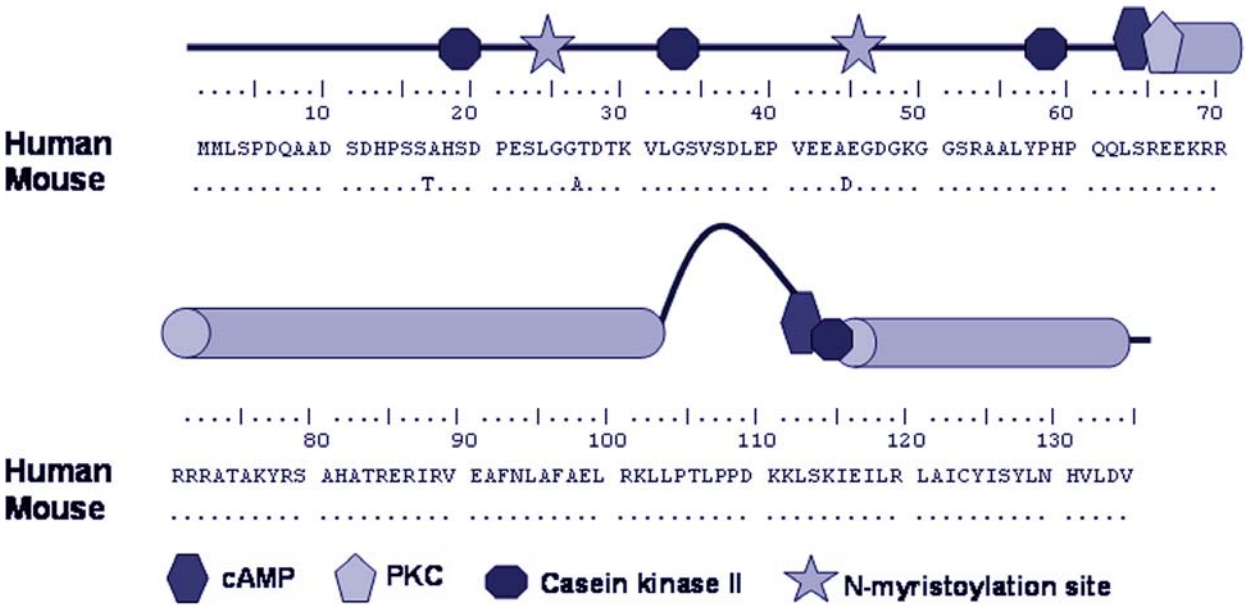

Figure 3. Schematic representation of the protein folding and putative regulatory motifs on Nhlh2. The published amino acid sequence of NHLH2 (Nhlh2, mouse) (accession # AAH96359 (human) and AAH58413 (mouse) are shown below a schematic representation of the linear N-terminus and C-terminal helix-loop-helix domains of the protein. Putative motifs, identified using ProSite (http://ca.expasy.org/prosite/) (57) are identified along the protein strand.

The protein sequence of Nhlh2 suggests that it could also be subject to post-translational protein modification by several different pathways (Figure 3). The human, mouse and bovine Nhlh2 protein sequences contain two putative cAMP-dependent protein kinase (PKA) sites—one within the basic DNA binding domain and the other within the loop domain of the bHLH motif. Both of these sites have the potential to influence DNA binding or protein interaction for Nhlh2. In muscle, PKA-mediated phosphorylation within the basic domain of the bHLH transcription factor MyoD activity leads to inhibition of muscle cell differentiation (53, 54). There is also a putative protein kinase C phosphorylation site in the basic domain of Nhlh2 and this could mediate DNA binding as well. Again, myogenin and MyoD both have a PKC site in their basic domain which appears to inhibit DNA binding by these bHLH transcription factors (55). The protein coding sequence of Nhlh2 contains four putative casein kinase (CKII) phosphorylation sites. The DNA binding activity of tal-1, a highly homologous bHLH transcription factor used in the original cloning scheme for Nhlh2 and the related Nhlh1 gene (12), is modulated by CKII phosphorylation (56). Finally, Nhlh2 contains two N-myristolation sites within its N-terminal activation and regulatory domain. To our knowledge, no mammalian bHLH factors is modulated by the addition or removal of myristoyl, although this still remains a possible route for post-transcriptional regulation, especially as a mechanism for sequestering the protein in the cytoplasm. Overall, regulation of Nhlh2 activity via post-translational modification of this transcription factor following leptin stimulation or other modulators of energy availability is a wide open area for investigation. Changes in the transcriptional activity and DNA binding affinity of Nhlh2 via protein modification could affect levels of PC1 and PC2, as well as other Nhlh2 target genes, leading to downstream changes in mature neuropeptide production and body weight control.

4. PERSPECTIVE

Post-translational processing of neuropeptides is an important step in the regulation of adult body weight. Key to this is the regulation of both the pro-neuropeptide products and the enzymes that process them. We have strong evidence that the neuronal bHLH transcription factor Nhlh2 regulates the mRNA levels for both the PC1 and PC2 neuropeptide processing enzymes and at least one mRNA for a pro-neuropeptide, TRH. Thus, Nhlh2 exerts its effect on both the transcriptional and post-transcriptional pathways that lead to expression of neuropeptides (Figure 2). For now, our evidence and that from other laboratories suggests that Nhlh2 mediates its control during periods of high energy availability, as Nhlh2 expression itself is increased in response to leptin or food intake and reduced when animals are food deprived. It is possible that other hypothalamic neuron subtypes could express Nhlh2 in a fashion opposite to what we have found to date. Much more work will be needed to examine these other populations to determine if Nhlh2 is expressed in the so-called orexigenic neurons such as melanin-concentrating hormone neurons in the lateral hypothalamus, and if so, if its regulatory pattern in these neurons differs from that in anorexigenic neurons, such as POMC or TRH neurons. To date, our evidence using whole hypothalamic mRNA patterns of expression suggests that Nhlh2 is confined to the energy sensing neurons that mediate an anorexigenic and energy saving response pattern. Our data support a hypothesis whereby Nhlh2 regulates expression level of the

processing enzymes needed to generate the energy saving response, both by directly regulating PC1 and PC2 mRNA transcriptionally, and in some cases, regulating expression of the neuropeptide genes themselves. Identification of other Nhlh2-target genes, and indirect targets via Nhlh2's transcriptional control of the PC1, PC2 and TRH genes, using microarray and proteomic approaches should yield additional information on the energy balance networks that depend on functional Nhlh2 transcription factor for downstream effects on body weight control.

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Abbreviations: PC1 (prohormone convertase I), PC2 (prohormone convertase II), POMC (pro-opiomelanocortin), Nhlh2 (nescient helix-loop-helix 2), TRH (thyrotropin releasing hormone). STAT3 (signal transducer and activator of transcription 3), NF kappa B (nuclear factor kappa B), SF1 (steroidogenic factor 1), JAK (janus kinase), AP1 (activator protein-1), bHLH (basic helix-loop-helix), N2KO (Nhlh2 knockout mice), ARC (arcuate nucleus of hypothalamus), PVN (paraventricular nucleus of hypothalamus), LH (lateral hypothalamus), VMH (ventromedial hypothalamus), miRNA (microRNA).

Key Words : PC1, PC2, POMC, Nhlh2, TRH, Leptin, Hypothalamus, Transcription, Post-Transcriptional Processing, Review

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