Nhlh2 is a Cold-Responsive Gene

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Abstract: Nescient helix-loop-helix 2 (Nhlh2) is a basic helix-loop-helix transcription factor that functions to communicate signals of energy availability. Based on the expression pattern of Nhlh2 in cold-sensitive areas of the hypothalamus, we hypothesized that mice containing a targeted deletion of Nhlh2 would be unable to maintain body temperature and that Nhlh2 gene expression would vary with cold exposure. Following cold exposure, Nhlh2 mRNA levels are significantly reduced throughout the hypothalamus. Furthermore, mice with a targeted deletion of Nhlh2 (N2KO mice) are unable to maintain body weight at 4°C. This paper attempts to describe these new results as they relate to the ongoing gene regulation that occurs in the hypothalamus following changes in energy availability brought about by food intake or cold exposure.

Keywords: NSCL-2, Hen2, hypothalamus, cold exposure, TRH, transcription factors, gene regulation.

INTRODUCTION

Food intake, metabolism, and energy expenditure are adjusted and maintained by central nervous system neurons responding to and modulating peripheral signals [1]. Hypothalamic neurons play a key role in sensing and regulating available energy stores during changing conditions. For example, following food intake there is strong activation of hypothalamic neurons, especially the pro-opiomelanocortin (POMC) neurons within the arcuate nucleus [1]. These same neurons respond to leptin, as do neurons in the paraventricular nucleus of the hypothalamus (PVN), ventromedial (VMH), dorsalmedial (DMH) and lateral hypothalamic regions [2]. Each area contains leptin receptors that account for the wide-spread hypothalamic targets [3]. There is also a hypothalamic response to cold exposure, which occurs in some of the same hypothalamic nuclei as the response to leptin. For example, c-fos expression is increased in both the PVN and ARC of rat exposed to just 1.5 hours of cold (4°C) [4]. Rodents exposed to long-term (4 weeks) or acute temperatures (2 hours) of 4°C have a significant reduction in serum leptin levels compared to room-temperature held control animals [5, 6]. Thus it is probable that leptin mediates the response to energy availability following changes in food intake and ambient temperature. In this review we will analyze the published support for this, as well as provide some new supporting data. We will also provide evidence that the basic helix-loop helix transcription factor, nescient helix-loop--helix 2 (Nhlh2) may be responsible for mediating some of the downstream gene regulatory changes that occur with changes in energy availability, including cold exposure.

Cold Sensitivity and Impaired Ability to Regulate Core Body Temperature is Present in Some, but not Other Obese Mouse Models

Early experiments to test the ability of an obese line of mice (later determined to carry an inactivating mutation in leptin) to utilize energy stores in response to a cold challenge resulted in severe drops in core body temperature and death [7, 8]. With these, and later experiments, it was hypothesized that animals with a defect in the leptin-signaling pathway would be sensitive to cold exposure. Indeed, mice lacking leptin receptor show a similar sensitivity to temperatures below ambient levels [9] and were completely rescued of this phenotype when leptin receptors were restored in the hypothalamus [10]. These experiments would support the notion that leptin signaling is necessary for cold tolerance. However, the entire leptin signaling pathway is not required for this response. This is clearly seen when examining melanocortin-4-receptor (Mc4r) knockout mice. These mice show adult-onset obesity but are cold-tolerant. Thus, the leptin-stimulated pathway involving POMC and αMSH secretion, followed by binding of αMSH to Mc4r receptor on PVN neurons may not be necessary for complete tolerance to cold temperatures. On the other hand, cold exposure results in increased thyrotropin-releasing hormone (TRH) mRNA levels in the PVN [11], and *Cpe*^{fat} mice which cannot adequately process TRH are cold-intolerant [12]. Again contrary to this finding is one showing that the leptin-leptin receptor pathway can directly stimulate TRH gene expression [13]. With leptin levels reduced during cold exposure, other mechanisms and signaling pathways must account for the increase in TRH gene expression following cold.

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Mice containing a targeted deletion of Nhlh2 (N2KO) display adult-onset obesity marked by reduced spontaneous physical activity, which precedes body weight gain and mild hyperphagia present in older animals [14, 15]. N2KO mice also have reduced hypothalamic levels of thyrotropin-releasing hormone (TRH) mRNA and protein [16]. Compared to the levels in wild type (WT) mice, TRH mRNA levels in N2KO mice respond unexpectedly to changes in energy availability with an increase in mRNA levels following 24-hour food deprivation [17]. Other reports have shown that TRH varies in response to other signaling pathways including food intake (Lechan, Fekete 2006) and cold exposure (Zoeller, Kabeer & Albers 1990, Zoeller, Rudeen 1992). The cold-intolerant mice carrying a natural mutation in carboxypeptidase E, *Cpe*$^{fat/fat}$ mice also have reduced TRH levels [12]. The expression of Nhlh2 in cold-responsive regions of the hypothalamus and the phenotype of the N2KO mice, adult-onset obesity and abnormal TRH levels, suggest that N2KO mice might show impaired sensitivity to cold compared to WT mice.

N2KO Mice Maintain Normal Body Temperature at the Expense of Body Weight at 4°C

To test the hypothesis that N2KO mice are cold intolerant, a total of N = 10 WT and N = 10 N2KO mice were individually housed mice at 4°C starting at 1300 hours for 24 hours. All mice were males and were 8-12 weeks of age, which is an age at which there is no significant difference in body fat, body temperature or body weight in the N2KO line [14]. Core body temperatures were measured in WT and N2KO mice beginning with an initial reading at time point zero, and then at 30 minutes and 1, 2, 4, 8, 12 and 24 hours. Surprisingly there was no significant difference in the ability of N2KO mice to maintain their body temperature during cold-exposure, when compared to WT mice (Fig. **1A**). Body weight was also measured at each of these time points. There is a significant correlation between changes in body weight and genotype with post-hoc testing showing a significant difference between WT and N2KO body weight at both 4 and 8 hours (Fig. **1B**). In addition, WT mice did not show significant changes in body weight over the 24 hour period, but N2KO mice showed a significant decrease in body weight starting at 1 hour and lasting throughout the experiment. During cold-exposure, 24-hour food intake between the two genotypes was not significantly different (data not shown). Thus, like Mc4r knockout mice [18], N2KO mice are able to maintain body temperature when exposed to cold. Body weight loss by the N2KO mice exposed to cold could be due to nonthermogenic heat production mechanisms such as shivering which would expend more energy than thermogenic mechanisms [19]. No studies have been done to determine if Mc4r KO mice maintain body temperature through shivering thermogenesis, or if they lose weight when exposed to cold.

Leptin Levels Rise in Cold-Exposed N2KO Mice

In normal animals, leptin levels fall with exposure to cold temperatures (for example [5, 9]). In testing the levels of leptin in ambient-held, cold exposed and cold-exposed/rewarmed animals, there was an overall significant interaction between genotype and experimental condition (Fig. **2**, $p \leq 0.0001$). Young pre-obese N2KO mice have

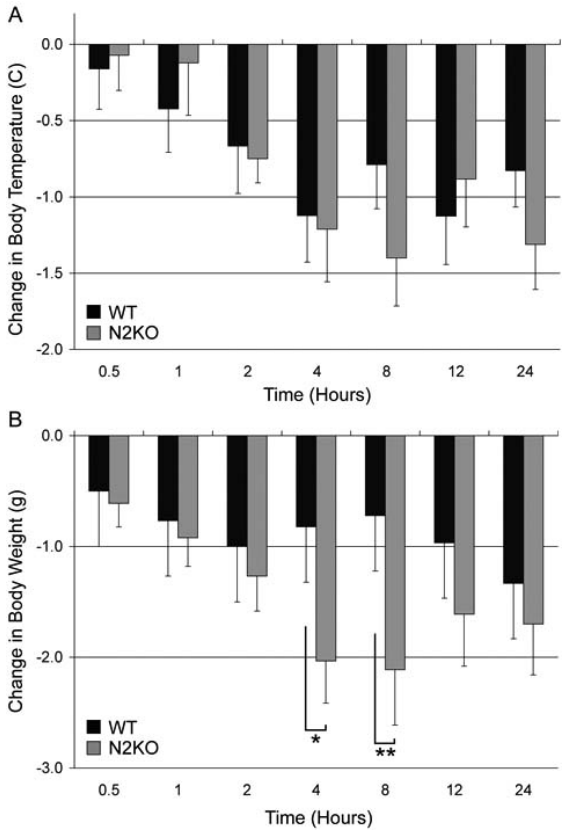

Fig. (1). N2KO mice can regulate body temperature, but not body weight during 24 hour cold exposure.

WT and N2KO mice were placed at 4°C for 24 hours. Body temperature and body weight were recorded at start (0 minutes), 30 minutes and 1, 2, 4, 8, and 12 hours. Total food intake was measured for the 24-hour period. **A,** Difference from starting body temperature (time point zero) in WT and N2KO over 24 hours is shown. **B,** Difference in starting body weight (time point zero) in WT and N2KO mice. **A, B,** Values displayed as mean ± SEM. * = $p \leq 0.05$ and ** = $p \leq 0.01$. Within genotype effects are described in the text.

significantly reduced serum leptin concentration compared to WT mice at room temperature (Fig. **2**, $p \leq 0.05$). Cold and Cold + RT N2KO mice have significantly elevated serum leptin levels compared to their experimentally matched WT controls (Fig. **2**, $p \leq 0.001$). Within genotype, WT mice experience a significant reduction in serum leptin levels with exposure to cold compared to RT mice and leptin levels return to normal with 2 hours re-warming (WT: RT vs Cold and Cold vs Cold + RT, $p \leq 0.01$). Conversely, serum leptin levels in N2KO mice rise significantly with exposure to cold and continue to rise with re-warming compared to RT controls (N2KO: RT vs Cold, Cold vs Cold + RT, and RT vs Cold + RT, $p \leq 0.001$). Low leptin levels generally signal the body to conserve energy stores. For example, serum leptin levels drop in response to food deprivation, signaling the individual to eat more and decrease energy expenditure if fat storages are low [20]. This is a protective mechanism which also activates release of energy stores for important physiological processes and reduction of energy consuming processes that are not essential for immediate survival.

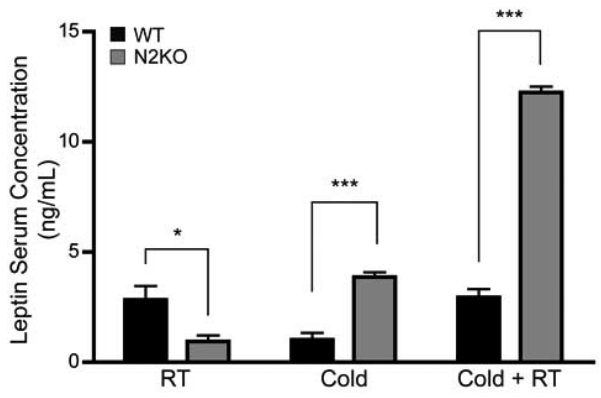

Fig. (2). Serum leptin levels are altered in N2KO mice.

Serum was collected from WT (black bars) and N2KO (gray bars) animals that were placed at room temperature for 8 hours (RT), at 4°C for 8 hours (Cold), or at 4°C for 8 hours followed by 2 hours at room temperature (Cold + RT). Serum leptin concentration is expressed as mean ± SEM (ng/ml). * = $p \leq 0.05$, *** = $p \leq 0.001$. Horizontal lines indicate significance between WT and N2KO experimental groups. Within genotype effects are describe in the text.

Reproductive processes decrease, body temperature drops to conserve energy used for metabolism, growth related processes decrease, the stress hormone levels increase to mobilize needed energy stores, and alternate fuels are employed through lipolysis [21]. The lack of a body temperature drop in cold-exposed N2KO mice may actually be due to the higher leptin levels seen in cold-exposed animals. These higher levels may prevent the normal conservation mechanisms from beginning, leading to the body weight loss seen.

Effect of Cold Exposure on Hypothalamic Gene Regulation

Within the hypothalamus, only a few genes are known to be differentially-regulated in response to a cold challenge. *C-fos* expression is induced in the arcuate and PVN in animals exposed to acute (1.5hour) cold conditions[4]. Under these same conditions, NPY mRNA levels in the arcuate are reduced [4]. While c-fos is a transcription factor, NPY and *c-fos* were not co-localized under these conditions, so it is likely that the reduction in NPY was not directly due to the induction of *c-fos*. To our knowledge, hypothalamic arcuate POMC mRNA levels have not been measured following cold-exposure, but POMC expression in the intermediate lobe of the pituitary is increased with a 30 minute exposure to cold [22]. Likewise, levels of PC1/3 in the arcuate have not been measured in response to cold exposure, although levels in the PVN increase, concomitant with increased thyrotropin-hormone releasing hormone (TRH) neuropeptide processing by PC1/3 [23]. TRH increases would be needed to stimulate the hypothalamic-pituitary-thyroid (HPT) axis for thermoregulation, so these increases in PC1/3 and TRH are expected. However, there remains a paradox with respect to PC1/3 and TRH gene regulation, as these genes are both increased in response to direct leptin stimulation and but also in response cold exposure, where leptin levels are reduced.

The National Center for Biotechnology Gene Expression Omnibus (NCBI GEO) allows researchers to search for microarray studies using keyword searches [24, 25]. A search of this database (terms hypothalamus, hypothermia; July 2009) identified no deposited studies where gene expression in the hypothalamus had been studies with respect to cold exposure. However, a study in the rat where macroarrays containing nearly 1200 rat genes revealed 43 genes whose levels were increased at least 2-fold in respond to cold exposure, and 4 genes whose levels were decreased long-term cold exposure [26]. Of these, some were known (e.g. corticotrophin releasing hormone [CRH], reduced 50% by cold exposure), and some were novel. Leptin receptor expression was not changed with cold exposure, although in the same study it was significantly increased with high fat feeding [26]. In this study only two mRNAs, NMDA receptor 2B (NMDAR2B) and GTP-binding protein G-alpha (GTPBPGα), were increased both by cold and high fat feeding [26]. These two represent only 0.17% of all the genes in the study, with all other modulated genes (213/1176 total genes) showing modulation with either high fat feeding or cold exposure, but not both conditions. TRH was not one of the genes analyzed in this study, but given the results from other studies [11, 13], it is likely that TRH expression would have also been modulated by both conditions.

Hypothalamic Nhlh2 mRNA Expression Levels Respond to Both Cold Exposure and Leptin

Nhlh2 mRNA expression in the PVN, arcuate nucleus, DMH, lateral hypothalamus and VMH of the hypothalamus is modulated by changes in energy availability using a food deprivation and food or leptin return paradigm [17]. Since the PVN and DMH are known to be cold-responsive areas of the hypothalamus [27, 28] and N2KO mice are unable to maintain body weight following a cold challenge, we hypothesized that hypothalamic Nhlh2 mRNA expression would change in response to cold exposure, as part of the signaling pathway necessary for induction of non-shivering thermogenesis. Control WT mice were kept at room temperature (RT) for 8 hours. Experimental WT mice were either placed at 4°C for 8 hours (Cold) or placed at 4°C for 8 hours and then at room temperature for 2 hours (Cold + RT). An 8-hour cold exposure was chosen because N2KO mice were unable to maintain body weight between 4 and 8 hours during initial experiments, revealing this time point to be critical (Fig. **1B**). A 2-hour re-warming period was chosen because previous studies in our laboratory found that Nhlh2 expression was maximal following 24 hours food deprivation and 2 hours of leptin exposure [17], and we had shown that 2 hours of rewarming resulted in a significant elevation of serum leptin levels in the N2KO mice (Fig. **2**). Using quantitative reverse-transcriptase PCR (Q-RT-PCR) on RNA isolated from whole hypothalamus Nhlh2 mRNA levels from mice held at 4°C for 8 hours were significantly reduced compared to levels of mice at room temperature and mice placed at room temperature following cold exposure ($p \leq 0.01$, Fig. **3**). *In situ* hybridization was then used to identify the hypothalamic nuclei in which Nhlh2 mRNA modulation occurs in response cold exposure. In the PVN and the DMH, the cold-responsive centers of the hypothalamus, Nhlh2 mRNA was significantly reduced in

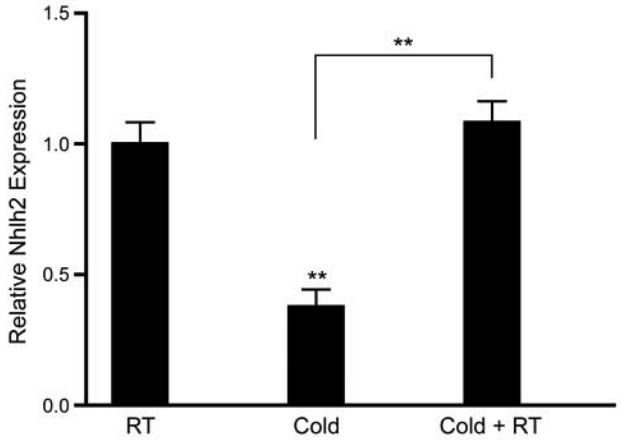

Fig. (3). Hypothalamic Nhlh2 mRNA expression responds to cold exposure.

Whole hypothalamic RNA was isolated from WT mice. Relative expression levels of Nhlh2 RNA via RT-QPCR using primers to Nhlh2 normalized to β-actin are displayed as the mean ± SEM and normalized to RT animals. Values displayed as mean ± SEM. ** = $p \leq 0.01$. Asterisks indicate statistical significance compared to RT mice. Horizontal lines indicate significance between Cold and Cold + RT groups.

mice exposed to 4°C for 8 hours compared to mice at room temperature ($p \leq 0.05$, Fig. **4, A-D, G-H**). In the PVN, 2 hours at room temperature following cold exposure restored Nhlh2 levels to RT levels and resulted in a significant 4.4-fold increase over Nhlh2 mRNA levels of cold exposed mice ($p \leq 0.05$, Fig. **4A, C, E,** and **G**). In the DMH, levels of Nhlh2 mRNA remained significantly decreased in mice placed at room temperature for 2 hours following cold exposure ($p \leq 0.001$, Fig. **4B, D, F,** and **H**). Nhlh2 mRNA levels are also modulated in the arcuate nucleus, lateral hypothalamus and VMH in response to changes in energy availability [17]. Likewise, these regions of the hypothalamus see similar significant decreases Nhlh2 expression in response to cold exposure ($p \leq 0.05$, Figure **5A-F, J-L**). Like what was seen in the DMH, Nhlh2 mRNA levels remain significantly decreased in the arcuate nucleus and VMH of mice placed at room temperature for 2 hours following cold exposure ($p \leq 0.05$, Fig. **5A-C, G-L**).

Given these new data, and the paucity of data analyzing differential gene expression in the hypothalamus of animals in different energy availability conditions, Nhlh2 is one of only four hypothalamic genes (TRH, NMDAR2B and GTPBPGα) that show differential regulation in conditions of both high (leptin, or high fat feeding) and low (cold-exposure) energy availability conditions. Of these, only the expression of the Nhlh2 gene correspond to serum levels—high serum leptin (leptin, food intake, room temperature exposure) equals high hypothalamic Nhlh2 expression while low serum leptin (food deprivation or cold exposure) equals reduced hypothalamic Nhlh2 expression. Furthermore, while *c-fos* transcription factor is induced with both cold and leptin treatment [4, 29], Nhlh2 is the only transcription factor to be induced by leptin, but reduced by cold-exposure or food deprivation throughout the hypothalamus.

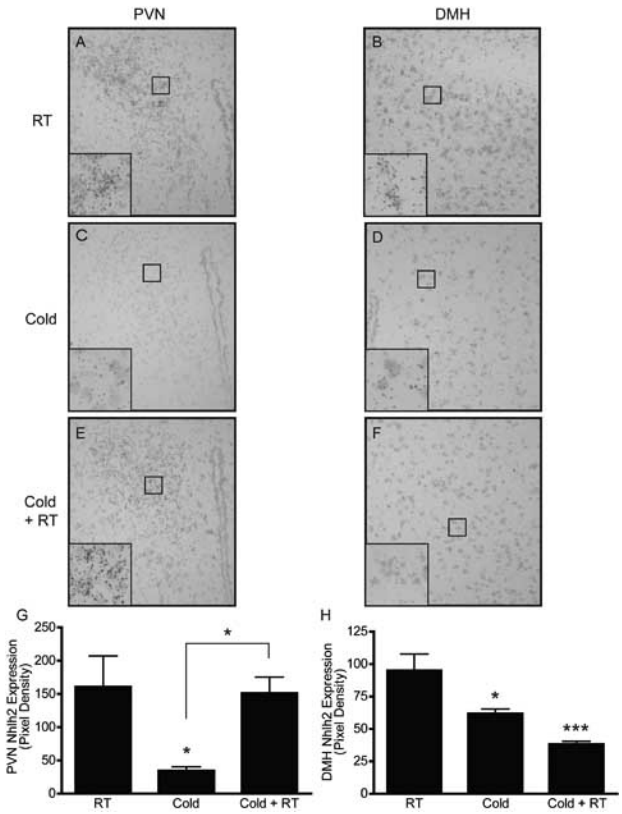

Fig. (4). Nhlh2 expression is modulated in cold-responsive hypothalamic nuclei.

A ^{33}P-labeled cRNA probe to Nhlh2 was used to label hypothalamic sections of brains from WT mice that were placed at room temperature (RT: **A**, PVN**, B,** DMH), exposed to 4°C for 8 hours (Cold: **C**, PVN**, D,** DMH), or exposed 4°C for 8 hours followed by 2 hours at room temperature (Cold + RT: **E**, PVN**, F,** DMH). 20X magnification is displayed with 40X inset. Nhlh2 expression density in the PVN (**G**) or DMH (**H**) is displayed as total pixel density and is expressed as mean ± SEM. * = $p \leq 0.05$ and *** = $p \leq 0.001$. Horizontal lines indicate significance between Cold and Cold + RT groups. All other asterisks indicate significance compared to RT animals.

Target Gene Regulation by Nhlh2

Nhlh2 is a transcription factor, and as such, changes in the hypothalamic levels of Nhlh2 should bring about changes in gene expression for downstream targets. Our laboratory has already shown that the PC1/3 gene is a direct transcriptional target for Nhlh2, and that Nhlh2 is required for leptin-stimulated transcriptional activation of the PC1/3 gene [30]. This activation of PC1/3 is downstream of the leptin receptor and requires STAT3 participation as a dimerization partner for Nhlh2. In addition, a full microarray analysis of Nhlh2 gene targets in a leptin-stimulation paradigm has been published [31]. WT and N2KO mice were subjected to three experimental conditions to simulate different levels of energy availability. Comparisons were made between and within genotypes exposed to ad lib feeding, food deprivation or leptin stimulation. As expected, food deprived animals had a relatively large number of genes

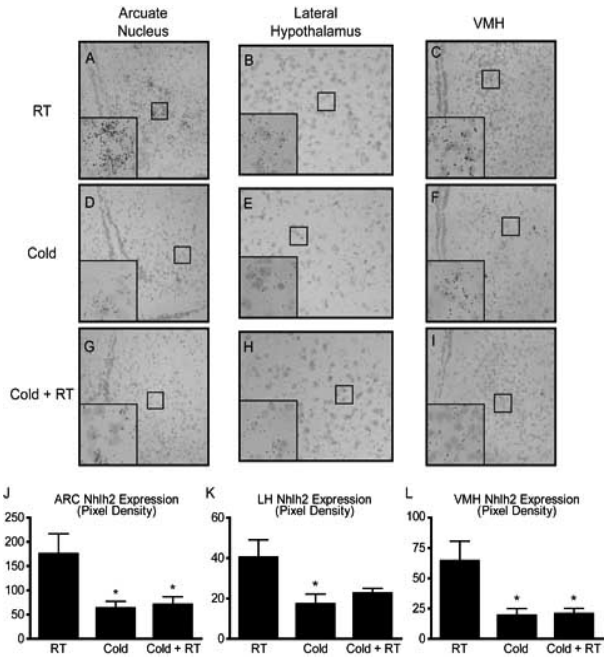

Fig. (5). Nhlh2 expression is modulated in other hypothalamic nuclei.

A ^{33}P-labeled cRNA probe to Nhlh2 was used to label hypothalamic sections of brains from WT mice that were placed at room temperature (RT: **A**, arcuate nucleus**, B,** lateral hypothalamus, **C**, VMH), exposed to 4^{o}C for 8 hours (Cold: **D**, arcuate nucleus**, E,** lateral hypothalamus, **F**, VMH), or exposed 4^{o}C for 8 hours followed by 2 hours at room temperature (Cold + RT: **G**, arcuate nucleus**, H,** lateral hypothalamus, **I**, VMH). 20X magnification is displayed with 40X inset. **J-L,** Nhlh2 expression density in arcuate nucleus (**J**), lateral hypothalamus (**K**) and VMH (**L**) of WT mice is displayed as total pixel density and is expressed as mean $\pm$ SEM. * = $p \leq 0.05$. Asterisks indicate statistical significance to RT animals.

with reduced expression (>94%) when compared both to *ad lib* fed and leptin-treated animals. Surprisingly, of the 4,762 genes identified as differentially expressed between WT and N2KO animals in the leptin-treatment condition, 4,526 of those genes were found to be over-expressed in the N2KO animals compared to 239 under-expressed genes. These data suggest that Nhlh2 can act as a transcriptional repressor in some situations. Alternatively, it may regulate another transcription factor that acts as a global transcriptional repressor following leptin stimulation. Indeed, gene ontogeny analysis of these differentially regulated genes revealed that 7% of them are transcriptional regulators [31]. Another interesting significant gene ontogeny category that was revealed during this study is category of motor activity which includes proteins involved cellular and molecular motors. While at this time we have not investigated any of the genes in this category further, we have previously shown that N2KO mice lack normal spontaneous wheel running activity [14] such that these potential Nhlh2 gene targets represent potential new directions for exploration.

Microarray analysis has not yet been used to identify gene regulatory changes following cold exposure in N2KO

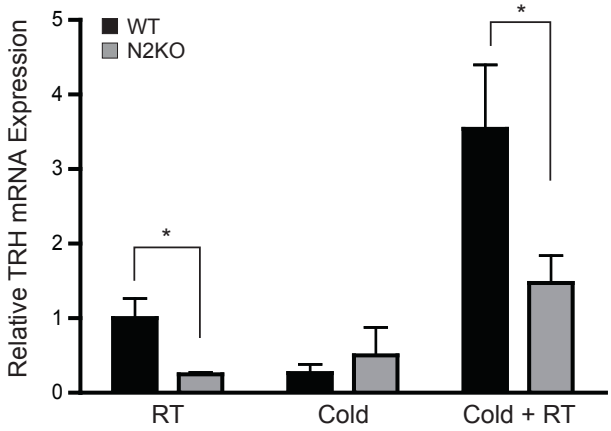

Fig. (6). TRH mRNA levels are dysregulated in N2KO mice.

Whole hypothalamic RNA was collected from WT (black bars) and N2KO (gray bars) animals that were placed at room temperature for 8 hours (RT), at 4^{o}C for 8 hours (Cold), or at 4^{o}C for 8 hours followed by 2 hours at room temperature (Cold + RT). Relative expression levels of TRH mRNA are normalized to β-actin mRNA expression. Expression levels are normalized to WT RT animals and expressed as mean $\pm$ SEM. * = $p \leq 0.05$. Horizontal lines indicate significance between WT and N2KO experimental groups. Within genotype effects are described in the text.

mice. However, TRH gene regulation was further investigated as we had previously shown dysregulation of TRH expression with food and leptin treatments. As compared to WT mice, N2KO mice have reduced TRH mRNA expression in the PVN during conditions of energy availability (leptin or food intake) and higher expression of TRH during energy deficit, opposite of WT animals[16, 17]. To analyze TRH levels in WT and N2KO animals following cold exposure or cold exposure with rewarming, Q-RT-PCR on whole hypothalamic mRNA was used. N2KO mice had significantly reduced TRH mRNA levels compared to WT mice at RT and Cold + RT (Fig. **6**). In WT mice, TRH mRNA was significantly reduced in mice exposed to cold for 8 hours. WT mice placed at room temperature following cold exposure had a 3.5-fold increase in TRH mRNA expression over mice left at room temperature and a 13.5-fold increase over mice placed at 4^{o}C for 8 hours. In N2KO mice, exposure to cold did not change TRH mRNA levels significantly compared to RT mice. The studies reported here are in contrast to those reported by Perello and colleagues [23] in that TRH levels in the WT are reduced, rather than increased in response to cold exposure. This difference is most likely due to the acute versus chronic cold treatment (1 hour in the study by Perello and colleagues [23] and 8 hours in this study). A time-course comparison of mRNA levels of TRH in WT and N2KO mice is needed to resolve this issue. Interestingly compared to cold-treatment, there is a rapid induction of TRH levels following 2 hours of re-warming for both genotypes, although the induction in N2KO mice does not reach significance. This change results in a significant reduction in TRH levels in re-warmed N2KO mice compared to WT animals. We have previously shown that Nhlh2 is expressed in ~40% of labeled TRH neurons in the PVN [16]; perhaps the remaining 60% of the neurons

respond normally to rewarming, leading to the difference captured in this study.

DISCUSSION

There are only a few hypothalamic transcription factors whose mRNA expression level is modulated with changes in energy availably. Other hypothalamic transcription factors, such as STAT3 appear to be modulated only post-translationally through protein modification and subcellular localization. The results presented identify the hypothalamic transcription factor Nhlh2 as a target of energy availability pathways activated by changes in environmental temperatures, and attempt to place Nhlh2 gene regulation in the context of global changes in energy availability signals, including leptin. The findings herein report a drop in Nhlh2 mRNA expression in all hypothalamic nuclei with 8-hour exposure to 4°C. Return to room temperature following cold exposure restores Nhlh2 mRNA levels to those of mice kept at room temperature only in the PVN. These data suggest that Nhlh2 gene expression in the PVN is modulated negatively in response to 8 hours of cold exposure and positively with a return to room temperature, corresponding to the fall and rise of leptin levels. Other work from our laboratory shows that hypothalamic Nhlh2 levels correlate with serum leptin levels in fasting, refeeding and leptin injection and that Nhlh2 gene expression is a direct target of the leptin-stimulating pathway [17]. Falling levels of hypothalamic Nhlh2 gene expression during cold-exposure in WT mice are most likely result in falling leptin levels but specific studies to address this (i.e. injection of leptin into cold-exposed mice) need to be done. Interestingly, there are differences in the response of Nhlh2 expression levels across different hypothalamic nuclei. It is unclear whether the delays seen in the arcuate, DMH, VMH and lateral hypothalamus represent different regulatory mechanisms compared to the PVN. If the difference was due to serum leptin concentrations only, the Nhlh2 levels in the arcuate would be expected to respond similarly to the levels in the PVN, but this is not the case.

The proximal 200 base pairs of the Nhlh2 promoter contains 5 putative STAT3 binding sites and an AP-1 site (Vella, Burnside, Abdulrazzaq Al-rayyanun and Good, unpublished). In leptin treated animals, there is activation of both STAT3 transcription factor and *c-fos*, a component of the AP-1 complex [29, 32], making it possible that one or both of these transcription factors result in leptin-induction of the Nhlh2. The mechanism for rewarming induction in the PVN may be similar as leptin levels return to room temperature conditions within the two hour time period (Fig. **2**). The mechanism for lack of return of Nhlh2 mRNA levels to levels at room temperature in the ARC and other hypothalamic areas is still under debate. Also unclear is the mechanism by which Nhlh2 expression is reduced with food deprivation or cold expression. Is this an active or a passive mechanism? In other words, is the reduction due simply to decay of the Nhlh2 mRNA in light of reduced gene transcription? Or is there an active degradation of Nhlh2 mRNA, perhaps by microRNA-mediated mechanisms? Yugami and colleagues have already shown that heterogeneous nuclear ribonucleoprotein-U (hnRNP-U) binds to and stabilizes the Nhlh2 mRNA in human 293T kidney cells [33]. In this study, the authors concluded that hnRNP-U increases expression of its target mRNAs by contributing to increased mRNA stability. The 3' tail of Nhlh2 contains 9 AU-rich elements (AREs), known to contribute to mRNA instability [34]. The 3' tail of Nhlh2 is also very long, relative to its coding region (408 bp coding region; 1445 bp 3' tail). In addition to the hnRNP-U binding site in its 3'tail, human NHLH2 contains 24 regions with the potential to interact with known microRNAs, while mouse Nhlh2 has 16 putative regions [35]. Thus the possibility exists for multiple regulatory pathways that could contribute to up- and down-regulation of the Nhlh2 in response to changing energy availability.

The real importance of Nhlh2 regulation in response to different energy levels is its effects on downstream gene regulatory targets and ultimately on the pathways that affect body weight through energy conservation and use. We have identified several targets of Nhlh2. For PC1/3, we have data to show exactly how Nhlh2 transcriptionally activates this gene. In response to leptin and food deprivation, we have data to suggest that Nhlh2 may actually act as a transcriptional repressor for the majority of its target genes.

In summary, Nhlh2 is a target of cold exposure signaling in the hypothalamus, most likely mediated by changing serum leptin levels. However, with respect to expression with re-warming following cold exposure, the mechanisms of Nhlh2 gene regulation in hypothalamic areas other than the PVN may be more complex. Nhlh2 expression is required for the correct expression of TRH mRNA in the hypothalamus both at room temperature and during cold exposure. This study provides evidence that Nhlh2 is necessary for communicating signals to maintain body weight homeostasis under thermogenic challenges.

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REFERENCES

[1] Morton GJ. Hypothalamic leptin regulation of energy homeostasis and glucose metabolism. J Physiol 2007; 583: 437-43.

[2] Munzberg H. Differential leptin access into the brain--a hierarchical organization of hypothalamic leptin target sites? Physiol Behav 2008; 94: 664-9.

[3] Robertson SA, Leinninger GM, Myers MG, Jr. Molecular and neural mediators of leptin action. Physiol Behav 2008; 94: 637-42.

[4] Park JJ, Lee HK, Shin MW, *et al.* Short-term cold exposure may cause a local decrease of neuropeptide Y in the rat hypothalamus. Mol Cells 2007; 23: 88-93.

[5] Korhonen T, Saarela S. Role of adiposity hormones in the mouse during fasting and winter-acclimatization. Comp Biochem Physiol A Mol Integr Physiol 2005; 140: 217-23.

[6] Trayhurn P, Duncan JS, Rayner DV. Acute cold-induced suppression of ob (obese) gene expression in white adipose tissue of mice: mediation by the sympathetic system. Biochem J 1995; 311 (Pt 3): 729-33.

[7] Davis TR, Mayer J. Imperfect homeothermia in the hereditary obese-hyperglycemic syndrome of mice. Am J Physiol 1954; 177: 222-6.

[8] Mayer J, Barrnett RJ. Sensitivity to cold in the hereditary obese-hyperglycemic syndrome of mice. Yale J Biol Med 1953; 26: 38-45.

[9] Trayhurn P. Thermoregulation in the diabetic-obese (db/db) mouse: the role of non-shivering thermogenesis in energy balance. Pflugers Arch 1979; 380: 227-32.
[10] de Luca C, Kowalski TJ, Zhang Y, *et al*. Complete rescue of obesity, diabetes, and infertility in db/db mice by neuron-specific LEPR-B transgenes. J Clin Invest 2005; 115: 3484-93.
[11] Arancibia S, Rage F, Astier H, Tapia-Arancibia L. Neuroendocrine and autonomous mechanisms underlying thermoregulation in cold environment. Neuroendocrinology 1996; 64: 257-67.
[12] Nillni EA, Xie W, Mulcahy L, Sanchez VC, Wetsel WC. Deficiencies in pro-thyrotropin-releasing hormone processing and abnormalities in thermoregulation in Cpefat/fat mice. J Biol Chem 2002; 277: 48587-95.
[13] Huo L, Munzberg H, Nillni EA, Bjorbaek C. Role of signal transducer and activator of transcription 3 in regulation of hypothalamic trh gene expression by leptin. Endocrinology 2004; 145: 2516-23.
[14] Coyle CA, Jing E, Hosmer T, Powers JB, Wade G, Good DJ. Reduced voluntary activity precedes adult-onset obesity in Nhlh2 knockout mice. Physiol Behav 2002; 77: 387-402.
[15] Good DJ, Porter FD, Mahon KA, Parlow AF, Westphal H, Kirsch IR. Hypogonadism and obesity in mice with a targeted deletion of the Nhlh2 gene. Nat Genet 1997; 15: 397-401.
[16] Jing E, Nillni EA, Sanchez VC, Stuart RC, Good DJ. Deletion of the Nhlh2 transcription factor decreases the levels of the anorexigenic peptides alpha melanocyte-stimulating hormone and thyrotropin-releasing hormone and implicates prohormone convertases I and II in obesity. Endocrinology 2004; 145: 1503-13.
[17] Vella KR, Burnside AS, Brennan KM, Good DJ. Expression of the hypothalamic transcription factor Nhlh2 is dependent on energy availability. J Neuroendocrinol 2007; 19: 499-510.
[18] Voss-Andreae A, Murphy JG, Ellacott KL, *et al*. Role of the central melanocortin circuitry in adaptive thermogenesis of brown adipose tissue. Endocrinology 2007; 148: 1550-60.
[19] Morrison SF, Nakamura K, Madden CJ. Central control of thermogenesis in mammals. Exp Physiol 2008; 93: 773-97.
[20] Flier JS. Obesity wars: molecular progress confronts an expanding epidemic. Cell 2004; 116: 337-50.
[21] Chan JL, Mantzoros CS. Role of leptin in energy-deprivation states: normal human physiology and clinical implications for hypothalamic amenorrhoea and anorexia nervosa. Lancet 2005; 366: 74-85.
[22] Wu P, Childs GV. Changes in rat pituitary POMC mRNA after exposure to cold or a novel environment, detected by *in situ* hybridization. J Histochem Cytochem 1991; 39: 843-52.
[23] Perello M, Stuart RC, Vaslet CA, Nillni EA. Cold exposure increases the biosynthesis and proteolytic processing of prothyrotropin-releasing hormone in the hypothalamic paraventricular nucleus *via* beta-adrenoreceptors. Endocrinology 2007; 148: 4952-64.
[24] Barrett T, Edgar R. Gene expression omnibus: microarray data storage, submission, retrieval, and analysis. Methods Enzymol 2006; 411: 352-69.
[25] Barrett T, Troup DB, Wilhite SE, *et al*. NCBI GEO: mining tens of millions of expression profiles--database and tools update. Nucleic Acids Res 2007; 35: D760-5.
[26] De Souza CT, Pereira-da-Silva M, Araujo EP, *et al.* Distinct subsets of hypothalamic genes are modulated by two different thermogenesis-inducing stimuli. Obesity (Silver Spring) 2008; 16: 1239-47.
[27] Dimicco JA, Zaretsky DV. The dorsomedial hypothalamus: a new player in thermoregulation. Am J Physiol Regul Integr Comp Physiol 2007; 292: R47-63.
[28] Zoeller RT, Rudeen PK. Ethanol blocks the cold-induced increase in thyrotropin-releasing hormone mRNA in paraventricular nuclei but not the cold-induced increase in thyrotropin. Brain Res Mol Brain Res 1992; 13: 321-30.
[29] Woods AJ, Stock MJ. Leptin activation in hypothalamus. Nature 1996; 381: 745.
[30] Fox DL, Good DJ. Nhlh2 Interacts with STAT3 to regulate transcription of prohormone convertase 1/3. Mol Endocrinol 2008; 22: 1438-48.
[31] Fox DL, Fox DLG, Jensen RV, Good DJ. Microarray technology uncovers biological pathways involved in the development of obesity in Nhlh2 knockout mice. In: Thangadurai DTW, and Pullaiah T, Eds. Genes, genomes and genomics. New Delhi: Regency Publications 2007.
[32] Ghilardi N, Ziegler S, Wiestner A, Stoffel R, Heim MH, Skoda RC. Defective STAT signaling by the leptin receptor in diabetic mice. Proc Natl Acad Sci USA 1996; 93: 6231-5.
[33] Yugami M, Kabe Y, Yamaguchi Y, Wada T, Handa H. hnRNP-U enhances the expression of specific genes by stabilizing mRNA. FEBS Lett 2007; 581: 1-7.
[34] Bolognani F, Perrone-Bizzozero NI. RNA-protein interactions and control of mRNA stability in neurons. J Neurosci Res 2008; 86: 481-9.
[35] Wang X. miRDB: a microRNA target prediction and functional annotation database with a wiki interface. RNA 2008; 14: 1012-7.

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