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A molecular conundrum involving hypothalamic responses to and roles of long non-coding RNAs following food deprivation

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Abstract

Long non-coding RNAs (lncRNAs) are one of most poorly understood RNA classes in the mammalian transcriptome. However, they are emerging as important players in transcriptional regulation, especially within the complexity of the nervous system. This review summarizes the known information about lncRNAs, and their roles in endocrine processes, as well as the lesser-known information about lncRNAs in the brain, and in the neuroendocrine hypothalamus. A “call-to-action” is presented for researchers to use archival transcriptome data to characterize differentially expressed lncRNA species within the hypothalamus. In accordance, we analyze for differential-expression of lncRNA between normal mice and mice with a targeted deletion of the nescient helix-loop-helix 2 gene, and between C57Bl/6 and 129Sv/J mice. Finally, strategies and approaches for researchers to analyze their own datasets or those on the NCBI GEO datasets repository are provided, in hopes that future studies will reveal many new roles for lncRNAs in hypothalamic physiological responses, solving this so-called “molecular conundrum” once and for all.

Keywords

long non-coding RNA; bioinformatics; Noncoder; microarray transcriptome; Nhlh2 knockout mice; strain differences

1. Introduction

In the last decade, genome wide high throughput studies revealed that only a small proportion (1–2%) of the mammalian genome are transcribed into protein-coding RNAs whereas the vast majority remain as transcripts only^{1–3}. In 2012, the Encyclopedia of DNA Elements (ENCODE) consortium debunked the notion that the majority of the human genome is filled with non-coding, do-nothing junk DNA with the publication of 30 research

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papers detailing the various findings that at 80.4% of the DNA we carry has a purpose⁴. While there were data to support this notion prior to 2012, the September 2012 publication of these papers was hailed as a major breakthrough in science. The ENCODE project defined all “functional elements” encoded by the human genome, meaning that they were either protein-coding, made a defined product (such as non-coding RNA, or ncRNA), or displayed a biochemical signature indicating that they were regulatory in nature⁵. Indeed, non-coding transcripts such as transfer RNAs (tRNA), ribosomal RNAs, and spliceosomal RNAs are associated with many critical cellular process and biology functions, and increasing evidence now supports the role of other longer non-protein transcripts in gene regulation at epigenetic, transcriptional and post-transcriptional levels, with medical implications. A total of 1,640 datasets representing 15 different cell lines were used for the ENCODE project, which identified 9,277 genes, producing 14,880 long non-coding RNA (lncRNA) transcripts⁶. However, these original datasets did not include any neuronal cell types—the two listed neuronal cell lines SK-N-SH, a neuroblastoma cell line, and H1 neurons, from an embryonic stem cell line, are in “Tier II” category, meaning they have subsequently been tested but were not in the original 2011–2012 data⁷. Surprisingly, there are only 338 papers on PubMed from the last 5 years, using the search terms “brain AND long non-coding RNA”, and delineating further to “hypothalamus AND long non-coding RNA” leaves us with 18 papers. This review attempts to provide some background on lncRNAs, and use the available literature, as well as some of our own, as yet unpublished data, to build a case for focusing efforts on the role of lncRNA in hypothalamic functions as it relates to body weight control and general signaling in the hypothalamus in order to solve this “molecular conundrum”.

2. What are long non-coding RNAs?

lncRNAs are defined as non-protein coding transcripts longer than 200 nucleotides in length. This is in contrast to other non-coding RNA types, such as the small non-coding RNA family of microRNA (19–24 bp), Piwi-interacting RNA (26–31 bp), and tRNA (76–90 bp); the mid-size non-coding RNA family of promoter-associated short RNAs (PASR, 22–200bp), small nuclear RNA (snRNA, 80–350 bp) and small nucleolar RNAs (snoRNA 60–300 bp)⁸; and the protein-coding transcripts, which include both the protein coding regions and introns. Using the genomic locations of lncRNA and (in some cases) its nearby or associated protein-coding gene, lncRNAs can be classified into different groups including intergenic lncRNAs, bidirectional lncRNAs, exonic lncRNAs, and intronic lncRNAs; as well as sense and anti-sense lncRNAs based on the directions of lncRNA transcripts relative to the protein-coding genes. In fact, up to one-half of the known lncRNA transcripts overlap a protein-coding region of the genome, either in the sense or anti-sense direction⁹. lncRNAs can regulate their target genes through a variety of mechanisms, including transcriptionally, through either *cis* (on neighbor genes in close proximity) or *trans* (on distantly located domains or chromosomes) mechanisms, and post-transcriptionally through mRNA processing and stability, or translation interference. Thus, depending on the regulatory mechanisms and the functions of lncRNAs on their associated protein-coding genes, lncRNAs can be placed into several sub categories such as pseudogenes, enhancer-associated lncRNAs, promoter-associated lncRNAs, and many others^{10,11}. Unlike other

short non-coding RNAs such as tRNAs, snRNAs, and some micro- and sno-RNAs which are transcribed by RNA polymerase III, the majority of lncRNA transcripts are transcribed by RNA polymerase II with the same transcription machinery as protein-coding RNAs¹². They also have a 7-methyl guanine cap, a 3'-polyadenylation, and can be spliced⁹. Controversy exists as to whether they can also, under some conditions, produce small 5–10 amino acid peptides, but the prevailing notion is that whether or not they can be translated, when one discusses lncRNA, they are discussing the RNA-specific function of the transcript.

To date, the nine publically available lncRNA databases have catalogued over 150,000 lncRNAs from multiple mammalian and non-mammalian species⁹. The GENCODE database, Version 24 has listed 15,941 human long non-coding genes with 28,031 lncRNA transcripts¹³, whereas another popular lncRNA database, NONCODE (<http://www.noncode.org/>), has 90062 human lncRNA genes with 141353 lncRNA transcripts in their most recent database. Although only very few of these lncRNAs have been well-studied, it is quite clear that these lncRNAs play important roles in various structural and regulatory biological processes, such as splicing regulation (e.g. MALAT1), transcriptional regulation (e.g. NEAT1) and translational regulation (e.g. BC1)^{8,10}. Within these mechanistic regulatory functions, it appears that subsets of lncRNAs are differentially expressed within certain tissues, and in response to certain external stimuli. Food deprivation (fasting), and endocrine/metabolic regulation will be further explored in this review.

3. lncRNAs in endocrine and metabolic processes, including food deprivation

3.1. lncRNAs in endocrine and metabolic processes

Emerging evidence has revealed important roles for lncRNAs in endocrine and metabolic processes. Transcriptome analysis on endocrine organs, including white and brown adipose tissues, pancreas, thyroid gland, and pineal gland, have identified groups of lncRNAs that associated with hormonal and metabolic changes in the endocrine system¹⁴. For example, hundreds of lncRNAs have been identified that are specifically regulated during adipogenesis using transcriptome analysis on mouse brown and white adipocytes. In particular, key adipogenic transcription factors such as peroxisome proliferator-activated receptor γ (PPAR γ) and CCAAT/enhancer-binding protein α (CEBP α) directly regulate the expression of an lncRNA, namely lnc-BATE1, in adipose tissue^{15,16}. In this study, several identified lncRNAs appear to be co-localized both in adipose and brain tissue¹⁶. NEAT1, a well characterized lncRNA which plays an important role in the nuclear paraspeckles^{17–19}, also participates in adipogenesis through a unique microRNA-lncRNA regulatory network, in conjunction with microRNA-140²⁰. Moreover, recent investigations have also shown the role of lncRNAs in glucose metabolism^{21–26} and lipid metabolism^{27–29}.

Some non-neural lncRNAs have been associated with food deprivation or changes in energy availability in several systems. For example, a starvation-induced lncRNA, ncns-1, has been identified by Hellwig and colleagues in *Caenorhabditis elegans*. In their study, ncns-1 which is expressed in intestine and hypodermis in *Caenorhabditis elegans*, showed an approximately 3-fold induction in response to acute food deprivation and reached to and

maintained at 6-fold enrichment after 10 hours long term food deprivation³⁰. Another study by Hirota and colleagues shows that the transcription of a cascade of lncRNAs is required for chromatin remodeling at the fission yeast *Schizosaccharomyces pombe fbp1*⁺ locus during transcriptional activation following glucose starvation³¹. Moreover, growing evidence has also unveiled the roles of lncRNAs in glucose homeostasis and diabetes mellitus. Kino and colleagues demonstrated that endogenous Human Gas5 in HeLa cells is a negative regulator of the glucocorticoid receptor (GR) induced cIAP2 and SGK1 mRNA expression in serum-free medium and concluded that Gas5 modulates the transcriptional activity of the GR during starvation²⁵. Likewise, it has been also reported that lncRNA liver GCK repressor (lncLGR) is induced by fasting in mouse liver and regulates glycogen storage by suppressing glucokinase activity³². Furthermore, another study on steroid receptor RNA activator (SRA) lncRNA demonstrates the role of SRA in glucose homeostasis by showing that depletion of SRA can reduce diet-induced obesity and improves glucose tolerance³³. Taken together, these studies suggest that endocrine processes, including food deprivation, modulate expression of various lncRNAs, and the processes themselves can be also influenced by differential level of lncRNAs. The question is whether there are roles for similar lncRNAs in neuroendocrine metabolic processes, or if the neuroendocrine lncRNA transcriptome is vastly different and unique when compared to peripheral tissues.

3.2. Neural lncRNAs in endocrine processes: Which ones, and where?

Comprehensive analysis of the mammalian whole RNA transcriptome has revealed that brain has a highly complex transcriptome, as compared to other tissue types, with up to 50% of all known RNA transcripts expressed at some point in brain tissue^{34,35}. However, while numerous small non-coding RNAs have been shown to regulate the development and function of the nervous system^{36,37}, only very few lncRNAs have been characterized in the nervous system.

One of the earliest studies profiling the number and type of lncRNA in the mammalian brain was a mouse study, where the mouse-specific data contained within the Allen Brain atlas was profiled using in silico methods to regionally map the expression of 151 ncRNAs to 11 neuroanatomical regions, including the hypothalamus³⁸. In their results, the expression of ncRNA in hypothalamic regions was low, compared to regions such as the cortex, cerebellum and olfactory bulbs. A paper published one year later identified and mapped 849 lncRNAs in the mouse brain, and provided a link for others to search the Allen Brain atlas for regional expression of ncRNA³⁹. A search of that database for hypothalamic ncRNA returned 1142 results and all of these link out to the ncRNA expression database, providing sequencing information of each of the results for further study⁴⁰.

While ENCODE did not use neuronal cell lines in the initial analysis, their published data on lncRNAs included an expression analysis of 9747 lncRNA transcripts within 5 cell lines, 9 brain regions, and 17 other tissues. For brain researchers, one of the most interesting outcome of this analysis was the cluster analysis revealing a differential set of lncRNA, of which, 40% showed strong brain-specificity⁶. Also of interest was the fact that lncRNA generally was expressed at a much lower level than protein-coding RNA examined in the

same study⁶. In this analysis, the hypothalamus was not one of the regions selected for further study, so it was unclear how hypothalamic expression of lncRNA would compare to that in the cortex, hippocampus and the other seven regions.

More recently, lncRNAs have been characterized in the developing human cortex, and again, were expressed at a level 13.6% lower than protein coding genes within the same region^{41,42}. This study identified 11,642 lncRNA species in the neocortex transcriptome. The authors attribute the relatively low expression level overall of lncRNAs (throughout whole neocortex samples, when compared to protein-coding RNAs) to very cell-specific expression of lncRNAs, which could then be demonstrated with single-cell enrichment of lncRNA, but were likely “washed out” by bulk analysis of the neocortex⁴¹. This highly cell-type specific expression of lncRNA, in one of the most cell-type diverse organs of the body, undoubtedly points to key roles for lncRNAs in not only neurodevelopment, but also normal adult functioning and responses of the nervous system.

The bottom line is that the lncRNAs are expressed in the brain, and for the purpose of this review, in the hypothalamus, both during development and in the adult. We do know some of the neural lncRNAs that are expressed, but at least some of the data indicates that the hypothalamic expression of lncRNA may be relatively lower than that of other brain regions. The question now becomes, what are these lncRNAs doing, and when are they expressed?

3.3. Some known roles for neural lncRNAs

As in other systems that are more thoroughly studied, lncRNAs participate in a number of physiological processes, including development, cell fate, and the response to external signals. While not as many functions have been characterized for the neural lncRNAs, we will discuss some of the highlights here. For example, specific lncRNAs have been characterized in brain development. Blackshaw and colleagues performed a genomic analysis of mouse retinal development, and identified a list of lncRNAs that are dynamically expressed in retinal progenitor and precursor cells⁴³. Later on, another group focused on lncRNA Taurine Upregulated Gene 1 (TUG1) suggesting that TUG1 is required for the photoreceptor formation and thus plays an important function role in retinal development⁴⁴. Interestingly, this paper went on to show that TUG1 is also expressed in multiple regions of the developing brain, such as cortex and hippocampus. Other lncRNAs, such as Gomafu and NEAT1, have a pattern of regional brain expression, which appears to be linked to neural stem cell fate decisions^{45,46}. As discussed previously, NEAT1 is found in nuclear paraspeckles^{17–19}, and forms a microRNA-lncRNA network that functions in transcriptional regulation²⁰. Another well characterized neural lncRNA, MALAT1, can regulate several synaptogenesis genes and is associated with synaptic density⁴⁷. Differential night/day expression of 112 lncRNAs was detected using RNA-seq on rat pineal gland total RNA, indicating a dynamic role of lncRNAs in the circadian clock system⁴⁸.

Several lncRNAs have been also implicated in diabetic neuropathies. For example, microarray profiling of lncRNAs in rat superior cervical ganglia nerve fibers revealed a significant induction of the lncRNA, uc.48 + in diabetic rats, and correlation of the induction with diabetic neuropathic pain⁴⁹. Alternatively, lncRNA Sox2OT may play a protective role in the retinal ganglia of diabetic rats with diabetes-related retinal neurodegeneration⁵⁰.

Other than the role of some lncRNAs in tumors of neuroendocrine origin, there are few published reports on the role of neural lncRNAs in obesity, or other similar conditions with a neuroendocrine basis ⁵¹. This likely reflects the novelty of the field, and more work will certainly identify conditions and signaling pathways influenced by or affecting regulation of lncRNAs.

3.4. Hypothalamic lncRNAs: what we do know

A PubMed search using the search term “hypothalamus AND lncRNA” gives a list of 18 publications. The first report of a hypothalamic lncRNA was in 1993, for Brain Cytoplasmic RNA 1 (BC1), which is also one of the earliest studied lncRNA in the nervous system ⁵². BC1 is only 152 nucleotides long, and while it doesn't fit the classic description of lncRNA being greater than 200 nucleotides in length, it is listed in the lncRNA database ⁵³. BC1 lncRNA is found in neurosecretory axon terminals from the hypothalamus that converge on the posterior pituitary. Further studies using rabbit reticulocyte lysates, and rat whole brain lysates shows that BC1 can complex with ribosomal proteins as part of a ribonuclear protein particle, and then acts to inhibit recruitment of the small ribosomal complex, ultimately inhibiting translation of targeted mRNAs ⁵⁴. While no studies have directly examined BC1 mRNA targets in the hypothalamus, within the hippocampus of BC1 knockout mice, the Fragile X mental retardation protein and the postsynaptic density protein 95 are abnormally expressed, leading to neuronal hyperexcitability, and suggesting that BC1 lncRNA directly targets these mRNAs ⁵⁵. However, the direct relevance of BC1 lncRNA mechanisms to humans is under dispute, as BC1 lncRNA likely arose from a retrotransposon event affecting the mouse tRNA for alanine, and is rodent-specific ⁵⁶.

The next lncRNA to be specifically detected in the hypothalamus was Gtl2 (Alias maternally expressed 3, or MEG3) ⁵⁷, although expression of this lncRNA is also widespread in non-neuronal tissues. As described in the lncRNA database, this lncRNA is derived from a much longer transcript (1.3 kb), is maternally-expressed, and is found in both the adult and developing nervous systems of humans, rodents and sheep ⁵³. MEG3 functions as part of a chromatin-remodeling complex, directing imprinting the maternal Dlk1 locus, and acting as a tumor suppressor gene through the p53 pathway ⁵⁸. Microarray analysis comparing embryonic whole brains from wildtype and MEG3 knockout mice identified Vascular Endothelial Growth Factor (VEGF) pathway genes as negative regulatory targets of MEG3, and found higher angiogenesis levels in the brains of the knockout mice ⁵⁹. The knockout mice (both the full knockouts and those heterozygous mice with the maternally-inherited allele) do not survive past birth, and so it is unclear if there would be further adult consequences of the deletion, such as hypothalamic-specific effects.

lncRNA sequences and regional expression patterns are conserved among different amniotes, as shown in a study where lncRNA AK082467 was found to have a highly conserved expression pattern among chicken, opossum, and mouse in the ventricular zone of the hippocampus and in the preoptic area of the hypothalamus. In the same study, expression of lncRNA, AK082072, was found for all three amniotes in the stria terminalis, a region that contains tracts originating from the amygdala and terminating in the hypothalamus and thalamus region ⁶⁰. Both findings indicate how conserved expression patterns of lncRNAs

might be ancient mechanisms in specifying the developing and functional regionalization of the hypothalamus.

As in other tissues, bioinformatics and transcriptome approaches have been used to identify some hypothalamic lncRNAs. For example, in a bioinformatics-based approach aimed at identifying lncRNA within Alzheimer's related genes, the hypothalamic-specific hypocretin gene was instead identified as a possible lncRNA-containing gene⁶¹. However, this publication failed to specifically name the lncRNA candidate within any of the Alzheimer's related genes, and a search of the hypocretin locus in both the GenBank and UCSC genome browser failed to show any transcripts arising from or even near the hypocretin exons, other than the hypocretin RNA transcripts themselves. While this could be a new lncRNA (i.e. one which is not currently listed in GenBank), there are no other publications currently supporting the finding. Likewise, in a publication by Wang and colleagues, RNA-seq was used to identify 1,520 rat hypothalamic lncRNAs. This study further characterized lncRNA TCONS_00090635 as a hypothalamic-enriched lncRNA⁶². Again though, no further studies on TCONS_00090635 or any of the other identified rat hypothalamic lncRNAs have been done to date. A similar transcriptome study on canine hypothalamus and cerebral cortex using RNA-seq has identified 328 non-coding RNAs including 57 long non-coding RNAs, but none of these have been further studied⁶³. Several other hypothalamic lncRNAs have been identified by different transcriptome and bioinformatics studies, including Narcolepsy candidate-region (NLC) 1-A and NLC1-C, which are expressed in the human hypothalamus⁶⁴, and Colorectal Neoplasia Differentially Expressed (Non-Protein Coding) (CRNDE), which is expressed in the human, rat and mouse hypothalamus at moderate high level⁶⁵. While the functions of these lncRNAs have been characterized in different biological processes^{21,66}, their roles in the hypothalamus have not been explored and are still poorly understood.

There is only one study where the adult expression patterns of hypothalamic lncRNAs under energy availability stress were studied, and that comes from our laboratory⁶⁷. Using Affymetrix exon arrays of WT ad lib fed and fasted mice, we identified over 12,000 lncRNA transcripts in the hypothalamus, 622 of which were differentially-regulated in response to 24-hour fasting. Using independent hypothalamic samples from ad lib and fasted mice, three of these lncRNAs were further tested by quantitative PCR, and two of them confirmed to be upregulated in response to food deprivation. AK038506 was upregulated by approximately 3-fold in fasted compared to ad lib fed mice, while AK049914 showed a nearly 6-fold increase. At the time when writing the original paper⁶⁷, there was no significant homology for AK038506 using a genomic blast, but a new Blast analysis, using a less stringent approach and a ref-seq genomic blast, revealed 99% overlap of 99% of the 1933 nucleotide sequence with two different sequences. The first is the RasA12 gene on mouse chromosome 1, with homology within an intron between exons 8 and 9. This is a ras-GTPase activating protein family member and has been implicated as a tumor suppressor gene in mainly in breast cancers⁶⁸. Of direct relevance to this review, a single nucleotide polymorphism, rs10913469, near the RasA12 gene, shows a significant linkage to BMI in a Mexican cohort [24]. The second and equally interesting homology occurs is a region of chromosome 1 with many repeats (AC119220.11). The region of overlap is one labeled as L1-like on mouse chromosome 1. L1 is a member of the "long interspersed repeat elements" family, of which

<500,000 copies exist in the human genome and there are about 3% more in the mouse genome⁶⁹. It is interesting that the blast of AK038506 only identifies this single L1 element on chromosome 1, and not any other LINE elements, but the significance of this finding is unknown. Rather than overstate the possible implications of either of these homologies, as none of these are confirmed interactions, we will simply state that the two possible targets and the resulting possible mechanisms by which lncRNA AK038506 could regulate either L1 or rasAI2 make AK038506 a valid target for lncRNA researchers to pursue.

The other hypothalamic lncRNA increased with fasting was AK049914, blasts to a region that sits between exons 3 and 4 of the ApoD gene⁶⁷. The ApoD gene, whose protein product is a component of high-density lipoproteins, is upregulated by fasting⁷⁰, suggesting that the placement of a fasting-regulated lncRNA might not be coincidental. Although several recent studies have unveiled the role of ApoD in different metabolism pathways and oxidative stress response in the central nervous system^{71–77}, the role that ApoD plays in the hypothalamus remains a mystery. Liu and colleagues have suggested that hypothalamic ApoD is involved in the leptin and the long form leptin receptor signal transduction pathway and modulates energy homeostasis⁷⁸. In their study, ApoD expression was decreased by low-fat diet. In extrapolating this response to the increase in hypothalamic AK049914 with fasting, allows us to speculate that lncRNA-mediated negative regulation of ApoD may occur in response to low leptin levels.

In sum, there is still only one well-characterized hypothalamic lncRNA (BC1), and this one has been studied functionally, both in the hypothalamus and in other regions of the nervous system. No other hypothalamic-specific or even general lncRNA has been functionally studied within hypothalamic cells or tissue. Transcriptome analysis has revealed hundreds of other expressed lncRNAs, some of which are differentially-regulated under the conditions tested, but their role and function within the hypothalamus remains a mystery. We would like to suggest a call-to-action for those scientists with transcriptome datasets available, who have not yet analyzed lncRNA transcriptome of those datasets. One method, which we use below, is to use the ‘Noncoder’ server⁷⁹, which can analyze CEL files from Affymetrix exon array transcriptome datasets, revealing differential lncRNA expression patterns which can be used for further study. This server also identifies the location of the lncRNA within the genome, which can reveal possible regulatory mechanisms for some lncRNAs. If investigators with these types of transcriptome dataset available do this simple bioinformatics analysis, the new analyses can yield new pathways and signals in the hypothalamus where lncRNAs are up- or down-regulated. Analysis of all components of the hypothalamic transcriptome will enable a better understanding of regulation within this complex tissue.

4. New studies

In taking our own advice, we re-analyzed a previously unpublished dataset of total RNA expression in the hypothalamus of 129Sv/J strain wildtype (WT) and Nhlh2 knockout (N2KO) mice, under *ad lib* and fasting conditions. Nhlh2 is a hypothalamic basic helix-loop-helix transcription factor that transcriptionally regulates several genes, including prohormone convertase I, melanocortin-4-receptor, and monoamine oxidase A^{5,80,81}. The

N2KO mice were developed nearly 20 years ago, and are characterized by adult-onset obesity, as well as reduced physical activity levels⁸². Furthermore, Nhlh2 itself is a target of energy availability pathways, such as fasting, cold-exposure and leptin signaling^{83–85}. In fact, our previous finding on leptin-induced Nhlh2 expression through Signal transducer and activator of transcription 3 (Stat3) rather than Nuclear factor kappa B (NFKB) indicates a possible indirect regulation of Nhlh2 expression by lncRNAs due to the fact that the transcriptional activity of both Stat3 and NFKB can be regulated by lncRNAs^{50,86–88}. For this study, the Affymetrix GeneChip® Mouse Exon 1.0 ST array was used in triplicate replicates of hypothalamic RNA from 10–12 week old male WT and N2KO mice, treated to either ad lib and 24-hour fasting conditions. A total of 12 mice, 6 for each genotype, were randomly assigned to ad lib or fasting group with 3 mice for each group. The CEL files from the arrays were uploaded to the ‘Noncoder’ server⁷⁹, and were processed using RMA normalization. The normalized raw data were extracted from the ‘Noncoder’, and a Student’s t-test was performed on each comparison between two genotypes and two food treatments. To survey for candidate lncRNA with differential expression, statistical criteria of *P*-value 0.05, fold change 1.5, and more than one (2) identified probes were used to identify differentially expressed lncRNAs.

A total of 60 lncRNAs are differentially regulated by fasting in WT mice, while 65 lncRNAs are differentially expressed between ad lib WT and N2KO mice, with 5 lncRNAs overlapping both comparisons (three shown in Figures 1–2). Meanwhile, 55 lncRNAs are differentially expressed between ad-lib and fasting in KO mice, and 58 between WT and N2KO by fasting. The top five up- and down-regulated for all conditions above are shown in Table 1. However, while most of the expression differences are modest (2-fold or less), none of the lncRNAs listed are yet characterized in the hypothalamus. In other words, a PubMed search or NONCODE search using their sequence name give us no information on their expression pattern, role/function, or DNA/RNA targets in the hypothalamus. To provide a sense of the types of data to be analyzed in future studies, we analyzed three differentially expressed lncRNAs which showed highly significant differences between WT and N2KO *ad lib* and fasted mice. These three are shown on Figure 1. As shown, relative expression levels are different between the three lncRNAs, even within the WT *ad lib* fed mice. In addition, deletion of Nhlh2 results in lack of a response to fasting, or an opposite response to fasting, compared to the WT animals. There are many other lncRNAs identified by our dataset, each with different levels in WT and N2KO mice, *ad lib* and fasting conditions (data not shown), and these will be worthy of further study. Of note, even though Nhlh2 is a hypothalamic transcription factor⁸², it is unlikely that it is responsible for transcription of any of these lncRNAs, and we are not making that assumption. However, deletion of Nhlh2 does affect energy availability, as sensed by hypothalamic neurons^{82,84}, and thus, we are implying that the changes to lncRNA expression in the N2KO mice may be part of either a rapid or long-term response of the hypothalamus to energy needs of the organism.

lncRNAs are predominately involved in transcriptional regulation, and can target and impact gene expression using cis-regulatory mechanisms, such as anti-sense RNA interference on the gene they overlap with, or using trans-regulatory mechanisms for distant loci. Thus, it is important to understand where the lncRNA gene is located in the genome. Noncoder provides this information for every lncRNA in the dataset. We analyzed the three

lncRNAs from Figure 1 for genome location to demonstrate this point (Figure 2). AK139892 is located in an intergenic location between Rpl221l and Gm1527 in the sense direction, and Gm17485 and Mecom in the anti-sense. As it is not directly within any protein coding gene, this suggests that AK13982 potentially acts in a trans-regulatory fashion. Conversely, AK089873 is located within an intron of the Tox gene, suggesting the possibility of cis-regulation. Tox, otherwise known as the thymocyte selection-associated high mobility group box factor is an evolutionarily conserved DNA-binding protein which regulates transcription by modifying local chromatin structure and modulating the formation of multi-protein complexes⁸⁹. There is no indication at this time that it is regulated by lncRNAs, but a recent publication has implicated Tox expression in neural stem cell commitment⁹⁰. Finally, AK082527 lncRNA is expressed in an anti-sense direction relative to the Icam5 gene, which is very suggestive of a cis-regulatory role for this lncRNA in Icam5 expression. Icam5 is a neural-specific cell adhesion protein which appears to be key to dendritic extension and functional synapse formation⁹¹. At this time, there has been no report of its regulation by lncRNA. Although further characterization on regulatory mechanism of three lncRNAs is necessary, this pilot analysis provides us a potential relationship of these three RNAs and their neighbor protein coding genes.

As the study on WT and N2KO mice was done in the 129Sv/J strain, compared to the C57Bl/6 strain used in our published paper⁶⁷, we were also able to compare expression of lncRNA between strains, and in response to fasting. As shown in Table 2, using statistical criteria of P-value 0.05, fold change of 1.5, and more than 2 identified probes for analysis, a total of 386 lncRNAs were found to be differentially regulated by food deprivation in C57Bl/6 with 305 of those up-regulated and 81 down-regulated by a 24-hour fast. In contrast, there was a more modest change of only 39 lncRNAs with increased expression level with fasting, and 21 decreased lncRNAs in 129Sv/J strain. Of these, only two lncRNAs are differentially up-regulated by fasting in both strains, and none of the down-regulated lncRNAs overlap between the two studies. We also compared the top 10 hypothalamically-expressed lncRNAs between C57Bl/6 and 129Sv/J strains and found that while there are slight differences in expression levels and the order of the top 10, the top 10 are identical between the two strains (Table 3). Of note, AK172585 which shows the highest expression for both strain, was also reported to be expressed in the nucleus accumbens and differentially down-regulated with methamphetamine treatment⁹². Given these data, it is clear that strain differences should be a consideration for future studies.

For the WT and N2KO mouse bioinformatics studies, using archival transcriptome datasets, several interesting lncRNAs were identified for further follow-up studies, and based on additional analyses, including position within the genomes (especially if located within protein-coding regions of genes), experimental studies can now be designed to further characterize the function of these candidates. This is a first step in starting to characterize the role of lncRNAs in fasting mechanisms within the hypothalamic transcriptome.

5. Final Thoughts

The role of lncRNAs in the neuroendocrine hypothalamus is in its infancy, but researchers interested in studying these non-coding RNAs can start by using their own hypothalamic

transcriptome datasets, or even by downloading datasets from the GEO datasets repository through NCBI ⁹³. A quick search of the GEO datasets revealed hypothalamic transcriptome datasets high and low weight gain C57Bl/6 mice, calorie-restricted rats, differential strain-specific mouse crosses, high fat diet-induced obese mice, and even post-mortem samples of human hypothalamus from males and females over 70 years of age at death (data not shown). RNA-seq or whole transcriptome files will be much better to use than older files where only known protein-coding genes were profiled, but even on those older datasets, some expressed sequence tags (ESTs) were included, and now may be annotated.

The complexity of the hypothalamus, with its various nuclei and neuronal subtypes suggests that there will also be complexity in the lncRNA transcriptome. In addition to nuclei-specific analysis of lncRNAs, an easier approach might be to examine lncRNAs using a defined hypothalamic cell line, such as those generated by the Belsham group ⁹⁴. Leptin-treatment, glucose deprivation, or other stressors can be used *in vitro*, with the cell-line transcriptome of lncRNAs confirmed in mice at a later time. This method would save time, would likely be less costly than animal studies, and would allow for functional characterization of the lncRNA within that cell line following transcriptome identification.

More work is also needed to understand the structure-function relationships that dictate how lncRNAs regulate their target genes. The secondary and tertiary structures of lncRNAs can be predicted (as shown, Figure 2), and while those structures appear to be conserved between animals, we still do not understand how the structure-function can be used to predict target genes and regulatory networks ⁵⁰. It may be easiest to start with lncRNAs located within protein-coding genes, as most evidence suggests that they exert cis-regulatory activities on those genes ⁸⁷. In closing, increasing the numbers of known hypothalamic-specific lncRNAs, as well as those with known functions will help to elucidate the roles of lncRNAs in energy balance, and body weight metabolism processes. We have hereby given a “call-to-action” to researchers with hypothalamic transcriptome datasets to analyze and use these datasets to help further characterize lncRNAs in each of the hypothalamic nuclei, and neuronal subtypes, and solve this so-called “molecular conundrum” once and for all.

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List of Abbreviations

cIAP2	Baculoviral IAP repeat containing 3
BC1	Brain Cytoplasmic RNA 1
CEBPα	CCAAT/enhancer-binding protein α
CRNDE	Colorectal neoplasia differentially expressed
Dlk1	Delta-like 1 homolog
ENCODE	Encyclopedia of DNA Elements

GEO	Gene Expression Omnibus
GR	Glucocorticoid receptor
Gas5	Growth arrest-specific 5
lncLGR	LncRNA liver GCK repressor
lncRNA	Long non-coding RNA
Gtl2	Maternally expressed 3, or MEG3
1Malat1	Metastasis associated lung adenocarcinoma transcript
GOMAFU	Myocardial infarction associated transcript
NLC	Narcolepsy candidate-region
NCBI	National Center for Biotechnology Information
Nhlh2	Nescient helix-loop-helix 2
N2KO	Nhlh2 knockout mice
ncRNA	Non-coding RNA
NEAT1	Nuclear paraspeckle assembly transcript 1
PPARγ	Peroxisome proliferator-activated receptor γ
PASR	Promoter-associated short RNA
SGK1	Serum/Glucocorticoid regulated kinase 1
snRNA	Small nuclear RNA
snoRNAs	Small nucleolar RNAs
SRA	Steroid receptor RNA activator
TUG1	Taurine Upregulated Gene 1
tRNA	Transfer RNA
VEGF	Vascular Endothelial Growth Factor
WT	Wildtype

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Highlights

- Long non-coding RNA identification and function in the hypothalamus is discussed.
- A call-to-action to use archival data for lncRNA discovery is proposed.
- New data on hypothalamic lncRNA expression in mice is provided.

Figure 1. Relative expression level of three differentially-expressed lncRNAs

Three different lncRNAs, found to be differentially-expressed in WT mice that were *ad lib* fed, compared to fasted were chosen for further analysis in N2KO mice (n=3 for each group). These three were chosen randomly among the 5 overlapped lncRNAs between Tables 1 and 2. All data comes from bioinformatic analysis only, and shows the average relative normalized expression data for the lncRNA on the array in each condition. *P*-values are shown between WT *ad lib* and fasted mice. There was no significant difference in expression between N2KO mice in the same conditions.

Figure 2. Noncoder analysis of lncRNAs

The three lncRNAs analyzed for Figure 1 were further analyzed using Noncoder online tools ⁷⁹. The RNA secondary structure, and lncRNA genomic location for AK139892 (A), AK089873 (B) and AK082527 (C) are shown. Genebank names, and distance in kilobases (kb) from the lncRNA loci are given.

LncRNAs showing significant differential expression to food deprivation and deletion of Nhlh2

Table 1

The top five up- and down-regulated lncRNAs are shown for the differentially-regulated lncRNAs identified for each condition. GenBank accession numbers are provided.

	GenBank Gene ID	WT Ad lib	WT Food Deprived	Fold Change ad lib/food deprived	P-value
Down-regulated	AK081323	8.31	22.12	0.38	0.01
	AK033006	22.01	49.39	0.45	0.01
	AK134166	17.04	37.23	0.46	0.02
	AK049876, AK021104	81.19	175.55	0.46	0.03
	AK018564	21.09	45.47	0.46	0.04
Up-regulated	AK046498	76.57	45.04	1.7	0.05
	AK156159	86.23	47.4	1.82	0.03
	AK015258, AK019564	24.33	13.21	1.84	0.00
	AK139947	33.61	18.16	1.85	0.02
	AK089873	28.62	13.44	2.13	0.02
	GenBank Gene ID	WT Ad lib	N2KO Ad lib	Fold Change WT/KO	P-value
Down-regulated	AK043724	32.82	87.06	0.38	0.02
	AK053298	64.88	143.17	0.45	0.01
	AK043219	32.18	62.44	0.52	0.04
	AK087042	29.8	53.57	0.56	0.01
	AK046886, AK013592	103.55	183.43	0.56	0.03
Up-regulated	AK156113	52.17	30.4	1.72	0.02
	AK138864	16.84	9.8	1.72	0.01
	AK087154	35.11	17.61	1.99	0.01
	AK089807	36.39	17.74	2.05	0.01
	AK038267	25.03	9.64	2.6	0.01
	GenBank Gene ID	N2KO Ad lib	N2KO Food Deprived	Fold Change ad lib/food deprived	P-value
Down-regulated	AK053409	21.79	54.48	0.4	0.01
	AK042236	20.49	41.4	0.5	0.02
	AK138864	9.8	18.64	0.53	0.03

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GenBank Gene ID	WT Ad lib	WT Food Deprived	Fold Change ad lib/food deprived	P-value
AK034825	24.31	45.87	0.53	0.04
AK083893	46.08	86.28	0.53	0.05
AK049712	40.92	22.45	1.82	0.02
AK162987	25.14	13.72	1.83	0.03
AK078079	19.51	10.65	1.83	0.02
AK134750	403.55	219.29	1.84	0.03
AK086100	36.88	18.05	2.04	0.01
GenBank Gene ID	WT Food Deprived	N2KO Food Deprived	Fold Change WT/KO	P-value
AK157258	80.17	144.79	0.55	0.00
AK081356	51.29	91.01	0.56	0.03
AK040341	100.42	172.39	0.58	0.04
^a	89.92	154.45	0.58	0.02
AK049824	60.15	100.14	0.6	0.02
AK162405	47.25	23.94	1.97	0.03
AK054026	25.24	12.7	1.99	0.02
AK142123	63.04	30.83	2.04	0.03
AK053225	76.74	37	2.07	0.00
AK084419	301.76	103.09	2.93	0.01

^aNONCODE ID NONMMUT041114.2 (no GenBank ID available)

Table 2

Strain-specific differentially expression of lncRNAs with fasting.

	C57Bl/6	129Sv/J	Overlapped
Up-regulated	305	39	2
Down-regulated	81	21	0
Total	386	60	2

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Table 3
Top Expressed Hypothalamic lncRNAs in C57Bl/6 and 129Sv/J strains

The relative ranking between the two strains is shown, with C57Bl/6 ranking in order and relative ranking of 129Sv/J shown in parenthesis.

GenBank Gene ID	C57Bl/6 Expression	129Sv/J Expression (rank in 129Sv/J)	region
AK172585	11781.05	15390.56 (1)	Intergenic
AK159184	6944.49	9745.51 (2)	Extension
AK155141	3874.19	6342.29 (3)	Intergenic
AK076188,NR_003270.2	3063.68	4931.37 (4)	Sense
AK156916	2247.74	2617.80 (8)	Antisense (exonic)
AK013768	2193.81	3731.56 (5)	Intergenic
AK139256	1840.65	2277.05 (9)	Sense
AK011460	1749.59	2974.77 (6)	Antisense (intronic)
^a	1544.10	2779.25 (7)	Intergenic
AK078154	1459.07	2228.62 (10)	Intergenic

^aNONCODE ID NONMMUT053481.2 (no GenBank ID available)