Transcriptional Regulation of Hypothalamic Energy Balance Genes

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Within the postnatal human brain, there are at least 4627 expressed transcription factors (Hawrylycz et al. 2012). These transcription factors are differentially expressed within the various lobes and structures of our brain, including the hypothalamus and its distinct set of nuclei. Data from my own laboratory indicates that there are as many as 2089 mRNAs coding for transcription factors within the hypothalamus alone (Jiang and Good, unpublished, referring to dataset within (Jiang et al. 2015)). When one begins to contemplate the enormity of having over 2000 different transcription factors to specify the thousands of proteins that make up the hypothalamus, it is clear that we are still a long way from completely figuring out how each of these are coordinated in response to energy balance signals. In addition to the transcription factors, thousands of noncoding RNAs mediate posttranscriptional regulatory signals, and both proteins and noncoding RNAs direct differential translation of mRNAs which specifies the hypothalamic proteome. This chapter provides an overview of what we know about transcriptional regulation of genes expressed within the hypothalamus, and discusses the questions that remain in order for us to fully understand the hypothalamic transcriptome and resulting proteome.

3.1 Transcriptional Mechanisms

In its simplest model, transcription of a gene involves RNA polymerase binding to or near the transcriptional start site of a gene and starting the process of making a new mRNA. It is, unfortunately, not that simple. Tissue-specific transcription is guided by the chromatin state (methylated/acetelyated/biotinylated, etc. DNA and histones), specific transcription factor binding sites within the promoter region of a gene, and the complement of transcription factors active within the nucleus at any given moment. While some researchers look at multiple transcription factors or transcribed genes at once, others focus on resolving individual gene-transcription factor interactions. Still others examine the delineation of regulatory regions—in other words, what constitutes a hypothalamic promoter? In this section, we will examine some of the ways that hypothalamic transcriptional mechanisms can be studied, using specific examples from the literature, and then piece together what is known about how changes in energy balance, including leptin or glucose signals, food intake, and food deprivation, change the transcriptional landscape.

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3.1.1 Models and Cell Lines for Studying Hypothalamic-Specific Transcription

3.1.1.1 Gene Expression Localization

Some of the first studies of gene expression in the hypothalamus utilized antibodies and immuno-histochemical localization of hypothalamic proteins, Northern blots of isolated hypothalamic RNA, and in situ hybridization probes to hypothalamic tissue sections to show localized expression of genes in response to stimuli. For example, Northern analysis of vasopressin mRNA from the hypothalamus of the Brattleboro rat was used to show that vasopressin was expressed but at a lower level and at a slightly different size than the Long-Evans rat (Majzoub et al. 1984). We now know that the Brattleboro rat carries a point mutation within the protein-coding region of vasopressin, which affects the open reading frame of the mRNA and leads to diabetes insipidus (Schmale and Richter 1984) The technique of in situ hybridization was used to show c-fos expression in the paraventricular nucleus of the hypothalamus in response to water deprivation of rats (Sagar et al. 1988). As stated by the authors of this historic paper, in situ hybridization allowed for cellular-level resolution of expression within these neurons, as opposed to antibody labeling, which could be more diffuse. Dual-label in situ hybridization techniques enabled co-localization of gene expression, while some RNA probe-based methods allowed researchers to look at specific transcripts of genes (i.e., differentially spliced genes), but none of these could get at the actual mechanisms of gene regulation—only the fact that mRNA and protein were expressed in hypothalamic locations and in response to various stimuli. For understanding mechanisms involved in hypothalamic gene expression, other model organisms and cell lines were developed.

3.1.1.2 Hypothalamic Cell Lines

One of the first established hypothalamic cell line was the HT9 cell line, established via SV-40 viral infection of primary embryonic hypothalamic cells in 1974 (De Vitry et al. 1974). This cell line expressed both vasopressin and neurophysin, suggesting that the cell line originated from neurosecretory cells of hypothalamic magnocellular neurons. However, few published studies used these lines, as indicated by only four citations to the original paper made since 1974. Several other lines were established in the 1980s and 1990s, including the GT1–7, gonadotropin-releasing hormone (GnRH) secreting cell line, which originated from the scattered GnRH neurons in the rostral hypothalamus (Mellon et al. 1990). The developers of this line used a transgenic mouse expressing SV-40 under the control of the GnRH promoter to direct the transforming oncogene, Large T, just to the neurons of interest—namely, the scattered GnRH neurons of the hypothalamus—and it worked! The GT1–7 line continues to be in use to this day, with a 2016 publication on enhancers and noncoding RNA involvement in GNRH promoter regulation (more about his later) (Huang et al. 2016). Each of these previous examples is of one specific hypothalamic cell type, but in 2004, Dr. Denise Belsham and her group published an article detailing 38 embryonic mouse hypothalamic cell lines, each representing different neuronal cell types of the hypothalamus (Belsham et al. 2004). These cell lines allowed individuals working in hypothalamic gene regulation to tailor the gene regulation studies to the specific hypothalamic neuron type that they were interested in, meaning that the gene expression and promoter analysis studies could be hypothalamic-specific, with regard to transcription factors, DNA promoter methylation and acetylation marks, and noncoding RNAs—all of which will be discussed in the next sections. The 2004 paper has been cited more than 128 times, indicating the utility of those defined hypothalamic cell lines for gene expression and other studies. Dr. Belsham's group has also developed rat hypothalamic lines—both adult and embryonic (Gingerich et al. 2009)—and mouse adult hypothalamic cell lines (Belsham et al. 2009) which allow researchers to do even more specific hypothalamic gene expression studies.

Why is the establishment of hypothalamic cell lines so important to studying hypothalamic gene regulation? Simply put, having cell lines that

match the tissue being studied allows one to do molecular "promoter-bashing" studies that provide quick and informative data on regions of the promoter needed for tissue-specific gene expression, and transcription factors involved in regulation of hypothalamic genes. Too many times the inappropriate cell line is used and later found not to represent the tissue that the researcher claimed to be studying. For example, while HeLa cells, derived from a human cervical carcinoma, have been used in many different gene expression studies, and certainly have helped establish some basic "rules" about gene expression (Landry et al. 2013), they would not be a good model for hypothalamic-specific gene expression.

Promoter-bashing refers to the use of cell lines transfected with fragments of DNA containing a promoter region of interest linked to a reporter gene such as luciferase to determine regions of DNA, and transcription factors that confer hypothalamic-specific expression patterns. We have used the Belsham hypothalamic cell lines in studies to examine melanocortin-4-receptor expression in paraventricular nucleus-like lines and the leptin responsiveness of the nescient helix-loop-helix 2 and prohormone convertase 1 promoters in arcuate nucleus-like lines (Al Rayyan et al. 2014; Fox and Good 2008; Wankhade and Good 2011). Without the cell lines, the work would have been done either using transgenic animals (see below), which are much more time-consuming, or would have had to be done in non-hypothalamic neuronal lines, which might not have been representative of hypothalamic gene expression.

3.1.1.3 In Vivo Methods and Models

Even with the best circumstances, cell lines do not give the most accurate profile of gene expression, as they are usually grown without glial cells, and on plastic in defined media, rather than on the extracellular matrix supplied by capillary blood containing growth factors, hormones, and other signals. Thus, the best mechanistic gene expression studies are those done using in vivo methods, such as developing promoter transgenes or examining DNA binding of transcrip-

tion factors using chromatin pull-down assays from tissues.

There are many ways to use transgenes, such as directing expression of a gene to a specific target tissue, but in this section, we will focus on using transgenes composed of a promoter linked to a reporter to examine hypothalamic-specific transcription and to identify minimal promoters and enhancer regions needed for this specificity. There are multiple examples of using this method in the literature, but studies using the minimal promoter and enhancer region from the GnRH promoter provide an excellent example. In these studies, cell lines had been previously used to identify both rat and mouse GnRH promoter regions that directed expression in the GnRH neuron cell line GT1–7 cells (Whyte et al. 1995). These data showed that a minimal promoter existed within the first ~173 base pairs prior to the start of transcription, and a hypothalamic-specific enhancer region could be found between −1571 and − 1863 (Lawson et al. 2002). Based on that knowledge, a minimally sized transgene was created, which contained both the minimal promoter and the enhancer within just a few base pairs of each other, and this transgene recapitulated hypothalamic expression in multiple lines of animals (Lawson et al. 2002). The use of transgenic promoters to study expression appears to have fallen out of favor in recent articles, likely due to the time and expenses needed to generate the constructs and the animals. However, the new methodology of CRISPR/Cas9 gene editing may provide a way to mutate a promoter or enhancer element in situ (i.e., within the genome, not as a transgene per se) and analyze the resulting effects on expression. While this procedure has not yet been done in vivo in hypothalamic tissues, a very recent article was able to successfully modify a gene in mouse retinal cells using CRISPR/Cas9 technology (Latella et al. 2016). A similar strategy could be employed for hypothalamic promoters, combining this in vivo method with current CRISPR/Cas9 promoter analysis methods (i.e., such as those used by Fulco et al. 2016, among others).

DNA binding of transcriptional elements and analysis of histone modifications in vivo can be

done using tissue chromatin immunohistochemistry methods. In this procedure, protein-bound chromatin are isolated from a tissue of interest and then immunoprecipitated with antibodies to the transcription factor or histone modification domain of interest. The protein-DNA complexes pulled down in the immunoprecipitation are further analyzed by PCR for the region of interest within the DNA. Quantitative PCR can be used to analyze relative binding as well. My laboratory has used this method to show Stat3 interactions on the hypothalamic Nhlh2 promoter occurring 2 h following leptin injection of mice (Al Rayyan et al. 2014). This method allows one to look at a snapshot of protein-DNA interactions in vivo but with the caveat that the DNA fragments in this method are about 200 base pairs in length and thus usually contain multiple possible sites for protein binding or histone modifications. Thus, the results must be confirmed with in vitro or cell-based studies.

3.1.1.4 Genomic Transcriptional Analysis

A number of databases exist online, which allow researchers to analyze data in silico before going to the bench. First, one can look directly at gene expression in online datasets in the NCBI GEO Profiles site: https://www.ncbi.nlm.nih.gov/geo-profiles/. By entering a gene name or tissue in the search box, previously submitted transcriptome analysis data will be displayed. For example, by entering the term "hypothalamus" in the search box, more than 804,000 entries are displayed. Adding the term "leptin" narrows the search down to a more reasonable 998 entries, and further narrowing the search by putting in the gene name STAT3 results in 91 entries. This type of data may be a place to start looking to design new experimental directions.

Another worthwhile analysis using completely online tools involves promoter analysis and comparison between species. One possible way to do this, even if one doesn't know the exact location or length of the promoter is through the gVISTA tool (http://genome.lbl.gov/cgi-bin/GenomeVista). An mRNA or genomic sequence for the gene of interest can be put into the query box, and the tool will align the sequence with human or any other species in the databases. One can zoom out from the initial comparison to see the upstream and downstream regions of homology. An example of this is shown on Fig. 3.1, where the gene for NHLH2 was originally screened and then the genome sequence surrounding NHLH2 compared between several species. As one can see, homologous noncoding regions (shown in pink) appear in both the proximal promoter, next to the transcription start site (light blue box, 5′ untranslated region), and in a region approximately 10,000 base pairs upstream which may represent an as yet uncharacterized enhancer region for the NHLH2 gene. One can use the sequences in this region to analyze putative transcription factor binding sites with the PROMO website: http://alggen.lsi.upc.es/cgi-bin/promo_v3/promo/promoinit.cgi?dirDB=TF_8.3 . This type of in silico analysis can be a starting point for cell- or animal-based studies. Other sites of use for promoter/transcription factor analysis include some of the sites listed at Epigenie: http://epigenie.com/epigenetic-tools-and-databases/. These online analysis tools provide information about histone methylation and acetylation and DNA methylation based on studies already done for multiple tissues and whole genomes. As methylation and acetylation of histones and methylation of GC dinucleotide in DNA can specify "active" and "inactive" areas of the genome, the in silico analysis can be a very good starting point for transcriptional analysis of a hypothalamic promoter.

3.1.2 Hypothalamic Promoter Regions

According to one paper that has functionally mapped human promoters, there are at least 400 different cell types in the human body, with essentially 1 genome to specify them all, and this specificity must occur through gene regulation (Consortium et al. 2014). Gene promoters have evolved to specify cell-specific gene expression, and from the work of the FANTOM consortium, neural promoters form a subgroup of these
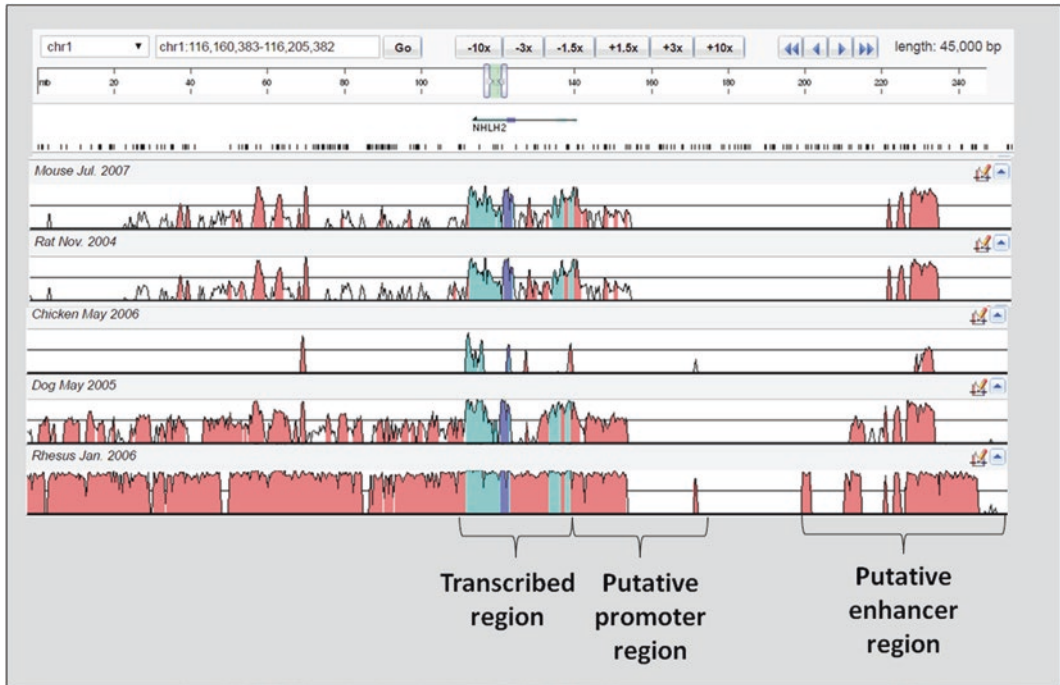
Fig. 3.1 Promoter analysis for NHLH2, using the online GenomeVISTA tool. The search term "NHLH2" was entered into the search bar (http://genome.lbl.gov/cgi-bin/GenomeVista), resulting in the following output. Pink regions are areas of homology between species, while light blue and dark blue regions are transcribed (light blue is untranslated, while dark blue is translated). The peak height indicates the strength of homology. Note that the NHLH2 gene is transcribed from the opposite strand of the genomic DNA, and runs from right to left

expression patterns (Consortium et al. 2014). In addition, there are multiple enhancer regions for each gene, located within 500 kilobases (kb) of the start of transcription (Andersson et al. 2014). Further use of the FANTOM site at http://fantom.gsc.riken.jp/5/ allows one to dig deeper into hypothalamic promoter analysis, finding a list of 1000 transcription factors that are enriched in adult diencephalon (Fig. 3.2, discussed more below). One can use a site like this to explore an individual promoter, or transcription factors, but the bulk of the confirmation work still needs to be done in the laboratory.

Some of the earliest studies on hypothalamic promoters used the pro-opiomelanocortin (POMC) gene as a model (Young et al. 1998). POMC's expression is limited to approximately 1000 POMC-specific neurons within the arcuate nucleus of the hypothalamus, as well as within hindbrain neurons and corticotrophs of the pituitary. Initial studies of the POMC promoter in pituitary cell lines found a very small ~500 base pair (bp) region directed expression in pituitary cells, and in the pituitary of transgenic mice, but not in the hypothalamus of those same animals (Rubinstein et al. 1993). Instead, a 15 kb region, containing ~13 kb of upstream sequence, was shown to direct hypothalamic-specific expression (Young et al. 1998). Further studies identified an enhancer region at ~12 kb upstream as the key regulatory element, and this region had high sequence conservation between human, mouse, dog, and bovine sequences, even though the latter two genomes were barely even started at that time (de Souza et al. 2005). Ten years later, two POMC gene enhancers, nPE1 and nPE2 located ~2 kb from each other and ~10 kb from the transcription start site, direct transcription of POMC within those 1000 POMC arcuate nucleus neurons (Lam et al. 2015). Interestingly, although deletion of just one of the enhancers still resulted in hypothalamic-specific expression, POMC

FF:10193-103E4

Name: diencephalon, adult
Species: Human (Homo sapiens)
Library ID: CNhs12610
Sample type: tissues
Genomic View: UCSC ⌗
MicroRNAs: link ⌗
RefEX: Specific genes ⌗
FANTOM CAT: 1 ⌗, 2 ⌗, 3 ⌗, 4 ⌗
CAGEd-oPOSSUM: link ⌗

Additional information [Expand]

Accession numbers [Expand]

Download raw sequence, BAM & CTSS [Expand]

Relative expression to median (log10)

Transcription factors with enriched expression in this sample ⓘ Copy CSV PDF

Show 10 ⌄ entries   Search: ▯

CAGE peaks ⇕	Log10(Relative expression over median) ▼	TPM ⇕	TF ⇕
p2@TSC22D4	2.89	770.50	TSC22D4
p1@OLIG1	2.61	406.45	OLIG1
p1@PEG3,p1@ZIM2	2.47	291.66	PEG3
p1@SOX10	2.28	187.41	SOX10
p1@C11orf9	2.18	307.35	C11orf9
p1@OLIG2	2.12	130.02	OLIG2
p1@SOX2	1.89	76.14	SOX2
p1@ST18	1.88	75.67	ST18
p3@ZEB2	1.83	111.28	ZEB2
p2@OLIG1	1.81	64.19	OLIG1

Showing 1 to 10 of 1,000 entries   ◀ Previous Next ▶

Fig. 3.2 Use of the FANTOM site (http://fantom.gsc.riken.jp/5/) to find hypothalamic transcription factors. As shown, search tools to narrow the tissue to adult diencephalon from adult tissues were used. The first 10, in a list of 1000, are shown, presented from highest expression to lowest expression

expression was decreased overall, suggesting the two enhancers have an additive effect on overall transcription. Furthermore, deletion of the nPE1 alone, which reduced expression of POMC and POMC cell numbers to 30% of normal, led to a significant increase in body weight and body fat in the mutant mice (Lam et al. 2015). This example alone makes a case for the relative importance of hypothalamic promoter function in maintaining POMC and other hypothalamic-specific genes needed for energy balance pathways. Expression levels and proper expression patterns are necessary for physiological maintenance of body weight and energy sensing. In a study examining single nucleotide polymorphisms within or near CpG islands, which can specify promoter activity via DNA methylation, 28 genes (including POMC) were found to have SNPs that affected promoter methylation level (Voisin et al. 2015). Studies that specifically examine differential methylation of the POMC promoter have shown a link between POMC methylation and offspring obesity in animals fed a high-fat/high-sucrose diet (Zheng et al. 2015). In a very recent

study, variable methylation of the POMC gene was shown to be established early in human fetal development within a POMC variable methylation region located within intron 2, where hypermethylation equals reduced POMC expression and increased body weight (Kuhnen et al. 2016). Thus one needs to consider not only the promoter but also enhancers and downstream regions within the gene for overall transcriptional regulation.

Polymorphisms in the promoter of the melanocortin-4-receptor (MC4R) gene, whose protein product binds to the POMC peptide alpha-melanocortin-stimulating hormone, have been linked to obesity in several studies (Muller et al. 2014; Tan et al. 2014; Valli-Jaakola et al. 2006; van den Berg et al. 2011). In one of these studies, the two variations studied were shown to reduce MC4R gene transcription (Tan et al. 2014). A 2 bp deletion in the MC4R promoter, found by another study (Valli-Jaakola et al. 2006), was used by our laboratory to show that the deletion affected Nhlh2 transcription factor binding and transcription of the MC4R gene (Wankhade and Good 2011).

It is likely that many more promoter-associated polymorphisms will be identified as more and more genomes are sequenced and more and more association studies are completed. At this time, the numbers of studies of this type are meager—just 39 published articles are found in PubMed using the search terms "hypothalamus, promoter, obesity, transcription." More work is needed both to confirm the transcriptional regulatory regions of obesity- and energy balance-associated genes and to determine if newly detected variants within the promoters or enhancer regions may affect an individual's ability to respond to energy availability.

3.1.3   Hypothalamic Transcription Factors

According to the FANTOM database, a total of 1762 human and 1516 mouse transcription factors have been identified to date (Consortium et al. 2014). Note that this number is lower than we found using exon arrays (unpublished data from (Jiang et al. 2015)). However, using the FANTOM database tools http://fantom.gsc.riken.jp/5/, the relative expression level of transcription factors expressed within the adult diencephalon can be analyzed. As shown on Fig. 3.2, of 1000 transcription factors with expression of at least $\log(10) = 0.33$ (~twofold) of the median expression over all of their studies, the top 10 in the list form a less than well-known list of transcription factors—at least as far as hypothalamic transcription factors go. So what does this mean for analyzing transcription within the hypothalamus? Is it possible that the highest expressed factors do not necessarily control hypothalamic specificity? Yes, it is possible. For example, the first factor on the list shown in Fig. 3.2 is TSC22D4, a leucine zipper transcription factor family member, which according to http://www.genecards.org (Stelzer et al. 2016) is at least as highly enriched in lymphocytes and liver and gland cells as it is in nervous system. Factors such as Nhlh2 (~twofold), Stat3 (not even listed), or FoxO1 (~3.3-fold)—all of which are transcription factors that whose "hypothalamic importance" has been shown—are expressed at much lower levels, at least within the snapshot of expression data used for the FANTOM studies.

So what is known about transcription factors within the hypothalamus—which are the "important" ones and which are more general and likely just performing housekeeping duties? One of the first transcription factors identified in the paraventricular and supraoptic hypothalamus was c-fos (Sagar et al. 1988). C-fos is part of the activator protein-1 (AP1) complex, and its expression and nuclear localization appear to be stimulated when nerves are activated. In the FANTOM database, basal expression is only about twofold above the median, but it is likely that stimulated neurons show much higher levels. This point is key to the discussion—the hypothalamus sits at the base of the brain, with a median eminence region that "sips" capillary blood and can integrate external signals—likely doing this through differential activation of transcription factors and

their target promoters. C-fos is likely one of those transcription factors that responds to external signals, such as water deprivation as was shown in that early study (Sagar et al. 1988). However, c-fos is expressed throughout the brain and in many different tissues, so while it is important for integrating signals, it is likely not key in mediating hypothalamic-specific responses.

Conversely, SOX2, which did make the top 10 list from the FANTOM expression analysis, is enriched in adult hypothalamic tissues at approximately 76-fold (log10 2.28) above the median gene expression levels (Fig. 3.2). SOX2 is normally thought of as a neuronal developmental transcription factor but, as shown by Hoefflin and Carter, is highly localized to the suprachiasmatic nucleus (SCN) and the periventricular nucleus (PeN), adjacent to the median eminence in adult rats (Hoefflin and Carter 2014). While the target genes for SOX2 within these hypothalamic nuclei have not been identified, the related transcription factors, SOX4 and SOX11, direct GnRH gene transcription within the hypothalamic-specific enhancer which is actually downstream of the transcription start site and within the first intron of the gene (Kim et al. 2011). Of note, this study used several of the cell lines and procedures described in the preceding section, including the GT1–7 cell line, two neuronal hypothalamic cell lines developed by the Belsham group, and ChIP analysis from hypothalamic lysates.

In summary, hypothalamic transcription factors, both those that are highly expressed and those that show a more targeted distribution with lower overall expression in the hypothalamus, require more analysis. Researchers certainly have not characterized the roles of many, if not most of the hypothalamic transcription factors that are expressed, nor understand what the hypothalamic nuclei-specific distribution of each of these might be. Further studies to identify gene targets of these transcription factors, and methods by which their expression is induced or repressed, as well as secondary modifications that might increase or decrease their activity or DNA binding, need to be elucidated.

3.1.4   DNA and Histone Modification

DNA methylation occurs via the addition of a methyl group to the 5′ cytosine of a dinucleotide CpG. DNA methyltransferase enzymes (Dnmt) perform this function—with Dnmt1, 3a, and 3b all being expressed in hypothalamic tissue (Benite-Ribeiro et al. 2016). While this simplified version makes sense from a mechanistic standpoint, the how, why, where, and when issues remain unanswered. What we know is that DNA is differentially methylated on different genes, in different conditions, and at different times, but what dictates these differences is still under intense investigation. Furthermore, in addition to DNA methylation, histones wrapping the DNA are also modified, with methylation, acetylation, and other less frequent marks like biotinylation (Benite-Ribeiro et al. 2016). The epigenetic marks of DNA methylation and histone modification can ultimately affect the level of gene expression at any point in time, irrespective of positively or negatively acting transcription factors.

Early studies using rats as model animals identified a CpG island with DNA methylation marks in the promoter region of the POMC gene and showed that the methylation of this area is inversely correlated to POMC mRNA expression (Plagemann et al. 2009). More interesting was the fact that rats that were overfed during the neonatal period showed increased body weight, accompanied by reduced POMC mRNA levels and higher POMC CpG methylation. This type of finding has been repeated in other models and appears to be consistent with the fact that overnutrition results in increased POMC promoter methylation, while undernutrition results in decreased POMC promoter methylation (Benite-Ribeiro et al. 2016). One study that has examined POMC promoter-associated histones in sheep that experienced maternal undernutrition found that there was a reduction in two different histone H3 methylation marks and an increase in histone H3 acetylation. However, these changes did not correlate into a difference in POMC mRNA expression (Begum et al. 2012). In a separate study, fasting of adult mice led to

increased expression in histone deacetylases −3 and − 4 and decreased expression of histone deacetylases −10 and − 11. The remaining seven histone deacetylases tested did not change with fasting (Funato et al. 2011). Just between these two studies, there is discordance in how undernutrition and fasting might affect overall acetylation—or is there? We don't quite understand how the 11 different deacetylases contribute to gene expression changes, nor how they are induced or repressed in response to energy availability. This last study did not examine gene expression for POMC—but did look at expression of histone deacetylases in POMC neurons—and was able to detect acetylated histones in POMC neurons both in the fed and fasted state (Funato et al. 2011). That was expected, as different promoters will be differentially regulated, some through histone modifications. However, the results do not tell us whether POMC expression is modulated by histone acetylation. Further studies discussed below will follow up on this question.

3.1.5 Transcriptional Up- and Downregulation Through Changes in Energy Availability

Undoubtedly, one of the key signals to energy availability regulation by the hypothalamus is the sensing of the level of circulating leptin. The response to serum leptin levels is also likely the most characterized hypothalamic energy balance-signaling pathway. Multiple studies have shown that increased serum leptin results in up- and downregulation of hypothalamic genes, including increasing the neuropeptide genes such as POMC (Munzberg et al. 2003), and thyrotropic releasing hormone (TRH) (Huo et al. 2004), increasing transcription factors such as Nhlh2 (Al Rayyan et al. 2014), and decreasing expression of the neuropeptide genes AgRP (Toorie and Nillni 2014) and NPY (Morrison et al. 2005). The overall effect is that an increase in energy availability, as signaled by increased circulating leptin, results in increased levels of anorexigenic and decreased levels of orexigenic neuropep-

tides. In this section, the responses of genes to energy availability will be discussed, specifically transcriptional modulation of gene expression through leptin-mediated and fasting-induced signaling in the hypothalamus. Some other hypothalamic regions have been investigated, but the POMC neurons have the best mix of information on them and thus will be the focus on this section.

3.1.6 POMC Neurons in Response to Increased Energy Availability

The POMC neurons lie within the arcuate nucleus of the hypothalamus and there can directly sense leptin signal from the median eminence. This sensing occurs through the long form leptin receptor (LepRb) through a Jak-Stat signaling pathway (Flak and Myers 2016). Of note, Stat3 is required for leptin signaling (Flak and Myers 2016), even though, as mentioned earlier, it is not even found on the top 1000 list of hypothalamic transcription factors in the FANTOM database for hypothalamus (diencephalon) (Consortium et al. 2014). As we have already discussed in general, Stat3 acts as a transcription factor, regulating the POMC gene, the NHLH2 gene, and the PCSK1 gene (Fig. 3.3) (Al Rayyan et al. 2014; Fox and Good 2008; Munzberg et al. 2003). Jak2, a tyrosine kinase, first phosphorylates LepRb following leptin binding, making the activated and phosphorylated LepRb complex more "attractive" to Stat transcription factors. Stat3 and Stat5 are recruited to the activated LepRb/Jak complex and phosphorylated, resulting in translocation of Stat3 and Stat5 to the nucleus (Flak and Myers 2016). Stat transcription factors form homo- and heterodimers with each other and other transcription factors (Reich 2007).

Stat5 is dispensable for leptin signaling (Flak and Myers 2016), so we'll focus on Stat3 transcriptional regulation. What are its target genes? The first leptin-responsive target gene identified was POMC (Munzberg et al. 2003). In this study, leptin was shown to stimulate POMC promoter expression in a kidney and in a pituitary cell line
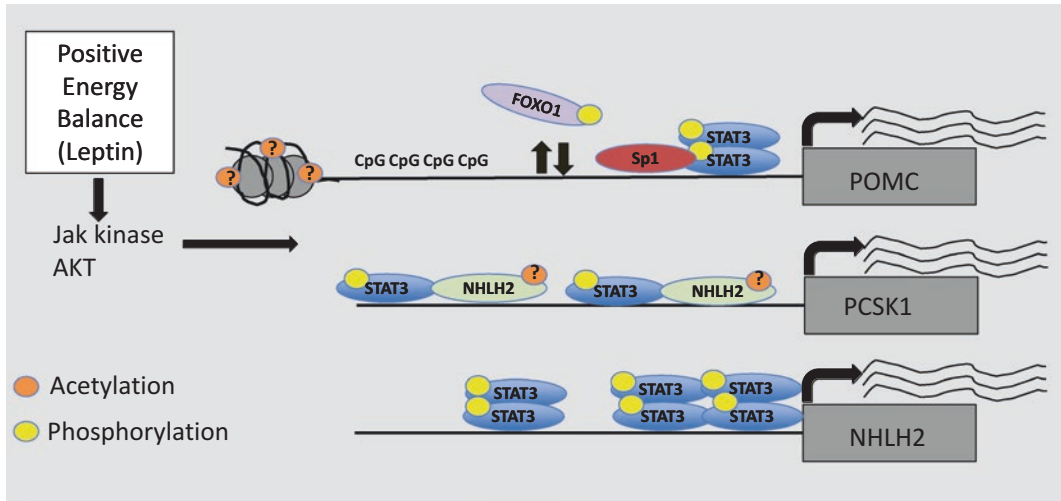
Fig. 3.3 Leptin-mediated induction of gene expression in POMC neurons. Three cartoons of gene promoters are shown with the possible set of transcription factors that regulate these genes in response to positive energy bal-ance, in particular leptin. The posttranscriptional modifi-cation status of the transcription factors is shown or hypothesized based on references within the text

(at the time, there we no good hypothalamic lines), and a 30 bp region of the POMC promoter between −91 and − 61 from the start of transcrip-tion was shown to be sufficient for leptin-mediated expression. However, while the study showed that Stat3 was activated (phosphorylated and translocated to the nucleus), Munzberg and colleagues were not able to detect Stat3 binding to that 30 bp element using electrophoretic mobility (gel shift) assays (Munzberg et al. 2003). The researchers speculated that this might be because Stat3 was interacting with another protein/transcription factor. Only later did another group show that this was indeed the case. Yang and colleagues narrowed down the region needed for Stat3 binding to a slightly larger 50 bp fragment located between −138 and − 88 of the POMC promoter. This region contained a speci-ficity protein 1 (Sp1) transcription factor binding site, but not a Stat3 binding site. However, immu-noprecipitation of Sp1 in leptin-activated cells could bring down phosphorylate Stat3 protein, indicating that Stat3 and Sp1 interacted (Yang et al. 2009). In the Munzberg paper, the original 30 bp element which was located just upstream of the Sp1 site did contain a putative Stat site (Munzberg et al. 2003), and it is likely (although not yet directly shown) that these two elements act together to mediate leptin responsiveness of the POMC promoter (depicted on Fig. 3.3). Interestingly, the Yang et al. article also showed that the transcription factor FoxO1 was involved in POMC regulation as well, namely, that FoxO1 appeared to sequester Stat3 from interacting with Sp1, and subsequent studies revealed that the FoxO1:Stat3 interaction domain was separate from the FoxO1 DNA binding domain and that the POMC promoter also had a FoxO1 binding element in its proximal promoter region (Ma et al. 2015).

FoxO1, which is found in the FANTOM list of hypothalamic transcription factors (Consortium et al. 2014), is a member of the forkhead tran-scription factor family and worth mentioning in the context of hypothalamic gene regulation. FoxO1 is a.

target of the AKT kinase, which itself is regu-lated by phosphoinositol-3-phosphate (PIP_3) lev-els. PIP_3 levels are elevated by LepR (both long and short form) signaling. Overall then, activa-tion of the leptin receptor through leptin results in phosphorylated FoxO1 protein, and this form of FoxO1 is not able to interact with Stat3 (Kwon et al. 2016) (Fig. 3.3).

Moving to the POMC enhancer regions discussed previously, these regions appear to specify hypothalamic tissue-specific transcription through homeodomain transcription factor binding motifs, and PE1 is needed for adult body weight regulation but only due to the fact that it allows a high level of hypothalamic POMC expression to occur (Lam et al. 2015). To date, no one has shown that the homeobox transcription factors that may bind in this region are leptin regulated. However, a region more proximal (at approximately −330 bp) appears to mediate POMC expression via differential DNA methylation and appears to be induced by excessive energy availability (Zheng et al. 2015). Additionally, changes in the levels of histone acetylases occur with fasting or high-fat feeding (Funato et al. 2011), suggesting that a histone-level gene regulation is occurring—but to date, this activity has not been specifically localized to the POMC promoter.

Slightly after leptin-mediated POMC gene regulation began to be characterized, regulation of the PCSK1 gene was characterized. The protein product of PCSK1 is prohormone convertase 1/3—the main neuropeptide processing enzyme for POMC. Thus, activation of PCSK1 gene by leptin makes sense, as the arcuate neurons need to not only increase POMC mRNA but make sure that the neuropeptide alpha-melanocyte-stimulating hormone is produced from the translated mRNA. In a similar fashion to POMC, Stat3 was shown to mediate PCSK1 (and the related gene PCSK2) expression following leptin stimulation (Sanchez et al. 2004). Only one promoter region containing a 971 bp fragment was used in the initial promoter analysis for this study, and although it contained Stat3 sites, there was no further specific characterization of the PCSK1 promoter at that time. However, during the same year, my laboratory showed that mice lacking the hypothalamic transcription factor Nhlh2 had low levels of PCSK1 mRNA, accompanied by low POMC peptide levels, with normal POMC RNA levels (Jing et al. 2004). This suggested that Nhlh2 might be a transcriptional regulator of the PCSK1 gene, which our laboratory was able to confirm in 2008 (Fox and Good 2008). As a

member of the basic helix-loop-helix (bHLH) transcription factor family, Nhlh2 can homodimerize (Fox and Good 2008) as well as form heterodimers with transcription factors. For PCSK1 leptin-mediated regulation, Nhlh2 appears to heterodimerize with Stat3 at two regions of the PCSK1 promoter, which contain adjacent E-box (bHLH binding) and Stat binding motifs (Fig. 3.3) (Fox and Good 2008). In a heterologous cell culture experiment, both Stat3 and Nhlh2 needed to be added to the cells for leptin-stimulated expression of PCSK1 to occur. In addition, in the absence of Stat3, Nhlh2 appeared to homodimerize, but addition of Stat3 led to heterodimerization of Stat3 and Nhlh2 in an in vitro pull-down assay (Fox and Good 2008). While Stat3 also homodimerizes, it is not clear at this time whether the heterodimer complex between a single Stat3 and a single Nhlh2 is sufficient or whether other factors, including other Stat3 molecules, are involved.

The NHLH2 gene, which is expressed within POMC neurons and throughout many other nuclei of the hypothalamus, is also induced by leptin (Vella et al. 2007), and this again makes sense if Nhlh2 is required for PCSK1 leptin-induced expression. The protein also appears to be regulated posttranscriptionally by the SIRT1 deacetylase (Libert et al. 2011). Indeed, the human and mouse Nhlh2 proteins contain seven lysine amino acids which could be the target of acetylases. Lysine #49 (K49) on Nhlh2 appears to be the deacetylation target of Sirt1, and deacetylation leads to loss of Nhlh2 promoter binding (Libert et al. 2011). While the studies by Libert and colleagues were on a negatively regulated Nhlh2 target which can only be expressed once, Nhlh2 is removed from the promoter; whether or not Nhlh2 is acetylated while binding to the PCSK1 promoter (Fig. 3.3) and whether or not acetylation of Nhlh2 is required for Stat3 interaction are still under investigation.

As mentioned above, the NHLH2 gene is also a known leptin-stimulated gene target in the hypothalamus. In order to further characterize elements in the NHLH2 promoter which might facilitate leptin-mediated regulation, we scanned the proximal 950 bps of the mouse and human

NHLH2 promoter and found multiple putative Stat3 binding sites (Al Rayyan et al. 2014). Two nuclear factor, NFκB sites were also identified, but do not appear to mediate leptin-stimulated gene expression—in fact, mutagenesis of the most distal NFκB resulted in even better leptin-mediated expression, suggesting that these sites could be negative regulators. Three of the five putative Stat3 sites were all required for leptin-induced expression of an NHLH2 heterologous promoter (Al Rayyan et al. 2014) (Fig. 3.3). In this set of experiments, the N29/2 cell line from the Belsham group was used, as this cell line best replicated a POMC neuronal cell line (Belsham et al. 2004). In this study we were also able to confirm that Stat3 interacted on the endogenous Nhlh2 promoter, by performing chromatin immunoprecipitation (ChIP) on hypothalamic nuclear extracts following leptin stimulation of mice (Al Rayyan et al. 2014). Quantification of the ChIP analysis by QPCR revealed that leptin treatment leads to a ~threefold increase in Stat3 occupation on the NHLH2 promoter (Al Rayyan et al. 2014). It is not clear if Sp1:Stat3 interactions, as seen on the POMC promoter, might also be involved in Nhlh2 regulation, as several putative Sp1 sites exist on the Nhlh2 promoter but have not been further investigated at this time.

For all of the genes discussed in this section, there are likely other transcription factors and DNA methylation/acetylation dynamics at play in leptin-stimulated expression. For now, the simplified version how the POMC, PSCK1, and NHLH2 promoters all are stimulated by leptin signals will have to suffice until more work is done to reveal any additional mechanisms.

3.1.7 Responses to Fasting and Reduced Energy Availability

According to studies completed by our laboratory (Jiang et al. 2015), serum leptin levels fall approximately 50% and glucose levels about 60% following a 24 h food deprivation, relative to ad-lib fed mice. In this whole hypothalamic analysis of total RNA, approximately equal num-bers of mRNAs were upregulated or downregulated by fasting (Jiang et al. 2015). Interestingly, although 16 microRNAs were upregulated and 6 microRNAs were downregulated with the microarray data, of 6 tested further, none of these could be confirmed via QPCR. Overall, we were able to detect 536 microRNA species within the hypothalamus, which is more than the 105 out of 717 reported for mouse midbrain by http://www.microrna.org/microrna/home.do. Long noncoding RNA (lncRNA) appears to make up more of the noncoding RNA species and has more of those that are differentially regulated. In this same study, and a subsequent one, we were able to identify 421 lncRNA species that were upregulated in response to fasting, and 201 lncRNA species that were downregulated in response to fasting, and were able to detect over 12,000 lncRNA species that were expressed in hypothalamic tissues (Jiang et al. 2015). Two of three lncRNA tested by QPCR were confirmed to be differentially regulated in response to fasting. In addition, using data from an older generation microarray, we were also able to identify 60 lncRNAs that were differentially expressed with fasting (Jiang and Good 2016). However, our knowledge of the role of lncRNAs in the hypothalamus and especially in response to energy balance is still evolving. We don't yet understand how many of these lncRNAs function, nor how they are differentially expressed (Jiang and Good 2016). However, it is important to mention that within the context of differential gene expression, one should not forget the noncoding RNAs.

So what about the "more common" mRNAs—as noted from our study—there were significant changes in the mRNA transcriptome following fasting (Jiang et al. 2015). Of these, gene ontology analysis revealed a number of genes in the cell cycle category were changed—many of them surprisingly upregulated in response to fasting. One of these was Cdkn1a, which codes for the p21 protein (Jiang et al. 2015). Of note, Cdkn1a had already been shown to be upregulated in specific hypothalamic nuclei with fasting, and one study showed that the FoxO1 transcription factor played a role in its upregulation (Tinkum et al. 2013), although the transcriptional analyses were

only done with the liver where p21 protein is also increased with fasting. However, as noted by these authors, Cdkn1a has FoxO1 binding sites in its proximal promoter, so it is likely that FoxO1, which is expressed in hypothalamus, mediates this gene expression upregulation. As noted previously, POMC promoter also has a FoxO1 binding element in its proximal promoter region (Ma et al. 2015), and overexpression of FoxO1 in POMC neurons reduces POMC expression and overall POMC neuron numbers (Plum et al. 2012). So, FoxO1, acting on different neurons, perhaps in different conditions, can either up- or downregulate target gene expression.

In considering gene regulation during reduced energy availability, it is important to look at how orexigenic genes, such as neuropeptide Y (NPY) and agouti-related protein (AgRP), are induced. The human and mouse promoter regions for AgRP were characterized in 2001 (Mayfield et al. 2001) and found to contain at least on Stat site, a CCAAT box, which can bind to factors of the CREB family of transcription factors, and an E-box motif (for bHLH transcription factors) which was also the site of a human DNA variant that reduced gene expression (Mayfield et al. 2001). In this study, Mayfield and colleagues were able to show, using GT1–7 hypothalamic cells, a significant reduction in promoter activity with the variant and an association of the variant genotype TT with obesity (Mayfield et al. 2001). Subsequent studies from this same laboratory did not follow up on the BHLH transcription factor in AgRP regulation, but rather showed that a Krüpple-like transcription factor, Klf4, directed expression of AgRP (Ilnytska et al. 2011). In these studies, the Belsham hypothalamic cell line N38 (Belsham et al. 2004) was used to show induction of both Klf4 and AgRP with fasting as well as direct binding of Klf4 to the AgRP promoter under these conditions (Ilnytska et al. 2011). AgRP is also regulated by FoxO1, which appears to be assisted by the activating transcription factor ATF3 (Lee et al. 2013) and antagonized by Stat3 (Kitamura et al. 2006). All of this is summarized in cartoon form in Fig. 3.4. Of note, while we know that Stat3 is likely phosphorylated, FoxO1 is normally acetylated when

inactive and deacetylated by Sirt1 when it is in the nucleus and regulating AgRP (Cakir et al. 2009). Phosphorylation of ATF3 and acetylation of KLF4 have been shown in other systems (e.g., Mayer et al. 2008; Meng et al. 2009), but not confirmed in hypothalamic AgRP neurons.

Despite the fact that one of the main human polymorphism in the NPY gene which is linked to metabolic phenotypes resides in the NPY promoter region at −399 (e.g., de Luis et al. 2016), relatively little is known about the transcriptional regulation of NPY in response to fasting. It has been known for more than 25 years that NPY gene expression is induced with fasting (Sanacora et al. 1990), but the mechanisms of this remain elusive. The polymorphism at −399 appears to affect an SP1 site, which is conserved in the bovine NPY gene as well (Alam et al. 2012). According to a very large study of the response to starvation in mice in which a transcriptional network analysis was performed, the "interactome" of transcriptional responses to starvation, SP1 is listed in 4 of the 5 tissues examined, notably absent from brain (Hakvoort et al. 2011). Thus it could be that SP1 did not reach the level of significance in this study but may in fact be a fasting−/starvation-induced transcription factor responsible for NPY expression (Fig. 3.4). The NPY promoter also contains two distal Stat3 sites, but the role of these in leptin-inhibited versus fasting-induced expression is not yet clear. Notably, in a review that this author wrote 17 years ago, there was still little known about NPY promoter regulation, but several studies suggested that its regulation was not transcriptional but posttranscriptional, with increased mRNA stability of the NPY mRNA contributing to its change in expression in response to fasting (Good 2000). With more knowledge about microRNA and lncRNA expression in the hypothalamus, as well as the known function of some noncoding RNA in mRNA stability, more work should be done on NPY mRNA stability versus transcriptional activation in response to fasting.

As mentioned above, several of the transcription factors mediating fasting response in the hypothalamus undergo posttranslational modifications such as phosphorylation and acetylation
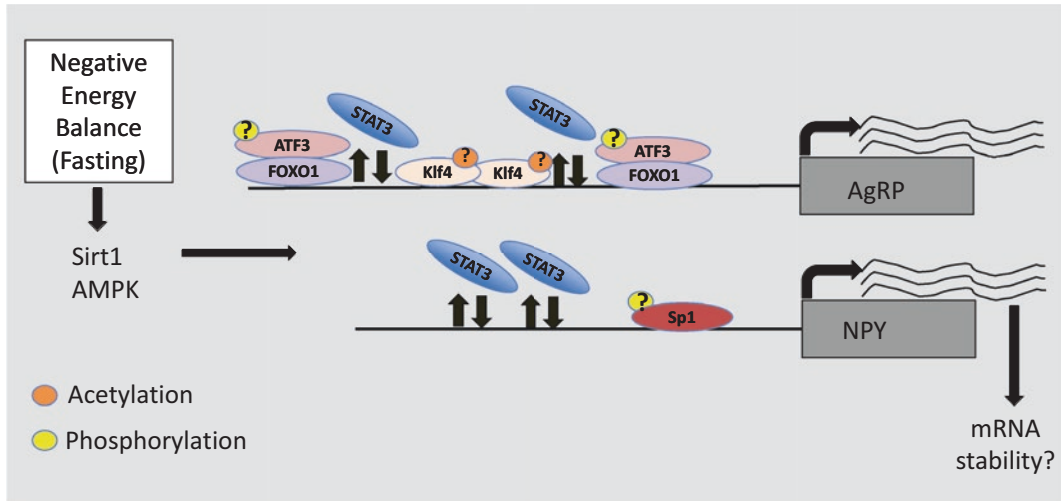
Fig. 3.4 Fasting-induced induction of gene expression in NPY neurons. AgRP and NPY genes, with their putative promoter regions, are shown as both of these genes are induced with fasting. The posttranscriptional modification status of the transcription factors is shown or hypothesized based on references within the text

(and removal of these modification) in order to either move to the nucleus or bind DNA. For example, AMP kinase and Sirt1 deacetylase are both active under conditions of low leptin, such as fasting, and both are responsive to metabolic signals (low ATP/high AMP for AMP kinase and low NADH/high NAD for Sirt1) which would be present during fasting conditions, and both target FoxO1 transcription factor (Minokoshi et al. 2008; Toorie and Nillni 2014). Thus, we are starting to build transcriptional signaling pathways to understand the molecular changes that occur in response to fasting. However, this area likely needs more attention to characterize more gene targets of fasting and how these gene targets are regulated. The roles of lncRNA should also be considered as several lines of evidence suggest that they may play a major role in energy balance control (Jiang and Good 2016).

3.2 Summary and Key Questions that Remain Unanswered

The tools are in place for researchers to build comprehensive knowledge on gene expression mechanisms in response to changes in energy balance, but much more needs to be done. It is clear, for example, that many more genes undergo transcriptional regulation by those transcription factors that we know are induced (FoxO1, Stat3, Nhlh2, etc.), and these genes need to be characterized to fully understand the response to feeding and fasting. Furthermore, there are likely many other transcription factors that have not been fully characterized; yet still many likely play important roles in energy balance regulation. One of the downsides of working with transcription factors is that they generally are not highly expressed and may be difficult to detect or missed with some previous methods used. Antibodies can sometimes detect the protein, but posttranslational modifications also interfere with immunodetection, depending on whether conformational changes occur with the modification. Many transcription factors, like Stat3, are not necessarily transcriptionally regulated but posttranscriptionally regulated through protein modifications, so studying their transcriptional regulation may be important, but may not tell us much about energy balance signaling. However, posttranslational modifications need to be more rigorously investigated for each neuron type within the hypothalamus and under condi-

tions of both fasting and ad-lib feeding to fully understand how the transcription factors mediate differential gene expression.

It is also clear that sexual dimorphism exists in hypothalamic transcriptional and responses (Loganathan and Belsham 2016), and yet few laboratories study this area. One should consider the "sex" or gender of the cell lines and then use specific and perhaps matched cell lines to establish and study sex-specific gene expression differences for many different hypothalamic promoters. In addition, once we begin to understand these mechanisms in cell lines, we need to extend it to humans, and specifically characterize transcription factor binding sites within human promoters, and then utilize online genomic databases to identify polymorphisms that might affect transcriptional regulation, as possible mechanism for genetic mechanisms of body weight control. Genome-wide association studies often identify associated polymorphisms that are not within gene coding regions and, thus, may instead be associated with gene regulatory changes.

No chapter on transcriptional mechanisms of gene regulation would be complete without at least mentioning posttranscriptional mechanisms, which have been done with NPY and mRNA stability, posttranslational modifications, and analysis of noncoding RNA expression with fasting in this chapter. However, many other posttranscriptional mechanisms exist than were discussed above, and given the number of different outcomes for an mRNA once it is transcribed, this topic really deserves a chapter of its own.

We finish his chapter by again contemplating the enormity of having over 2000 different transcription factors to regulate the thousands of genes that are needed for energy balance control by the hypothalamus. While researchers have made some headway into this topic, it is also clear that there is still a lot to be done to understand the complexity of transcription (and posttranscriptional) control of the hypothalamic transcriptome and ultimately its resulting proteome.

Questions to Consider

1. Compare and contrast studying gene expression in hepatocytes to gene expression within the hypothalamic arcuate POMC neurons. What are the challenges and benefits for each tissue type?
2. Take a look at the NCBI gene expression profiles database (https://www-ncbi-nlm-nih-gov.ezproxy.lib.vt.edu/geoprofiles/), and put a search term into the box (such as hypothalamus, a nucleus of the hypothalamus, or a gene name). What kind of data is returned and how might one use this information to develop a research question?
3. Analyze the actual data from one or two of the papers referenced in this chapter in which transcriptional regulation of a hypothalamic gene was characterized. What would be the next step in this type of research? What tools would you need to conduct this type of research?
4. Is it better to study gene expression in cell lines or in animals? Compare and contrast these two models.
5. Can knowledge about gene expression be used in personalized medicine? Are there any known treatments to date that take gene expression patterns into account?

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