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A Molecular Dissection of the Ventromedial Hypothalamic Nucleus

A thesis presented to the faculty of

The Rockefeller University

in partial fulfillment of the requirements for

the degree of Doctor of Philosophy

by

Jeremy P. Segal

The Rockefeller University

New York

June 2006

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Dedication

This thesis is dedicated to my wife, Jennifer. Without her love and support, the lack of a thesis would be the least of my worries.

Acknowledgements

There are many people to acknowledge for their support, guidance and friendship during my graduate studies. First and foremost I would like to thank Jeff Friedman, who has been a dedicated mentor, friend and role model throughout my four years in the laboratory. While Jeff's overwhelming success as a scientist can make walking in his footsteps seem like a daunting task, it is a credit to his encouraging mentorship and his investment in his students that I leave his laboratory convinced that I possess the capabilities to admirably do so.

Over the years, the members of the Friedman lab have provided a great deal of scientific advice, discussions, friendship and fun times. Shirly Pinto, my bay-mate for three years, took me under her wing when I arrived as a somewhat hopeless fledgling scientist. She's been a great friend, and I owe so much for teaching me so many concepts and techniques. The old-school crowd (Alex Soukas, Jason Montez, Paul Cohen, Mak Ishii, Jeff DeFalco, and Mike Eisenstein) was a one-of-a-kind group. In addition to being universally great scientists, they were always up for a good time (often in lab, where JD would routinely kick my butt at Quake). Scott Sternson and Hiro Hiyoshi are both remarkably good scientists. I'll miss their excellent advice, but more than that I'll miss our golf and batting cage outings. Thanks to Susan Korres for doing so many things to keep the lab running smoothly.

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The members of my committee, Drs. Bob Darnell, Nat Heintz, and Tim Ryan, have all provided valuable suggestions and guidance throughout this process, and I am grateful for all their help directing and shaping the work presented in this thesis.

Finally, one last thanks to Jen, Mom, Dad, Jan, Mr. And Mrs. J, and all my other family and friends who have supported and encouraged me throughout this wonderful, extended, academic process.

Table of Contents

Dedication *iii*

Acknowledgements *iv*

Table of Contents *vi*

Directory of Figures *viii*

Directory of Tables *x*

List of Publications *xi*

Abstract 1

Chapter 1: Introduction 4
- Obesity 4
- Hypothalamic Control of Food Intake 10
- The VMH 11
- Leptin 15
- Leptin Receptor 18
- Leptin Signaling 20
- Neuropeptides 24
- Marker Genes and the Leptin Feeding Circuit 33
- Specific Aims 42

Chapter 2: Materials and Methods 44
- Animal Care and Maintenance 44
- Northern Blotting 44
- Southern Blotting 45
- Quantitative Real-Time PCR 45
- Laser-Capture Microdissection 46
- Microarrays 48
- *In situ* Hybridization Histochemistry 50
- FACS-Sorting of SF-1 Neurons 52
- Expression of LBH2-eGFP fusions protein in 293T Cells 52
- Generation of LBH2-eGFP BAC Transgenic Mice 54
- Immunohistochemistry 56
- Pseudorabies Virus Ba2001TK+ 59
- Microscopy 60

Chapter 3: Results 62
Microdissection of Hypothalamic Nuclei 62
Amplification and Microarray Hybridization 64
Patterns of Transcription in Hypothalamic Nuclei 68
VMH Marker Genes 68
Validation by *in situ* Hybridization 73
Distribution of Cbln1 in the Mouse Brain 78
SF-1 Transcriptional Network 78
Analysis of VMH Morphology in Slit3 Knockout Mice 84
LBH2 87
LBH2 Antiserum 91
LBH2-eGFP BAC Transgenic Mice 91
Tracing VMH -> ARC Projections Using Pseudorabies Virus 105

Chapter 4: Discussion 109
VMH Marker Genes 109
Steroidogenic Factor 1 112
LBH2-eGFP Mice: Markers as Tools to Understand the VMH 114
Conditional Pseudorabies Virus Tracing 116

Conclusion 119

References 120

Directory of Figures

Figure 1. Health Risks Associated with Overweight and Obesity 6

Figure 2. Electolytic Lesioning of the VMH causes Hyperphagia and Obesity 12

Figure 3. Mutation of the Obese (ob) Gene Causes Profound Obesity 16

Figure 4. Overview of Intracellular Leptin Receptor Signaling 21

Figure 5. Proposed Wiring Diagram of Hypothalamic Feeding Circuitry 37

Figure 6. Laser-Capture Microdissection of Hypothalamic Nuclei 63

Figure 7. RNA Analysis, Amplification, and Microarray Hybridization 65

Figure 8. Microarray Analysis of RNA from Hypothalamic Nuclei 67

Figure 9. Analysis of Marker Gene Expression in Hypothalamic Nuclei 69

Figure 10. Confirmation of Microarray Results by Real-Time PCR 72

Figure 11. Expression of VMH Marker Genes in Fed and Fasted Mouse Hypothalamus 74

Figure 12. Northern Blot Analysis of LBH2, Cbln1, and PACAP 76

Figure 13. Comparison of PACAP, Cbln1, and LBH2 mRNA Distributions in the VMH 77

Figure 14. Distribution of Cerebellin 1 mRNA in the Mouse Brain 79

Figure 15. Disordered VMH Neurons in the SF-1 Knockout Mouse 81

Figure 16. Expression of VMH Marker Genes in SF-1 Knockout Mice 82

Figure 17. Cerebellin 1 Expression in WT and SF-1 KO Hypothalamus 83

Figure 18. Distribution of VMH Neurons in Slit3 Knockout Mice 85

Figure 19. Hypothalamic Estrogen Receptor-alpha Distribution in WT and Slit3 Knockout Mice 86

Figure 20. LBH2 Family Protein Sequence Comparisons 88

Figure 21. Expression of LBH2-eGFP Fusion Protein in Cell-Culture 89

Figure 22. Distribution of LBH2 mRNA in the Mouse Brain 90

Figure 23. Cytoplasmic Localization of LBH2 Protein in VMH Neurons 92

Figure 24. LBH2-eGFP BAC Modification 93

Figure 25. Hypothalamic eGFP Expression in LBH2-eGFP Transgenic Mice 95

Figure 26. Natural eGFP Fluorescence in LBH2-eGFP Mice 96

Figure 27. Validation of LBH2-eGFP BAC Transgenic Mice 97

Figure 28. Co-Visualization of LBH2-eGFP and NeuN in the VMH 100

Figure 29. Co-Visualization of LBH2-eGFP and SF-1 in the VMH 101

Figure 30. Co-Visualized VMH Distributions of LBH2 and Estrogen Receptor-alpha 103

Figure 31. Leptin Responsiveness of VMH LBH2 Neurons 104

Figure 32. Ba2001TK+ Infection of AgRP-Cre and WT Mice 107

Directory of Tables

Table 1. Sequences of Taqman Real-Time PCR Probe/Primer Sets 47

Table 2. Top VMH-Enriched Genes 69

List of Publications

Segal JP, Stallings NR, Lee CE, Zhao L, Socci N, Viale A, Harris TM, Soares MB, Childs G, Elmquist JK, Parker KL, Friedman JM. Use of laser-capture microdissection for the identification of marker genes for the ventromedial hypothalamic nucleus. J Neurosci. 2005 Apr 20;25(16):4181-8.

Segal JP, Pinto S, Stanley S, Cai X, Viale A, Perez C, DeFalco, J, Heisler LK, Friedman JM. Analysis of the central neuronal circuitry underlying efferent outputs to the liver and white adipose tissue. (In Preparation).

Abstract

The ventromedial hypothalamic nucleus (VMH) plays an important role in the regulation of food intake, glucose metabolism, and body weight. However, in contrast to other hypothalamic nuclei that are also known to regulate energy homeostasis, there is a paucity of nucleus-specific marker genes for the VMH that can be used to label its constituent neurons. This represents a significant impediment to the application of molecular approaches for analyzing VMH circuitry and function.

Thus, we conducted a microarray screen in order to identify VMH-specific genes that could be used to label populations of VMH neurons. Laser-capture microdissection was used to isolate RNA from the VMH and from two adjacent hypothalamic nuclei known to play a role in energy balance, the arcuate (ARC) and dorsomedial hypothalamic nucleus (DMH). Amplified RNA from these three nuclei were intercompared to identify genes with VMH-enriched expression. The top 12 VMH marker gene candidates were screened by real-time PCR, and three genes (Cerebellin 1, PACAP, and a novel gene we characterized, LBH2) were examined by in situ hybridization for further validation and examination of their subnuclear expression profile.

One of the VMH markers, steroidogenic factor 1 (SF-1), is an orphan nuclear receptor with few known target genes. As this transcription factor is responsible for proper developmental formation of the VMH and also for normal energy homeostasis, we endeavored to determine whether any of the VMH marker genes may be regulated by SF-1. The expression of 4 markers was significantly altered in VMH neurons of SF-1

knockout mice, and this result was confirmed by an in situ hybridization study of cerebellin 1 expression in brain-specific SF-1 knockout mice.

One of the VMH markers was a previous undescribed gene that we further characterized and named LBH2 owing to its similarity to a presumed transcription factor called limb bud and heart (LBH). To further examine the expression of this gene in the VMH, and to begin to describe VMH neuronal populations, we created LBH2-GFP BAC transgenic mouse line expressing GFP under the control of the LBH2 promoter. These mice were validated for correct expression of the transgene and examined for overlap with other populations of interest in the VMH, including estrogen receptor-alpha neurons and leptin-responsive neurons.

Finally, we used a TK+ strain of PRV Bartha2001 to trace neuronal inputs from AgRP neurons in the ARC using AgRP-cre mice. Using this technique, it could be determined that the dorsomedial and intermediate VMH sends significant outputs to this population of ARC neurons. This technique, together with the marker genes discovered, may now be used to identify and catalog individual VMH neuronal subsets that project to these neurons.

In conclusion, we have discovered a set of marker genes for the VMH using laser-capture microdissection coupled with cDNA microarray analysis. This combination of techniques represents a powerful approach for the identification of genes enriched in specific, anatomically-defined brain regions. The discovery of multiple genes regulated by SF-1 also suggests this technique may be useful for identifying nucleus-specific transcriptional networks. The VMH-enriched genes identified here, in conjunction with LBH2-GFP mice and other transgenic animals, will provide a basis for a full

characterization of VMH neurons, and will prove greatly useful for future neuroanatomic and transgenic-based studies of this important nucleus.

Chapter 1: Introduction

Obesity

Obesity is fast becoming an urgent worldwide health problem. No longer just restricted to the United States and the rest of the developed world, obesity is now commonplace among the world's developing countries, often co-existing side-by-side with undernutrition (WHO, 1997; Caballero, 2001).The scope and pace of the global obesity epidemic, as well as its serious health and financial consequences, highlight the demand for rapid, effective research and action.

According to the most recent study by the National Center for Health Statistics (1999-2002), 30% of adults in the United States are obese, with an additional 35% overweight (Flegal et al., 2002). This represents a substantial increase over the previous survey conducted between 1988-1994, when 23% and 33% of adults were found to be obese and overweight, respectively (Kuczmarski et al., 1994). While the United States is the most overweight large industrialized country, the US numbers are being mirrored by developed countries worldwide (Saitoh et al., 1999; Seidell, 2002). Recently, developing countries have begun to be affected as well, and in many the incidence of overweight and obesity is higher than that of undernutrition (Monteiro et al., 2004). For example, the incidence of overweight exceeds 30% in Peru, Tunisia, Brazil, Colombia, and Costa Rica, according to a 1997 World Health Organization survey (WHO, 1997). Additionally, more than 60% of the population of urban Samoa fits the definition of obesity (Hodge et al., 1994). In total, it is estimated that there were 300 million obese people worldwide in the year 2000, and this number continues to rise (WHO, 2000).

Perhaps most disturbing of all are the statistics regarding young people. According to the NHANES study, 16% of US youths 6-19 were found to be overweight, a sharp increase from 11% in 1994 and 6% in 1980 (Ogden et al., 2002; Hedley et al., 2004). This is of particular concern, as childhood adiposity is a strong predictor for adult adiposity and its concomitant risks (Steinberger et al., 2001). These trends are now being observed worldwide (Dehghan et al., 2005).

Obesity is a complex disease with a multitude of deleterious consequences. Obese individuals are significantly at risk for a number of series diseases and afflictions, including but not limited to type 2 diabetes mellitus, hypertension, coronary heart disease, cancer, osteoarthritis, cholelithiasis, and sleep apnea (Fig. 1) (Kopelman, 1994, 2000). Additionally, overweight and obese individuals may suffer from arthritis, aches and pains, sleep disturbance, dyspnea, and social stigmatization, all potentially leading to depression and poor quality of life (Myers and Rosen, 1999; Lawrence and Kopelman, 2004). There are a number of different ways to quantify weight status and obesity. However, the most commonly used is the body mass index (BMI) scale, whereby BMI equals the weight in kilograms divided by the square of the height in meters. BMI of 18.5 – 24.9 is considered normal, whereas a BMI of 25.0 – 29.9 is considered overweight and BMI of 30 or more is considered obese (WHO, 1995; Willett et al., 1999). In the Framingham Heart study of ~115,000 women, obesity (BMI>30) was specifically linked to overall mortality and mortality from cardiovascular disease (Hubert et al., 1983). In a separate study of men, weight elevated 20% above normal was shown to be associated with elevated mortality (Garrison et al., 1983). Mortality alone does not adequately represent the full scale of the problem, because a number of the sequelae experienced by

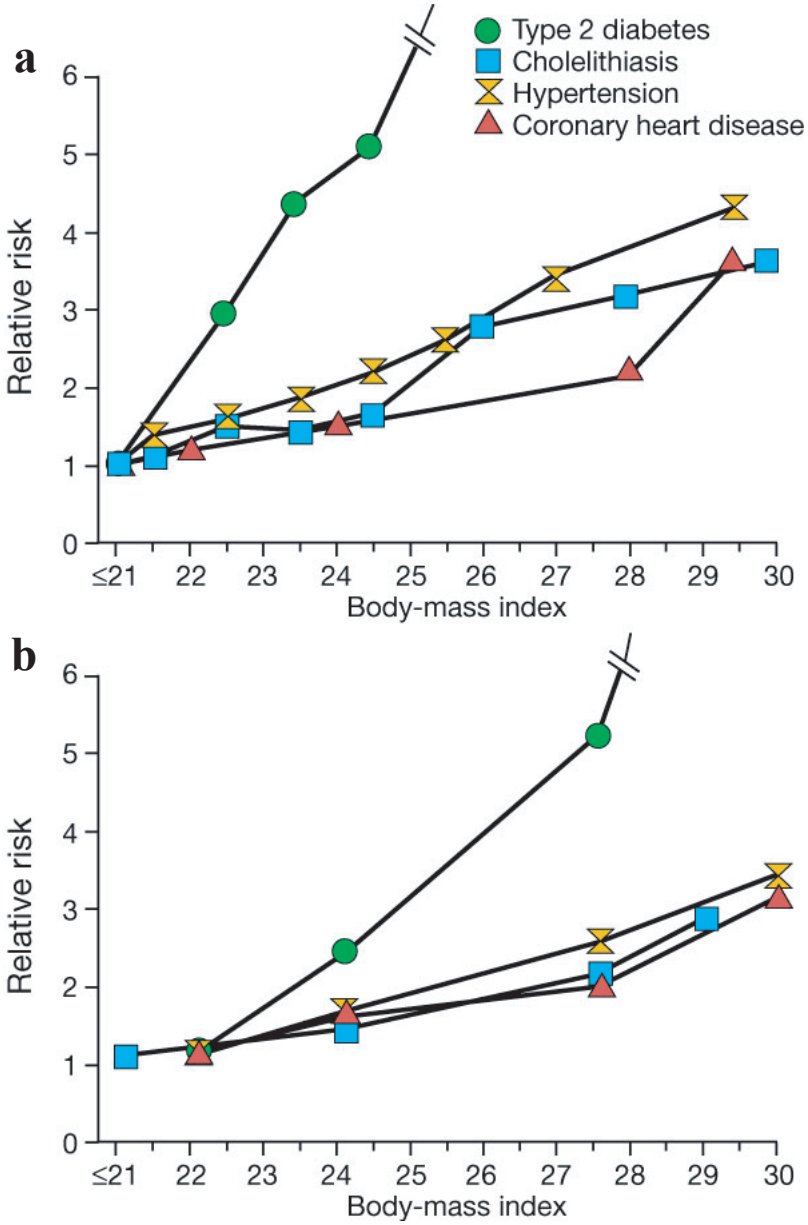


Figure 1 *Health Risks Associated with Overweight and Obesity*
Relationships between BMI and relative risk of type 2 diabetes, cholelithiasis, hypertension, and coronary heart disease. (a) Women, age 30 to 55, followed for 18 years. (b) Men, initially 40 to 65 years old, followed for 10 years. Adapted from Willett et al. 1999.

obese patients are held at bay by expensive medical treatments. Recently, the costs attributable to obesity were estimated to have eclipsed 78.5 billion dollars annually, representing 9.1% of all US medical expenditures (Finkelstein et al., 2003).

The historical perspective on obesity is that it is the result of laziness and a lack of willpower. This notion is one of the main sources of the stigma experienced by overweight people (Myers and Rosen, 1999). However, the bulk of the evidence does not support this theory, instead pointing unmistakably to the conclusion that human body weight is under strict genetic control (Friedman, 2000). It has long been observed that obesity tends to cluster within families, but such findings are open to criticism along the age-old lines of genes vs. environment. Studies of twins have yielded much stronger, irrefutable data. In one study, the concordance rates for height, weight, and BMI were found to be twice as high in monozygotic twin pairs as in dizygotic twins, highlighting the importance of identical vs. similar genetic material within the same environmental context (Stunkard et al., 1986). Additionally, a study of twins separated at birth and raised apart found that BMI was correlated with a coefficient of 0.70 for men and 0.66 for women (Stunkard et al., 1990). This is a higher level of correlation than that observed for other conditions, such as schizophrenia, commonly regarded as being under genetic control (Allison et al., 1996). Additionally, because the twin pairs showed such highly correlated weights despite growing up in different environments, these results suggest that environment may not be the most important determinant of body weight.

This evidence, taken together, is the basis for what is termed the "set point" theory of body weight, whereby each person has a particular set-point weight that is strictly adhered to by the body without, and often despite, conscious control (Schwartz

and Seeley, 1997). This idea is reinforced by the failure of diet schemes to effect significant weight loss over long periods of time. All schemes tested to date have resulted in compensatory weight gain following an initial period of weight loss (Wadden, 1993).

So, if body weight is under strict genetic control, why the recent surge in the incidence of obesity? Clearly, population genetics cannot vary on such a short time scale as one or two generations, and there must be some input from the environment.

Humans living in the developed post-industrial world find themselves in a situation for which their evolutionary history has left them wholly unprepared. Since the dawn of evolution, organisms have struggled with the pressure to reproduce, evade predators, and consume and store adequate nutrients. Those organisms that could best complete these tasks went on to dominate over their weaker competitors. However, modern affluent humans have largely escaped from each of these selective pressures. In particular, ever-improving food production and storage technology have made food readily available to nearly all of our citizens, regardless of economic status.

However, this dramatic success has had far-reaching consequences for our culture. For example, an entire scientific, economically-driven industry has emerged whose sole purpose is to best cater to the taste-buds and sensibilities of consumers. Food manufacturers and restaurants compete with each other to create the best food and flavor combinations, and the successful competitors survive and grow while the rest perish. Today, it is the food that competes for the mouths, rather than the other way around. The result of this is a steady increase in the proportion of fat in the western diet, a factor

strongly linked with weight gain in multiple studies (Sonne-Holm and Sorensen, 1977; Curb and Marcus, 1991; Bray and Popkin, 1998).

Taken together, it may be hypothesized that genetic factors set a strict body weight within a particular environmental context. Stated another way, genetic make-up determines whether an individual will succumb to obesity within a certain environment. Clearly, if an individual were to grow up in an environment where food was not plentiful, obesity would not be a likely outcome regardless of genetics. But, if a thin person were to move to a country with different food options, availability and culture, the susceptibility might reveal itself. This hypothesis is born out by studies of immigrants. In one 2004 study, immigrants to the United States were found to have a far lower rate of obesity than US-born individuals (8% vs. 22%, respectively) (Goel et al., 2004). However, among immigrants who had lived in the United States for more than 15 years, the rate of obesity rose to 19% (Goel et al., 2004). Another study showed that second and third-generation Asian-American and Hispanic children born in the US were more than twice as likely to be obese as first generation residents (Popkin and Udry, 1998). Interestingly, people descended from predominantly hunter-gatherer cultures seem to be much more susceptible to developing obesity when introduced to a high-fat western diet than people of agricultural European descent (Friedman, 2003). For example, more than 60% of the Pima Native American tribe living in the Gila River valley of Arizona are clinically obese, with BMI > 30 (Knowler et al., 1991). Many Pacific island nations, such as Samoa, have also been severely affected as a result of the introduction of Western diet following WWII. The common explanation for this phenomenon is that hunter-gatherers were evolutionarily forced to place a higher premium on energy storage,

because they faced more routine periods of food deprivation (Neel et al., 1998; Neel, 1999).

Most people would assume that their food intake is voluntarily controlled, and that they decide when they eat, what they eat, and how much. But, people's weights do not fluctuate over the long term, except to perhaps slowly drift upwards, indicating precise control of caloric intake to within 99.5% (Friedman, 2003). This would tend to deny the possibility of long-term conscious control of food intake, and necessitate that each person's body performs a constant accounting of energy stores and requirements, dictating exactly how much is eaten over the long-term. If that is the case, how does this system work?

Hypothalamic Control of Food Intake

Researchers have long been interested in feeding motivation. Originally, it was not known whether the sensation of hunger was a peripheral or central phenomenon. The first evidence for CNS control of food intake came from the observation that a significant proportion of patients with pituitary tumors displayed uncharacteristic hyperphagia and obesity (Frohlich, 1901). But, while these studies demonstrated that the brain played a major role in forming and responding to hunger, it was unclear whether damage to the pituitary itself, or to the overlying hypothalamus, was responsible for the hyperphagia.

Conclusive evidence for hypothalamic regulation of food intake and body weight came decades later, in the form of classic lesioning studies first performed on rats by Hetherington and Ranson (Hetherington and Ranson, 1940). Electrolytic lesions to the ventromedial hypothalamic nucleus (VMH) produced hyperphagia and massive obesity

(Fig. 2), while lesions to the lateral hypothalamic area (LH) produced aphagia and weight loss (Hetherington and Ranson, 1940; Anand and Brobeck, 1951). These studies formed the basis for the dual-center model, whereby the VMH acts as the satiety center and the LH acts as the feeding center. According to this model, the interplay between the two regions would then be responsible for determining minute to minute feeding decisions (Elmquist et al., 1999).

Today, the hypothalamus is recognized as the great homeostatic regulator of the body (Kandel and Schwartz, 1991). The hypothalamus receives information related to nearly all homeostatically regulated parameters, such as salt and water levels, blood pressure, blood glucose, hormone concentrations, and nutrients, and makes compensatory changes designed to keep them within desirable limits. At the microscopic scale, the hypothalamus is composed of a number of discrete, densely-packed populations of neurons called nuclei, which are developmentally regulated to have consistent size, shape, composition, localization and gene-expression. The hypothalamus exerts its homeostatic influence either by signaling via axons to distal brain sites, including autonomic nervous system centers, and by directing hormone release from the pituitary, to which it is physically connected via the infundibulum.

The VMH

Although the lesioning experiments did conclusively demonstrate a central role for the hypothalamus in energy homeostasis, the dual-center model was much criticized. Many argued that the lesions produced by Hetherington and Ranson were large enough to damage a number of other proximal nuclei (such as the adjacent arcuate nucleus (ARC)),

Figure 2 *Electolytic Lesioning of the VMH causes Hyperphagia and Obesity*
(Right) Rat with bilateral VMH lesions produced using a Horsley-Clark stereotaxic apparatus develops hyperphagia and massive obesity. (Left) Control rat. Adapted from Hetherington and Ranson, 1940.

or possibly white matter tracts passing through the region (Gold, 1973). Further research, though, would more concretely verify the role of the VMH in feeding and body weight regulation, and provide evidence for the involvement of the VMH in a number of metabolically related functions.

To overcome the criticisms of the electrolytic lesioning work, investigators took advantage of chemicals to produce more specific lesions. Lesions induced by ibotenic acid produce the same phenotypes as electrolytic lesions, but with much less non-specific damage (Grossman et al., 1978; Shimizu et al., 1987). Also, gold-thiogluocose, when administered intraperitoneally to mice and rats, produces hyperphagia and massive obesity secondary to lesions of the VMH (Marshall et al., 1955; Marshall and Mayer, 1956).

Perhaps the most specific VMH lesion is that which fortuitously resulted from targeted deletion of the gene Steroidogenic factor-1 (SF-1). SF-1 is an orphan nuclear receptor, originally identified as a binding factor to a 5' flanking sequence common to a number of the cytochrome P450 steroid hydroxylases (Lala et al., 1992; Parker et al., 2002). SF-1 knockout mice suffer from adrenal and gonadal agenesis and gonadotrope disfunction, leading to death during the first week of birth from adrenal insufficiency (Ingraham et al., 1994; Luo et al., 1994). Rescue of these mice with exogenous corticosteroids and adrenal transplants resulted in a maturity onset obesity phenotype characterized by normophagia in the context of significantly reduced energy expenditure (Majdic et al., 2002). Examination of the hypothalamus of these animals reveals a significantly disordered VMH (Ikeda et al., 1995). Initially termed "VMH agenesis", in reality these mice have VMH neurons, as monitored by lineage tracing using an SF-1-

EGFP transgene, but these neurons fail to coalesce into their proper location and architecture during development, instead remaining spread throughout the hypothalamus (Davis et al., 2004). These neurons also fail to terminally mature in the absence of SF-1 (Tran et al., 2003).

A number of studies have implicated the VMH as a central player in glucose homeostasis. A glycemic-clamp study showed that ibotenic acid-induced lesions to the VMH, but not the LH, specifically interfered with an important physiological counterregulatory response to hyperglycemia, characterized by increased release of glucagon from the pancreas and catecholamines from the adrenal gland (Borg et al., 1994). Subsequently, infusion of a glucose analog, 2-deoxyglucose, into the VMH of rats via cannula was found to be sufficient to cause counterregulatory hormone release in the context of systemic normoglycemia (Borg et al., 1999). Local perfusion of glucose into the VMH was also capable of blocking the response in hypoglycemic rats (Borg et al., 1997). The ability of the VMH to sense and respond to systemic glucose levels is attributable to its large population of glucose sensing neurons. By some estimates, as many as 60% of VMH neurons are either activated by or inhibited by increased glucose concentrations (Kang et al., 2004).

A number of molecules are implicated in glucosensing in the VMH. In an analogous system to pancreatic beta cells blood glucose monitoring, involvement of the low-affinity glucose transporter GLUT2 and the glucose-phosphorylating enzyme glucokinase have been suggested. Consistent with this possibility, the expression of both GLUT2 and a glucokinase isoform have been reported in the VMH (Leloup et al., 1994; Lynch et al., 2000). Glucokinase has been shown to be a very good predictive marker for

glucosensing ability by VMH neurons (Kang et al., 2004). ATP-sensitive potassium channels have also been implicated in glucose sensing, as they offer a means to couple membrane potential to internal cell metabolism (Ashford et al., 1990a, b). Targeted deletion of the K_{ATP} channel Kir6.2 resulted in normoglycemic mice that displayed severely blunted glucagon release following hypoglycemia imposed by insulin administration (Miki et al., 2001). By electrophysiological analysis, VMH neurons from these mice were not responsive to glucose.

These studies all clearly showed a significant role for the VMH, and by extension the hypothalamus, in the regulation of food intake, body weight, and metabolism. However, fundamental questions about the hypothalamic control of food intake remained unanswered. Specifically, it remained entirely unclear how the hypothalamus accounted for peripheral energy stores, and how this information was incorporated into feeding decisions. This type of accounting would have to be a critical component of any model attempting to explain the exquisitely tight regulation of body weight observed in both animals and humans.

Leptin

In the summer of 1949, a spontaneous mutation was observed in the V mouse stock at Jackson Laboratories. Mice homozygous for this recessive mutation, *obese* (*ob*) were more than three times the weight of their unaffected littermates at 10 months of age, the result of massive hyperphagia and reduced energy expenditure (Fig. 3) (Ingalls et al., 1950). Additionally, these mice displayed sterility, insulin resistance, and significant disturbances in hormonal axes and thermoregulation (Batt and Mialhe, 1966). In 1966,

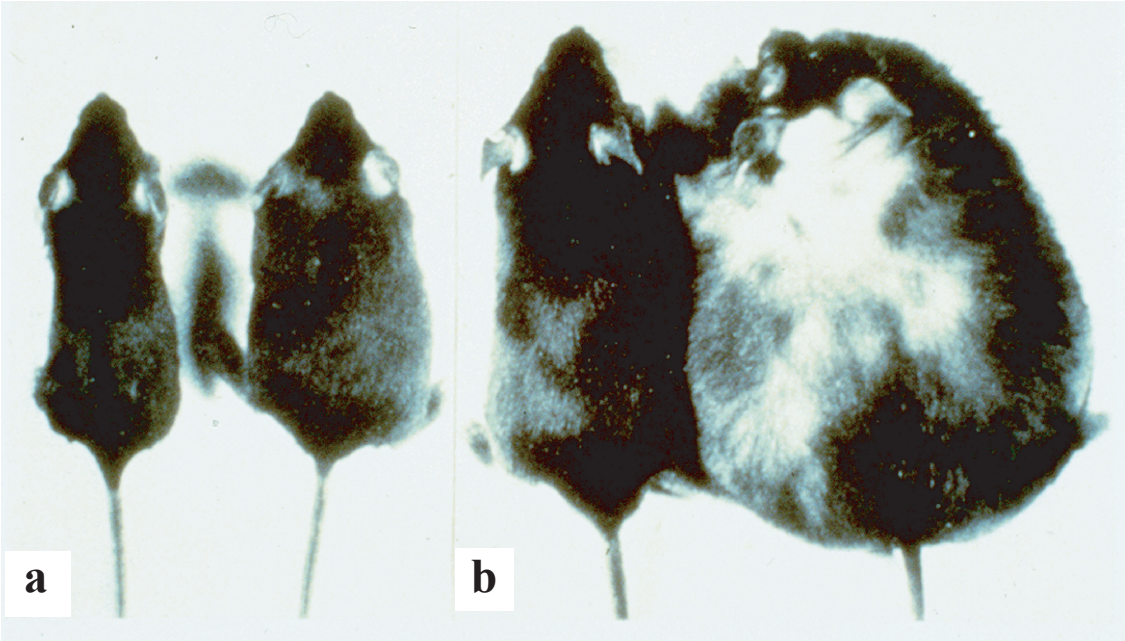


Figure 3 *Mutation of the Obese (ob) Gene Causes Profound Obesity*
(a) Normal control (12g) and *ob/ob* mouse (16g) at 3 weeks of age. (b) Normal control (29g) and *ob/ob* mouse (90g) at 10 months of age. Adapted from Ingalls et al. 1950.

another spontaneously obese strain arose, displaying a similar adiposity phenotype to the *ob/ob* mouse. However, this mouse was more hyperglycemic and insulin resistant, and was thus given the name *diabetes* (*db*) (Hummel et al., 1966).

Because of the similarity of the two phenotypes, it was thought that the two mutations might play a role in the same pathway, and indeed the differences between the two phenotypes were shown to be largely the result of background strain effects (Coleman and Hummel, 1973). This supposition was greatly supported and expanded upon by classic parabiosis studies performed by Coleman. In these experiments, mice were literally stitched together to promote the formation of a shared circulatory system. When *ob/ob* mice were parabiosed to wild-type control mice, they reduced their food intake to that of their parabiosed partners. The same was not observed for *db/db* mice when parabiosed to normal mice or *ob/ob* mice (Coleman, 1973). Instead, parabiosed partners of *db/db* mice, whether they were normal or *ob/ob* mice, would reduce their food intake, becoming hypoglycemic and dying of starvation (Coleman and Hummel, 1969). The experiments suggested the existence of a powerful negative feedback system regulating food intake, mediated by a factor circulating in plasma. Additionally, Coleman hypothesized that the *ob/ob* mouse was deficient in its ability to produce this factor, while the *db/db* mouse lacked the ability to respond to the factor. Two decades later, he was proven correct.

The positional cloning of the *ob* gene in 1994 represented an enormous breakthrough for molecular obesity research (Zhang et al., 1994). The product of the *ob* gene is a 16kd (167 amino acid) secreted protein, named leptin. The original obese mouse, ob/ob^{1j}, was shown to bear a nonsense mutation, resulting in a truncated, non-

functional protein. Consistent with the results of the parabiosis studies, leptin was shown to dramatically inhibit feeding in *ob/ob* and wild-type mice upon IP administration, whereas it had no effect on *db/db* mice . Indeed, leptin administration was able to correct nearly all of the abnormalities of the *ob/ob* mouse, including the food intake, weight, energy expenditure, fertility, and insulin resistance phenotypes (Campfield et al., 1995; Halaas et al., 1995; Pelleymounter et al., 1995). Interestingly, mice treated with leptin lost significantly more weight than pair-fed control mice, indicating that leptin has a significant effect on metabolism separate from its impact on food intake (Levin et al., 1996). Leptin is produced and excreted predominantly by adipose tissue, though expression is also seen in the placenta, gastric epithelium, and skeletal muscle (Masuzaki et al., 1997; Bado et al., 1998; Wang et al., 1998). Plasma leptin is undetectable in *ob/ob* mice, whereas in *db/db* mice the levels are massively elevated. In normal mice and humans, leptin levels are roughly correlated with adipose tissue mass (Maffei et al., 1995; Zachwieja et al., 1997). Thus, circulating leptin levels represent an accurate read-out of aggregate energy storage.

Leptin Receptor

Once leptin was discovered, it was used as a tool by Tartaglia et al. to identify its own receptor (Tartaglia et al., 1995). The group made several leptin-alkaline phosphatase fusion constructs (as well as I^{125}Leptin), and used them to screen a variety of cell lines and tissue samples. Choroid plexus was identified as having a high affinity for leptin, and a cDNA library was constructed from this tissue. Subsequent screening of this library for binding to the leptin receptor fusion constructs led to the identification of the

receptor for leptin, Ob-R. Ob-R is a single membrane-spanning receptor, sharing significant homology with the gp130 signal-transducing component of the IL-6 receptor, G-CSF receptor, and LIF receptor, making it a member of the class I cytokine receptor family (Tartaglia et al., 1995).

There at least six spliceoforms of the leptin receptor (Ob-Ra – Ob-Rf), each of which differs only with regards to the C-terminal portion (Lee et al., 1996). The extracellular portion of all six receptor forms is identical. Of the six, only the Ob-Rb variant possesses the full spectrum of intracellular signaling components, including Jak-Stat binding motifs and three tyrosines that, when phosphorylated, activate SH2-containing proteins. Following the cloning of Ob-R, it was discovered that the original C57Bl/K *db* mutant bore a premature stop codon that affected only the Ob-Rb isoform (Lee et al., 1996). Because the phenotype of the original mutant was indistinguishable from that of other later-discovered *db* mutants that were defective in the production of all six isoforms, it was reasoned that the Ob-Rb isoform was of pivotal importance for leptin-responsive signal transduction.

The various leptin receptor isoforms are widely expressed throughout the body, but the long variant, Ob-Rb, is expressed most strongly in the hypothalamus, and in the very same nuclei that had previously been implicated by lesioning studies to play a role in the regulation of food intake and body weight (Lee et al., 1996). *In situ* hybridization with an Ob-Rb specific probe showed strong expression in the ARC, VMH, LH, dorsomedial hypothalamic nucleus (DMH), and ventral premammillary nucleus, and to a somewhat lesser extent in the periventricular nucleus (Mercer et al., 1996; Fei et al., 1997; Elmquist et al., 1998).

When leptin and its receptor were discovered, it was hoped that leptin would represent a miracle cure for obesity. However, it turned out that most obese humans already had high levels of plasma leptin, indicating resistance to leptin's effects (McGregor et al., 1996). The same was found to be the case for high-fat diet-induced obese animals (Frederich et al., 1995). This concept of leptin resistance is still not fully understood, nor is it known whether, in humans, leptin resistance is a primary failing or a byproduct of adiposity. This unexpected discovery represents a transitional point in obesity research. The work of many different subfields of obesity research have been unified by the discovery of the elegant leptin feedback loop, answering many of the fundamental questions about how the body performs energy accounting. But, at the same time, the surprising findings regarding leptin resistance served to highlight how little was still known about both the mechanism of entry of leptin into the brain as well as the complex neuronal circuitry that must integrate and process such information in order to produce feeding decisions.

Leptin Signaling

The leptin receptor appears to exert its effects through a number of different intracellular signaling pathways (Fig. 4). As previously mentioned, the long form of the receptor, Ob-Rb, is a member of the class I cytokine receptor family, whose members possess cytoplasmic Box 1 and Box 2 domains that are involved in the recruitment of Janus kinases (JAKs) (Ihle and Kerr, 1995; Kloek et al., 2002). Upon binding of leptin to its receptor, the receptor oligomerizes, leading to the recruitment and cross-phosphorylation of JAK2 proteins, which subsequently phosphorylate tyrosine residues

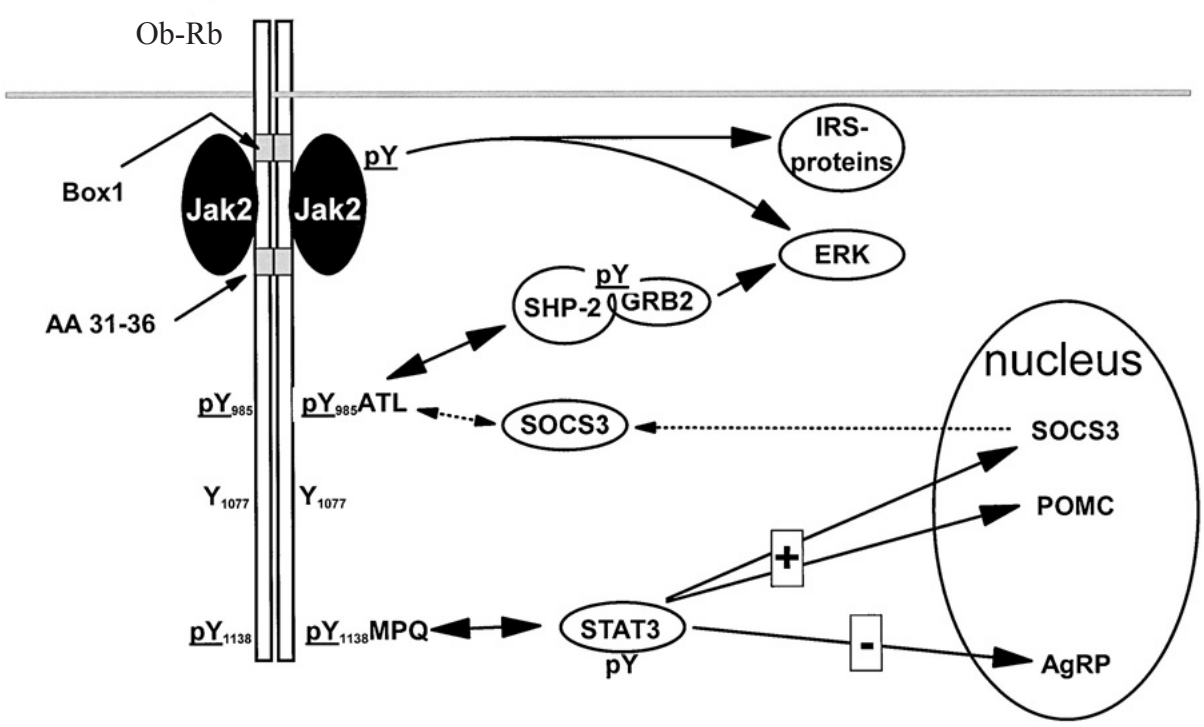


Figure 4 *Overview of Intracellular Leptin Receptor Signaling*
Binding of leptin to the long form of the leptin receptor (Ob-Rb) causes activation of Jak2 molecules associated with the Box 1 domain of the receptor. Activated Jak2 phosporylates tyrosines 985 and 1138 on the receptor. Signal transducer and activator of transcription 3 (Stat3) binds pY1138, leading to Stat3 phosphorylation, activation, and nuclear translocation. pStat3 causes increased expression of POMC and SOCS3 mRNA, and depresses the expression of AgRP mRNA. pY985 recruits SHP-2, which becomes phosphorylated and binds Grb-2, leading eventually to activation of extracellular signal-regulated kinase (ERK). After prolonged receptor stimulation, an unidentified event allows the inhibitory molecule SOCS3 to bind to the receptor at pY985, where it interferes with signal transduction. Signals mediated via unidentified tyrosine phosphorylation sites on Ob-Rb include the tyrosine phosphorylation of insulin receptor substrate (IRS) proteins (leading to activation of the phosphoinositol-3-kinase (PI3K) pathway) and a minor component of ERK activation. Adapted from Myers 2004.

on the receptor (at positions 985, 1077 and 1138) (Ghilardi and Skoda, 1997; Nakashima et al., 1997; White et al., 1997). Tyr1138 has been shown to be crucial for the subsequent recruitment of the Src-homology 2 (SH-2) domain-containing STAT (signal transducer and activator of transcription) proteins (Ghilardi et al., 1996; Bjorbaek et al., 1997). STAT proteins, once bound to phospo-tyrosine residues, are then phosphorylated by JAKs, leading to STAT dimerization and transport to the nucleus, where they activate transcriptional programs (Schindler, 1999). Of the STAT proteins, the one most closely associated with leptin receptor signaling is STAT3. STAT3 phosphorylation has been observed in the hypothalami of both wild-type and *ob/ob*, but not *db/db*, mice following leptin administration (Vaisse et al., 1996). Interestingly, when Tyr1138 of the leptin receptor was replaced with a serine reside via a knock-in approach, an interesting mixed phenotype was observed. The Ser1138 animals were hyperphagic and obese, like *db* mice, but were not infertile (Bates et al., 2003). Additionally, they were less hyperglycemic and exhibited normal body length, different from the stunted growth observed in the *db* animal. This suggests that STAT3 activation is required for leptin-dependent energy homeostasis, but that a separate leptin receptor dependent signaling pathway or pathways are utilized for control of fertility, lean body growth, and glucose homeostasis.

Other intracellular signaling pathways have been implicated in the response to leptin. The phosphatidylinositol-3-OH (PI3) kinase pathway has been shown to be activated *in vivo* by leptin administration (Kellerer et al., 1997), and icv administration of a PI3K inhibitor significantly attenuated the anorexic effect of leptin administration (Banks et al., 2000). In a number of cell culture studies, binding of leptin to its receptor

has been documented to activate the mitogen-activated protein kinase (MAPK) pathway (Bjorbaek et al., 1997; Takahashi et al., 1997). Subsequently, it was shown that MAPK is phosphorylated in the hypothalamus in response to leptin, and that this process requires the action of the phosphatase SHP-2, which can dock at phosphotyrosine 985 of Ob-Rb (Banks et al., 2000). It is unknown what *in vivo* function is played by the MAPK pathway with regards to the response to leptin, but this pathway is implicated in the upregulation of c-fos, a marker of neuronal activation which is upregulated in response to leptin stimulation (Bjorbaek et al., 2001). By electrophysiological examination, leptin has also been found to hyperpolarize leptin-responsive neurons in the Arc and VMH via the actions of an ATP-sensitive potassium pump (Spanswick et al., 1997). The mechanism of activation of this pump is not clear.

Leptin signaling is specifically inhibited by a couple of known pathways. The expression of suppressor of cytokine signaling 3 (SOCS3) is specifically upregulated in leptin-responsive Arc and DMH neurons of WT and *ob/ob*, but not *db/db*, mice in response to leptin (Bjorbaek et al., 1998). Additionally, in DIO mice, SOCS3 was found to be upregulated in the Arc (Munzberg et al., 2004). SOCS3 has been shown in cell culture to interfere with leptin signaling by binding to phosphotyrosine 985 of the Ob-Rb (Bjorbaek et al., 2000). Another molecule, protein-tyrosine phosphatase 1B (PTP1B), has been shown to negatively regulate leptin signaling. PTP1B is expressed in some of the same nuclei as Ob-Rb, including the Arc and VMH, and in cell culture, PTP1B selectively dephosphorylates JAK2 (Cheng et al., 2002; Zabolotny et al., 2002). PTP1B -/- mice are resistant to obesity, displaying hypersensitivity to leptin treatment and resistance to diet-induced obesity (Elchebly et al., 1999; Klaman et al., 2000). Because

of their ability to specifically interfere with leptin signaling in the hypothalamus, both SOCS3 and PTP1B have been strongly implicated in the development of leptin resistance.

Neuropeptides

Recent research has been revealing an ever expanding, complex set of neuropeptides that are expressed in various hypothalamic nuclei, some of which are implicated in the regulation of food intake. What follows is a brief overview of what has become a vast and complicated field of research.

Melanin concentrating hormone (MCH), a cyclic 19 amino acid peptide originally studied for its ability to concentrate melanin in the melanophores of fish species (Wilkes et al., 1984), is expressed in the LH and zona incerta of the rodent hypothalamus (Skofitsch et al., 1985; Bittencourt et al., 1992), where it is now regarded as having a significant impact on food intake. MCH mRNA is expressed at elevated levels in the hypothalami of *ob/ob* mice, compared with *ob/+* controls (Qu et al., 1996). It is also elevated in fasted mice, compared with control mice fed *ad libitum*. Additionally, injection of MCH peptide into the lateral ventricles of rats led to significantly increased food intake (Qu et al., 1996). Targeted deletion of the MCH gene results in mice which display leanness resulting from decreased food intake and increased energy expenditure, despite decreased levels of leptin, certifying this orexigenic peptide as a downstream target of the leptin pathway (Shimada et al., 1998).

Two MCH receptors have been currently identified. The first was a previously orphan G-protein coupled receptor (GPCR), SLC-1, now referred to as MCHR1

(Chambers et al., 1999; Lembo et al., 1999). MCHR1 is widely expressed throughout the CNS, and within the hypothalamus, MCHR1 is localized to numerous nuclei, including the LH, ARC, VMH and DMH (Lembo et al., 1999; Hervieu et al., 2000). MCHR1-/- mice are roughly normal weight, but are lean with reduced fat mass (Chen et al., 2002; Marsh et al., 2002). These mice are also hyperphagic, but this hyperphagia is offset by increased energy expenditure. After the discovery of the first MCH receptor, another similar receptor, MCH2R was cloned (An et al., 2001; Hill et al., 2001). However, this receptor may not be as physiologically relevant, as many non-human mammals (including rat, mouse, hamster, guinea pig, and rabbit) either do not express functional MCHR2 receptors, or express a non-functional MCHR2 pseudogene (Tan et al., 2002).

The orexins (orexin-A and orexin-B), also referred to as the hypocretins, are another important neuropeptide type expressed in the LH along with MCH, but in a mutually exclusive group of neurons (Broberger et al., 1998). The orexins are short peptides, which originate from the same precursor molecule, Prepro-Orexin (de Lecea et al., 1998). Like MCH, icv administration of orexin peptides leads to a significant dose-dependent induction of food-intake (Sakurai et al., 1998). Prepro-Orexin mRNA levels are increased by fasting, but were observed to be downregulated in *ob/ob* and *db/db* hypothalami by *in situ* hybridization, indicating that their regulation is different from that of MCH (Yamamoto et al., 1999; Yamamoto et al., 2000). Targeted deletion of the Prepro-Orexin gene results in a phenotype similar to human and canine narcolepsy (Chemelli et al., 1999). This suggests that the orexins are really modulators of sleep/wake states and alertness. Prepro-Orexin knockout animals are of normal body

weight, though they do display hypophagia. Transgenic mice in which the orexin neurons are ablated by expression of a truncated Machado-Joseph disease gene product (ataxin-3) display similar phenotype to the Prepro-Orexin knockout, displaying narcolepsy and hypophagia, but these mice actually develop late-onset obesity despite the hypophagia (Hara et al., 2001). There is ample evidence that the orexins are involved in both sleep/wake/alertness as well as energy homeostasis, revealing an interesting, complex link between these two systems.

Two orexin receptors have been identified (OX1R and OX2R), which were both previously orphan GPCRs (Sakurai et al., 1998). They display broad and somewhat overlapping patterns of expression within the CNS, and are expressed highly in hypothalamic nuclei implicated in energy homeostasis. Within the hypothalamus, OX1R is mostly restricted to the VMH and DMH, while OX2R mRNA is expressed mainly in the Arc, VMH, DMH, and paraventricular nucleus (PVN) (Trivedi et al., 1998; Lu et al., 2000).

Taken together, the mutually exclusive orexin and MCH neuronal populations within the LH display all of the properties of the LH feeding center predicted from the original lesioning studies (Elmquist et al., 1999). This refinement of a processing center into its genetically defined component neuronal populations is a common theme of recent progress towards the elucidation of leptin-responsive pathways in the CNS.

Neuropeptide Y (NPY) is a 36 amino acid peptide that shares significant homology with the gut peptides pancreatic polypeptide and peptide YY (Tatemoto et al., 1982). NPY is widely expressed throughout the brain, and is expressed strongly in the Arc, where it is found in neurons that express the leptin receptor (Allen et al., 1983).

NPY has been implicated in, among other things, hypothalamic-pituitary signaling (Colmers and Wahlestedt, 1993), cardiovascular physiology (McDermott et al., 1993), sympathetic nervous system function (Stjarne et al., 1986), and modulation of mood (Wahlestedt et al., 1993). Notably, icv administration of NPY peptide into rats produces a significant increase in feeding (Clark et al., 1984), while long-term infusion actually results in obesity (Zarjevski et al., 1993). Fasted mice, as well as ob/ob and db/db mice, display elevated levels of NPY mRNA in the Arc (Wilding et al., 1993). Significant reduction in hypothalamic NPY mRNA and NPY release are observed following treatment with IP or icv leptin (Stephens et al., 1995; Schwartz et al., 1996b).

Interestingly, though the data supports a central role for NPY in processing and transmission of the leptin signal, mice with targeted deletion of the NPY gene show only the mildest phenotype related to energy homeostasis (Erickson et al., 1996b). NPY knockout mice are of normal size and weight. However, these mice do display slight hypersensitivity to treatment with leptin, eating less and losing more weight during the first two days of chronic leptin treatment than WT control mice. However, by day 5, total weight loss between the two groups was identical. NPY -/- mice also displayed slightly stunted re-feeding responses following a 2-day fast. However, this effect was also short-lived, and none of these effects were observed without substantial back-crossing onto C57Bl6 background. A more significant phenotype was observed when NPY -/- mice were bred onto an *ob/ob* background. The resulting mice displayed significant attenuation of all aspects of the *ob/ob* phenotype, with reduced adiposity and food intake and improved fertility, diabetes, and energy expenditure. (Erickson et al., 1996a). This data clearly establishes NPY as an important mediator of all of the

physiological effects of leptin. However, the lack of severity of the knockout phenotype is still puzzling.

The neuropeptide Y family members (NPY, peptide YY (PYY), and pancreatic polypeptide), bind to the Yx family of GPCRs (Y1 through Y5 and PYY-preferring receptor). A study of the pharmacological profiles of various receptor agonists suggested that the Y5 receptor was the most likely to mediate the orexigenic effect of icv NPY administration (Gerald et al., 1996). However, Y1-/- and Y5-/- mice have been generated, and display minimal metabolic phenotypes aside from mild late-onset obesity (Kushi et al., 1998; Marsh et al., 1998). Intriguingly, chronic icv administration of NPY to Y1-/- or Y5-/- mice produced equivalent hyperphagia and weight gain to that observed in NPY-treated WT mice (Raposinho et al., 2004). Thus, there remains uncertainty regarding the true effector/effectors of NPY action. It is likely that there is significant redundancy between the receptors, and that at the very least, receptors Y1 and Y5 work together to transduce signals from icv injected NPY. The Y1, Y2, Y4, and Y5 receptor subtypes are all abundantly expressed in hypothalamic feeding nuclei, with considerable overlap between Y1 and Y5 expression, enforcing the theory of redundancy and cooperation between these receptors (Gustafson et al., 1997; Jacques et al., 1998; Parker and Herzog, 1999; Kishi et al., 2005).

Over the years, a number of spontaneous mutations have been observed which result in yellow coat color, obesity, diabetes, and increased susceptibility to cancer. These include the lethal yellow (A^y) and viable yellow (A^{vy}) mutations, which are alleles of the agouti (A) locus (Dickies, 1962, 1969). Agouti is named after the banding pattern of the South American rodent of the same name. Cloning of the *agouti* gene revealed

that it is a 131 amino acid paracrine signaling factor, produced in hair follicles, that acts at melanocortin 1 receptors (MC1R) on melanocytes in order to inhibit eumelanin (black) production in favor of production of pheomelanin (yellow) (Furumura et al., 1996). In A^y and A^{vy} mice, agouti is constitutively produced in the hair follicles, (where its constitutive inhibition of MCR1 produces pure yellow fur), as well as in many other tissues. In the brain, this excess agouti protein acts at other melanocortin receptors, MC3R and MC4R, which are present at high levels in the hypothalamus, and this interaction is the cause of the obesity phenotype of these animals (Mountjoy et al., 1994; Lu et al., 1994; Klebig et al., 1995).

A search was conducted to find expressed sequence tags (ESTs) related to agouti, resulting in the identification of a transcript that produces another 131 amino acid protein with 25% homology to agouti. Agouti-related peptide (AgRP, or agouti-related transcript, ART) is expressed predominantly within the Arc, median eminence, and adrenal medulla in mice, and within the Arc, AgRP shows completely overlapping expression with NPY (Shutter et al., 1997; Hahn et al., 1998). *AgRP* mRNA expression in the Arc is approximately 10-fold elevated in *ob/ob* and *db/db* mice. AgRP was found to inhibit melanocortin action selectively at melanocortin 3 and 4 receptors, with 100-fold more potency than agouti protein (Fong et al., 1997; Ollmann et al., 1997).

Mice with targeted deletion of the AgRP gene, like NPY knockout mice, have little or no observable phenotype. AgRP-/- mice display normal food intake, growth curves, energy expenditure, and body composition (Qian et al., 2002). It was thought that perhaps AgRP and NPY play redundant functions within Arc neurons, and so the Agrp and NPY knockout mice were bred together to create AgRP-/-; NPY-/- double mutants.

Strikingly, there was no additive effect of double mutation, and AgRP-/-; NPY-/- mice were not shown to have any severe metabolic abnormalities (Qian et al., 2002). The bulk of the data still suggests an important role for these peptides in feeding, but in that case the redundancy inherent to the hypothalamic energy homeostatic system is extremely robust.

The biology of the agouti protein and its relative, AgRP, clearly implicated the melanocortin system as a central component of the system that regulates food intake and body weight. The melanocortins, adrenocorticotropin (ACTH) and the alpha, beta, and gamma melanocyte-stimulating hormones, are implicated in an extremely diverse set of biological functions, precluding a full summary (Strand, 1999). Implication of the melanocortins in body weight regulation was first suggested by a study showing a significant reduction in spontaneous feeding following icv injection of ACTH(1-24) (Poggioli et al., 1986). Of the remaining melanocortins, icv administration of alpha- and beta-MSH produces a significant inhibitory effect on post-fast feeding in rats, whereas gamma-MSH has no effect (Abbott et al., 2000). All four melanocortins are cleaved from a single precursor protein, pro-opiomelanocortin (POMC). Hypothalamic POMC mRNA is reduced by fasting, and is upregulated in response to leptin treatment, and ob/ob and db/db show reduced levels of hypothalamic POMC expression (Mizuno et al., 1998a). Within the hypothalamus, POMC mRNA is expressed highly in the Arc, and subsequent work showed that POMC and NPY/AgRP are expressed in mutually exclusive populations of neurons within the Arc (Elias et al., 1999). This separation between the orexigenic and anorexigenic peptides in the Arc suggests the possibility of

negative feedback between the two systems, which will be discussed in more detail in the following section.

The melanocortins act through a family of five G-protein coupled receptors (MC1R through MC5R) (Adan and Gispen, 1997). In the hypothalamus, the predominant receptor forms are MC3R and MC4R (Roselli-Rehfuss et al., 1993; Mountjoy et al., 1994), and it is MC4R that is most intimately connected with the regulation of feeding, as both alpha- and beta-MSH act largely at this receptor (Abbott et al., 2000). MC4R knockout mice display a striking metabolic phenotype, developing maturity-onset obesity in the context of hyperphagia, hyperinsulinemia, and hyperglycemia (Huszar et al., 1997). This mouse largely recapitulates the metabolic phenotype of the obese *agouti* mutants, supporting the model that the obesity in these mice is the result of chronic MC4R antagonism. Because gamma-MSH acts primarily at MC3R and has no significant effect on feeding, it was postulated that this receptor would not be critically involved in this pathway (Abbott et al., 2000). Indeed, while MC3R knockout mice do display a modest increase in fat mass and a concomitant decrease in energy expenditure, the phenotype of these mice is far less striking than that of the MC4R knockout (Butler et al., 2000).

Cocaine and amphetamine-regulated transcript (CART) is a potent anorexigenic peptide, discovered via a PCR differential display screen for genes upregulated by these psychomotor stimulants (Douglass and Daoud, 1996). CART is widely expressed throughout the CNS, and in the hypothalamus it is expressed strongly within the Arc and DMH (Kristensen et al., 1998). Fasted mice show reduced levels of Arc CART expression, and in *ob/ob* and *db/db* mice Arc CART expression is almost completely

absent. icv infusion of CART peptide into rats inhibits both normal and post-fasting food intake (Kristensen et al., 1998). Interestingly, in the Arc, CART was found to be almost totally colocalized with POMC, indicating that this molecule most likely has its influence on energy homeostasis at the same level of the pathway as the melanocortins (Elias et al., 1998a).

There are a number of other neuropeptides that are implicated in the central regulation of feeding, but not to the degree of those described above. Two related peptides, galanin and galanin-like peptide (GALP), are both expressed in feeding nuclei (Skofitsch and Jacobowitz, 1985; Ohtaki et al., 1999; Larm and Gundlach, 2000), including the Arc, and central administration of both peptides stimulates feeding in rodents (Kyrkouli et al., 1986). Whereas chronic injection with NPY, for example, produces a long-term change in body weight, the same is not the case for galanin (Smith et al., 1994). Nevertheless, the discovery that some galanin- and GALP-expressing neurons in the Arc also express the leptin receptor and modulate their level of galanin/GALP mRNA in response to leptin treatment has led to renewed interest in these peptides (Hakansson et al., 1998; Sahu, 1998; Jureus et al., 2000). Corticotropin-releasing hormone (CRH) is expressed in the paraventricular, supraoptic, medial and periventricular preoptic, and premammillary nuclei of the hypothalamus (Merchenthaler et al., 1982). CRH neurons in the paraventricular nucleus project to the pituitary, where they regulate the excretion of ACTH, a key regulator of adrenal cortisol production (Cone, 2000). icv injection of CRH causes a significant reduction in food intake (Gosnell et al., 1983). Neuromedin U (NMU) is a neuropeptide produced mainly within the Arc in the hypothalamus in neurons that also express the leptin receptor. icv administration of

NMU sharply reduces food intake, and injection of an anti-NMH antibody increases food intake (Howard et al., 2000; Kojima et al., 2000). NMU knockout mice display obesity, hyperphagia, and decreased food intake and energy expenditure (Hanada et al., 2004). Interestingly, administration of exogenous leptin led to reduced adiposity, suggesting that NMU may act, at least in part, via a leptin-independent pathway.

Marker Genes and the Leptin Feeding Circuit

Clearly, this bewildering array of neuropeptides is integrally involved in the regulation of food intake and energy homeostasis, but the diverse expression profiles of these peptides and their receptors within the hypothalamus suggest a much deeper level of complexity at the level of neuronal organization and integration. It has become apparent that classical models of feeding regulation based on antagonistic phamacological properties of neuropeptides are fundamentally insufficient, in that they provide little foundation upon which to build complex models of neuronal circuitry. In order to begin to understand the operation of a complex neuronal circuit, the first step must be to define the component players and understand how they are interconnected. Thus, discrete neuronal cell types of interest must be identified, catalogued, and studied. From such studies identifying functional components, a working model may then be created that would be robust enough to allow for further refining and further integration of new components.

Schemes for defining neuronal cell types were historically based on neuronal morphology. For example, neurons in the PVN and many other nuclei are commonly broken down into magnocellular and parvocellular groups, etc (Armstrong et al., 1980).

However, morphology is an extremely blunt tool with which to dissect the differences between neurons. Recently, it has emerged that discriminating neuronal cell types on the basis of gene expression may be the most powerful and functionally relevant method. For example, within the ARC are two mutually exclusive groups of neurons, the NPY/AgRP and POMC/CART subpopulations. These populations of neurons express neuropeptide sets known to be functionally antagonistic, and yet these two groups of neurons cannot be differentiated on the basis of size, localization, or morphology. Indeed, it is through intensive study of these very two populations, defined on the basis of gene expression, that most of the recent progress towards understanding this system has been made.

Because the NPY/AgRP and POMC/CART neurons express neuropeptides with opposing pharmacological function, it was assumed that these populations of neurons would have antagonistic functions. However, both populations were found to express significant amounts of the long form of the leptin receptor, Ob-Rb, which at first glance appeared paradoxical. However, this was resolved when it was observed that treatment with leptin activates only the POMC neurons, as measured by the expression of the activation marker c-fos (Elias et al., 1999). Presumably, leptin inhibits NPY/AgRP neurons, as NPY mRNA levels drop following leptin treatment, while the expression of POMC is increased in POMC/CART neurons (Schwartz et al., 1996a). Thus, leptin acts as a tuner, modulating the activity of these two important populations of neurons. But, if that is the case, and the interplay between these groups are responsible for regulation of feeding, how do they exert their downstream effects?

ARC NPY/AgRP and POMC/CART neurons both project to the same regions, among them the LH and the PVN (Kiss et al., 1984; Elias et al., 1999). At downstream sites expressing the MC4 receptor, these projections exert their opposing influence, and their relative contributions are integrated (Elmquist, 2001). The PVN is recognized as perhaps the major output center of the hypothalamus. The PVN expresses a number of hormones (including CRH, thyrotropin releasing hormone (TRH), and oxytocin), and has dense projections to the pituitary, where it is a major regulator of hormone release (Kandel and Schwartz, 1991). The PVN is also heavily implicated in signaling to autonomic centers. In fact, two anatomically and functionally segregated groups of neurons within the PVN are responsible for significant outputs to both sympathetic and parasympathetic nervous system centers (Buijs et al., 2003). In the PVN, the same neurons that express the MC4 receptor and receive inputs from Arc NPY/AgRP and POMC/CART neurons also express pre-TRH RNA (Legradi and Lechan, 1999; Fekete et al., 2000a; Fekete et al., 2000b). This suggests that some of the metabolic effects of leptin are attributable to modulation of the hypothalamic-thyroid axis.

In the LH, both the orexin and MCH expressing neuronal subtypes receive dense innervation by NPY/AgRP and alpha-MSH containing fibers (Broberger et al., 1998; Elias et al., 1998b). Because AgRP expression in the CNS is localized exclusively to the Arc, the NPY/AgRP fibers clearly originate from there. Additionally, stereotaxic injection of the retrograde tracer fluorogold into the LH led to positive staining of both NPY/AgRP and POMC/CART neurons in the Arc (Elias et al., 1999). Both the MCH and orexin neuronal populations in the LH project to a wide range of intra- and extra-hypothalamic sites, including autonomic system centers and to the cortex (Saper et al.,

1976b; Saper, 1985; Saper et al., 1986). According to a model proposed by the authors, these cortical projections might represent an important link between this largely unconscious regulatory system and consciousness itself. An overview of proposed hypothalamic feeding circuitry connections is shown (Fig. 5).

This initial data regarding the NPY/AgRP and POMC/CART neurons confirmed the utility of approaching this circuitry problem from the perspective of discrete neuronal subtypes defined on the basis of gene expression. Interestingly, as a result of this approach, the ability of the neuropeptides to label discrete neuronal populations has become far more valuable to researchers than their pharmacological activities. In fact, because of the multitude of neuropeptide knockouts with minimal or no phenotype, it seems likely that neurons are much more than just the sum of the neuropeptides that they express. It stands to reason then that neurons do not need to express a pharmacologically-validated feeding peptide to be critically involved in the leptin-responsive circuit. Such neurons may be just as important, and a gene that is specifically expressed in that neuronal compartment, which can be used to mark or label those neurons and not surrounding neurons, may be as important as a neuropeptide to the overall task of developing a working model of the circuit.

The experiments described above utilized the marker genes to label their component neurons by either immunohistochemistry or in situ hybridization. However, these techniques represent only the tip of the iceberg as far as the utility of the marker genes is concerned. Their true value is in their use as genetic tools to express transgenes of interest specifically to the cell types that they label, by hijacking the promoter elements of the markers themselves. This strategy has been used to learn substantially

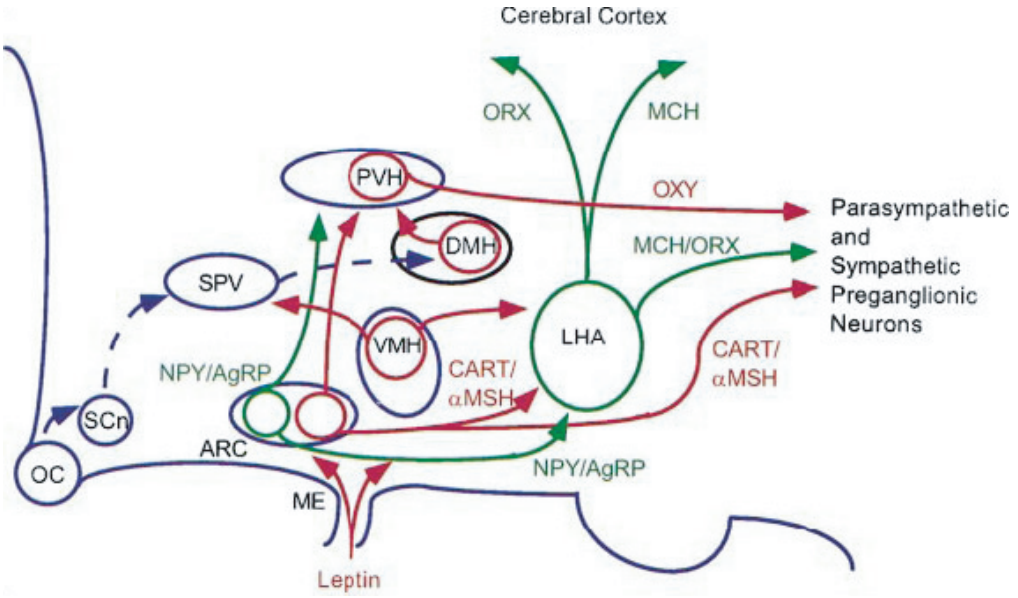


Figure 5 *Proposed Wiring Diagram of Hypothalamic Feeding Circuitry* Pathways inhibited by leptin, and thus presumably anorexic, are indicated by red arrows. Pathways activated by leptin (presumably phagic) are denoted by green arrows. Circadian inputs are denoted by dashed blue lines. OC, optic chiasm; SCn, suprachiasmatic nucleus; SPV, subparaventricular zone; PVH, paraventricular hypothalamic nucleus; DMH, dorsomedial hypothalamic nucleus; VMH, ventromedial hypothalamic nucleus; ARC, arcuate nucleus; LHA, lateral hypothalamic area. Adapted from Elmquist et al. 1999.

more about populations of interest in the hypothalamic feeding circuit, as discussed below.

Transgenic and knock-in mice expressing transgenes in specific component neuronal populations within the feeding circuitry have proved themselves to be invaluable tools for assaying not only the biology of these neurons, but also the connectivity, function, and plasticity of the circuit as a whole. For example, knock-in mice expressing cre recombinase in Ob-Rb neurons (Ob-Rb cre mice) have been used in elegant tracing experiments utilizing a conditional strain of pseudorabies virus to map the multi-synaptic inputs to leptin receptor neurons within the ARC (DeFalco et al., 2001). This study highlights the extraordinary potential for using such mice in conjunction with conditional tracing methods to generate an extremely detailed circuitry map for this system. While PRV infects primarily in a retrograde manner via synaptic connections (Strack et al., 1989b), highlighting inputs to neurons of interest, there is no reason the cre/lox strategy could not also be applied to anterograde tracers, such as lectins or wheat-germ agglutinin (Braz et al., 2002).

Because knock-in mice are difficult and slow to generate, recent efforts have focused on transgenic methods to express transgenes under the control of marker gene promoters. While traditional transgenic constructs, in which sections of 5' flanking DNA are inserted upstream of transgenes to drive expression, have been used to drive the expression of transgenes, the resulting expression is highly sensitive to positional effects (Yang et al., 1997). Additionally, distal enhancers and repressor sequences are often absent, leading to incorrect expression patterns. To counter these problems, bacterial artificial chromosome (BAC) based transgenesis has gained in prominence. In BAC

transgenesis, a large chunk of genomic DNA (usually greater than 100kb), containing the gene of interest, is modified in *Escherichia coli*, such that the transgene follows the ATG translation initiation site, rather than the coding sequence of the original gene. The whole BAC is then used as a transgenic construct, so that the resulting progeny mice express the transgene under the full set of promoter elements of the original gene (Yang et al., 1997; Gong et al., 2002). This tends to produce transgene expression that much more closely parallels the distribution of expression of the original marker gene, with less variation due to positional effects.

Individual BAC transgenic mouse lines have been created that express fluorescent proteins of different colors in a number of feeding-related neuronal subsets (Gong et al., 2003). Because these mice allow easy identification of labeled neurons, they have been essential for studies of co-localization, gene expression, electrophysiology, and plasticity (Liu et al., 2003a; Pinto et al., 2004; Roseberry et al., 2004). In one study of mice expressing different GFP variants under the control of the NPY and POMC promoters, it was discovered that the numbers of inhibitory and excitatory axosomatic synaptic connections and postsynaptic currents onto these ARC neuronal populations were different between wild-type and *ob/ob* mice (Pinto et al., 2004). Most strikingly, these differences were corrected by administration of leptin to *ob/ob* mice, an effect which was observed as early as 6 hours post-treatment. While the source of the axosomatic contacts and postsynaptic currents onto these cell populations remains unclear, this study clearly revealed a new dimension to the complexity of the hypothalamic feeding circuit. At the same time, it concretely demonstrated the profound utility of marker genes, and the

marker gene-based transgenic approach, for the study of this, or any, system of neuronal circuitry.

While most BAC transgenic mice created to date have been engineered to express GFP variants or cre recombinase in neuronal populations of interest, there is no technical limit to the variety of transgenes that might be used to investigate the biology of the feeding circuitry. Efforts are already underway to express modulators of neuronal activity in specific cell types. Neuronal activity could be modulated by a number of different transgenic strategies, for example by expressing ivermectin-sensitive chloride channels to allow for temporospatial inhibition of neuronal activity (Slimko et al., 2002). Forcible activation or inhibition of specific neuronal populations will be a critically important strategy for determining the contribution of these populations to behavior, and for investigating their impact on downstream neurons.

Undoubtedly, the study of NPY/AgRP and POMC/CART neurons has greatly increased our understanding of the leptin-responsive feeding circuit and the regulation of food intake and body weight. However, because of the vast array of significant data regarding these ARC populations, it became customary to consider the ARC as the most important first order leptin-responsive site within the hypothalamus. In fact, the data is beginning to suggest that other nuclei that express the leptin receptor may play an equally important role in sensing and responding to leptin. POMC-specific deletion of the leptin receptor (POMC-cre X flox-ObR-flox mice) does result in hyperphagia and increased adiposity, but the phenotype is only a small fraction of that observed for the *db/db* mouse (Balthasar et al., 2004). Interestingly, knockout of the receptor in VMH neurons, using an SF-1-cre transgenic line, leads to a similar adiposity phenotype (H. Dhillon, personal

communication). These results have led to the emerging realization that all Ob-Rb expressing hypothalamic nuclei likely play an important role in the response to leptin, and thus are all of great importance to the overall task of energy homeostasis.

Specific Aims

Traditional approaches to studying the hypothalamic control of feeding and body weight revolved around lesioning experiments to identify nuclei of interest that specifically contributed to feeding behavior. However, it has slowly come to light that brain regions such as hypothalamic nuclei, though they may appear to be composed of homogeneous populations of neurons, may in fact be extremely complex, not only in structure, but in gene expression, connectivity and function. In the ARC, two opposing populations of neurons, with differing gene expression, function, and connectivity, are co-mingled with similar sub-nuclear patterns of localization. In addition to labeling these populations, the genetic markers for these populations, NPY/AgRP and POMC/CART, have been successfully used to drive transgene expression in these very subsets, allowing for detailed experimentation and analysis. Thus, the definition of neuronal subtypes based on the expression of marker gene expression rather than anatomic localization or morphology is advantageous not only from the point of view of improved neuronal discrimination, but also in the potential for targeted study. Indeed, the subpopulations in the ARC have been so successfully analyzed that the ARC is now commonly regarded as the primary site of leptin action in the hypothalamus. Recent evidence suggesting a similarly important role for the VMH in the first-order response to leptin suggests a critical role for this nucleus in leptin sensing. If this is the case, then full understanding of the leptin response and hypothalamic energy homeostasis demands intensive study of this nucleus in an analogous fashion to study of the ARC.

However, while several lines of evidence have established that the VMH plays an important role in controlling food intake, glucose metabolism and body weight, efforts to determine how specific classes of VMH neurons mediate these processes have been hampered by the lack of a set of marker genes that could facilitate a molecular analysis of their function. To that end, this thesis aims to investigate gene expression in the VMH with the aim of discovering genes that may be used as markers for its component neurons. As nucleus-specific marker genes are most useful as drivers for transgene expression, particularly for tracing experiments and studies of gene expression, we endeavored to define VMH markers through microarray comparison of VMH RNA with RNA from two adjacent nuclei, the ARC and DMH, isolated using laser-capture microdissection. The results of this intial screen, validation, and follow-up experimentation on the VMH marker genes discovered are included.

Chapter 2: Materials and Methods

Animal Care and Maintenance

All mice were either purchased from The Jackson Laboratory (Bar Harbor, ME) or bred at Rockefeller University. Animals were housed at the Rockefeller Laboratory Animal Research Center (LARC), subject to a 12 hour light:dark cycle (600-1800 hr light, 1800-600 hr dark). All animals were cared for according to institutional guidelines.

Northern Blotting

Tissues were dissected from mice using sterile instruments, and total RNA was isolated using Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer's protocol. 15µg of RNA from each sample were run on a 1% agarose, 1X MOPS, 18% formaldehyde gel. After running, the gel was washed in DEPC-H20, washed for 15 min in 0.05N NaOH, 2x 15 min in 10X SSC, and transferred in 10X SSC onto a Hybond-N+ membrane (Amersham, Buckinghamshire, UK). All solutions were made with DEPC-H20. Each 32P-radiolabeled probe was generated by PCR, and the membrane was hybridized with the probe at a concentration of 10^6 cpm/ml in Rapid-Hyb (Amersham, Buckinghamshire, UK) overnight at 65^0C. The next day, the membrane was washed for 15 min at room temperature in 2x SSC/0.1% SDS, followed by washing for up to 2 hours in 0.1X SSC/0.1% SDS at 65^0C. The membrane was exposed to BIOMAX MR x-ray film (Fisher Scientific, Hampton, NH) at -80^0C for 1-7 days, and then developed. The probes for LBH2, Cbln1, and PACAP were created using modified T3 and T7 primers (modified T7 primer 5'-CGACTCACTATAGGGAATTTGGC-3', modified T3 primer

5'-CTAAAGGGAATAAGCTTGCGGCC-3'), using the original NMHY clone plasmids as templates.

Southern Blotting

10μg of LBH2 BAC DNA was digested in with HindIII overnight at 37^0C, and then run on a 1% agarose 1X TAE gel. After running, the gel was treated with denaturing solution (1.5M NaCl, 0.5N NaOH) for 20 min, followed by 20 min treatment in neutralization solution (1.5M NaCl, 1mM EDTA, 0.5M Tris pH 8.0), followed by a brief rinse in 10X SSC. The gel was then transferred to a Hybond-N+ membrane (Amersham, Buckinghamshire, UK) in 10X SSC. Each 32P-radiolabeled probe was generated by PCR, and the membrane was hybridized with the probe at a concentration of 10^6 cpm/ml in Rapid-Hyb (Amersham, Buckinghamshire, UK) overnight at 65C. The next day, the membrane was washed for 15 min at room temperature in 2x SSC, followed by washing for up to 2 hours in 0.1X SSC at 65^0C. The membrane was exposed to BIOMAX MR x-ray film (Fisher Scientific, Hampton, NH) at -80^0C for 1-7 days, and then developed.

Quantitative Real-Time PCR

Quantitative real-time PCR was performed on RNA from microdissected hypothalamic nuclei. First, roughly 2ng RNA from each sample were reverse transcribed with random hexamers using Taqman Reverse Transcription Reagents (Applied Biosystems, Foster City, CA) to produce cDNA. Real-time PCR was performed using Taqman Universal PCR Master Mix (Applied Biosystems, Foster City, CA) on an ABI Prism 7700 Sequence Detection System (Applied Biosystems, Foster City, CA). Control RNA was

prepared from whole hypothalamus using Trizol reagent (Invitrogen, Carlsbad, CA) according to the instructions of the manufacturer. Data analysis was performed using SDS 1.9.1 software (Applied Biosystems, Foster City, CA). The primer and probe sets used are summarized in Table 1. Cyclophilin primers and probes were used as a control, and each sample was normalized against cyclophilin data. Each sample was run in duplicate, and the results are the average of three independent experiments.

Laser-Capture Microdissection

Tissue preparation and staining. 8 week old female C57Bl6/J mice were sacrificed, and brains were manually dissected and frozen immediately in Tissue-Tek O.C.T 4583 (Sakura, Santa Rosa, CA). The frozen blocks were sectioned on a cryostat (Leica, St. Gallen, Switzerland) to a thickness of 10 microns, and immediately adhered to plain, uncharged slides (VWR, West Chester, PA). Sections on slides were fixed for 1 minute in 100% EtOH, and then rehydrated with a graded alcohol series (95%, 75% 50%, 30 s each) and stained for 1 minute in filtered 0.5% cresyl violet acetate (Sigma-Aldrich, St. Louis, MO). Next, the sections were again dehydrated with EtOH (50% for 30 s, 75% for 30 s, 95% for 30 s, 100% for 1 min) and finally cleared with Histo-Clear (AGTC Bioproducts, Beverley, UK) for 1 minute. Before microdissection, slides were allowed to thoroughly dry, as the presence of water in the sections interferes with cap adhesion. All solutions were made with autoclaved 0.1% DEPC water.

Table 1 Sequences of Taqman Real-Time PCR Probe/Primer Sets

Gene	Forward Primer	Probe Sequence	Reverse Primer
PACAP	5'-CTGCAGAAGCCAGCTCTGTTC-3'	5'-GGAAGGAGGGTCTCCAGAAAATCCACAA -3'	5'-AATGCATGAGGGCAAGGGT-3'
Guanine Deaminase	5'-TGGAAAGCAGGTCGTTCCA -3'	5'-TCTCCAGCTCAGTGTAAGGACCTTGGACA -3'	5'-TCCCAGGAGAATGTCGCAG -3'
Cerebellin 1	5'-ACACAGGAAAGGCAAAGGGA -3'	5'-CCTGCTACGGGAGACAGCGCAGA -3'	5'-GCCTTCTTCGCAGAGCCAT -3'
Steroidogenic Factor 1	5'-AGCTGCTGGTGTTGGACCA -3'	5'-CGCCAGGTCCAGTACGGCAAGG -3'	5'-TCCAGTAACCAGCAGGATGCT -3'
D123	5'-GCAGCCCAGCCCCTATCT -3'	5'-AGCTTCGGGCTGCCCAAGGACTT -3'	5'-TCCCCGGTGGAAAGGTCTAC -3'
Slit3	5'-GTGCAACGCCTGCCTCTC -3'	5'-AGCCCGTGCAAGAACAACGGCA -3'	5'-CACGGGATCCTGACTGCAA -3'
LBH2	5'-TGCTCCAGTAACAGATGCGG -3'	5'-GGTGGTCAGAGAGTAGAGCCTGCCACA -3'	5'-TCCTGGGATGGAGGTCCTCT -3'
P450 2J9	5'-CTGCATGGGAGACACTGGTG -3'	5'-CTCCCATTTGAGCCGGTAAAATTAAGGTGAC -3'	5'-TCTTAGTCTCATTGCACGCACTC -3'
P450 2JX	5'-GAAAACGTGCTTGTCTTGGAGA -3'	5'-TGATAAAAATGAACAGCTCAGACCTGGCCAG -3'	5'-GGGCTTGAAGGTAAATTTTTGGA -3'
ARP-1	5'-TGCAGATCACGGACCAGGT -3'	5'-GCCCTCCTTCGCCTCACCTGG -3'	5'-CGCATTCAACACGAACAGCT -3'
Ten-m2	5'-AGACAGAATGAGATGGGAAAGAGG -3'	5'-AACAAAATAACCTGCTGCCACCTCTTCTCTG -3'	5'-GTTGCTCCTGCTGAGCCAC -3'
3-OST4	5'-CCTGAGGTTGGCAAAGATGAA -3'	5'-GCTTGTAACTGTGGGTATCAGGCAAACCC -3'	5'-GCCACCCACCAAGAGGCT -3'
EST AA982708	5'-GCCTACATGATACCAGAAGGGTG -3'	5'-TGCTCTACCAGCTCCACACATGCTTGA -3'	5'-GCCAGCAGCCAAGACTTTAAA -3'
Cyclophilin	5'-TGTGCCAGGGTGGTGACTT -3'	5'-ACACGCCATAATGGCACTGGTGG -3'	5'-TCAAATTTCTCTCCGTAGATGGACTT -3'

Microdissection. ARC, VMH, and DMH tissue were microdissected out of a series of 10 evenly spaced Nissl-stained sections covering the region from 1.46 mm to 1.94 mm caudal to bregma as defined by The Mouse Brain in Stereotaxic Coordinates (Franklin and Paxinos) using a Pixcell II Laser-Capture Microdissection System (Arcturus, Mountain View, CA). Microdissection was performed using a beam-width of 15µm, using maximum power and a beam duration of 100 ms. Each sample of collected tissue, adherent to the underside of the LCM cap, was then dissolved in 50µl of digestion buffer (Stratagene, La Jolla, CA) and frozen at -80^0C. Later, RNA was extracted from these samples using the Absolutely RNA microprep kit (Stratagene, La Jolla, CA). The digested tissue from all ten evenly spaced sections were pooled prior to RNA extraction to eliminate any potential rostral-caudal gene expression bias in subsequent analysis. Typical pools contained roughly 20 ng of RNA.

Microarrays

RNA Amplification and Hybridization. RNA collected from microdissected tissue was amplified using the MessageAmp system (Ambion, Austin, TX). This system utilizes T7 polymerase to produce a linear amplification, with large quantities of antisense RNA as product. This aRNA was labeled with either Cy3 or Cye5 dyes via the following protocol. For each sample, 8 µg of aRNA added to 10µg of random hexamers (Promega, Madison, WI) in a total volume of 24µl in DEPC water. Samples were incubated at 70^0C for 10 min, chilled on ice and spun down. To each sample was added 10µl 1st strand buffer for Superscript II (Invitrogen, Carlsbad, CA), 5µl of 0.1M DTT, 1µl of 25mM d(AGC)TP, 2µl of 1mM dUTP-Cy3 or Cy5 (Amersham, Buckinghamshire, UK), 2µl of

1mM dTTP, 2µl of Rnasin (Promega, Madison, WI), and 4µl Superscript II (Invitrogen, Carlsbad, CA). The samples were incubated at room temperature for 10 min, followed by incubation at 37^0C for 2 hours. Subsequently, the samples were treated with two units of Rnase H (Promega, Madison, WI), for 20 min at 37^0C. The labeled cDNA was then purified using the QiaQuick PCR purification kit (Qiagen, Los Angeles, CA) according to the manufacturer's instructions, with elution in two rounds totaling 80µl. The eluted cDNA was then concentrated down to less than 20 µl using Microcon YM-10 columns (Millipore, Billerica, MA), spun at 12,000g.

Microarray Hybridization. Labeled probes were hybridized onto cDNA arrays in a Genetac cDNA Hybridization Station (Genomic Solutions, Ann Arbor, MI) according to the manufacturer's instructions. Two different cDNA arrays were probed. The first contained 27,588 features comprised of Incyte, NCI, and Image Consortium clones with 4 bacterial genes as controls. The second array was made from a normalized mouse hypothalamic cDNA library comprised of roughly 5000 clones, constructed by Dr. Marcelo Soares. This library was the result of subtraction of a parent hypothalamic library with a mouse total brain library to select for hypothalamic-enriched clones. Both arrays were printed at the Albert Einstein College of Medicine microarray facility. Following washing, slides were imaged using a Scanarray Lite scanner (GSI Lumonics, Northville, MI) and ScanArray Express software (PerkinElmer, Wellesley, MA) at 532nm and 635nm. Thus, for each slide separate TIF files were produce for the Cy3 and Cy5 signals.

Data Analysis. Slide images were analyzed using GenePix 3.0 software (Molecular Devices, Sunnyvale, CA). Results from each slide were normalized via an intensity dependent normalization using a rank-invariant technique to select the normalization set (Tseng et al., 2001; Yang et al., 2002). Results are averages of two separate sets of experiments.

In Situ Hybridization Histochemistry

Tissue Preparation. Expression of candidate VMH marker genes was verified by in situ hybridization histochemistry as previously described (Marcus et al., 2001; Liu et al., 2003a), using 8 week old female C57Bl6/J mice. For studies of Cbln1 expression in SF-1 knockout mice, mice with a brain-specific KO of SF-1 were generated using a conditional SF-1 allele and the nestin-Cre transgene (The Jackson Laboratory, Bar Harbor, ME) and sections from the mediobasal hypothalamus were analyzed by in situ hybridization with the Cbln1 probe described below. Mice were anesthetized with 600 mg/kg intraperitoneal (IP) chloral hydrate (Sigma-Aldrich, St. Louis, MO) and perfused intracardially with 10% neutral buffered formalin solution (Sigma-Aldrich, St. Louis, MO). Brains were dissected out and post-fixed for 4 hours in 10% formalin at RT. Subsequently, the brains were dehydrated overnight at 4^0C with 30% sucrose in 1X PBS. The brains were then sectioned to a thickness of 25µm using a cryostat (Leica, St. Gallen, Switzerland). Sections were stored in antifreeze solution (5 parts 10% neutral buffered formalin, 3 parts ethylene glycol, 2 parts glycerol) at -20^0C until needed, at which time they were mounted on Superfrost slides (Fisher Scientific, Hampton, NH), dessicated and frozen until use at -20^0C.

Probe Manufacturing. Probes for ISHH were manufactured by cloning fragments of the LBH2, Cbln1, and PACAP cDNAs amplified by PCR first into the pGEM-T vector (Promega, Madison, WI), and then they were excised by EcoR1 digestion and cloned into the EcoR1 site of pBluescript IIKS+ (Promega, Madison, WI). Depending on insertion, plasmids were then cut with either Sal1 or Not1, and both sense and antisense ^{35}S probes were created by transcription with either T7 or T3 polymerases (Promega, Madison, WI) according to the manufacturer's protocol.

Hybridization. Slides were fixed in 4% paraformaldehyde in DEPC-treated 1X PBS at 4^0C for 20 min, dehydrated with a graded alcohol series (3 min each of 50%, 70%, and 95% EtOH, quick wash in 100% EtOH, and then 3 min in 100% EtOH), cleared with xylene for 15 minutes, rehydrated with the reverse ethanol series, and placed in prewarmed sodium citrate buffer (95^0C, pH 6.0), and microwaved in a commercial oven (1100 watts) for 10 minutes at 70% power. The slides were then dehydrated with the same alcohol series and air-dried. Probes were diluted to 10^6 cpm/ml in hybridization solution (50% formamide, 10 mM Tris-Hcl pH 8.0, 5.0 mg tRNA (Roche Diagnostics, Indianapolis, IN), 10mM DTT, 10% dextran sulfate, 0.3M NaCl, 1mM EDTA pH 8.0, 1X Denhardt's solution (Sigma-Aldrich, St. Louis, MO)). Hybridization solution was applied to the slides, and they were coverslipped and incubated overnight at 57^0C. Subsequently, the coverslips were removed, and the slides were washed with 2X sodium chloride/sodium citrate buffer pH7.0 and incubated with 0.002% RNAse A (Promega, Madison, WI) in 0.5M NaCl, 10mM Tris-HCl pH 8.0, 1mM EDTA for 30 min. Sections

were rinsed in SSC (2X for 1 hour at 50^0C, 0.2X for 1 hour at 55C, and 0.2X for 1 hour at 60^0C). They were then dehydrated with an ethanol series containing 0.3M NH4Oac followed by 100% ethanol. The slides were air-dried and exposed to VMR-2 film (Kodak, NY), for 2-3 days. Next, the films were developed, and the slides were dipped in NTB2 photographic emulsion (Kodak, Rochester, NY), dried and stored in sealed boxes for roughly 2 weeks. Slides were developed with D-19 developer (Kodak, Rochester, NY), counterstained with thionin, and coverslipped with Permaslip (Alban Scientific, St. Louis, MO).

FACS-sorting of SF-1 Neurons

Mice bearing an eGFP transgene under the control of the SF-1 promoter (SF-1-eGFP) were previously described (Stallings et al., 2002). These mice were bred onto WT, SF-1 +/- (Het), or SF-1 -/- (KO) backgrounds and males were sacrificed at E16.5. Brains were dissected and trypsinized, and eGFP-positive neurons were resolved by FACS essentially as described (Motoike et al., 2000). RNA was isolated using Trizol (Invitrogen, Carlsbad, CA), and was reverse transcribed using SuperScript III reverse transcriptase (Invitrogen, Carlsbad, CA) according to the manufacturer's instructions. Real-time PCR was performed on the cDNA as described above.

Expression of LBH2-EGFP fusions protein in 293T Cells

Cloning. A 1 kb fragment of the EGFP cDNA (lacking the ATG) was amplified by PCR (forward primer 5'-CGGAATTCTCGTGAGCAAGGGCGAGGA-3' with a 5' EcoR1 site, reverse primer 5'-CCGCTCGAGCGATTTCGGCCTATTGG-3' with a 5' Xho1

site), and ligated into the vector pcDNA3.1(+) following digestion with EcoR1 and Xho1 to produce the plasmid pcDNA.xEGFP. Next, a 700 bp fragment of the LBH2 cDNA, containing 120 bp of 5' untranslated sequence and lacking a stop codon, was amplified by PCR (forward primer 5'-CGGGATCCGACCAGGGAGGCGAG-3' with 5' BamH1 site, reverse primer 5'-CGGAATTCCGAGAGTAGAGCCTGCCACA-3' with 5' EcoR1 site) and ligated into pcDNA.xEGFP following digestion with BamH1 and EcoR1, producing the vector pcDNA.LBH2xEGFP. The resulting plasmid expresses a fusion protein of LBH2 with EGFP, with the initiator methionine of EGFP replaced by a Gly-Ile linker, under the control of the CMV promoter. The upstream region of LBH2, as well as the LBH2 Kozak sequence and initiation codon are intact, in order to test their effectiveness *in vitro*.

Cell Culture and Transfection. HEK-293T cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 μg/ml streptomycin sulfate, and 250 ng/ml amphotericin B (Invitrogen, Carlsbad, CA) in a 37^0C incubator with 5% CO_2. Cells were grown on 10 cm plates to 90% confluence and transfected with 20 μg of either pcDNA.LBH2xEGFP or the control plasmid pcDNA.xEGFP using Lipofectamine 2000 (Invitrogen, Carlsbad, CA), according to the manufacturer's instructions. Cells were imaged the following day for EGFP fluorescence using an Axiovert 200 inverted fluorescence microscope (Zeiss, Oberkochen, Germany), fitted with an LD A-Plan 40X objective (Zeiss, Oberkochen, Germany).

Generation of LBH2-GFP BAC Transgenic Mice

Creation of Shuttle Vector. The BAC RP24-94019, containing the LBH2 gene and roughly 100kb both upstream and downstream, was purchased from the BACPAC Resource Center of the Children's Hospital Oakland Research Institute (Oakland, CA). A ~450 bp fragment (LBH2 homology box) of genomic DNA directly 5' to the ATG start site of LBH2 was amplified by PCR (forward primer 5'-TTGGCGCGCCAGCCACCTGACTCAAACTTG-3' including a 5' Asc1 restriction site, reverse primer 5'-GCTGTTGCTATTGCTACTGGGC-3'). This PCR fragment was digested with Asc1 enzyme and ligated into the shuttle vector pLD53.SCA-E-B, which had been digested with Asc1 and Sma1.

BAC Modification. BAC modification was performed as previously described (Gong et al.). E. coli harboring BAC RP24-94019 were grown in Luria broth (LB) (Invitrogen, Carlsbad, CA), supplemented with chloramphenicol (20 μg/ml) to an OD600 of 0.6-0.8. Cells were harvested by centrifugation at 3,000 x g for 10 min at 4^0C, and were resuspended in 5ml of ice-cold 50mM $CaCl_2$. Centrifugation and resuspension were repeated. Finally, the cells were resuspended in 300 μl of 50mM $CaCl_2$ plus 20% glycerol, yielding BAC competent cells. 100 μl of competent cells were transformed with 40 ng of the RecA expressing vector pSV1.RecA, and the cells were shaken at 30^0C for 1 hr following addition of 1 ml of SOC medium (Invitrogen, Carlsbad, CA). 5 ml of LB supplemented with 10 μg/ml tetracycline and 20 μg/ml chloramphenicol (Sigma-Aldrich, St. Louis, MO) was added, and the cells were incubated overnight in a 30^0C shaker. The next day, 1 ml of overnight culture was added to 50 ml LB with 10 μg/ml

tetracycline and 20 μg/ml chloramphenicol, and the cells were grown at 30^0C with shaking to an OD600 of 0.6 – 0.8. Cells were harvested by centrifugation at 3000 rpm for 10 min at 4^0C, and resuspended in 50 ml of cold 10% glycerol. This washing step was repeated, and then finally the cells were spun down and resuspended in 200 μl of 10% cold glycerol. Next, these competent cells were transformed with the modified shuttle vector. 1 μg of shuttle vector DNA was added to 40 μl of competent cells, and the mixture was incubated on ice for 1 min. Next, the mixture was added to a 0.1 cm cuvette (BIO-RAD, Hercules, CA) and electroporated with the following conditions: capacitance 25 μF, voltage 1.8 kV, resistance 200 Ohms. After electroporation, 1 ml of SOC medium was added to the cells, and they were shaken in 15 ml tubes at 30^0C for 1 hr. Transformed cells were selected by adding 5 ml of LB containing 10 μg/ml tetracycline, 20 μg/ml chloramphenicol, and 50 μg/ml ampicillin, with shaking overnight at 30^0C. The following day, 100 μl of cells were spread onto LB agar plates containing 20 μg/ml chloramphenicol and 50 μg/ml ampicillin, and the plates were incubated overnight at 43^0C. Genomic DNA for genotyping was procured using the Wizard SV Genomic DNA Purification System (Promega, Madison, WI) according to the manufacturer's instructions.

Screening. Colonies from the above plates were screened to find clones which had undergone the proper targeted recombination. Screening of BAC DNA was performed by Southern blot as described above, using the original LBH2 homology box as a probe. Original unmodified BAC was used as a control. Positive clones were those for which the original band had been replaced by two new bands of different size.

BAC Purification, Transgenesis, and Genotyping. The correctly modified BAC was purified via the cesium chloride method and injected into FVB/NJ pronuclei by the staff at GENSAT (Rockefeller University, NY). Founder mice, and subsequent progeny, were screened by PCR (forward primer 5'-CCTACGGCGTGCAGTGCTTCAGC-3', reverse primer 5'-CGGCGAGCTGCACGCTGCGTCCTC-3') for the presence of a ~500 bp band.

Immunohistochemistry

Tissue Preparation. 6-10 week old mice were anesthetized with 600 mg/kg chloral hydrate (Sigma-Aldrich, St. Louis, MO) IP and perfused intracardially with 4% paraformaldehyde in 1X phophate-buffered saline (PBS). Brains were removed and post-fixed overnight in 4% paraformaldehyde. For vibratome sectioning, the brains could either be immediately sectioned after post-fixation, or could be stored indefinitely at 4^0C in 1X PBS. For cryostat sectioning, brains were dehydrated following post-fixation by immersion for two days in 30% sucrose in 1X PBS. Brains could be stored in 30% sucrose/1X PBS for up to two weeks before sectioning.

Basic Immunostaining. Free-floating 50 µm vibratome sections or 20 µm cryostat sections of mouse brain, prepared as above, were washed 3 x 15 min in PBS, followed by blocking for 1 hr at room temperature in blocking solution composed of 3% bovine serum albumen (Cat# A9647, Sigma-Aldrich, St. Louis, MO), 2% goat serum (Cat# G9023, Sigma-Aldrich, St. Louis, MO), and 0.1% Triton X-100. Next, sections were incubated

overnight at 4^0C with primary antibody diluted in blocking solution. The following day, the sections were washed 3 x 15 min in PBS, and then incubated at room temperature for 2 hours in blocking solution plus secondary antibody antibody (goat anti-mouse or goat anti-rabbit antibody conjugated to either Alexa 488 or Alexa 594, Molecular Probes, Eugene, OR) at a concentration of 1:1000. Subsequently, the sections were washed 3 x 15 min in PBS, mounted on 25mm x 75mm Superfrost/Plus slides (Fisher Scientific, Hampton, NH), and coverslipped using Fluoromount-G (Southern Biotech, Birmingham, AL). Primary antibodies used with this protocol include rabbit anti-GFP antibody (Cat# A6455, Molecular Probes, Eugene, OR) diluted 1:1000, mouse monoclonal anti-GFP antibody (Cat# MAB3580, Chemicon, Temecula, CA) diluted 1:1000, custom made rabbit anti-LBH2 antibody (Washington Biotech, Columbia, MD) diluted 1:1000, and mouse anti-NeuN antibody (Cat# MAB377, Chemicon, Temecula, CA) diluted 1:2000.

Anti-pSTAT3 Immunostaining. 8-10 week old mice were injected with 1 mg/kg leptin in 1x PBS 45 minutes prior to perfusion with 4% paraformaldehyde. Following overnight post-fixation and cryoprotection with 30% sucrose in 1X PBS, 20 µm brain sections were cut on a cryostat and washed in PBS for 15 minutes. The sections were then treated with 1% H202/1% NaOH in H20 for 20 minutes, 0.3% glycine for 10 min, and 0.03% SDS for 10 min, all at room temperature. Sections were then incubated at room temperature for one hour with blocking solution, consisting of 3% goat serum, 0.25% Triton X-100, and 0.2% sodium azide in 1X PBS. Sections were then incubated for 48 hours at 4^0C with rabbit anti-pSTAT3 antibody (Cat#9131S, Cell Signaling Technology, Cambridge, MA), at a dilution of 1:3000 in blocking solution. Next, the sections were washed 3 x 15 min

in PBS and incubated with biotinylated goat anti-rabbit secondary antibody (Cat# BA-1000, Vector Laboratories, Burlingame, CA) diluted 1:1000 in the same blocking solution for 1 hr at room temperature. The sections were then washed 3 x 15 min in PBS, and treated for 30 minutes at room temperature with avidin-biotin-horseradish peroxidase (ABC) complex (Vector Laboratories, Burlingame, CA). Sections were washed for 10 min in PBS, and then treated for 2-5 minutes with the peroxidase substrate DAB (Vector Laboratories, Burlingame, CA). The sections were mounted onto 25mm x 75mm Superfrost/Plus slides (Fisher Scientific, Hampton, NH), and were left to air-dry overnight. The following day, the slides were dehydrated with a graded ethanol series (50%, 75%, 95%, and 100%, each for 2 min), followed by a 2 min immersion in citrus clearing solvent (Richard Allan Scientific, Kalamazoo, MI). Following a thorough drying, the slides were coverslipped using Permount (Fisher Scientific, Hampton, NH). When coupling this staining protocol with anti-GFP immunofluorescence staining, the sections were washed in PBS following DAB staining, and then were transferred into the basic immunostaining blocking solution and then stained exactly as described above. Following fluorescent secondary antibody staining, the sections were mounted, dehydrated and coverslipped with Permount as described in this section.

Anti-Estrogen Receptor-Alpha Immunostaining. 20 μm brain sections were cut on a cryostat and washed 3 x 15 min in PBS. Sections were then blocked for 1 hr in blocking solution consisting of 1% goat serum and 4% (yes, 4.0%) Triton X-100 in PBS. The sections were then incubated with rabbit anti-estrogen receptor alpha antibody (Upstate, Lake Placid, NY), diluted 1:20,000 in the same blocking solution, for 48 hrs at 4^0C.

Next, the sections were washed 3 x 15 min in PBS, and then were incubated with goat anti-rabbit Alexa 594-conjugated secondary antibody (Molecular Probes, Eugene, OR) for 2 hrs at a dilution of 1:1000 in PBS supplemented with 1% goat serum. Following washing for 3 x 15 min in PBS, the sections were mounted on 25mm x 75mm Superfrost/Plus slides (Fisher Scientific, Hampton, NH), and coverslipped using Fluoromount-G (Southern Biotech, Birmingham, AL). If this protocol was used for dual immunofluorescence staining, the other primary antibody (such as mouse monoclonal anti-GFP (1:1000 dilution, Chemicon, Temecula, CA), was added along with the anti-ER-alpha primary antibody, and an additional appropriate secondary antibody was used along with the goat anti-rabbit 594 conjugate.

Pseudorabies Virus Ba2001TK+

Virus culture and preparation. PK15 cells were cultured in Dulbecco's Modified Eagle's Medium (Invitrogen, Carlsbad, CA), supplemented with 10% fetal bovine serum, 100 U/ml penicillin G, 100 µg/ml streptomycin sulfate, and 250 ng/ml amphotericin B (Invitrogen, Carlsbad, CA) in a 37^0C incubator with 5% CO_2. Cells were grown on 10 cm plates to 90% confluence and infected with Ba2001TK+ at an MOI of 1:100. After roughly two days, when cell lysis was nearly complete, the cells and medium were collected and centrifuged for 2 min at 1000 x g. All but 2 ml of supernatant was discarded, the pellet was resuspended, and the solution was subjected to two rounds of freeze-thaw. Subsequently, 100 µl aliquots were dispensed into eppendorf tubes, and these were frozen at -80^0C until use. Typical titers used were 1 x 10^8 pfu/ml.

Stereotaxic injection of Ba2001TK+. 8-12 week old male AgRP-cre mice or C57Bl6/J control mice were anesthetized with IP ketamine/xylazine (60µl of a 25mg/ml ketamine and 25 mg/ml xylazine cocktail). They were then placed in a stereotaxic apparatus (Kopf Instruments, Tujunga, CA), restrained by ear bars. An incision was made in the scalp, and the fascia was removed by swabbing with 3% hydrogen peroxide, revealing the major skull landmarks bregma and lambda. Using the stereotaxic apparatus, a small hole was drilled in the skull, and a Hamilton syringe containing Ba2001TK+ was passed through so that it would reach the coordinates 1.45 mm caudal to bregma, 0.25 mm lateral to bregma, and 5.7 mm beneath the surface of the skull, the correct coordinates for the ARC. 0.15 µl of virus was injected over a period of 15 minutes, and the needle was left to rest for 5 minutes. Subsequently, the needle was removed slowly and the incision was sutured. At or before first observance of symptoms (including irritability, lethargy and weight loss), animals were perfused intracardially with 4% paraformaldehyde and brains were processed for immunohistochemistry as described above.

Microscopy

In Situ Hybridization and Immunofluorescence Imaging. Slides were imaged on an Axioplan 2 imaging microscope (Zeiss, Oberkochen, Germany) fitted with a Plan-Neofluar 5x/0.15NA objective using either a Spot Insight QE color digital camera (OpelCo, Dulles, VA) or an AxioCam MRm CCD camera (Zeiss, Oberkochen, Germany). Images were collected using AxioVision 3.1 Zeiss software.

Confocal Imaging. Slides were imaged on an Inverted Zeiss Axiovert 200 confocal

microscope using LSM 510 software (Zeiss, Oberkochen, Germany), fitted with a C-Apochromat 40x/1.2 NA water immersion objective (Zeiss, Oberkochen, Germany).

Chapter 3: Results

Microdissection of Hypothalamic Nuclei

In order to isolate VMH-enriched transcripts, we conducted a gene expression comparison of hypothalamic nuclei. Laser-capture microdissection (LCM) was used to isolate tissue from hypothalamic nuclei. In this technique, a cap covered with a thermoplastic transfer film is laid over a slide on which is mounted dehydrated, stained, cryostat-sectioned tissue. When cells of interest within the tissue are identified via microscopy, a near-infrared laser is activated, focally bonding the tissue to the film. When the cap is removed, the cells of interest remain bound to the transfer film on the cap, while the remaining tissue stays on the slide. The cap can then be placed on an eppendorf tube containing digestion buffer to extract nucleic acids from the dissected tissue. LCM allows for far greater precision and reproducibility than punch-out approaches, making it well suited for applications that require extremely accurate dissection of complex three-dimensional structures (Emmert-Buck et al., 1996; Bonaventure et al., 2002). In this case, because the comparisons were to be between closely adjacent nuclei, a very high premium was placed on accuracy.

LCM was used to isolate VMH, ARC, and DMH tissue from Nissl-stained hypothalamic sections of 8-week-old female C57BL/6J mice. The dissected tissue and the residual sections from a typical microdissection are shown (Fig. 6). We focused on the dorsomedial VMH for two reasons. First, this area contains nearly all of the leptin receptor in this nucleus, implicating this area in the response to leptin and in energy homeostasis (Mercer et al., 1996). Second, it is also the most neuron-dense region of the

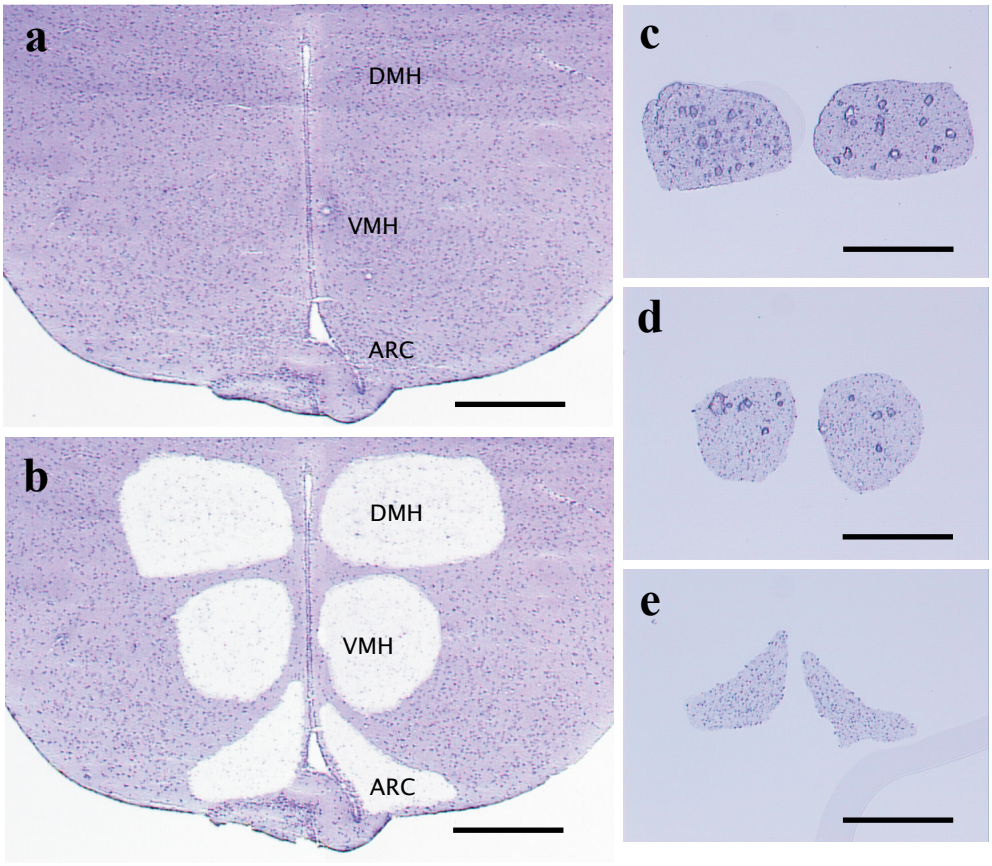


Figure 6 *Laser-Capture Microdissection of Hypothalamic Nuclei*
(a - b) A Nissl-stained section of mouse hypothalamus before (a) and after (b) sequential dissection of VMH, DMH, and ARC tissue. (c - e) Images of dissected DMH (c), VMH (d), and ARC (e) tissue, which is adherent to the cap. Scale bars, 500 µm.

VMH. Dissecting only this subset of the VMH minimized glial contamination, which was found in some cases to dilute the contributions from VMH neurons. As sources of RNA for comparison with the VMH, the ARC was chosen because several ARC-specific genes have already been identified and because this region is adjacent to the VMH (Elmquist, 2001). We also analyzed the DMH, another mediobasal nucleus adjacent to the VMH with similar neuron density also implicated in energy balance and other physiologic processes (Bernardis and Goldman, 1976; Gallo, 1981). For each sample, 10 evenly spaced sections were dissected and the collected nuclear tissue was pooled to minimize anterior-posterior bias. RNA was collected from the tissue using a commercial kit. The total mass of RNA in each pooled sample was approximately 20ng.

Amplification and Microarray Hybridization

To assay RNA integrity, a large sample of tissue was collected from a single slide, in order to yield a large enough sample of RNA for analysis. When this RNA was run on a 2100 BioAnalyzer (Agilent, Palo Alto, CA), 28S and 18S rRNA peaks were clearly visible (Fig. 7a-b), indicating that the RNA isolated from LCM-dissected samples was relatively intact.

RNA was amplified using a T7-based system. T7-based systems amplify RNA in a linear manner, in contrast with the exponential amplification produced by PCR, allowing for more accurate microarray results. Amplification was performed in two rounds, the first round with a poly-T-T7 primer, and the second with random primers (Fig. 7c). This amplification scheme produced antisense RNA products that were heavily 3' weighted, averaging 400-600 bp in length. However, this type of product is quite compatible with

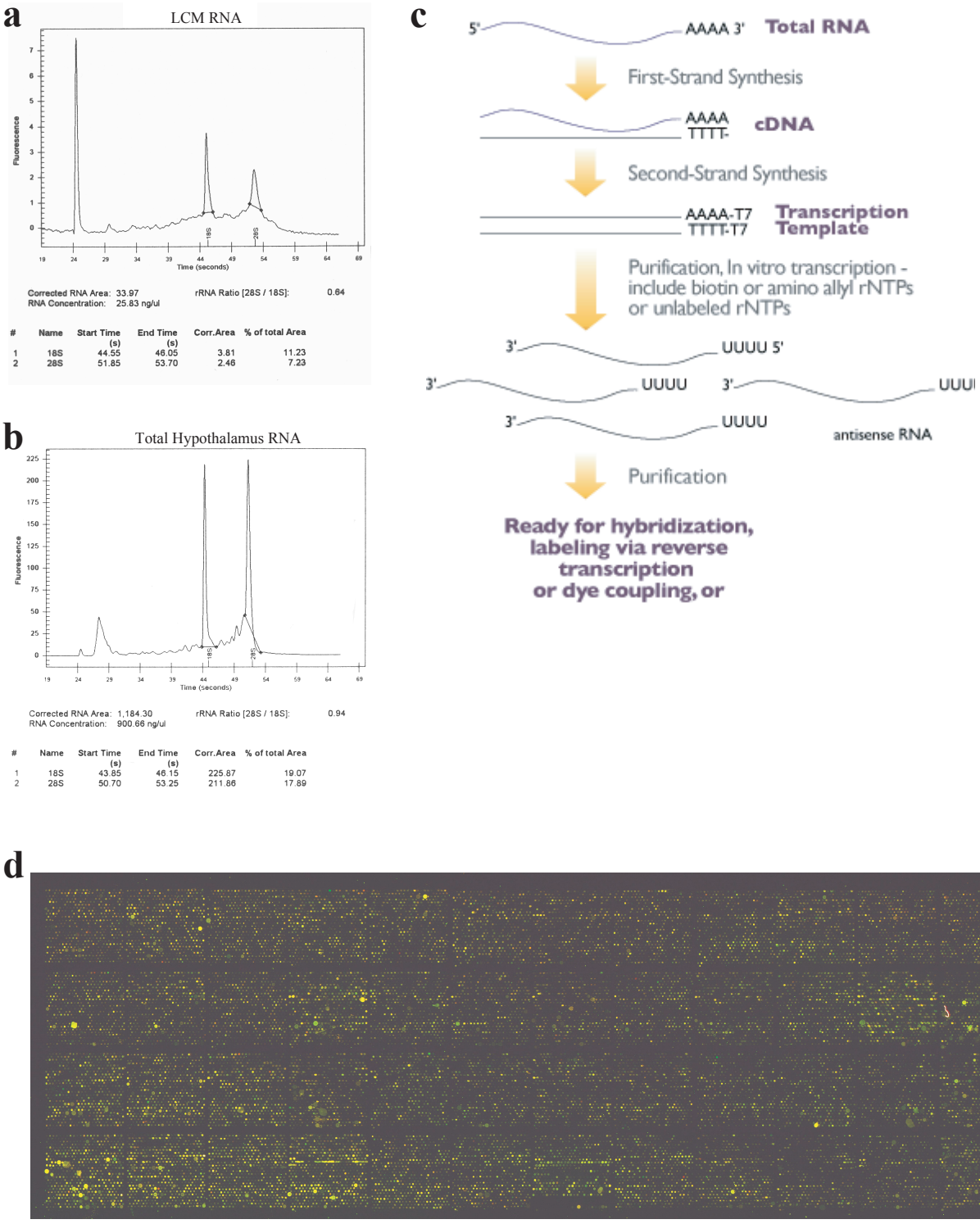


Figure 7 *RNA Analysis, Amplification, and Microarray Hybridization*
An oversized LCM-dissected RNA sample (a) and total hypothalamus RNA (b) were compared on an Agilent 2100 Bioanalyzer. The LCM sample shows distinct 28S and 18S peaks, indicating relatively intact RNA with modest degradation. (c) Schematic of the T7-based linear amplification scheme used to amplify RNA collected from LCM samples (adapted from Ambion). LCM RNA was amplified in two independent rounds. (d) Typical microarray hybridization result. A 28,000 gene M2 slide hybridized with labeled amplified ARC (Cy3, green) and VMH (Cy5, red) probes.

cDNA microarrays, because cDNA libraries tend to have the same 3' bias.

RNA products from each nucleus were labeled with either Cy3 or Cy5 dye and used to probe each of two different cDNA microarrays. The first array (named M2) was a collection of 28,000 cDNA clones comprising 20,000 unique genes (Fig. 7d). This array was composed mostly of embryonic and total mouse cDNA clones. The second array (NMHY) was composed of a set of 5000 cDNA clones from a normalized mouse hypothalamic cDNA library, which was selected for hypothalamic enrichment by subtraction against a library from total brain. The hypothalamic-specific array was used in combination with the general mouse arrays in order to yield the best possible gene coverage.

The labeled RNA from each of the three nuclei was compared in pairwise manner against labeled RNA from each of the other two, using reverse-color hybridization as an added control. Microarray results for each of the comparisons are shown (Fig. 8). Overall, it was evident that the vast majority of genes (>99%) are expressed at similar levels among the three nuclei. Nonetheless, it was possible to identify individual gene products that were enriched in specific nuclei. For example, in the arcuate nucleus, a number of previously identified ARC-specific genes were revealed, including POMC, NPY, and neurokinin B (Gee et al., 1983; Gehlert et al., 1987; Marksteiner et al., 1992). This was a good first confirmation that the method was effective for identifying nucleus-specific genes. For each of the nuclei, the up-regulated genes were analyzed further with respect to their relative levels of expression.

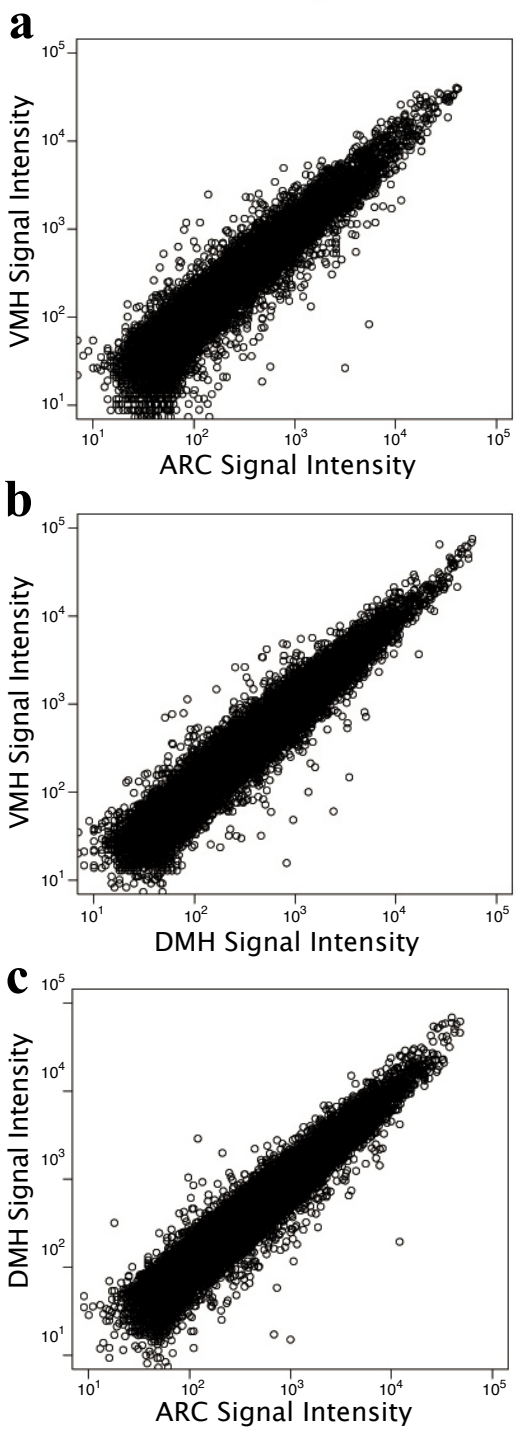


Figure 8 *Microarray Analysis of RNA from Hypothalamic Nuclei* Normalized ARC versus VMH (a), DMH versus VMH (b), and ARC versus DMH (c) cDNA microarray results on log-log intensity-value plots.

Patterns of Transcription in Hypothalamic Nuclei

A macroscopic analysis of these genes revealed interesting information with respect to transcription in these nuclei. The data revealed a substantial overlap between the set of genes with high ARC/DMH ratios and those with high ARC/VMH ratios (Fig 9a). This was expected because many ARC-enriched genes have already been identified. This same pattern was evident when analyzing the expression profile of the VMH-enriched genes, insofar as genes with high VMH/ARC ratios were also enriched in the VMH versus the DMH (i.e., they had high VMH/DMH ratios) (Fig. 9b) In contrast, there was very little overlap between genes with high DMH/ARC ratios and those with high DMH/VMH ratios (Fig. 9c). Together, this suggests that the ARC and VMH express a number of nucleus-enriched genes relative to adjacent areas of the mediobasal hypothalamus. In contrast, it was substantially more difficult to identify RNAs in DMH that were not expressed in either the VMH or ARC. Thus, the VMH, like the ARC, should have its own unique transcriptional network, whereas the DMH seems to share its transcriptional profile with other adjacent nuclei.

VMH Marker Genes

The genes with increased signals in the VMH samples compared with both the DMH and ARC were further analyzed to confirm that they were in fact VMH-enriched marker genes. In these experiments, we were especially interested in genes that showed the highest geometric mean of VMH/ARC and VMH/DMH ratios (i.e., a VMH score). Table 2 lists the genes with a VMH score >4.0. The list, which is headed by the canonical VMH marker gene SF-1, includes neuropeptides, ESTs, and several other genes. To

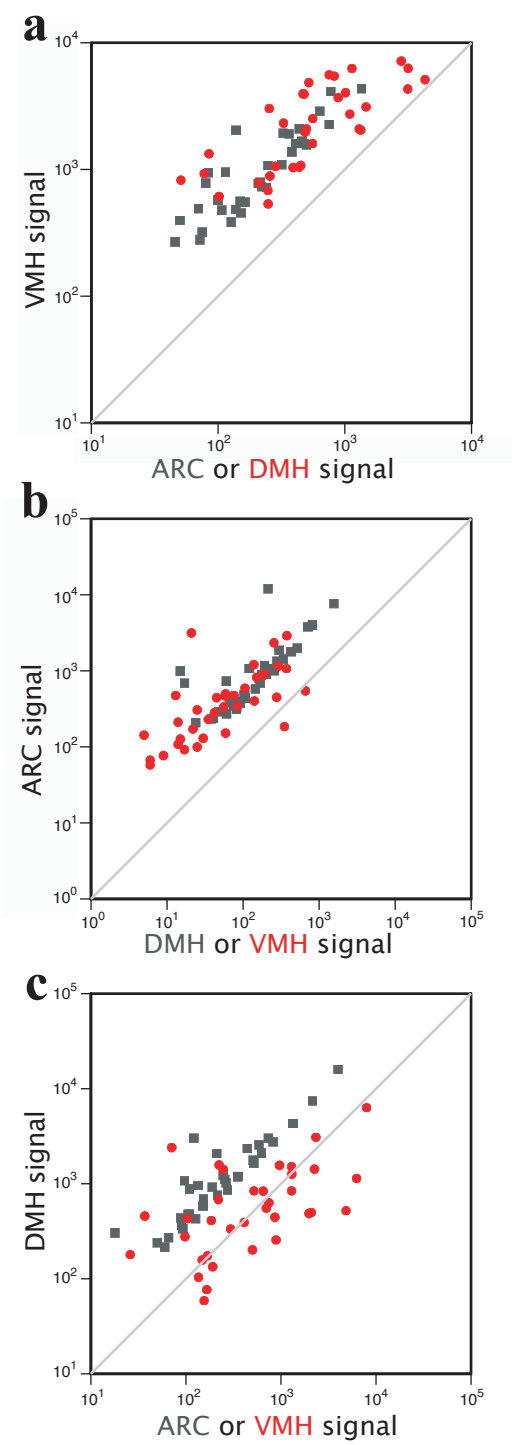


Figure 9 *Analysis of Marker Gene Expression in Hypothalamic Nuclei*
Marker gene comparisons using intentisy-value plots. (a) Genes with the highest VMH/ARC ratios (gray squares) also display high VMH/DMH ratios (red circles). (b) Genes with highest ARC/DMH ratios (gray squares also display high ARC/VMH ratios (red circles). (c) In contrast, only a small number of genes with high DMH/ARC ratios (gray squares) display high DMH/VMH ratios (red circles).

Table 2 - Top VMH-Enriched Genes

VMH marker candidates are listed by VMH score (geometric mean of VMH/ARC and VMH/DMH ratio). Common names and accession numbers are given, as well as the arrays on which the genes were found (28,000 gene total mouse and embryonic clone array, 5,000 gene subtracted hypothalamus array, or both).

Gene Name	Accession Number	Array	VMH/ARC ratio	VMH/DMH ratio	VMH Score
Steroidogenic factor 1	S65878	28k	7.92	15.66	11.14
Mouse homolog of Rattus norvegicus D123 gene product	BC024787	28k	7.00	16.18	10.64
Mus musculus cytochrome P450 CYP2J9 mRNA	AF336850	Hyp	16.41	5.75	9.71
Mus musculus cerebellin 1 (Cbln1) precursor protein	BC055730	Both	12.25	7.40	9.52
Slit homolog 3 protein precursor (Slit3)	AF144629	28k	7.07	11.91	9.18
Pituitary Adenylate Cyclase Activating Polypeptide (PACAP)	AB010149	Both	11.24	7.01	8.88
Mus musculus similar to limb-bud and heart (LBH2)	XM_354697	Hyp	6.62	9.50	7.93
Guanine deaminase	AF174583	28k	5.59	7.835	6.62
Heparan sulfate 3-O-sulfotransferase 4	AA030506	28k	5.75	6.02	5.88
EST - no significant homology	AA982708	Hyp	5.79	5.37	5.58
Mus musculus mRNA for Ten-m2	AB025411	28k	5.65	3.78	4.62
Apolipoprotein AI regulatory protein-1 (ARP-1)	X76653	28k	3.42	5.52	4.34

validate these microarray data, real-time PCR was performed for each of the top 12 VMH candidate genes on independent samples of RNA collected from the ARC, VMH, and DMH using LCM. These data confirmed the microarray results for 9 of these 12 genes: *SF-1, PACAP, Cbln1*, EST XM_354697, *Slit3, guanine deaminase, TenM2, 3-O-sulfo-transferase 4* (*3-OST-4*), and EST AA982708 (Fig. 10). Three of the nine VMH marker candidates were present exclusively on the 5000 gene hypothalamus-enriched array, highlighting the utility of this library and suggesting that some VMH genes are not expressed or are expressed at low levels elsewhere in the brain.

Although the real-time PCR data were consistent with the original microarray results for these nine genes, thus validating the screening approach, the data for *cytochrome P450 CYP2J9, D123*, and *ARP-1* did not recapitulate the array results. The basis for the apparent discrepancy for these three genes may be a result of cross-hybridization of RNAs with cDNA probes for other genes with sequence similarity. However, the consistency of the microarray results, especially with respect to *cytochrome P450 CYP2J9*, suggests the presence of real VMH-enriched transcripts. The cytochrome P450 family in particular is very large, with a number of genes with high degrees of sequence homology. Real-time PCR was performed on ARC, VMH, and DMH RNA using probes to unique and shared regions of CYP2J9 and closely related family members, but the family member responsible for the VMH-specific signal could not be isolated (data not shown).

Because the VMH is integrally involved in the regulation of food intake, and because other hypothalamic nuclei express genes that are regulated at the level of expression in response to changes in fed state, the validated VMH marker genes were

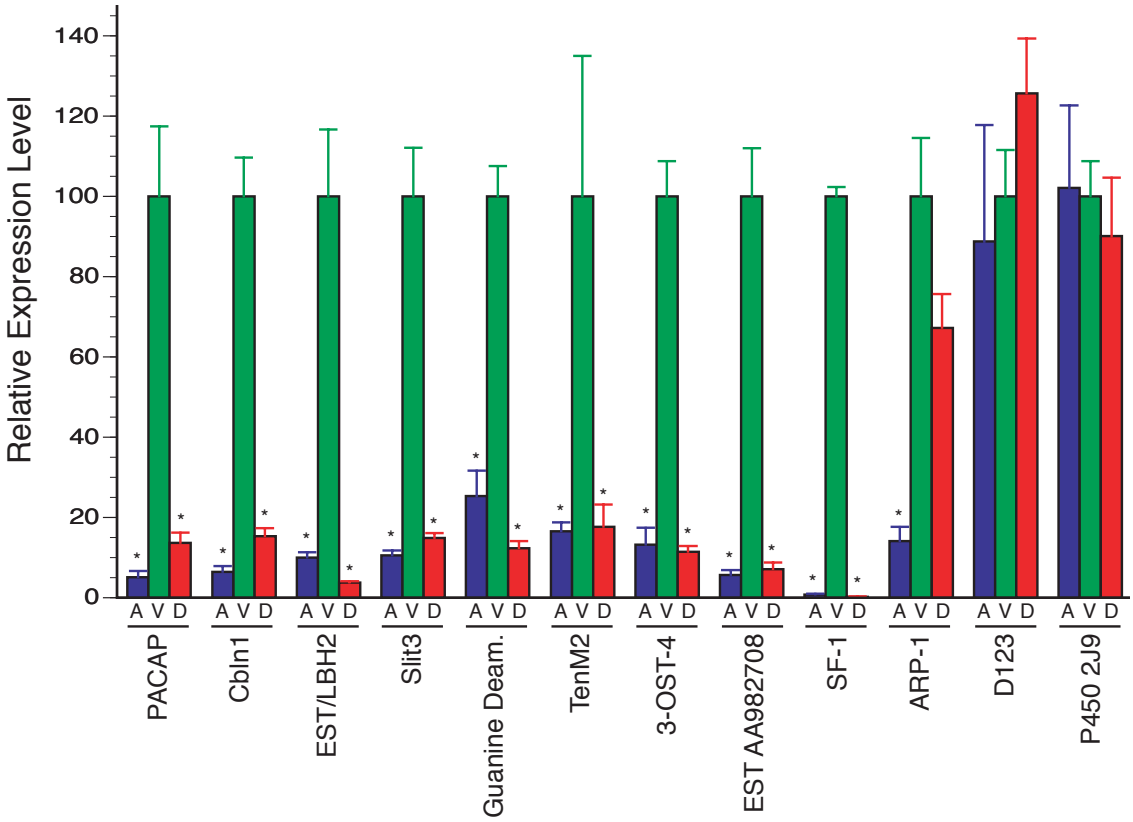


Figure 10 *Confirmation of Microarray Results by Real-Time PCR*
Twelve VMH candidate markers (*PACAP, Cbln1, EST/LBH2, Slit3, guanine deaminase, TenM2, 3-OST-4*, EST 982708, *SF-1*, *ARP-1, D123, and cytochrome P450 CYP2J9*) were tested by real-time PCR analysis using RNA from microdissected ARC (A), VMH (V), and DMH (D) tissue. For each gene, expression was normalized to cyclophilin, and results from different nuclei were then normalized to the VMH, with VMH expression set at 100. Values are expressed as mean +/- SEM. Statistical significance, *p< 0.05.

analyzed for modulation of gene expression in response to fasting. Whole hypothalamus RNA was isolated from 8 week old female mice which were either fasted for 24 hours or fed ad libitum, and the expression of the VMH markers was analyzed in these RNA samples. None of the genes showed differential expression in response to fasting (Fig. 11).

Validation by *In Situ* Hybridization

For the reasons stated above, the original dissection focused on the dorsomedial VMH, raising the possibility that these cDNAs might be localized in this region of the VMH. To further ensure that this screening system indeed produced legitimate VMH markers and to establish their distribution within the VMH, we endeavored to determine the general, and more specifically the hypothalamic, distribution of three of the genes: *PACAP, Cbln1*, and the hypothalamic array EST XM_354697 (discussed in detail below).

Cbln1 was initially isolated from rat cerebellum as a 16 aa peptide, and is derived from what was originally believed to be a precursor molecule, precerebellin (Umrath and Silberbauer, 1967; Urade et al., 1991). However, there are similarities between this precursor and circulating complement C1q (Urade et al., 1991). Additionally, the precursor molecules of Cbln1 and its family members have been shown to multimerize, indicating that the originally isolated 16 aa peptide may be a cleavage product of a larger, functional molecule (Pang et al., 2000). Cbln1 is known to be expressed in Purkinje cells of the cerebellum and cartwheel cells in the dorsal cochlear nucleus (Slemmon et al., 1984; Mugnaini and Morgan, 1987). Previously, Cbln1 was shown to be expressed at

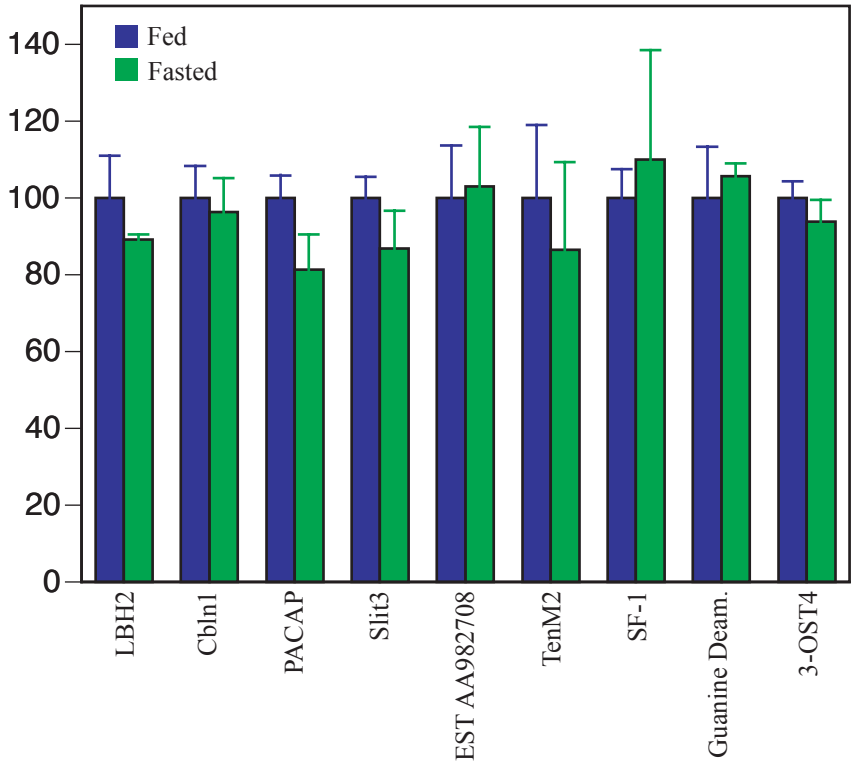


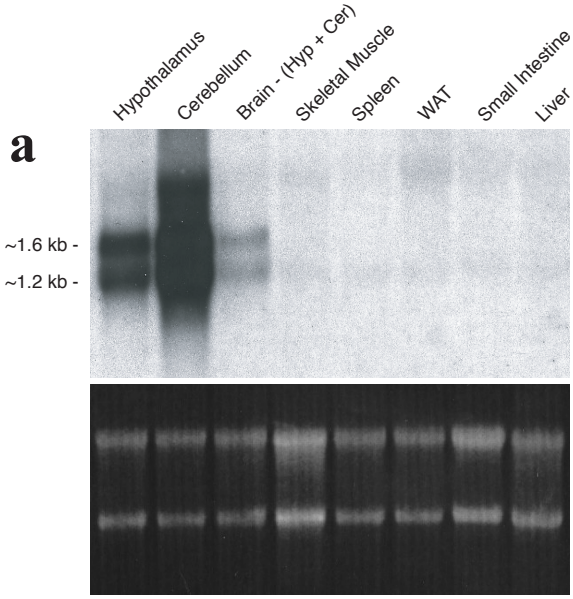
Figure 11 *Expression of VMH Marker Genes in Fed and Fasted Mouse Hypothalamus* Real-time PCR performed on total RNA from hypothalami of mice fed *ad libitum* (blue) or fasted for 24 hrs (green). Genes tested were *LBH2, Cbln1, PACAP, Slit3,* EST AA982708*, TenM2, SF-1, Guanine Deaminase, and 3-OST4*. For each sample, the resulting value was normalized against cyclophilin. Values for fed animals were set at 100. Values are expressed as mean +/- SEM.

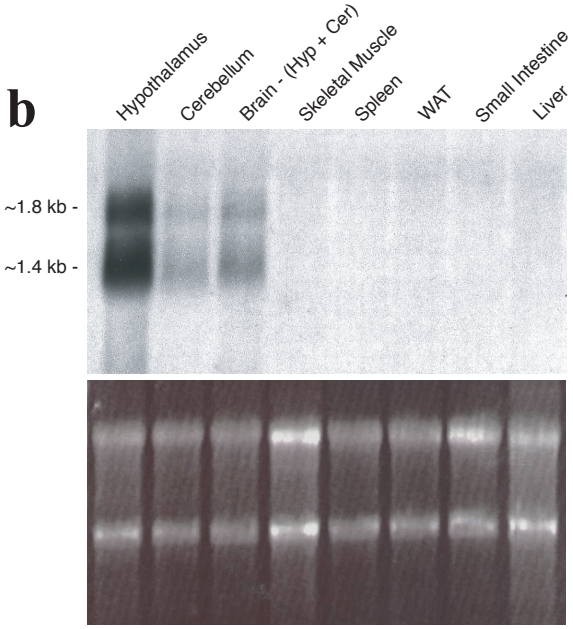
high levels in the cerebellum and hypothalamus by whole tissue radioimmunoassay (Morgan et al., 1988), but the details of its localization within the hypothalamus and most other brain regions have not been described. Tissue panel northern blot revealed that Cbln1 is expressed in the hypothalamus and cerebellin, and at lesser levels in other areas of the brain, but expression was not observed in any of the peripheral organs tested (Fig. 12a).

PACAP is a neuropeptide which comes in 27 aa and 38 aa forms, cleaved from a larger precursor protein, proPacap (Miyata et al., 1989). The CNS distribution of PACAP has previously been reported. In the brain, PACAP is expressed in a number of brain regions, including the hippocampus, the purkinje cell layer of the cerebellum, a number of brainstem nuclei, and in the hypothalamus, prominently in the VMH (Hannibal, 2002). By northern blot, PACAP is expressed strongly in the hypothalamus and to a lesser extent in other brain regions (Fig. 12b). PACAP signal was not observed in any of the peripheral tissues tested.

The EST from the hypothalamic library was listed in GenBank under the accession number XM_354697 and the name "similar to limb-bud and heart." Its characteristics are described in greater detail below. By northern blot, this gene is expressed most strongly in the hypothalamus, and to a lesser extent in the remainder of the CNS (Fig. 12c). Of the peripheral tissues tested, the expression of this gene was detected (albeit at low level) only in the spleen.

These three genes were analyzed by in situ hybridization, confirming that, within the hypothalamus, they are all highly enriched in the VMH, with very low expression in other areas of the hypothalamus, including the ARC and DMH (Fig. 13). Within the





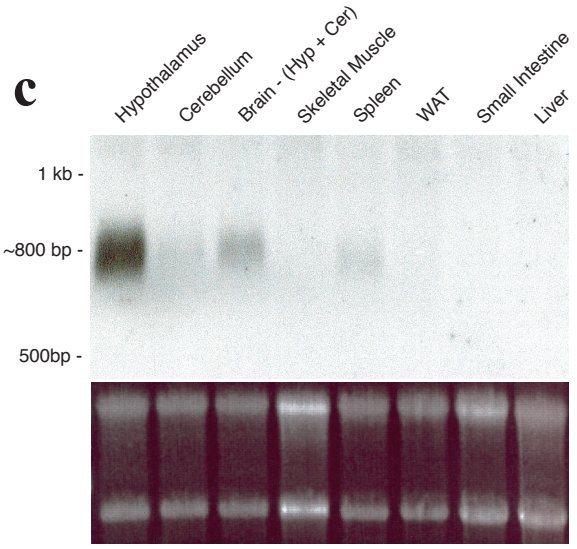


Figure 12 *Northern Blot Analysis of LBH2, Cbln1, and PACAP*
Tissue panel Northern blots for Cbln1 (a), PACAP (b), and EST XM_354697/LBH2 (c). Ethidium bromide staining of 28S and 18S ribosomal bands is shown as a loading control. Cer, Cerebellum; Hyp, hypothalamus; WAT, white adipose tissue.

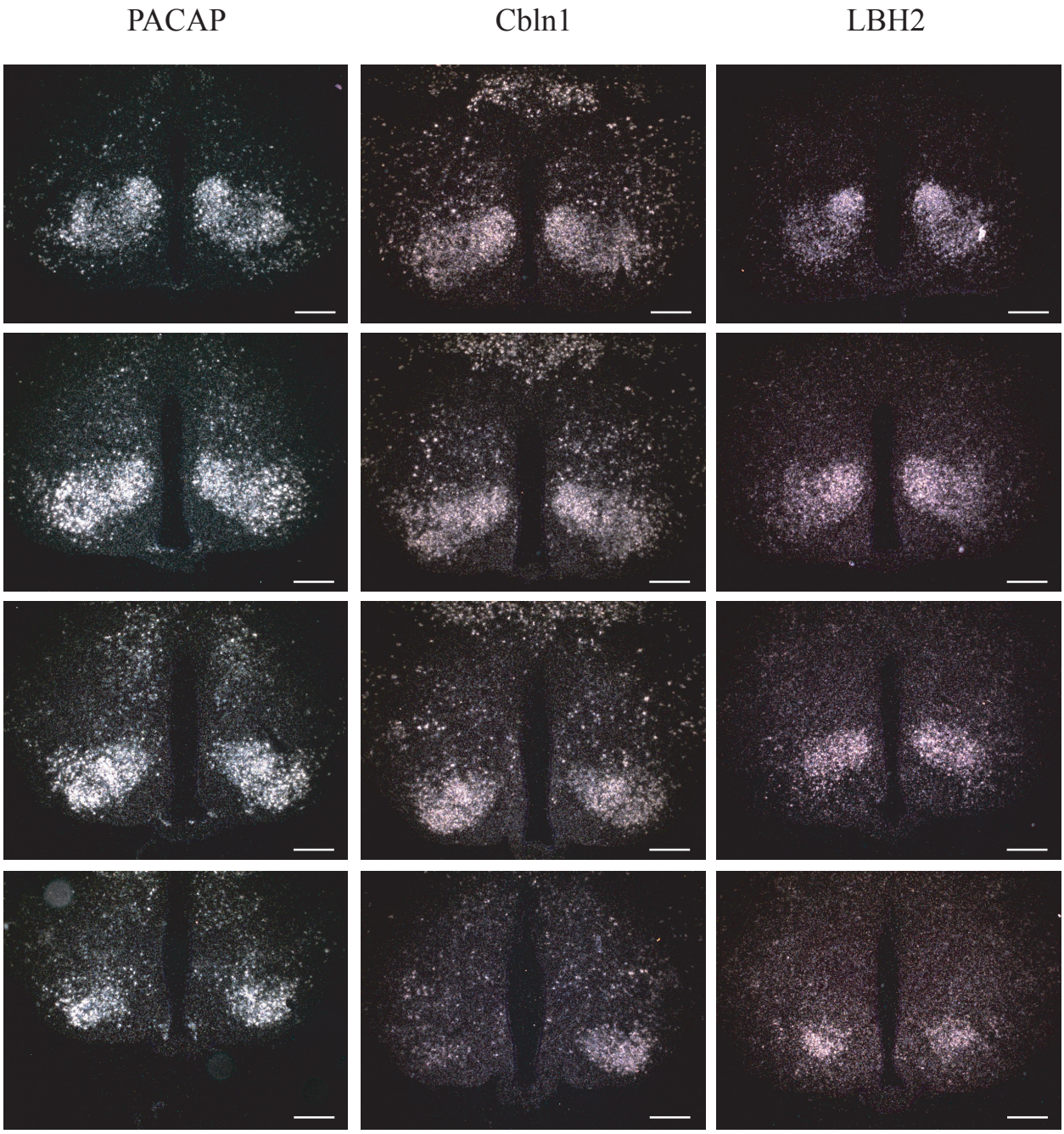


Figure 13 *Comparison of PACAP, Cbln1, and LBH2 mRNA Distributions in the VMH*
In situ hybridization images of PACAP (left), Cbln1 (middle), and LBH2 (right) expression in the mouse VMH. Images are organized from rostral (top) to caudal (bottom). PACAP and Cbln1 are expressed in all subcompartments of the VMH, including the dmVMH, intVMH, and vlVMH. LBH2 expression is restricted to the dmVMH and intMVH, and is not expressed in the vlVMH. Scale bars, 200 µm.

VMH, there are distinct differences in their localization. While PACAP and Cbln1 are expressed at high levels in all subdivisions of the VMH (dorsomedial (dmVMH), intermediate (intVMH), and ventrolateral (vmVMH)), EST XM_354697 appears to be expressed exclusively in the dmVMH and intVMH. Even in the most caudal sections, when VMH neurons are predominantly laterally located, EST XM_354697 mRNA was observed only in the intVMH.

Distribution of Cbln1 in the Mouse Brain

While the distribution of PACAP in the rodent brain has been previously reported (Hannibal, 2002), no such study of the brain distribution of Cbln1 has been performed. Cbln1 was expressed widely throughout the CNS (Fig. 14). Most notably, in addition to expression in the VMH and cerebellum, strong Cbln1 hybridization was observed in the lateral orbital cortex, dorsal endopiriform nucleus, ventromedial preoptic nucleus, thalamic stria medullaris, triangular septal nucleus, a wide array of thalamic nuclei, the latero-anterior and periventricular hypothalamic nuclei, ventral tegmental area, ventral cochlear nucleus, and parviceullular part of the medial vestibular nucleus.

SF-1 Transcriptional Network

The identification of the *SF-1* gene in this screen, along with a list of genes that share its hypothalamic localization, suggested that this transcription factor might regulate some of the other VMH-enriched markers. As discussed, SF-1 is a transcription factor first identified based on its ability to bind to a common upstream region shared by a group of cytochrome P450 steroid hydroxylases in the adrenal cortex (Lala et al., 1992).

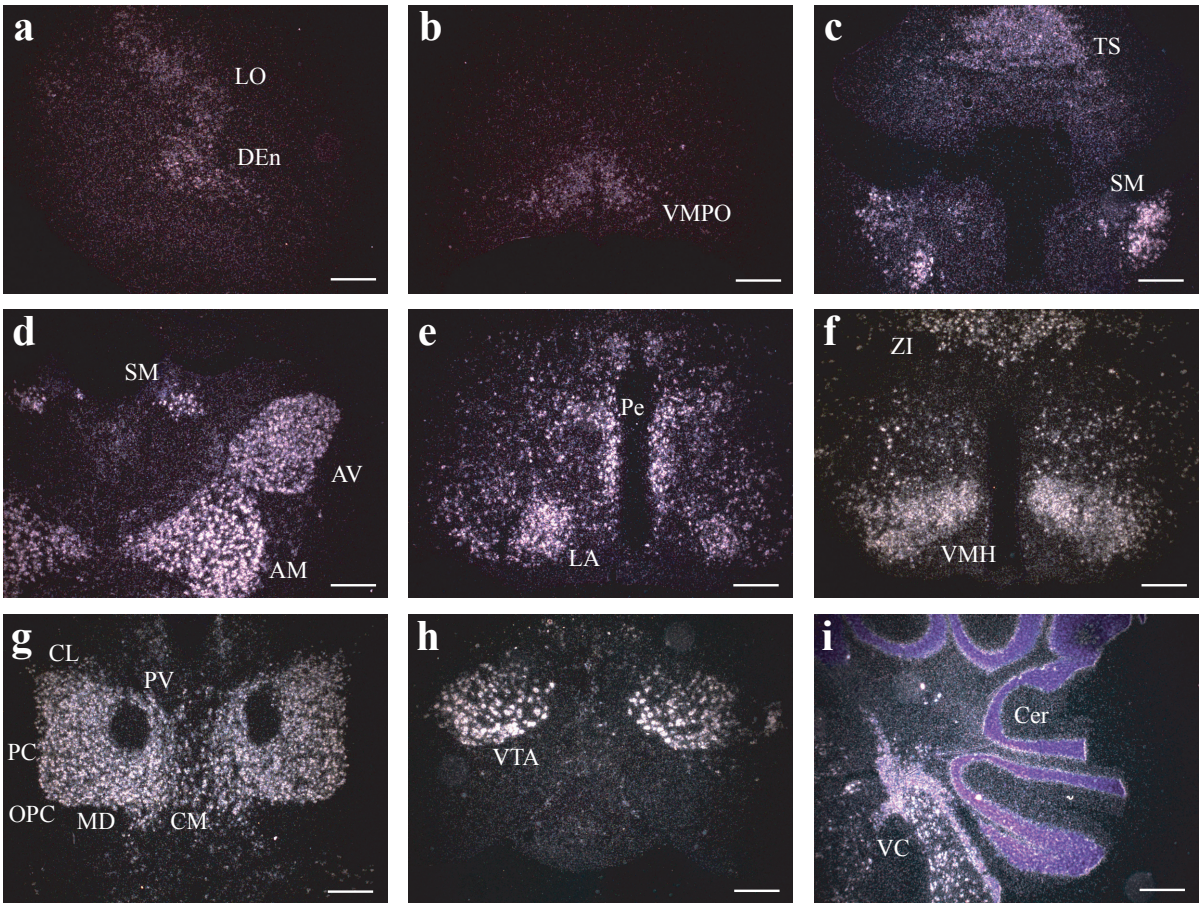


Figure 14 *Distribution of Cbln1 mRNA in the Mouse Brain*
Distribution of Cbln1 mRNA in mouse brain as determined by in situ hybridization. Strong Cbln1 hybridization was observed in the lateral orbital cortex (LO) and dorsal endopiriform nucleus (DEn) (a), ventromedial preoptic nucleus (VMPO) (b), thalamic stria medullaris (SM) and triangular septal nucleus (TS) (c), antero-ventral (AV) and anteromedial (AM) thalamic nucleus (d), latero-anterior (LA) and periventricular (Pe) hypothalamic nuclei (e), zona incerta (ZI) and ventromedial hypothalamic nucleus (VMH) (f), paracentral (PC), oval paracentral (OPC), mediodorsal (MD), para-ventricular (PV), centrolateral (CL), and centromedial (CM) thalamic nuclei (g), ventral tegmental area (h), ventral cochlear nucleus (VC) and cerebellum (Cer) (i), and parvicellular medial vestibular nucleus (MVePC) (j). Scale bars, 200 μm.

In SF-1 knock-out mice, there is a diffuse, disorganized collection of poorly developed VMH neurons outside their normal anatomic position, as determined by the Parker group using an SF-1-eGFP transgene (Fig. 15) (Davis et al., 2004). As a result of the abnormal development of this nucleus, these mice develop late-onset obesity despite normal food intake (Majdic et al., 2002).

Thus, as a first effort to define a genetic network that might mediate VMH-enriched expression, we analyzed the expression of the confirmed VMH-enriched transcripts in SF-1 knockout mice. Genes that are dysregulated in SF-1 neurons would represent potential SF-1 target genes that might play a role in the proper development of this nucleus. Using a previously described SF-1-eGFP transgenic line, we FACS sorted SF-1 neurons from WT and SF-1 knock-out and SF-1 heterozygous mice, as reported previously (Stallings et al., 2002). Real-time PCR for each of the bona fide VMH marker genes was then performed on RNA from these sorted neurons. The RNA levels for four of the eight genes (*Cbln1, Slit3, TenM2*, and EST AA982708) were found to be markedly reduced in samples from SF-1-/- mutant neurons versus wild-type or heterozygote neurons (Fig. 16). In contrast, PACAP and *guanine deaminase* seemed to be induced in the GFP-labeled neurons from the mutant animals, perhaps revealing a lack of feedback signaling.

To confirm this result, we performed ISHH for *Cbln1* on brain sections from wild-type and brain-specific SF-1 knock-out mice (Fig. 17). In sections from KO mice, the SF-1 neurons expressed significantly less Cbln1 mRNA compared with sections from wild-type animals. The signal intensity for Cbln1 was unaffected in the neighboring zona incerta.

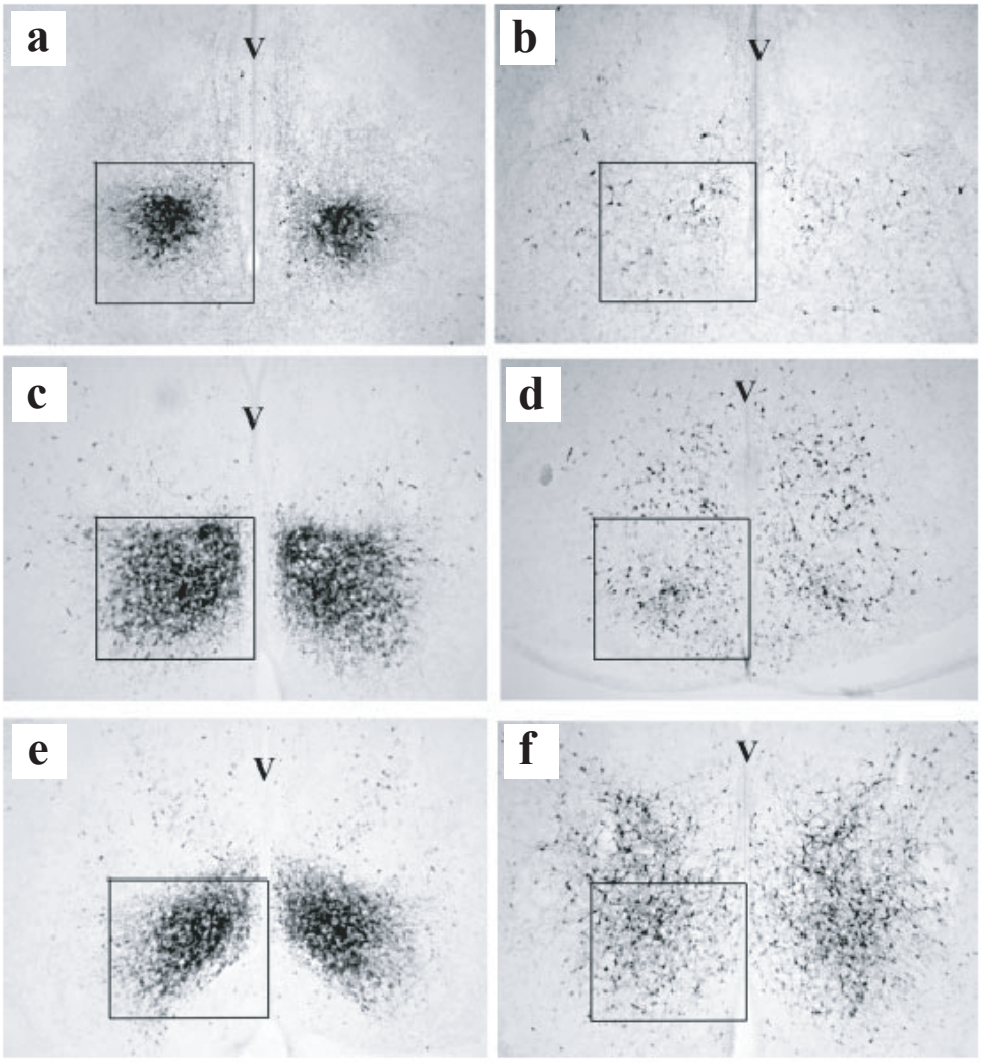


Figure 15 *Disordered VMH Neurons in the SF-1 Knockout Mouse*
eGFP immunohistochemistry of hypothalamic sections of SF-1-eGFP mice bred onto WT (a, c, e) or SF-1 knockout background (b, d, f). The box indicates the area containing the VMH in WT animals. Adapted from Davis et al. 2003.

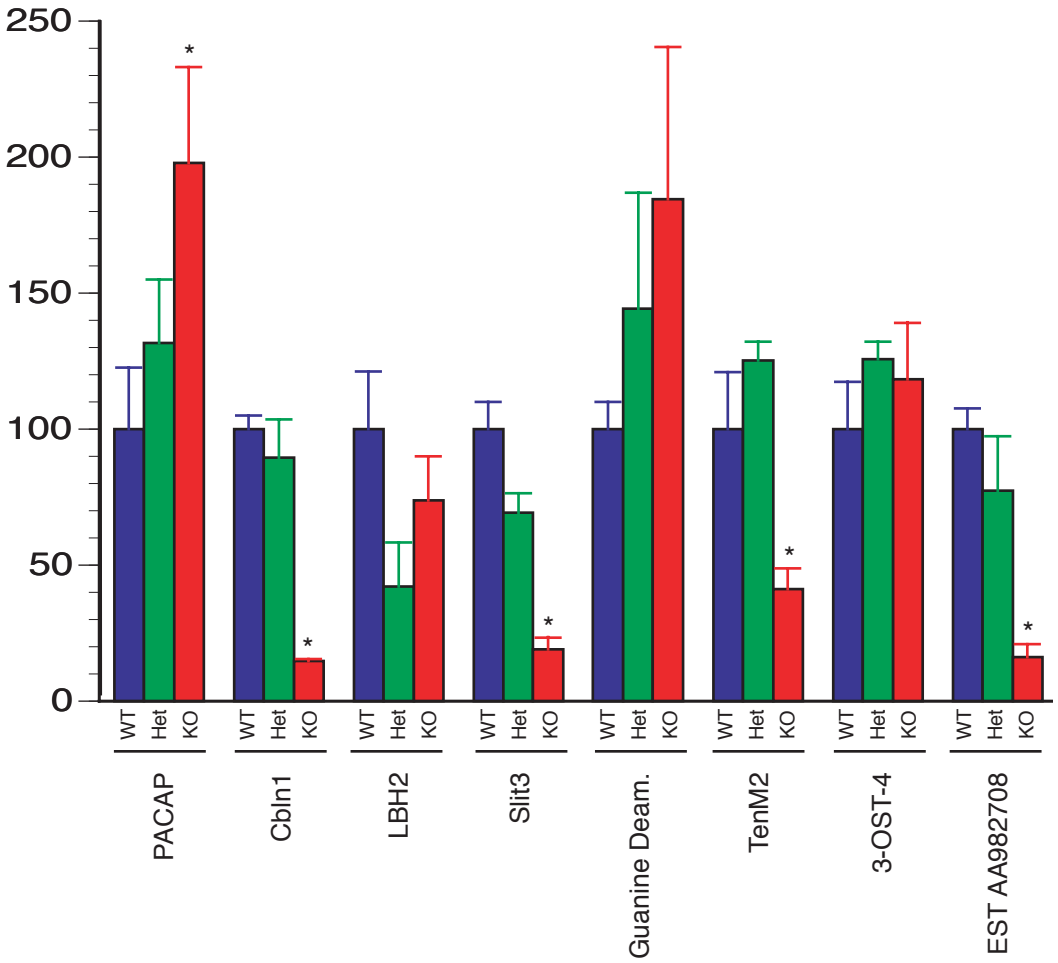


Figure 16 *Expression of VMH Marker Genes in SF-1 Knockout Mice*
Real-time PCR results for VMH marker genes on RNA from FACS-sorted SF-1-eGFP neurons on WT, SF-1 heterozygote (Het), and SF-1 knockout background (KO). All data points are normalized to cyclophilin, with WT values set at 100. Values are expressed as mean +/- SEM. Statistical significance, *p< 0.05.

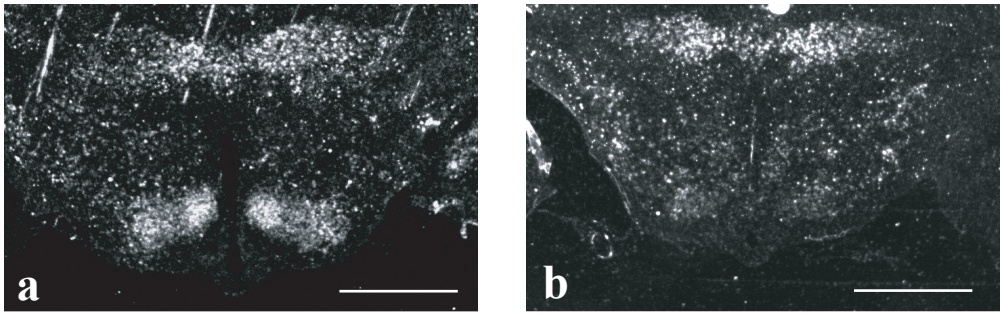


Figure 17 *Cerebellin 1 Expression in WT and SF-1 KO Hypothalamus*
Darkfield images (5x) of hypothalamic sections of WT control mice (a) and brain-specific SF-1 KO mice (b) hybridized with a Cbln1 antisense probe. Scale bars represent 1 mm.

Analysis of VMH Morphology in Slit3 Knockout mice

Of the candidate VMH marker SF-1 target genes, the most striking from the point of view of neuronal organization and migration was the gene *Slit3*. The Slit proteins (Slit1, Slit2, and Slit3) are secreted proteins, which bind to Robo family receptors. There is abundant data that Slit proteins are integrally involved in neuronal migration and axon guidance (Zinn and Sun, 1999). For instance, Slit1 repels neuronal precursors migrating from the anterior subventricular zone in the telencephalon to the olfactory bulb (Wu et al., 1999), while Slit2 has a chemorepulsive effect on cerebral cortical neurons and olfactory interneuron precursors (Hu, 1999). While there is no data concerning the activity of Slit3 in these processes, targeted deletion of Slit3 produces congenital diaphragmatic hernia, kidney agenesis and cardiac defects, indicating significant activity in tissue morphogenesis (Liu et al., 2003b).

Because of its tissue localization, regulation by SF-1, and suspected involvement in neuronal modulation, we examined Slit3 knockout mice for evidence of similar VMH irregularities as those observed in SF-1 knockout mice. However, immunostaining with an antibody against the pan-neuronal marker NeuN revealed identical distributions of VMH neurons in both Slit3 -/- and wild-type control mice (Fig. 18). As a follow-up, we examined the distribution of estrogen receptor-alpha (ER-alpha) in these mice. The ER-alpha neuronal distribution is profoundly affected by the loss of SF-1, as this group of neurons, which normally occupies the extreme vlVMH, is found near the 3rd ventricle in SF-1 knockout mice (Dellovade et al., 2000). However, the distribution of ER-alpha was unaffected by deletion of Slit3 (Fig. 19).

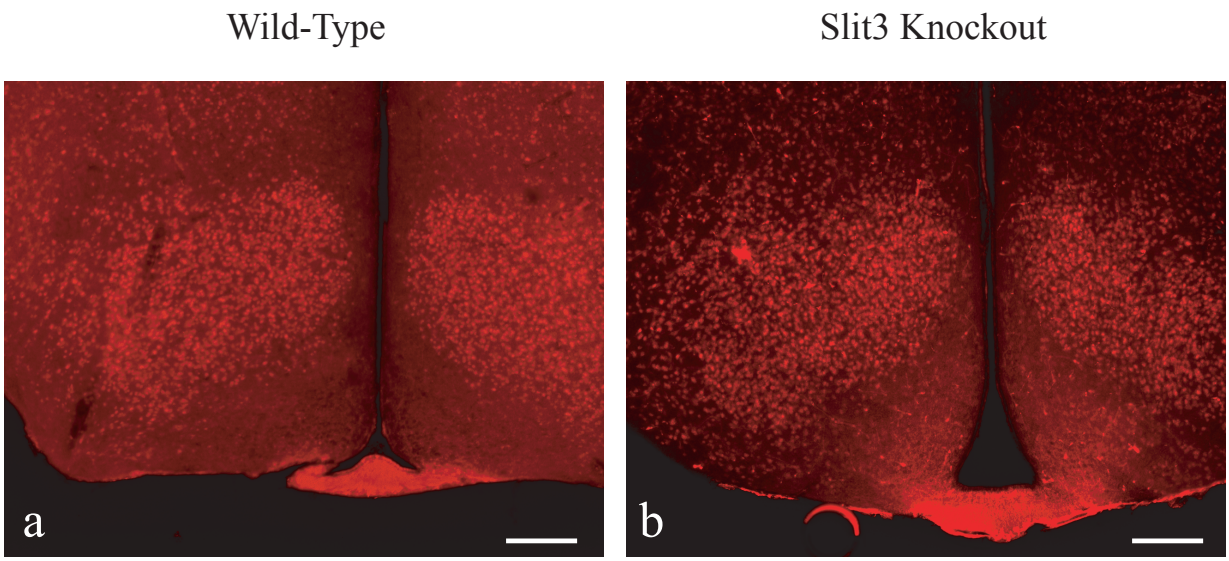


Figure 18 *Distribution of VMH neurons in Slit3 Knockout Mice*
VMH neurons are visualized by fluorescence immunostaining with an antibody against NeuN, a pan-neuronal marker in wild-type (a) and Slit3 -/- (b) hypothalamus.
Scale bars, 200 μm.

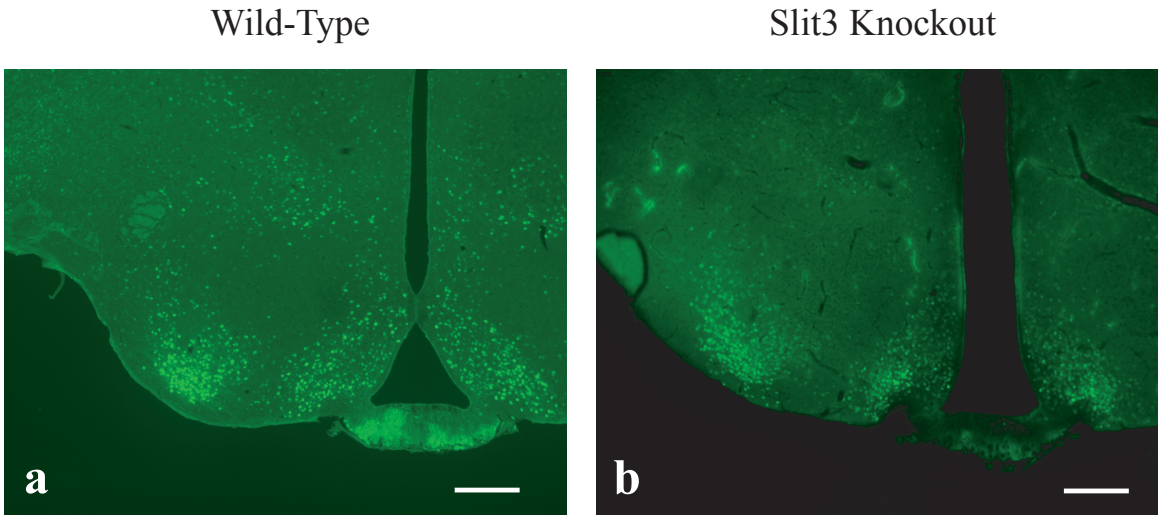


Figure 19 *Hypothalamic ER-alpha Distribution in WT and Slit3 Knockout Mice*
Fluorescence immunohistochemistry performed on hypothalamic sections from WT (a) and Slit3-/- (b) mouse brains using an antibody against estrogen receptor-alpha. Scale bars, 200μm.

LBH2

The VMH-enriched EST XM_354697 had not been characterized previously. This gene was present in multiple copies on the hypothalamus-enriched array, but was not a constituent of the 28,000 gene library. The transcript of this gene is roughly 800 bp, as determined by northern blot (Fig. 12c).

The corresponding gene is composed of 4 exons, occupying 5.3 kb of the "+" strand of chromosome 12 (qF1). The predicted product of this transcript is a 114 aa protein, which shares a 29 aa stretch of 86% identity with limb bud and heart (LBH), a putative transcription factor thought to play a role in limb and heart development (Briegel and Joyner, 2001). Thus, based on the guidelines of the International Committee on Standardized Genetic Nomenclature for Mice, we have named this gene *LBH2*. A comparison of the protein sequences of LBH2 with LBH and the homologous proteins "rat similar to XLCL2 protein" and "EST human LOC350101 protein" shows that this gene is evolutionarily conserved, especially within a middle stretch of 50 aa (Fig. 20). An LBH2-eGFP fusion protein expressed in 293T cells under the control of the cytomegalovirus promoter showed that the native Kozak sequence and ATG were indeed functional, and the distribution of the fusion protein within the cells was cytoplasmic (Fig. 21).

Within the brain, LBH2 expression is mainly limited to only a few areas, as determined by *in situ* hybridization. Specifically, LBH2 is expressed in the lateral septal nucleus, the amygdala, and the VMH (Fig. 22). Low-level expression was also observed in the exteral cortex of the inferior colliculus. As discussed previously, LBH2 expression in the VMH is almost totally restricted to the dmVMH and intVMH, with almost no

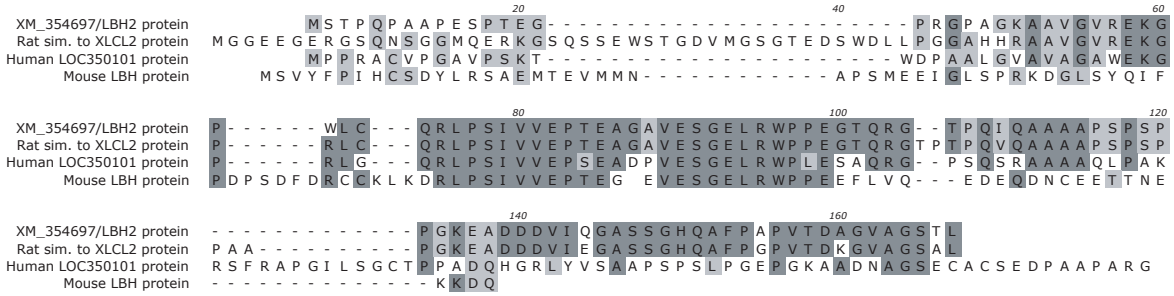


Figure 20 *LBH2 Family Protein Sequence Comparisons*
Protein sequence alignments of LBH2, rat similar to XLCL2, Human LOC350101, and mouse LBH. Light gray boxes indicate similarity, while dark gray boxes indicate identity. All proteins share a high degree of identity between amino acids 76-104.

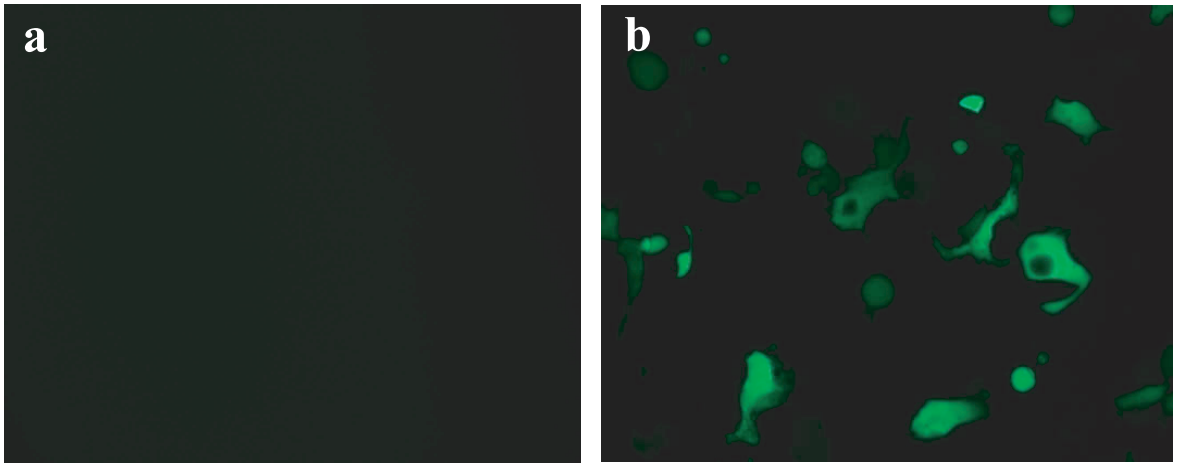


Figure 21 *Expression of LBH2-eGFP Fusion Protein in Cell-Culture*
Fluorescence images of HEK 293T cells transfected with pcDNA-xEGFP (a) or pcDNA-LBH2-xEGFP (b). Bright green fluorescence resulting from pcDNA-LBH2-xEGFP transfection indicates that the predicted ATG and Kozak sequences of LBH2 are functional.

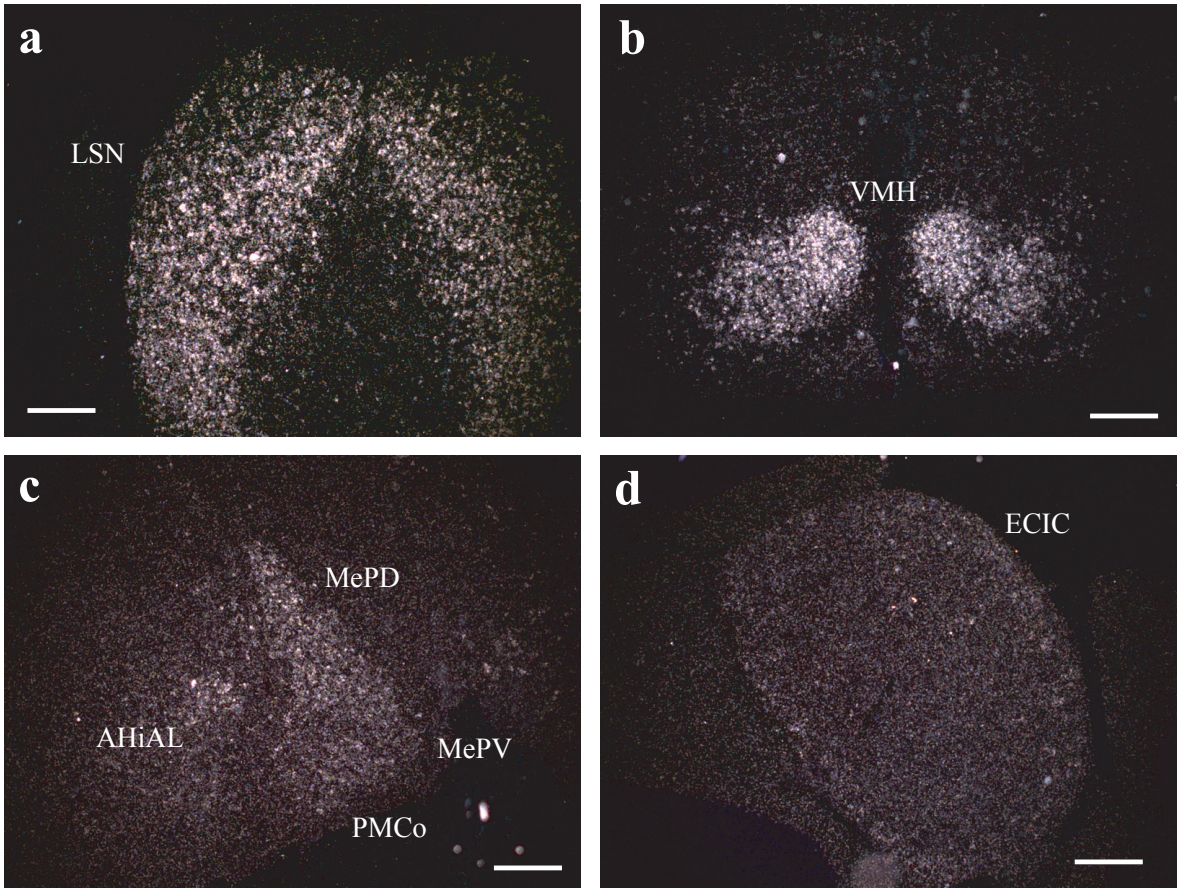


Figure 22 *Distribution of LBH2 mRNA in the Mouse Brain*
Distribution of LBH2 mRNA in the mouse brain as determined by in situ hybridization. Strong hybrization signal was observed in the lateral septal nucleus (LSN) (a), ventromedial hypothalamic nucleus (VMH) (b), posteroventral (MePV) and posterodorsal (MePD) medial amygdaloid nuclei, posteromedial cortical amygdaloid nucleus (PMCo), and anterolateral amygdalohippocampal area (AHiAL) (c), and the external cortex of the inferior colliculus (ECIC) (d). Scale bars, 200 μm.

expression in the vlVMH.

LBH2 Antiserum

In order to analyze the distribution of LBH2 within the VMH in detail, rabbit anti-LBH2 antiserum was created by injection of the peptide N-STPQPAAPESPTEGPRG-C (representing amino acids 2-18 of LBH2) into rabbits. Antisera were affinity purified. The resulting antisera was capable of labeling LBH2 neurons by immunohistochemistry, illuminating the expression of this gene in much greater detail. The expression pattern of LBH2 in the VMH and other brain regions as measured by immunohistochemistry recapitulated the in situ hybridization results (discussed below). Additionally, confocal imaging of LBH2 neurons in the VMH confirmed the predicted cytoplasmic localization of the protein product of this gene (Fig. 23).

LBH2-GFP BAC Transgenic Mice

In order to learn more about VMH subpopulations, we endeavored to create transgenic mice that expressed GFP under the control of the LBH2 promoter. A BAC transgenic approach was taken, as this method allows for expression of a given transgene under the full set of promoter elements of a gene of interest, whether they be proximal or distal to the transcriptional start site. A 185 kb BAC (RP24-90O19), with roughly 130 kb up upstream DNA and 40 kb of downstream DNA, was modified such that an EGFP-polyA construct was placed immediately following the native ATG site (Fig. 24). This construct was injected into strain FVB/NJ pronuclei, resulting in the production of 45 founder mice. Of these founders, one had focal expression of GFP in the VMH, and

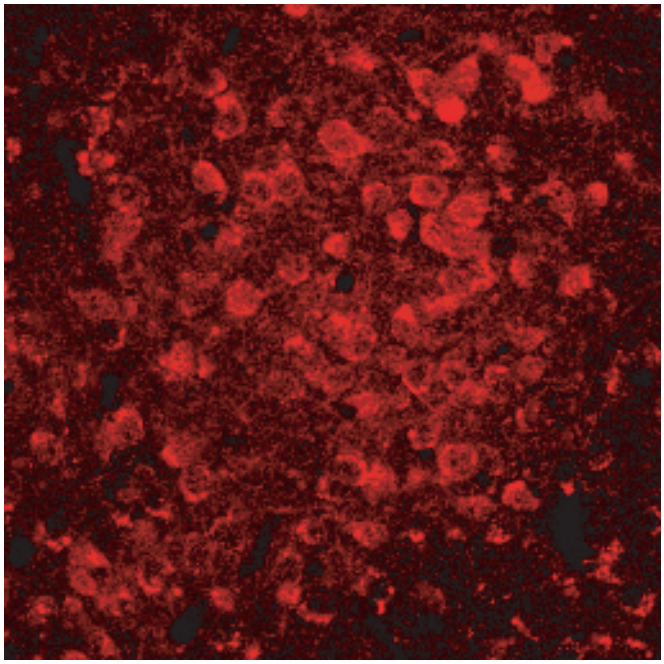


Figure 23 *Cytoplasmic Localization of LBH2 Protein in VMH Neurons*
Confocal fluorescence image of the dmVMH of wild-type hypothalamic brain section immunostained with an antibody raised against LBH2 peptide. The observed halo staining pattern is indicative of cytoplasmic expression.

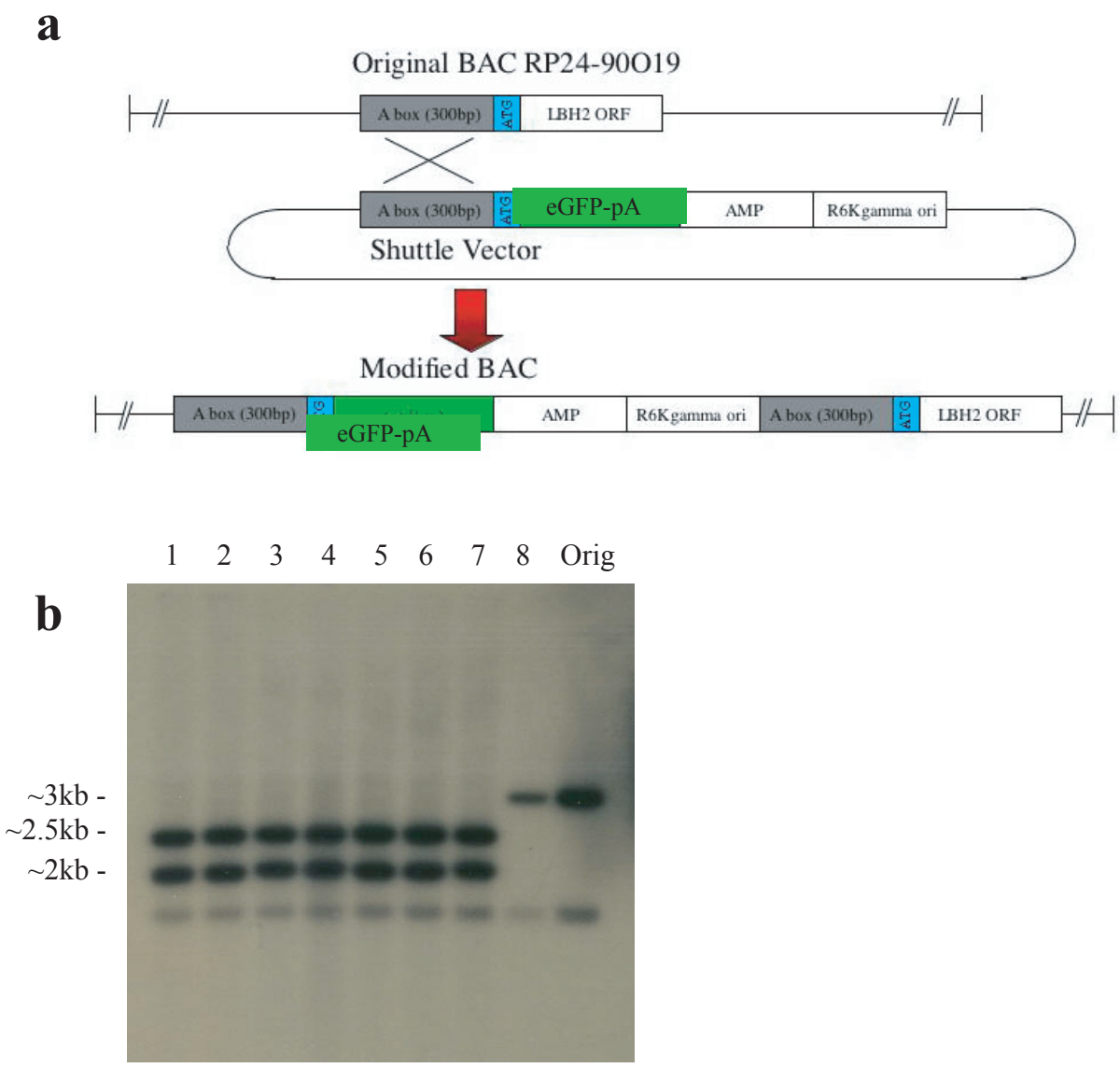


Fig 24 *LBH2-eGFP BAC Modification*
(a) Schematic of modification of LBH2-containing BAC RP24-90O19. Shown is the original BAC, shuttle vector containing the homology box A and the eGFP-poly A construct, AMP resistance cassette and R6Kgamma origin of replication.Homologous recombination within the "A box" produces the modified BAC (bottom). (b) Southern blot of HindIII digest of original BAC and 8 potential modified BAC clones, probed with ^{32}P-labeled A box. Modified clones 1-7 were successful, and clone #1 was injected into FVB/NJ pronuclei.

produced pups with the expected Mendelian ratio. This mouse line was thus designated LBH2-GFP.

Analysis of the LBH2-GFP mouse, by anti-GFP immunofluorescence, revealed the expression of the transgene in a number of brain areas, in good agreement with the *in situ* hybridization results. Specifically, LBH2-GFP mice express GFP in the lateral septal nucleus, the VMH, the amygdala, and the piriform cortex (Fig. 25). Of these sites, all are consistent with the expression profile revealed by *in situ* hybridization and immunohistochemistry, except for the piriform cortex expression.

Bright GFP fluorescence in neurons of GFP transgenic mice is an essential prerequisite to a number of different types of studies, including those involving cell sorting and electrophysiology. To analyze the GFP expression of the LBH2-GFP mice, fresh unstained sections were imaged by fluorescence microscopy. Native un-amplified GFP fluorescence was clearly visible (Fig. 26).

While the LBH2-GFP transgene was clearly expressed in the correct areas of the brain, it was still unclear whether the correct neurons in those areas were labeled. To verify the expression of the transgene, dual immunofluorescence was performed, using anti-GFP and anti-LBH2 antibodies. Confocal microscopy was used, because visualization of thin optical sections allowed for the most accurate possible investigation of gene expression in discrete neurons. Within the VMH, there was excellent overlap of anti-GFP signal with anti-LBH2 staining (Fig. 27 top). In general, all VMH neurons that were positive for GFP were also found to stain positive with the anti-LBH2 antibody. However, there appeared to be a low rate of observance of LBH2-expressing neurons that could not be positively visualized by GFP. However, GFP could still be expressed in

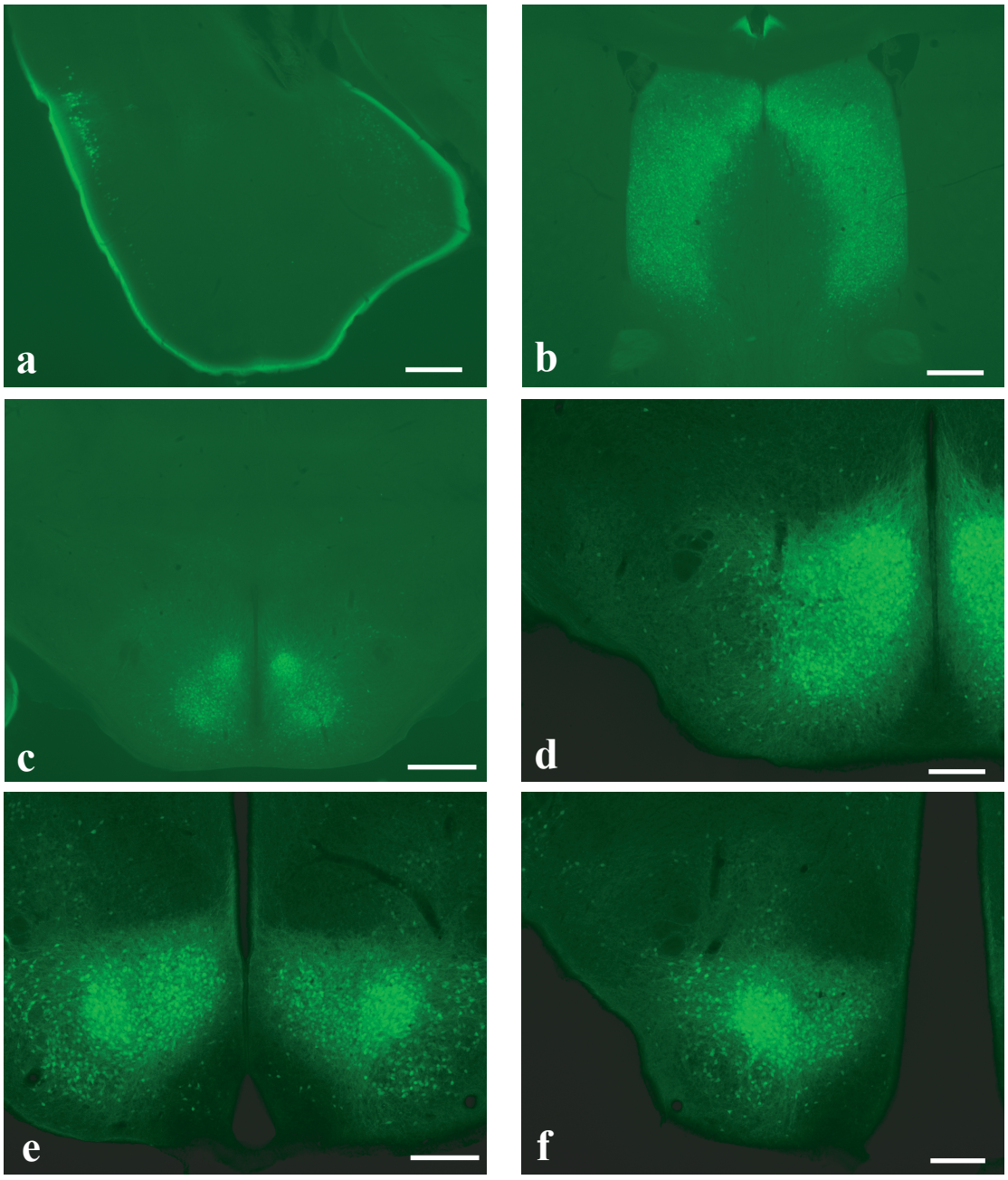


Figure 25 *Hypothalamic eGFP Expression in LBH2-eGFP Transgenic Mice*
Anti-GFP fluorescence immunostaining of brain sections from LBH2-GFP BAC transgenic mice. Expression can be observed in the piriform cortex and amygdala (a), lateral septal nucleus (b), and VMH (c-f). (d) In rostral VMH sections, eGFP is expressed predominantly in the dorsomedial division of the VMH. (e) In middle VMH sections, eGFP is expressed in all division of the VMH, most prominently in the intermediate VMH. (f) In caudal VMH sections, eGFP is expressed almost exclusively within the intermediate VMH. Scale bars, 400 μm (a-c), 200 μm (d-f).

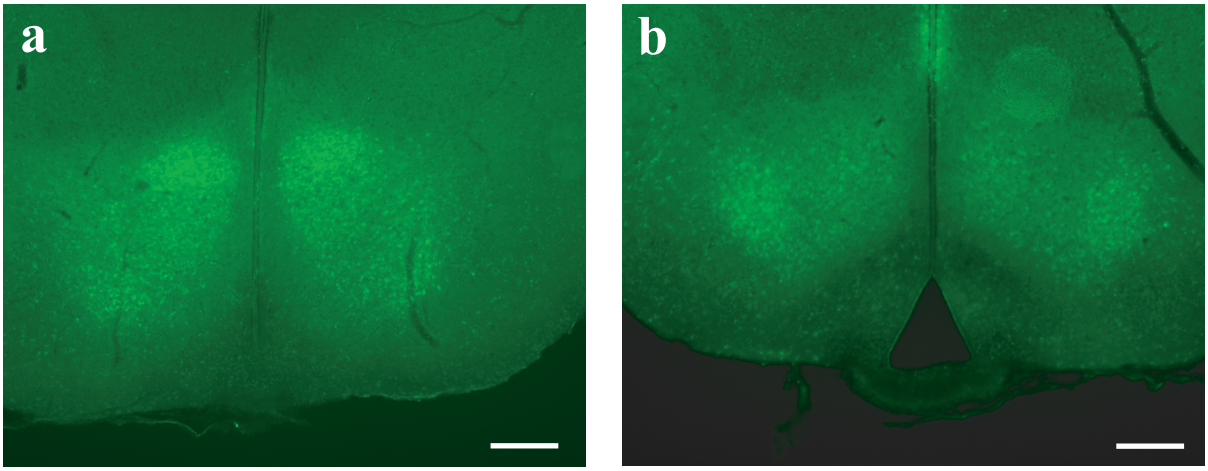


Figure 26 *Natural eGFP Fluorescence in LBH2-eGFP Mice*
Natural unstained fluorescence in the rostral VMH (a) and caudal VMH (b) in LBH2-eGFP BAC transgenic mice. Scale bars, 200 μm.

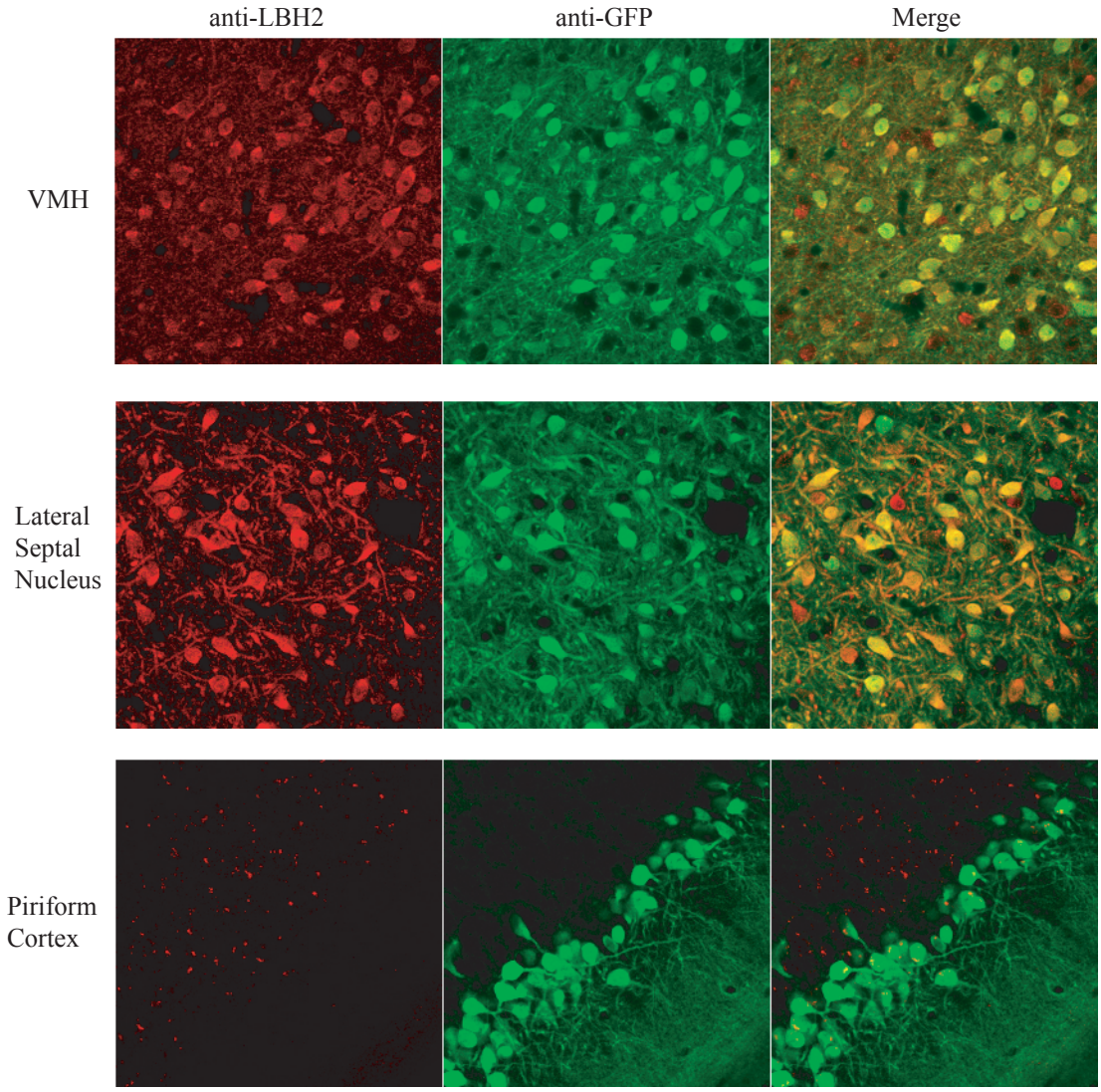


Figure 27 *Validation of LBH2-eGFP BAC Transgenic Mice*
Dual-fluorescence confocal images of LBH2-eGFP mouse brain sections immunostained with both anti-LBH2 (red) and anti-GFP (green) antibodies. Red (left), green (center), and merged (right) images are shown for staining of the VMH (top), lateral septal nucleus (middle) and piriform cortex (bottom).

these neurons, albeit at low, undetectable levels. In the dorsomedial VMH, almost all LBH2-expressing neurons were observed to be clearly labeled with GFP, at a rate approaching 90%. This rate was equally high for LBH2-expressing neurons in the intermediate VMH, but was slightly lower (roughly 80%), in the furthest lateral LBH2-expressing neurons.

In the lateral septal nucleus, overlap between LBH2-eGFP and anti-LBH2 immunostaining was also quite good (Fig. 27 middle). Roughly 90% of LBH2 neurons expressed the LBH2 transgene. However, in this nucleus, rare neurons could be found that expressed eGFP but did not stain positive for LBH2. It is possible that the LBH2 antibody (which is quite weak) was not able to visualize legitimate expression in these neurons, but that is not certain. In contrast, in the piriform cortcx, the situation was quite different. As expected from the *in situ* hybridization data, there was no LBH2 expression in this area as determined by anti-LBH2 immunohistochemistry (Fig. 27 bottom). Thus, the eGFP expression in this compartment is an artifact. However, this erroneous piriform cortex eGFP expression does not mitigate the utility of this transgene for the study of the VMH. Especially weak staining with the anti-LBH2 antibody in the amygdala precluded an assessment of trasngene expression in this nucleus. It is not susprising that this was so, considering the relatively weaker expression of LBH2 measured by *in situ* hybridization and the atypically weak expression of the LBH2-eGFP transgene in this region.

All in all, LBH2 appeared to be expressed in the vast majority of dmVMH and intVMH neurons. Expression in the ventrolateral segment of the VMH was less prominent, as expected. Next, we attempted to determine what proportion of VMH

neurons in different VMH subcompartments expressed LBH2. To do so, we performed dual label immunohistochemistry on LBH2-GFP mice using the anti-GFP antibody and an antibody against the pan-neuronal marker, NeuN (Fig. 28). Analysis of stained sections by confocal microscopy revealed that LBH2-expressing neurons accounted for roughly 80% of all neurons in the dorsomedial division of the hypothalamus. LBH2 neurons were found less frequently in the intermediate division of the VMH (roughly 80%), and even less in the lateral section of the VMH (<5%).

We also co-stained LBH2-GFP sections with a rabbit anti-SF-1 antibody to determine the extent of co-localization of LBH2 with this important VMH molecule. In rostral VMH sections, SF-1 appeared to be localized to every division of the VMH (dmVMH, intVMH, and vlVMH), whereas LBH2 was only present in the dmVMH and intVMH (Fig. 29). The LBH2 neuronal population appears to be a subset of the SF-1 population, as no LBH2 neurons were observed that did not express SF-1. In caudal VMH sections, neither LBH2 nor SF-1 appeared to be expressed in the vlVMH, the location of the estrogen receptor-alpha neuronal population (Simerly et al., 1990). Instead, both proteins were predominantly located in the intVMH. Again, no LBH2 neurons were observed that did not also express SF-1. In general, it appears that LBH2 is expressed in roughly 70 percent of SF-1 neurons in the VMH.

Because of the lack of prominence of LBH2 staining in the vlVMH, we investigated to what extent LBH2 expression overlapped to any extent with that of estrogen receptor-alpha. ERalpha is expressed widely throughout the hypothalamus, but within the VMH is expressed at low levels in the dmVMH and at extremely high levels in the vlVMH (Simerly et al., 1990). Estrogen receptor neurons in the vlVMH are

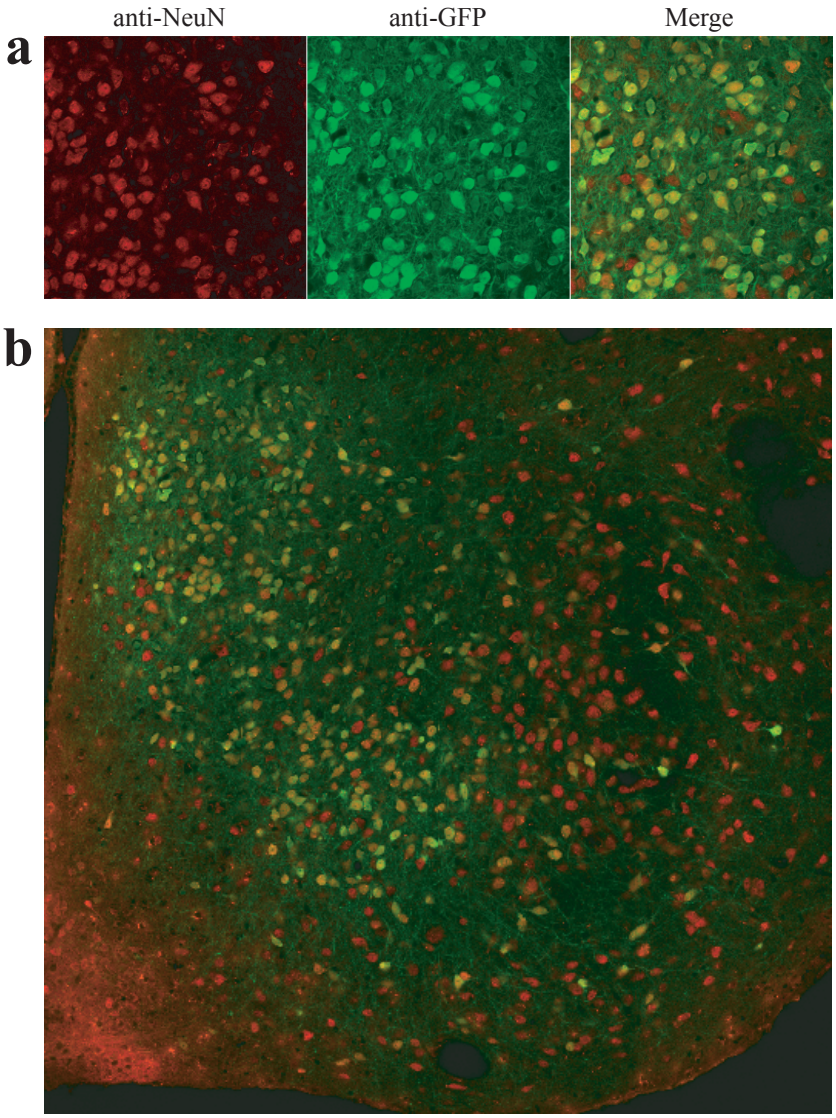


Figure 28 *Co-visualization of LBH2-eGFP and NeuN in the VMH*
Dual-fluorescence confocal images of LBH2-eGFP brain sections stained with both anti-NeuN (red) and anti-GFP (green). (a) 40x images of dmVMH. (b) Merged larger composite image containing the entire VMH.

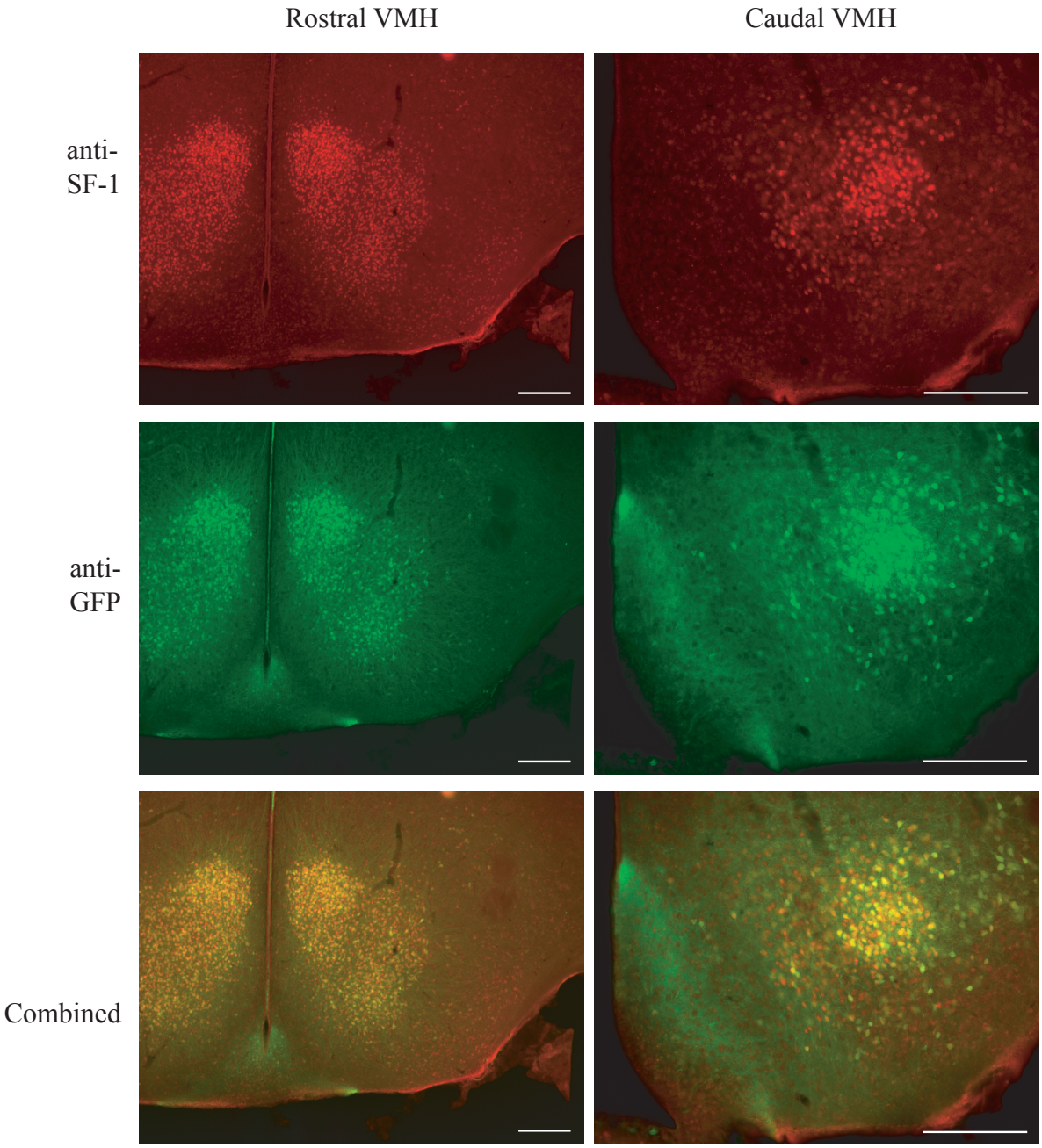


Figure 29 *Co-Visualization of LBH2-eGFP and SF-1 in the VMH*
Double-label immunohistochemistry with mouse anti-GFP and rabbit anti-SF-1 antibodies performed on rostral (left) and caudal (right) hypothalamic sections from LBH2-eGFP BAC transgenic mice. Scale bars, 200 μm.

implicated in a number of physiological functions, but most particularly in the initiation of the female sexual response lordosis (Etgen, 1987). We examined ER-alpha expression in LBH2-GFP mice by double label immunohistochemistry. In the dmVMH, where ER-alpha is expressed at low levels, there was significant overlap with LBH2. However, in the vlVMH, where ER-alpha expression is most prominent, no overlap between LBH2-GFP and ERalpha expression was observed, even though LBH2 is expressed in a small number of vlVMH neurons (Fig. 30).

Because LBH2 is prominently expressed in the dmVMH, we next asked to what extent LBH2 expression was associated with leptin responsiveness. Because there are no antibodies available that can be used to visualize leptin receptor neurons by immunohistochemistry, the best available means of identifying leptin responsive neurons is to treat animals with leptin and perform immunohistochemistry with an antibody specific for activated, phosphorylated STAT3, visualized in the nucleus by horseradish peroxidase-diaminobenzidine (DAB) staining. Animals were fasted for 24 hours prior to leptin treatment in order to reduce endogenous leptin levels, and then treated with 1mg/kg leptin IP. After waiting for 45 minutes for optimal activation of STAT3, the animals were perfused and processed for immunohistochemistry. Animals treated with leptin displayed abundant pSTAT3 immunoreactivity, within the VMH restricted primarily to the dmVMH. When this staining was performed on LBH2-GFP animals, in conjunction with fluorescent anti-GFP immunohistochemistry, significant overlap was observed between LBH2 expression and pSTAT3 signal. The pStat3 DAB staining diminished the GFP fluorescence of LBH2-GFP neurons in the perinuclear area, revealing dual-positive neurons by a characteristic halo (Fig. 31). However, this phenomemon is difficult to

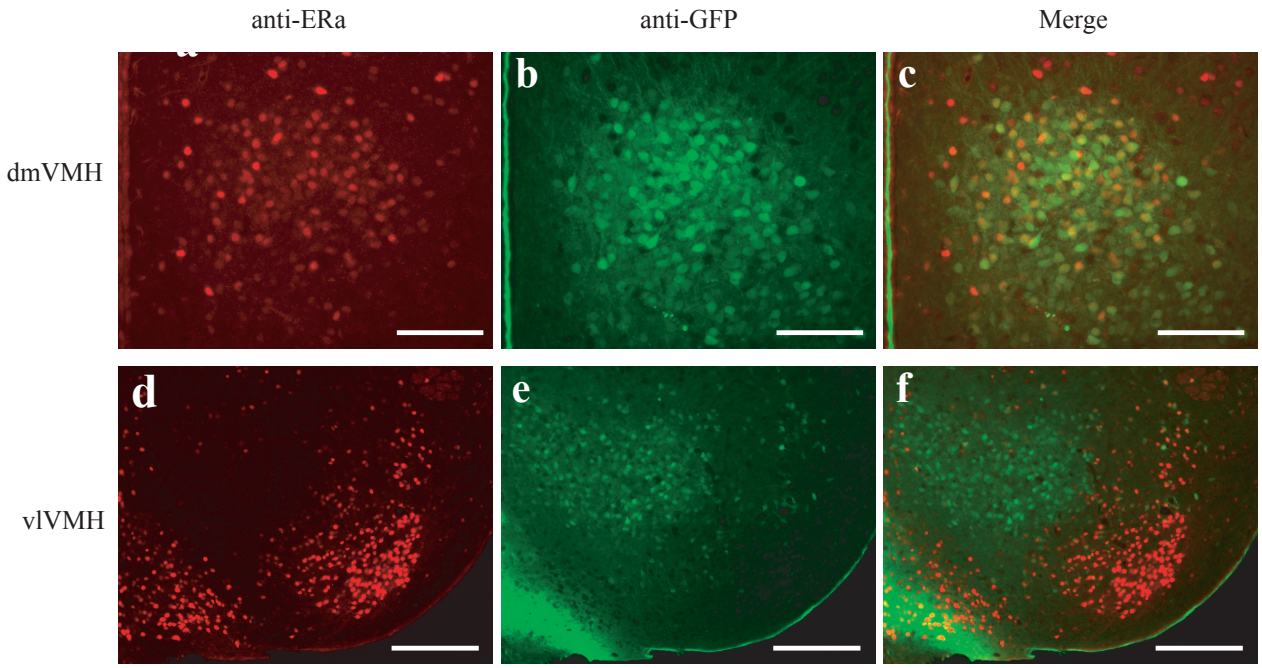


Figure 30 *Co-Visualized VMH Distributions of LBH2 and Estrogen Receptor-alpha* (a-c) Dual-color fluorescence images of dmVMH stained with anti-estrogen receptor-alpha (red) and anti-GFP (green). Magnification, 20x; scale bars, 100 µm. (d-f) Dual-color fluorescence images of dmVMH stained with anti-estrogen receptor-alpha (red) and anti-GFP (green). Magnification, 10x; scale bars, 50 µm.

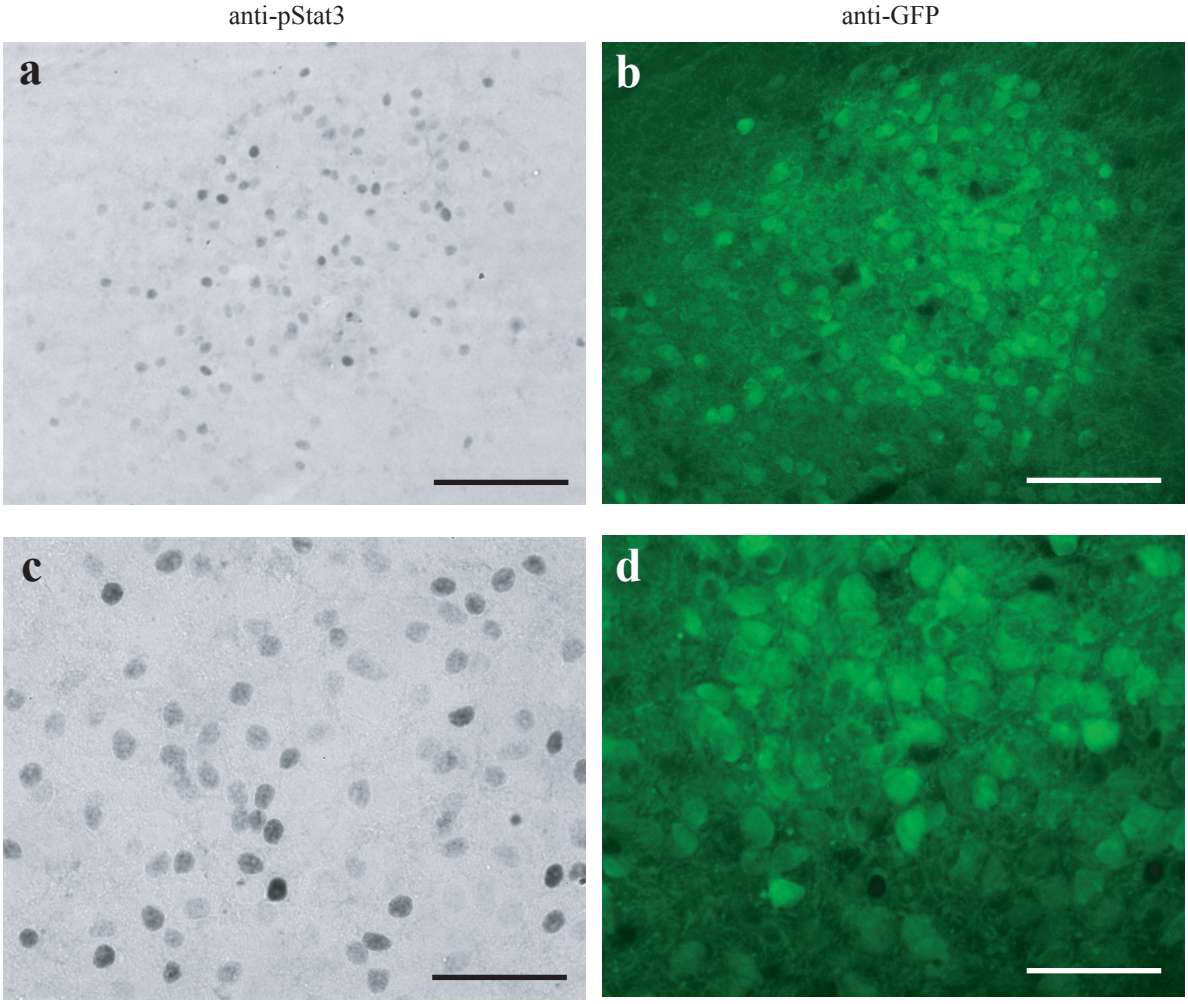


Figure 31 *Leptin Responsiveness of VMH LBH2 Neurons*
Simultaneous visualization of pStat3 and GFP in the VMH of LBH2-eGFP BAC transgenic mice. pStat3 (a, c) is visualized following 24 hour fast and 45 min treatment with 1 mg/kg leptin IP by anti-pStat3 antibody and ABC-DAB staining. GFP (b, d) is visualized by anti-GFP immunofluorescence. Green halos represent double-stained neurons. (a-b) Brightfield and fluorescence images of dmVMH at 20x. Scale bars, 100 µm. (c-d) Brightfield and fluorescence images of dmVMH at 40x. Scale bars, 50 µm.

quantitate, thus it was difficult to determine whether all pSTAT3 containing neurons were LBH2 positive, but at least 90% of leptin responsive neurons were found to be in the LBH2-expressing population.

Tracing VMH -> ARC Projections Using Pseudorabies Virus

It has been a source of debate in recent years how the VMH is integrated into the neuronal circuit regulating feeding and body weight. Staining of VMH neurons reveals an axonal field that extends around the area of the nucleus, reaching the VMH/ARC and VMH/DMH boundaries, but not progressing further (Saper et al., 1976a). The initial assumption was that VMH neurons did not synapse with ARC or DMH neurons. However, analysis of ARC neurons showed that the ARC was heavily populated by neurons that extended dendrites to this same internuclear zone. Recently, Sternson et al., using a laser-scanning photostimulation (LSPS) technique incorporating electrophysiology and focal release of caged glutamate, identified extensive connections between the VMH and the ARC (Sternson et al., 2005). Specifically, it was determined that POMC neurons in the ARC received inputs from medial VMH neurons, while NPY/AgRP neurons in the ARC received inputs from the intVMH and vlVMH. Though this approach revealed a beautiful array of connectivity between these two nuclei, the technique precludes the possibility of identifying specific neurons in the VMH that project to the ARC. To this end, we attempted to reproduce some of the LSPS findings using a different tracing system, pseudorabies virus.

Pseudorabies virus (PRV) represents an extremely powerful tool for tracing neuronal pathways (Strack et al., 1989a). PRV infects in a step-wise manner from neuron

to neuron via synaptic connections, in a retrograde- or bi-directional direction, depending on the strain. PRV has been used extensively to map connections in the CNS and outputs to various peripheral organs. However, the use of PRV was traditionally limited by the inability to initiated infection in specific cells, thus observing the inputs to particular defined sets of neurons. This problem was overcome by the development of a cre-dependent virus strain, Ba2001, which requires cre for expression of the essential viral gene thymidine kinase (TK) and a marker, GFP (DeFalco et al., 2001). Using this virus, DeFalco et al. traced the inputs of leptin receptor neurons in the arcuate nucleus using an Ob-Rb cre mouse line.

To trace VMH projections to the ARC, we utilized AgRP-cre mice (kind gift of Dr. Bob McKenzie, Wayne State University, Detroit, MI). Because AgRP is expressed exclusively in the ARC within the entire mouse brain, we were able to use a TK+ variant of Ba2001. This virus is infectious and kills both wild-type and cre animals, but it is totally conditional for GFP expression. Using the TK+ variant eliminated the necessity of total accuracy of stereotaxic injection into the ARC. An infection into a nearby area will spread to the ARC, where GFP expression will become activated by the cre recombinase, and subsequently any neurons infected by the virus from those cre neurons will also express GFP.

Ba2001TK+ was stereotaxicly injected into the ARC of both wild-type and AgRP-cre mice. In AgRP-cre mice infected with Ba2001TK+, strong GFP fluorescence was observed in the ARC, VMH, PVN, amygdala, lateral septal nucleus, superoptic nucleus, and nucleus of the solitary tract (Fig. 32 a-f). In contrast, no GFP was observed anywhere in the brains of wild-type control animals injected with the Ba2001TK+ virus

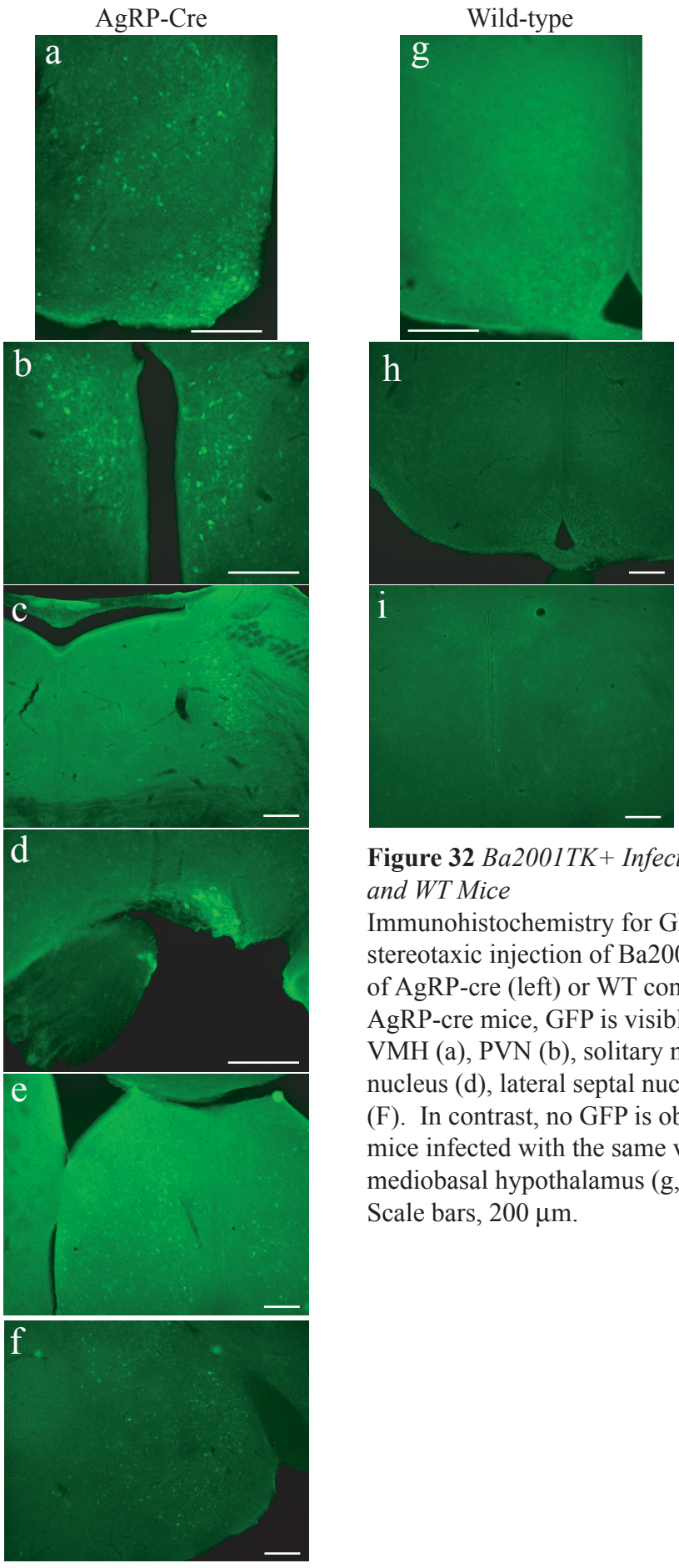


Figure 32 *Ba2001TK+ Infection of AgRP-Cre and WT Mice*
Immunohistochemistry for GFP 3 days following stereotaxic injection of Ba2001TK+ into the ARC of AgRP-cre (left) or WT control mice (right). In AgRP-cre mice, GFP is visible in the ARC and VMH (a), PVN (b), solitary nucleus (c), superoptic nucleus (d), lateral septal nucleus (e), and amygdala (F). In contrast, no GFP is observed in WT control mice infected with the same virus. Shown is the mediobasal hypothalamus (g, h) and PVN (i). Scale bars, 200 μm.

(Fig. 32 g-i). In the VMH, GFP was observed most prominently in the intVMH, with appreciable staining in the dmVMH. Less signal was observed in the vlVMH.

Chapter 4: Discussion

VMH Marker Genes

The use of marker genes to dissect complex neural circuits has previously been applied in studies of several brain regions revealing important and in some cases divergent responses of specific neurons in specific brain regions (Elias et al., 1999; Pinto et al., 2004). While several lines of evidence have established that the VMH plays an important role in controlling food intake, glucose metabolism and body weight (Elmquist et al., 1999), efforts to determine how specific classes of VMH neurons mediate these processes have been hampered by the lack of a set of marker genes that could facilitate a molecular analysis of their function. The importance of understanding the functional roles of specific neurons in this nucleus is further amplified by the observation that VMH-specific knockout of the leptin receptor leads to increased adiposity and body weight (H. Dhillon, personal communication). In these studies it was not known which VMH neurons are responsible for this effect.

We have successfully used LCM coupled with cDNA microarrays to generate an initial set of VMH enriched genes. In the future, this set may be expanded using a similar approach in combination with microarrays composed of different clones from those used here. Of the twelve top VMH candidates predicted to be VMH-enriched from the microarray results, nine were successfully validated by real-time PCR analysis of RNA from freshly microdissected ARC, VMH, and DMH tissue. These genes include *SF-1, PACAP, Cbln1, LBH2, Slit3, guanine deaminase, TenM2, 3-OST-4*, and EST AA982708. None of these genes were regulated in the hypothalamus in response to fasting.

It is noteworthy that the expression levels of most genes on the arrays were quite similar among the three nuclei analyzed. This was to be expected, as the tissues being compared are physically quite similar to each other. In terms of general expression patterns in hypothalamic nuclei, it was observed that, whereas the ARC and VMH appeared to have a significant number of uniquely expressed genes, this was less true for the DMH. It is possible that gene expression in the DMH is more promiscuous, and that the DMH may lack its own unique transcriptional network.

In situ hybridization was used to examine the subnuclear expression patterns of three of the nine VMH enriched genes (*Cbln1*, *PACAP*, and a novel gene we have named *LBH2)*, as well as to further validate the results of the real-time PCR. These genes were chosen based on their robust VMH expression as well as their potential functional role(s). *PACAP* encodes a neuropeptide that has previously been strongly implicated in metabolic function. PACAP peptide, when administered to mice via the third ventricle, leads to a sharp reduction of food intake (Mizuno et al., 1998b). Additionally, PACAP knockout mice exhibit severe metabolic disorders, including impairments to thermogenesis and the counter-regulatory response to hypoglycemia (Gray et al., 2001; Gray et al., 2002). The VMH has been shown to play a role in both of these processes, suggesting that PACAP may contribute to some portion of these VMH functions (Perkins et al., 1981; Borg et al., 1997). Cerebellin 1 was originally identified as a 16 aa peptide, isolated from rat cerebellum (Slemmon et al., 1984). The precursor protein from which this 16 aa peptide is derived displays some similarity to circulating complement C1q, suggesting that this precursor protein may be the authentic ligand for cerebellin signaling (Urade et al., 1991). While Cbln1 has been shown by radioimmunoassay to be expressed in the hypothalamus,

its distribution within this region has not been examined (Satoh et al., 1997). Here we show that the main site of *Cbln1* expression in the hypothalamus is indeed the VMH. Cerebellin is believed to exert a neuromodulatory activity, and has been shown to increase the steroidogenic capacity of the adrenal gland. It is possible that, because of this profile of activity, it may operate either in the same pathway or a parallel pathway to SF-1 (Albertin et al., 2000; Hochol et al., 2001). *LBH2* is a novel gene which shares significant homology with *LBH*, a presumed transcription factor postulated to play a role in limb and heart development (Briegel and Joyner, 2001). Sequence alignment shows this new gene to be evolutionarily conserved among a number of species. The mRNA for this gene is short, ~800 bp, and the vast majority of its expression in the body is confined to the VMH. The function of this VMH enriched transcript awaits further studies, currently underway.

The expression patterns of the three genes tested showed some variation in the pattern of subnuclear expression within the VMH. While PACAP and Cbln1 were observed to be strongly expressed in all major compartments of the VMH, including the dmVMH, intVMH and vlVMH, LBH2 expression was almost completely absent in the vlVMH. Similarly, in the most caudal sections of the VMH, while PACAP and Cbln1 were observed most strongly in the vlVMH, LBH2 was mostly restricted to the intVMH This proves a signficant variability in gene expression within the VMH. On a larger scale, the near-total restriction of the tested RNAs to the VMH within the hypothalamus validates the approach and suggests that the other genes enriched in the VMH using microarray analysis followed by real-time PCR are likely also legitimate VMH markers. These genes include *Slit3*, *3-OST-4* , *TenM2*, *Guanine Deaminase*, and EST AA982708.

Among these genes, *3-OST-4* belongs to a family of sulfotransferases which chemically modify heparan sulfate proteoglycans (HSPGs) (Shworak et al., 1999). These are large cell-surface complexes that bind extracellular ligands and are believed to play a role in, among other things, the modulation of intercellular signaling (Bernfield et al., 1999). Transgenic mice expressing another HSPG, syndecan-1, in multiple tissues including the hypothalamus, display obesity secondary to impairment of alpha-MSH signaling in the hypothalamus (Reizes et al., 2001; Reizes et al., 2003). HSPGs and their modifying enzymes are often tightly regulated to respond to stimuli, suggesting that 3-OST-4 may play a role in signal modification in the VMH. Little is known about EST AA982708 (located on mouse chromosome 2) but the fact that the level of its RNA is reduced in hypothalamus from SF-1 knockout mice suggests that it too may merit further study. Like LBH2, this transcript was present only on the subtracted hypothalamic cDNA array and not on the 28,000 gene array which was largely composed of embryonic and adult mouse cDNA clones. Thus it appears that, as was the case for LBH2, the hypothalamus (and by extension the VMH) is the primary site of expression of this EST in the mouse.

Steroidogenic Factor 1

Several of the VMH enriched genes were dysregulated in SF-1 knockout mice. SF-1 is an orphan nuclear receptor that plays a critical role in the embryonic development of the VMH (Ikeda et al., 1995). Proper differentiation and migration of VMH neurons during development is presumed to be associated with the activation of a number of SF-1 target genes. While direct targets of SF-1 have been identified in the adrenal gland including cholesterol side chain cleavage enzyme, steroid 21-hydroxylase and the

aldosterone synthase isozyme of steroid 11 beta-hydroxylase, few of its VMH targets have been identified (Parker and Schimmer, 1997). Four out of eight VMH markers tested (*Cbln1*, *Slit3*, *TenM2*, and EST AA982708) were significantly downregulated in mutant neurons.

The proper formation of the VMH during development clearly relies on a downstream SF-1 regulated gene. Without SF-1, the VMH neurons are diffusely spread throughout the hypothalamus. It is difficult to imagine a mechanism for co-migration and coalescence of a specific group of neurons without invoking the existence of a specific signaling molecule or molecules (either a secreted factor, receptor, or intracellular signaling molecule, or any combination of the above) expressed only by those specific neurons. Such a factor would necessarily be expressed by VMH neurons, but not by their non-VMH neighbors, and must also be regulated by SF-1. By this reasoning, those genes that comprise the subset of VMH markers that are regulated by SF-1 all represent strong candidates for involvement in the development of the VMH and the resulting phenotype of the SF-1 knockout mouse.

Among these genes, *Slit3* especially stood out, as the *Slit* genes, along with their *Robo* receptors, are known regulators of cell migration and axon guidance (Wu et al., 1999). However, examination of the VMH neuron distribution in Slit3 knockout mice revealed no obvious abnormalities. The VMH appeared grossly normal by staining with an anti-NeuN antibody, and the estrogen receptor-alpha neuron population was of normal appearance. Because the Slit genes are implicated in both neuronal migration and axon guidance, it remains possible that the VMH neurons of Slit3 knockout mice have an axon guidance phenotype. However, it was not possible to observe the neuronal connectivity

in the fixed brain tissue available. In the future, to examine the connectivity of these neurons in Slit3 knockout mice, VMH projections could be examined using the axonal tracer DiI in analagous studies to those used on the ARC (Bouret et al., 2004a, b). Alternatively, Slit3 -/- mice could be crossed to AgRP-cre or POMC-cre mice in order to test the VMH -> ARC connections using a conditional PRV strain. Even in the event that Slit3 plays no role in the organization of the VMH, it is likely that some or all these genes are direct or indirect targets of *SF-1*. As such, there is a chance that each of them may play some kind of role in VMH development. All of these genes merit further study.

LBH2-GFP Mice: Markers as Tools to Understand the VMH

The marker genes reported here thus constitute a set of reagents that can be used to attempt to define distinct cell types in the VMH. Knowledge about specific cell types would make possible future efforts to study their function. The definition of neuronal subtypes based on the expression of marker gene expression rather than anatomic localization or morphology offers a number of experimental advantages. For example, it has been shown that the POMC and NPY neurons in the ARC, which are not distinguishable on the basis of localization or morphology, display opposing responses to treatment with leptin, both with regard to activity and plasticity (Elias et al., 1999; Pinto et al., 2004). Defining neuronal subpopulations on the basis of gene expression is additionally advantageous because it allows for the introduction of a marker or other genes specifically to those neurons using BAC-mediated transgenesis. Bacterial artificial chromosomes (BACs) can be easily modified to express a transgene usually under the control of the full set of regulatory elements necessary for the eutopic expression of that

gene (Gong et al., 2002). BAC transgenic mice expressing GFP in specific classes of neurons have proven extremely useful for electrophysiological studies, electron microscopy-based assays of neuronal plasticity and synaptogenesis, cell culture assays, and gene expression studies of nuclear subpopulations (Pinto et al., 2004; Roseberry et al., 2004).

To this end, we produced BAC transgenic mice expressing eGFP under the control of the LBH2 promoter (LBH2-eGFP). These mice display bright green fluorescence in all of the sites of expression of LBH2 as determined by in situ hybridization. They do exhibit spurious expression in the piriform cortex. In the VMH, all eGFP-expressing neurons do in fact express LBH2, though there appear to be a small number of unlabeled LBH2 neurons. LBH2 appears to be expressed in roughly 80% of dmVMH neurons, and a lesser number (roughly 60%) of intVMH neurons. LBH2 is only expressed rarely in the vlVMH, where it shows a nearly complete non-overlapping profile with estrogen receptor-alpha. A comparison of LBH2 expression and SF-1 expression shows that, as expected, they show substantial overlap in the dmVMH and intVMH. In rostral VMH sections, SF-1 is significantly expressed in the vlVMH, while LBH2 is not. However, in the caudal vlVMH, neither SF-1 nor LBH2 appear to be expressed (it is possible that SF-1 is expressed here at low levels), and they show significant overlap in the intVMH. In all sections, LBH2 neurons represent only a subset of SF-1 neurons (with similar numbers as described above), as SF-1 appears to be expressed in all VMH neurons in the regions where it is present. The fact that LBH2 shows a more medial expression pattern than SF-1, PACAP, and Cbln1 suggests that it may serve as a useful driver for transgenes targeted to only the dmVMH and vlVMH. As

discussed, these regions are primarily implicated in the response to leptin and the regulation of food intake and body weight, while the vlVMH is most implicated in sexual behavior. Thus, LBH2 might represent a powerful tool to dissect the differences between these segments of the VMH.

Clearly, there are many avenues for further research on the VMH made possible as a result of these results. Other BAC transgenic mice expressing fluorescent proteins under the control of other VMH markers would be extremely useful for the combinatowrial studies necessary to fully define VMH neuronal subsets on the basis of gene expression. There are limitless options available for use of the marker genes to express of a wide array of transgenes. GFP mice, in addition to neuroanatomical studies, would be extremely useful for electrophysiological studies and studies of neuronal plasticity. Mice expressing cre recombinase in particular subsets would allow for tracing experiments using a variety of retrograde and anterograde transneuronal tracers. Perhaps most intriguing, expression of transgenes designed to modulate neuronal function and activity. The ability to exert spatiotemporal control over the activation or inhibition of specific neuronal populations would allow direct observations of their impact on the output of a circuit, whether it be behavioral or metabolic.

Conditional Pseudorabies Virus Tracing

Pseudorabies virus has been used extensively to trace neuronal circuitry, owing to its advantageous characteristic of infecting in a retrograde manner via synaptic connections. However, the advent of conditional strains of PRV that are conditional upon cre recombinase expression for viability and/or GFP expression have brought this

technology to a new level, allowing detailed transneuronal tracing from discrete populations of neurons (DeFalco et al., 2001).

Here, we have used a viable, TK+ strain of Ba2001 that is conditional for GFP expression only. The absolute restriction of the cre enzyme to one region of interest in the brain is a specific requirement for the use of this virus for tracing initiated in the CNS. If the cre enzyme were expressed in other areas, the virus would be able to spread there and then become GFP-positive at multiple sites. In such a case, it would be impossible to determine the origin of GFP-positive staining at a distal second-order site.

In wild-type control animals, minimal to no GFP expression is observed following infection with Ba2001TK+. In AgRP-cre animals, bright fluorescence was observed in the ARC and several upstream sites, including the VMH. This represents significant corroborating evidence for the finding that the VMH sends significant projections to the ARC. More excitingly, because this system allows for definitive identification of individual neurons that send outputs to cre-expressing neurons, this system can be used in conjunction with the set of VMH markers identified here to determine the identity of VMH neurons that project to ARC AgRP neurons. Using traditional Ba2001 in conjunction with POMC-cre mice would, in an analagous fashion, be useful for learning about VMH subpopulations that project to these important anorexigenic neurons. Knowledge about the particular VMH neuronal subtypes that project to ARC neurons would be extremely valuable. While it is clear that the VMH is profoundly important for energy homeostasis and for responding to leptin, it is wholly unclear how the VMH accomplishes these tasks. Detailed information about VMH neuronal wiring would allow for future experiments designed to understand the importance of particular VMH outputs

to other nuclei. For example, if subsets of VMH neurons that project to ARC neurons could be identified, these populations could be modulated to begin to understand whether VMH outputs to the ARC are the essential mediators of leptin's action on the VMH. Studies such as these will be essential steps along the path to a full description of hypothalamic feeding pathways that respond to leptin and other central and peripheral signals.

Conclusion

The ventromedial hypothalamic nucleus plays a central role in energy homeostasis, but study of this nucleus has been hampered by a lack of available marker genes to label its constituent neurons. We have used LCM in conjunction with cDNA microarray technology to develop a set of markers, and have begun to use those markers to learn about the component populations that make up this important nucleus. Additional studies of SF-1 knockout mice addressed the regulation of these genes, suggesting that LCM coupled with microarray analysis may be effective for uncovering nucleus-specific transcriptional networks. The techniques used in this thesis are not restricted to study of the VMH. They may be just as easily applied to other nuclei and subnuclei, whether they be in the hypothalamus or other regions of the brain. Marker identification, followed by definition of nuclear subtypes and subsequent transgenic approaches to study those subtypes, represents a generally applicable strategy to dissecting the composition, connectivity and function of any poorly characterized brain region.

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