Immortalized neurons for the study of hypothalamic function

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OVER THE PAST TWO DECADES, obesity has become a pandemic affecting millions of people globally (249). Obesity-related complications, such as type 2 diabetes, hypertension, atherosclerosis, stroke, and heart disease, contribute to the global burden of chronic disease and disability and increase mortality risks among all age groups in the human population (82). Enhanced intake of high-calorie food and beverages, coupled with sedentary and stressful lifestyles, as well as significant contributions of genetic factors, seems to be responsible for the dysregulation of energy balance that leads to the development of obesity (13, 47). Energy balance is largely regulated by the hypothalamus, which consists of neurons that control central neuroendocrine function, including, but not limited to, energy homeostasis (72). In complex, multicellular organisms, individual cells need a continuous supply of oxygen and energy-rich nutrients, which are provided by a steady flow from the systemic circulation. The coordination of these processes, including the ingestion, digestion, and absorption of nutrients and their disposal, utilization, and storage by various tissues and organs, is collectively known as energy homeostasis and is tightly coordinated by the hypothalamus via cross talk between peripheral signals and hypothalamic factors. The neuroendocrine hypothalamus integrates afferent signals from the periphery and processes efferent signals that modulate food intake and energy expenditure to maintain energy stores at appropriate levels for given environmental conditions (255). Any perturbations in this homeostatic system, which is centrally regulated by the hypothalamus, may lead to metabolic disorders resulting in major health problems, such as obesity, and related complications, such as type 2 diabetes and infertility (144).
The hypothalamus is subdivided into many regions, including several nuclei that regulate feeding and energy metabolism, such as the arcuate nucleus (ARC), paraventricular nucleus (PVN), ventromedial nucleus (VMH), dorsomedial nucleus (DMH), lateral hypothalamus (LH), and suprachiasmatic nucleus (SCN) (Fig. 1). The ARC, PVN, DMH, and SCN are among the most important hypothalamic nuclei involved in the regulation of energy intake and expenditure. Experimental lesioning and stimulation have suggested that the VMH and LH control satiety and hunger, respectively (7, 22, 28, 86, 206). All these hypothalamic regions and nuclei are interconnected by neuronal pathways to form a complex network of orexigenic (appetite-stimulating) and anorexigenic (appetite-suppressing) circuits, which regulate food intake and energy expenditure to maintain body weight (72). Extensive basic and clinical research has demonstrated that a delicate balance between appetite-regulating hypothalamic factors and neuronal circuits plays a critical role in maintaining energy homeostasis. Numerous neuropeptides, such as neuropeptide Y (NPY), agouti-related peptide (AgRP), melanin-concentrating hormone (MCH), α-melanocyte-stimulating hormone (α-MSH), corticotropin-releasing hormone (CRH), cocaine- and amphetamine-regulated transcript (CART), brain-derived neurotrophic factor (BDNF), orexin A and B, glucagon-like peptide (GLP)-1, GLP-2, galanin, ghrelin, and neurotensin (NT), are involved in orexigenic and anorexigenic processes in the hypothalamus (2). Apart from these factors, estrogen is critical, not only for the maintenance of normal reproductive behavior and function but also for its anorexigenic action in the prevention of obesity (50, 246), suggesting a dual role for estrogen, in reproduction as well as feeding. In recent years, major advances have been made in investigating the role of these hormones and peptides in energy homeostasis and in the development of obesity through the use of many rodent lesion, diet, and genetic models (15). Although the roles of different hypothalamic neuropeptideergic neurons, such as NPY/AgRP, proopiomelanocortin (POMC)/CART, MCH, orexin A and B, BDNF, and gonadotropin-releasing hormone (GnRH) neurons, in the vital functions of appetite control, energy metabolism, and reproductive function have been described extensively in the past decades (for reviews, see Refs. 20, 150, 183, 215), the precise molecular mechanisms involved in neuropeptide and receptor gene expression and regulation remain largely unknown. To investigate these mechanisms, studies on the whole brain are difficult because of the complex structure and heterogeneity of hypothalamic neurons, whereas use of primary hypothalamic

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Fig. 1. Schematic of the hypothalamic and brain stem regions that express neuropeptides involved in energy homeostasis (coronal sections). Hypothalamic function is regulated by complex neuronal connections between the hypothalamus, brain stem, and higher cortical centers. Adiposity signals (leptin and insulin), satiety signals (gut hormones and peptides), and nutrients (glucose and amino and fatty acids) act on neurons in the arcuate nucleus (ARC) and nucleus tractus solitarius (NTS) via the bloodstream because of an incomplete blood-brain barrier (BBB; dashed line) at the median eminence (ME) and area postrema (AP). Peripheral signals may also act directly on the brain stem and hypothalamus via the vagus nerve. AgRP, agouti-related peptide; CART, cocaine- and amphetamine-regulated transcript; CB1, endocannabinoid receptor 1; CCK, cholecystokinin; CRH, corticotropin-releasing factor; DMH, dorsomedial hypothalamic nucleus; GLP-1, glucagon-like peptide-1; GLP-2, glucagon-like peptide-2; LH, lateral hypothalamus; MCH, melanin-concentrating hormone; NPY, neuropeptide Y; NT, neurotensin; Ob-Rb, leptin receptor; OC, optic chiasm; OXM, oxyntomodulin; OXY, oxytocin; Pit, pituitary gland; POMC, proopiomelanocortin; PVN, paraventricular nucleus; PYY, peptide YY; SCN, suprachiasmatic nucleus; 3V, third cerebral ventricle; TRH, thyrotropin-releasing hormone; VMH, ventromedial hypothalamic nucleus.
culture is quite challenging. Suitable hypothalamic cell models were therefore needed to elucidate the action of neuropeptides in the hypothalamus to determine their role in energy homeostasis. We speculate that the recently generated embryonic- and adult-derived immortalized, clonal, hypothalamic mouse cell lines will serve as appropriate models to conduct these studies and provide information on the role of the hypothalamus in energy balance (18, 19, 78, 164). These are the first hypothalamic neuronal models that endogenously express functional hormone receptors and neuropeptides involved in energy homeostasis and reproduction and, thus, can be used to study signaling mechanisms initiated by receptor activation and regulation of neuropeptides by multiple stimuli in the hypothalamus. The primary purpose of this review is to provide updated information on the role of the currently available hypothalamic cell models in strengthening our knowledge of hypothalamic function. Specifically, this review will 1) comment on the structure, development, neurogenesis, and plasticity of the hypothalamus, 2) recapitulate the generation and use of embryonic and adult hypothalamic cell models, and 3) shed light on aspects of energy homeostasis, including glucose sensing, as well as reproduction.

Central Regions Involved in the Regulation of Energy Homeostasis

The concept of the hypothalamus as a distinct neurological entity concerned with a variety of regulatory processes, such as energy homeostasis, biological rhythms, hormone production, autonomic functions, and behavior, emerged by the end of the 19th century. At the turn of the 20th century, there was limited knowledge of the function of this part of the brain surrounding the third ventricle based primarily on various pathological and clinical observations (180). Since then, extensive experimental evidence indicates that the hypothalamus contains the control systems that are critically involved in a wide range of homeostatic and rheostatic regulatory processes (29, 185, 235). Among these are the control of water balance, food ingestion and energy metabolism, body temperature and neuroendocrine secretion, and regulation of reproduction and various emotional states (76). One of the most important functions of the hypothalamus is linking the nervous system to the endocrine system via the pituitary gland. The magnocellular neuroendocrine neurons in the supraoptic nucleus (SON) and PVN of the hypothalamus project to the posterior pituitary gland through the pituitary stalk and cause the release of oxytocin or vasoressin into the venous sinusoid of the posterior pituitary (12). The parvocellular neurosecretory neurons project to the median eminence (ME) and transmit releasing or inhibiting hormones into the portal blood vessels of the anterior pituitary, thus linking the hypothalamus to the anterior pituitary (99, 214). At least six parvocellular cell types are recognized: they produce thyrotropin-releasing hormone, CRH, growth hormone-releasing hormone, somatostatin, GnRH, and dopamine (236). Apart from these hormones, a large number of neuromodulators, including the classic neurotransmitters, and a wide variety of neuropeptides are present in the hypothalamus and have been shown to play a critical role in energy homeostasis (2, 127, 171, 181).

Anatomically, the human hypothalamus is confined anteriorly by the lamina terminalis, posteriorly by the midbrain tegmentum, and superiorly by the hypothalamic sulcus (26). The hypothalamus, because of its complex cellular arrangement and multitude of afferent and efferent projections, is conventionally distinguished into three main regions: 1) the chiasmic (preoptic) region, which contains the SCN, sexually dimorphic nucleus, SON, and PVN; 2) the tuberal region, which contains the VMH, DMH, ARC, lateral tuberal nucleus, and tuberomammillary nucleus; and 3) the mamillary complex (26, 27, 219, 235). The hypothalamus is extensively connected with the higher centers, such as the cerebral cortex and limbic region, and the brain stem. The main areas of the CNS involved in the regulation of appetite and reproduction are schematically represented in Fig. 1.

The anatomic structure of the hypothalamus is indeed well characterized, and much research has been performed to delineate the function of the hypothalamic regions and nuclei. The original stimulation and lesioning experiments concluded that some of the hypothalamic nuclei act as “hunger” and “satiety” centers, and it has recently become clear that hypothalamic nuclei comprise a complex array of distinct neuronal populations, expressing a specific complement of neuropeptides, neurotransmitters, and receptors that are involved in the regulation of many vital functions (76). Several recent studies using transgenic animals demonstrated the importance of hypothalamic neuronal subtypes in regulating energy homeostasis. For example, genetic ablation of AgRP neurons was shown to result in an acute reduction of feeding, and genetic ablation of orexin neurons causes narcolepsy, hypophagia, and late-onset obesity, suggesting a critical role of these neurons in the regulation of energy homeostasis (97, 103). Another study demonstrated that leptin partially regulates body weight homeostasis by activating leptin receptors on POMC neurons (11). Similarly, it has been shown that melanocortin-4 receptor (MC4R) knockout mice exhibit hyperphagia and obesity (116). On the basis of these significant findings, it is evident that genetically modified animal models have been instrumental in providing novel insights into the hypothalamic neuronal functions. However, further investigation focusing on the regulation of gene expression of these and several other neuropeptides, neurotransmitters, and their receptors remains to be performed. Specifically, in vivo gene transfer for gene promoter studies is needed; however, this is quite challenging work. This problem can be circumvented and such questions can be investigated at the molecular and cellular levels by establishment of clonal, immortalized hypothalamic neuronal cell lines that endogenously express the cellular markers of interest. For example, it has been shown that leptin’s inhibitory regulation of feeding is mediated at least partly through NT neurons (136, 216); however, it is difficult to determine, using in vivo models, whether the effect of leptin on NT neurons is direct or occurs through afferent neurons. To address this issue, we used immortalized cell lines that endogenously expressed NT and the functional leptin receptor Ob-Rb to analyze leptin responses within these NT cell lines and define molecular mechanisms involved in leptin-mediated regulation of NT gene expression (53, 54). Thus, animal and cell line models serve as complementary tools that, together, allow for a detailed characterization and understanding of native individual hypothalamic neurons responsible for regulating specific physiological processes.
Hypothalamic Development, Neurogenesis, and Plasticity

To better understand the function of the hypothalamus and native individual neuronal phenotype, it is essential to understand their temporal origin, maturation, migration, synaptic plasticity, and de novo neurogenesis. Neurogenesis is the process of generating functionally integrated neurons from progenitor cells or existing neurons. Just recently, the century-old dogma that neurogenesis occurs exclusively during embryonic stages in the mammalian CNS has been discarded, and investigators have established that active neurogenesis continues throughout life in discrete regions of the CNS of all mammals (98, 134, 155). Normal embryonic hypothalamic neurogenesis is critical, because developmental defects of the hypothalamus may serve as potential factors responsible for multiple diseases, including obesity. Using tritiated thymidine, a radioactive precursor of DNA, in pregnant mice on different days of gestation, Shimada and Nakamura (226) found that hypothalamic neurons are produced in the matrix layer surrounding the third ventricle between embryonic day 10 (E10) and E16. Neurons in most nuclei are formed between E11 and E14, with lateral nuclei typically being generated earlier than medially placed nuclei such as the ARC, PVN, and periventricular nucleus, which are formed during the later stage of embryonic life. This suggests a lateromedial gradient in the formation of hypothalamic nuclei (119, 226). Similar to rodents, in human embryos, the earliest observable structural differentiation of the hypothalamus takes place in the LH zone, which gives rise to the LH and posterior hypothalamus during the early gestational period, whereas structures such as the ARC, SCN, and PVN develop from the periventricular neuroepithelium and become evident only during the late period of gestation (145). Despite extensive research, the developmental and molecular events that make neuroepithelial cells differentiate into neuroendocrine cells, the factors that cause some neurons to migrate away from their place of origin, the distinct differences between hypothalamic neurons, and the signals that cause sexual differences in the development of hypothalamic nuclei remain largely unknown. Importantly, how embryonic neurons differ from adult neurons with respect to phenotypic neuropeptide gene expression, transcriptional regulation, and secretion is still not clear.

In most mammals, adult neurogenesis actively occurs in the subventricular zone of the lateral ventricle and in the subgranular zone of the dentate gyrus in the hippocampus. A number of growth factors and neurotrophic factors have been shown to regulate neural stem cells and progenitor proliferation in the adult CNS (101, 157). Originally, it was thought that neurogenesis outside the subventricular zone and subgranular zone, including the adult hypothalamus, is limited or even nonexistent (176), until it was discovered that cilary neurotrophic growth factor (CNTF) induces de novo neurogenesis in the adult mouse hypothalamus (142) via recruitment of GLP-1 (19). The CNTF-induced neurogenesis that occurs in hypothalamic feeding centers to reset the energy balance set point and to induce sustained weight loss suggests a critical role for neurogenesis in the regulation of energy homeostasis and, possibly, other hypothalamic functions such as reproduction. Other growth factors and neurotrophic factors such as BDNF, epidermal growth factor, basic fibroblast growth factor, and insulin-like growth factor I induce de novo neurogenesis in the adult hypothalamus, suggesting that this part of the CNS has potential to generate new neurons when stimulated under specific conditions (199, 201, 256). Adult neurogenesis in the hypothalamus may play an important adaptive role in replenishing neurons degenerated by aging. It has been shown that the multitude of changes in the hypothalamus during aging forms the basis of many physiological, endocrine, and behavioral changes in elderly people (5, 112). Indeed, some hypothalamic nuclei show a dramatic functional decline with aging, whereas others seem to become more active in old age (112). Apart from aging, environmental factors, such as exposure to high-fat diet, induce hypothalamic endoplasmic reticulum stress and neuronal apoptosis, which may play a role in the development of obesity (179, 193, 259), and as elegantly shown by Kokoeva et al. (142), hypothalamic CNTF-induced de novo neurogenesis plays a key role in its prevention. Thus, hypothalamic de novo neurogenesis, which was not recognized until recently, is an essential phenomenon that enables the hypothalamus to maintain its vital role.

Similar to de novo neurogenesis, neuronal plasticity in the hypothalamus also plays an essential role in the regulation of energy balance (113). It was shown that leptin exerts potent and rapid effects on the afferent inputs and synaptic connections of key neurons such as NPY and POMC neurons, suggesting that leptin can potentially change the threshold response to stimuli that regulate body weight (204). Similarly, leptin functions as an essential factor for brain development during the neonatal critical period, promoting formation of hypothalamic pathways projecting specifically from the ARC and, thus, directing the development of central circuits involved in energy homeostasis during postnatal life (113). Further studies into the mechanisms underlying the development of anatomic structures, adult neurogenesis, and neuronal plasticity of the mammalian hypothalamus could provide important insights into hormone and neuropeptide action, the regulation of feeding, the pathogenesis of neurodegenerative disorders, and the regulation of other complex behaviors.

Embryonic and Adult Hypothalamic Neuronal Cell Models to Study Hypothalamic Function

Structurally, the hypothalamus consists of a multitude of fully differentiated functional neurons regulating many vital functions that include energy homeostasis (76). These hypothalamic neurons express neuropeptides that are controlled by internal, as well as external, stimuli (76). The mechanistic studies of neuropeptide gene regulation and signal transduction events occurring in these hypothalamic neurons are quite challenging to perform in vivo because of the complex anatomy of the hypothalamic. Historical methods and manipulations, such as bilateral stereotactic or electrical stimulation or lesioning, were used to study the functions of specific hypothalamic nuclei; however, these methods could typically stimulate or destroy a wide range of hypothalamic neuronal subtypes, as well as activate or disrupt afferent or efferent neuronal terminals, thereby producing erroneous results. Also, classical in vivo approaches cannot be used to investigate any direct effect of an agent on specific hypothalamic neuronal subtypes or on neuropeptide gene transcription, synthesis, or secretion, largely because of the multitude of synaptic inputs received from other adjacent neurons. Although rodent genetic
models have recently been used to examine the consequences of eliminating neuropeptides or ablating neurons that endogenously express these neuropeptides, these models have limitations due to the challenges in creating hypothalamus-specific knockouts. On the contrary, in our opinion, established cell lines, derived from hypothalamic tumors or immortalized from primary cultures, provide a simpler model that lacks the complexity and integrated network of neuronal inputs, connections, and signaling and make it feasible to study those areas found to be challenging to investigate in vivo. Immortalized cell lines represent an unlimited homogeneous, clonal population of specific neuronal cell types and can be maintained in a controlled environment with fewer uncontrolled variables than in vivo models. Another advantage is that cell lines can be screened, and different neuronal phenotypes can be used to investigate the expression of specific genes or proteins; such investigation is difficult in vivo because of the numerous neuronal phenotypes present in a given hypothalamic region. For all these reasons, scientists have been generating and using immortalized cell models but have had limited success over the last 30 years (37, 38, 59). Using a similar technology of retroviral transfer of SV40 T antigen (37), our laboratory successfully generated an array of immortalized cell models from the mouse embryonic hypothalamus (18). Many of these clonal cell models express specific neuropeptides and receptors and have been extensively used in scientific laboratories worldwide (for review, see Refs. 17, 78, 164). Although these embryonic hypothalamic models are useful in understanding cellular biology of specific neuroendocrine cells, it is imperative to know whether these embryonic neurons differ from adult neurons in the basic control mechanisms involved in neuronal function. Unlike embryonic cells, which are proliferating, immortalization of fully differentiated, nonproliferating adult hypothalamic neurons seemed impossible, since immortalization using SV40 T antigen transfer can be achieved only in proliferating cells (37, 59). However, on the basis of a novel finding that CNTF induces neuronal proliferation in vivo (142), our group successfully immortalized adult-derived mouse hypothalamic neurons (17, 78, 164).

Generation of Embryonic and Adult Hypothalamic Neuronal Cell Models

Historically, there have been attempts to generate hypothalamic cell lines; however, these cell lines were not well characterized, and few studies utilized these models (131, 207). Thus the lack of appropriate neuronal cell models representative of unique hypothalamic neurons encouraged our research group to generate embryonic, clonal hypothalamic mouse cell lines (18). To immortalize embryonic neurons, we transformed primary hypothalamic cultures obtained from fetal mice on E15, E17, and E18 utilizing retroviral SV40 T antigen transfer to generate a heterogeneous mixed population of neurons. These immortalized embryonic neuronal cultures were further subcloned until we obtained several single cells that were further grown into homogeneous, clonal cell populations. Initially, we generated a total of 38 mouse embryonic, clonal, hypothalamic cell lines that were originally labeled N-“clone number”; later, we designated them mHypoE-“clone number” (mouse, hypothalamic, embryonic-“clone number”) to distinguish them from other newly created cell lines. We have generated >60 clonal hypothalamic cell lines. Although these cell lines commonly express mature neuronal markers, such as neuron-specific enolase (NSE) and neurofilament, but not glial fibrillary acidic protein specific to neuroglia, each cell line exhibits a distinct neuronal phenotype, demonstrating potential diversity within the original heterogeneous population. The cells also possess markers of neurosecretory machinery, such as syntaxin, contain a large number of neurosecretory granules, and exhibit an intracellular calcium response following potassium chloride-induced depolarization (18). These cell lines were further characterized and found to endogenously express hormone receptors and neuropeptides associated with regulation of energy homeostasis and reproduction. Some of the key neuropeptides and receptors expressed in these mouse embryonic cells are NPY, AgRP, POMC, CART, NT, MCH, vasoactive intestinal peptide, CRH, GLP-1, insulin receptor, leptin receptor, GLP-1 receptor (GLP-1R), GLP-2 receptor (GLP-2R), melanocortin-3 receptor, and MC4R. These cell lines have provided a valid model system for molecular and biochemical investigations on the regulation of hypothalamic neuropeptides involved in the regulation of vital functions, including energy balance and reproduction (17, 18, 78, 164).

Apart from the mouse model, the rat is another important animal model commonly used in biomedical research. Because of the lack of rat hypothalamic cell lines, our research team recently generated 33 rat embryonic hypothalamic cell lines (rHypoE-“clone number”) using the previously described immortalization technique (89). Similar to the mouse embryonic hypothalamic cell models, each rat cell line has a distinct phenotype and a unique array of genes involved in several neuroendocrine functions, representing a unique neuronal subtype from the hypothalamus. Many of the cells express receptors and neuropeptides involved in the regulation of reproduction, such as estrogen receptor (ER)-α and -β, kisspeptin, neurokinin B, GnRH, RF-amide-related peptide-3 (RFRP-3), and the kisspeptin receptor GPR54, whereas others express neuropeptides involved in nutrition such as NPY, AgRP, POMC, MCH, and receptors for insulin, leptin, and melanocortins (89) (http://www.CELLutionsBiosystems.com). Thus we speculate that using these cell lines, it is possible to dissect complex cellular regulatory mechanisms involved in the hypothalamic control of reproduction and nutrition and their cross-regulation.

After their generation, the embryonic hypothalamic neuronal cell models proved to be useful in dissecting the molecular mechanisms of specific neuroendocrine cells in the regulation of energy homeostasis and reproduction (18). Although our embryonic neuronal models exhibit the characteristics of mature neurons, it is possible that they may not accurately represent fully differentiated adult neurons because of differences in their physiological and developmental properties. This has been difficult to investigate, primarily because of the lack of adult hypothalamic neuronal cell models. Additionally, it is important to understand how adult neurons function and respond to drugs and hormones, which may not be possible using embryonic cell models. For this reason, our laboratory group was prompted to develop adult hypothalamic neuronal cell models. Historically, it has been difficult to generate these models because of the lack of naturally occurring CNS tumors and the challenges associated with immortalizing fully differentiated, nonproliferating adult neurons from primary culture
Table 1. Peptide and peptide-receptor genes expressed in adult mouse hypothalamic cell lines

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<tr>
<th>Peptides</th>
<th>Cell Lines</th>
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<tbody>
<tr>
<td></td>
<td>mHypoA-1/2</td>
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<tr>
<td>Agouti-related peptide (AgRP)</td>
<td>+</td>
</tr>
<tr>
<td>Arginine vasopressin (AVP)</td>
<td>+</td>
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<tr>
<td>Corticotropin-releasing hormone (CRH)</td>
<td>-</td>
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<tr>
<td>Glutamylpeptidase 4 (DPP4)</td>
<td>+</td>
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<tr>
<td>Galanin</td>
<td>+</td>
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<tr>
<td>Gherlin</td>
<td>+</td>
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<tr>
<td>Neurokinin B (NK-B)</td>
<td>+</td>
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<tr>
<td>Neurotensin (NT)</td>
<td>-</td>
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<tr>
<td>Neuropeptide Y (NPY)</td>
<td>+</td>
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<tr>
<td>Oxytocin (OXT)</td>
<td>+</td>
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<tr>
<td>Proglucagon (ProGlu)</td>
<td>+</td>
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<tr>
<td>Proopiomelanocortin (POMC)</td>
<td>-</td>
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<tr>
<td>Tyrosine hydroxylase (TH)</td>
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<td>Urocortin (Ucn)</td>
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<th>Peptide receptors</th>
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<tbody>
<tr>
<td>Androgen receptor (AR)</td>
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<tr>
<td>Ciliary neurotrophic factor receptor (CNTF-R)</td>
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<tr>
<td>Estrogen receptor-α (ERα)</td>
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<td>Ghrerin receptor (GHSR)</td>
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<tr>
<td>Glucagon-like peptide receptor 1 (Glp1R)</td>
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<tr>
<td>Glucagon-like peptide receptor 2 (Glp2R)</td>
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<tr>
<td>Gpr 54 (Kiss-1 receptor)</td>
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<td>Insulin receptor (InsR)</td>
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<td>Leptin receptor (ObRb)</td>
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<tr>
<td>Melanocortin-3 receptor (MC3R)</td>
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<tr>
<td>Melanocortin-4 receptor (MC4R)</td>
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<tr>
<td>Neuropeptide Y Y1 receptor (NPY Y1R)</td>
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<td>Neuropeptide Y Y5 receptor (NPY Y5R)</td>
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Characterization of Hypothalamic Cell Models

Having been removed from their native environment, cell models are often challenged on the basis of their likeness to their endogenous counterparts. For this reason, our laboratory has completed extensive profiling of our hypothalamic neuronal models. Our findings demonstrate that these cell lines not only maintain expression of key neuronal markers but that they also possess ultrastructural properties characteristic of mature endogenous neurons, such as neurites and secretory granules. In addition, a number of cell lines exhibit the potential to produce major neurotransmitters. Several of our cell lines have been shown to express tyrosine hydroxylase, a marker of catecholaminergic neurons, and, thus, have the potential to...
produce dopamine, norepinephrine, and epinephrine, depending on the complement of downstream catalytic enzymes. Furthermore, a number of other cell lines express tryptophan hydroxylase, the rate-limiting enzyme of serotonin production, while others express glutamate decarboxylase, an enzyme involved in GABA synthesis (17, 18). Consistent with the heterogeneity that defines the mammalian hypothalamus, each cell line possesses a unique expression profile that includes the expression of hormone-releasing and -inhibiting factors, as well as neuropeptides and receptors known to be involved in the regulation of homeostatic function (17–19, 89). Our experience has demonstrated that these expression profiles remain consistent up to ~30 passages and that expression studies are best performed within a specified passage range.

Nonetheless, there has been some debate as to whether these cell models represent true neuronal populations within the hypothalamus. For example, a couple of neuronal cell lines appear to express both POMC and NPY, neuropeptides widely believed to be expressed by mutually exclusive neuronal populations within the ARC. We have found that this may be due to subcloning more than one neuron, such as in the original mHypoE-43 cell line. Once this line was further subcloned, the clonal lines now only express NPY or POMC. Whether this is the case for the other lines is yet to be determined. For example, it is also known that, despite this apparent contradictory finding to current evidence, a recently published genetic lineage tracing study suggests that this phenomenon may be physiologically sound. Padilla et al. (194) demonstrated that POMC and NPY neurons share a common ontogeny and derive from a common POMC-expressing progenitor. As a subpopulation of these progenitors develops into mature NPY neurons, there are periods during gestation and the postnatal period that POMC and NPY are coexpressed within these neurons (194). Interestingly, in agreement with this idea, a limited number of the magnocellular neuroendocrine neurons in the hypothalamus have been found to express both oxytocin and vasopressin, although it was previously believed that these two peptides were exclusively released from individual neurons (140). A similar example is found in the small intestine. Originally thought to be produced by distinct intestinal endocrine cell populations, GLP-1 and glucose-dependent insulinotropic polypeptide were later discovered to be coexpressed within midintestinal regions in several species (182). Furthermore, it should be recognized that the limitations of current in vivo methods of detection, such as multilabel immunocytochemistry and in situ hybridization techniques, leave much unknown about neuropeptide coexpression in the hypothalamus. As such, a lack of in vivo evidence should not preclude the possibility of an unconventional coexpression of two functionally distinct genes.

Genetic modification of these neurons by introduction of SV40 T antigen may also potentially alter the cellular phenotype of our neuronal models. To investigate this, our laboratory has performed short-hairpin RNA-mediated T antigen knockdown studies. These experiments demonstrate that SV40 T antigen upregulates endogenous expression of what appears to be a limited number of neuropeptides, such as AgRP and oxytocin, and enhances the basal activity of several phospho-proteins, such as JAK/STAT and 5’-AMP-activated protein kinase (AMPK) (Belsham, unpublished data). However, these phenotypic alterations are not likely to impinge on the validity of our cell models, given that the appropriate controls are in place. For example, to reduce the basal activity of these key signaling proteins, a common practice in our laboratory has been to culture neurons in serum-free, low-glucose medium for several hours prior to treatment. As these studies are still preliminary in nature, further characterization is underway, so more distinct conclusions on the role of T antigen in cultured cell lines can be drawn from these studies.

Mechanisms Underlying CNTF-Induced Adult Neurogenesis

The novel technique that we have developed to immortalize adult hypothalamic neurons can be utilized to develop new models for neuroendocrine research. Using this technique, we have generated cell lines from adult mouse hippocampus (88), and we are in the process of immortalizing hypothalamic region-specific neuropeptide-expressing neurons. While establishing this unique immortalization technique, we studied the underlying mechanism by which CNTF induces neurogenesis, thereby permitting the immortalization of primary cells. Serendipitously, we observed that CNTF treatment of primary hypothalamic cultures upregulated neuronal expression of the proglucagon gene. This was further confirmed in vivo in the intact hypothalamus by immunohistochemical analysis of brain sections obtained from C57/BL6 mice that had undergone intracerebroventricular treatment with CNTF for 7 days. We found that CNTF induced the expression of GLP-1 (a product of posttranslational processing of prohormone proglucagon) in the hypothalamus, particularly in the periventricular nucleus and DMH of the hypothalamus (Fig. 2). Furthermore, using one of the newly generated adult hypothalamic clonal cell models mHypoA-2/10, we studied the regulation of proglucagon gene expression by CNTF and also signaling pathways activated by CNTF receptor. Using quantitative real-time RTPCR, we found that CNTF upregulated proglucagon gene expression between 8 and 12 h after treatment (Fig. 3B), and using Western blot analysis, we found that CNTF activated JAK2 and STAT3, the classical signaling molecules activated by CNTF receptor stimulation (Fig. 3A). To further investigate which proglucagon-derived peptide (PGDP) was involved in mediating CNTF-induced neurogenesis, we analyzed the effects of GLP-1 on primary hypothalamic neurons. Among several PGDPs that are generated by posttranslational processing of proglucagon by prohormone convertase 1 and prohormone convertase 2, two main peptides, GLP-1 and GLP-2, are capable of inducing cellular proliferation. On the basis of the findings that GLP-1 inhibits pancreatic β-cell apoptosis and stimulates their proliferation (77) and that GLP-2 induces intestinal epithelial proliferation (64), it is likely that both may play an important role in exerting CNTF-induced neurogenesis. However, it is more likely that GLP-1 primarily induces proliferation because of the high levels and wide expression of GLP-1R compared with the low levels and limited expression of GLP-2R in the hypothalamus (30, 147, 173, 237). This information, as well as the availability of exendin-4, a long-acting GLP-1R agonist (74), exendin 9-39, an antagonist of GLP-1R (208), and primary hypothalamic culture from GLP-1R knockout mice, prompted an investigation into the role of GLP-1 in CNTF-induced neurogenesis (223). Thus we treated primary adult hypothalamic cultures with exendin-4 for 1 wk and then administered bromodeoxyuridine or Ki67 to
detect proliferating cells by immunohistochemical analysis. We found that exendin-4 treatment led to a significant twofold increase in the number of proliferating neurons, whereas pre-treatment of the primary cultures with exendin 9-39, the antagonist of GLP-1R, inhibited this proliferating effect of exendin-4. Importantly, CNTF treatment of the primary hypothalamic cultures derived from GLP-1R-knockout mice did not exhibit proliferation, confirming that CNTF acts through an induction of proglucagon expression and through GLP-1R signaling to promote neurogenesis within the adult hypothalamus.

It is known that CNTF is a cytokine released from the glial cells that exerts neurotrophic effects in the CNS (245). In 1996, CNTF was discovered to decrease appetite and induce weight loss in animals and humans (6). The anorexigenic effect of CNTF was not associated with stress responses, and the cessation of CNTF treatment did not result in immediate rebound weight gain (142, 233). Thus, CNTF bears great potential to become a promising antiobesity drug. However, attempts to develop CNTF analogs to treat obesity have been unsuccessful; for example, axokine, a modified version of human CNTF, becomes ineffective 3 mo after treatment because of the production of neutralizing antibodies (75). Although it is possible to redesign CNTF analogs with lower immunogenicity than axokine, our novel finding that CNTF mediates its effects via GLP-1R signaling renders GLP-1R agonists as another alternative to CNTF analogs as antiobesity drugs. GLP-1R agonists such as exendin-4 and long-acting GLP-1 analogs such as liraglutide are used as antidiabetic drugs (188, 196). It is possible that, apart from their hypoglycemic and β-cell-protective, antiapoptotic, and proliferative effects, long-acting GLP-1R agonists, which easily cross the blood-brain barrier (132), may be involved in de novo neurogenesis in the hypothalamus.
thalamus to regulate feeding and glucose homeostasis. In addition, dipeptidylpeptidase-4 inhibitors, which prolong the half-life of endogenous GLP-1, are also approved for antidiabetic treatment (135). Therefore, it is imperative to investigate the effect of these novel antidiabetic drugs on hypothalamic neurogenesis and the modulation of appetite-regulating neuropeptides. Hypothalamic cell models could be quite useful for these studies and for the design of novel antiobesity treatments.

Prospective Cell Models: Hypothalamic Glial Cells and Brain Stem Neurons

Numerous recent studies on animal models of diet-induced obesity implicate inflammatory activation within the hypothalamus as a key factor in the development of obesity (58, 114, 202, 257). These studies demonstrate that consumption of a high-fat diet activates the expression or the activity of inflammatory-responsive proteins such as SOCS3, IKK, JNK, and protein tyrosine phosphatase 1B, which impair hypothalamic leptin and insulin signaling, the main adipostatic routes that maintain a stable body fat mass. This is an important mechanism, because genetic and pharmacological inhibition of inflammatory signaling in the hypothalamus can reverse or prevent the development of diet-induced obesity (58, 114, 202, 257). Cytokines such as TNF-α and IL-1β, which are highly expressed in diet-induced obesity, are proinflammatory and proapoptotic (58), whereas cytokines such as CNTF exert anti-inflammatory and antiapoptotic effects (170). The exact mechanisms of these pro- and anti-inflammatory cytokines remain unclear because of the heterogeneity of the hypothalamus. Indeed, the hypothalamus comprises astrocytes, oligodendrocytes, tanyctyes, microglia, endothelial cells, and ependymal cells, along with numerous other neuronal subgroups (Fig. 4). For this reason, it is not possible with the currently available research tools to determine precisely which cells are responsible for the activation or suppression of inflammatory signals in response to physiological and environmental stimuli.

Thorough and rigorous evaluation of the progression of the inflammatory signaling cascade within specific hypothalamic cell types will be the next key step toward deciphering the mystery surrounding the effect of inflammation on energy homeostasis. To this end, hypothalamic glial cell models are required.

Although glial cell biology has been studied through the analysis of primary astrocyte and oligodendrocyte cell cultures, the main disadvantage of this method is that these cells are postmitotic and cannot be serially passaged or preserved. Therefore, to circumvent this problem, tumor or hybrid cell lines representative of neuroglia have been generated. One of the earlier hybrid cell models is MO3.13, an immortal human-human hybrid cell line obtained from fusion of a rhabdomyosarcoma cell (Te671) with an adult human oligodendrocyte that expresses phenotypic characteristics of primary oligodendrocytes (167, 168) (http://www.CELLutionsBiosystems.com). Another cell line, CHME-5, was obtained from human fetal microglia by transfection with the SV40 T antigen (120). Several other human or rat tumor-derived cell lines have also been generated, including glioma cell lines such as C6, F98, and RG2; glioblastoma cell lines such as U-138, M059K, and M059J; and astrocytoma cell lines such as U-87 MG, U-373 MG, and GL-15 (24) (http://www.atcc.org). These cell models present as useful tools in delineating functions of neuroglia. However, some of these cell lines represent undifferentiated oligodendrocytes arrested in an immature developmental stage (31) or are derived from benign or malignant tumors, such that they may not truly represent the physiological morphology and biochemistry of glial cells. Apart from these issues, the exact origin of these tumor-originated cell lines is not clear, unlike the cells immortalized from the hypothalamic primary cultures. Also there are no cell models representing ependymal cells, which have been reported to proliferate (42) or control subventricular neurogenesis (156). Importantly, glial cell lines originating from the glial cell-rich periventricular region or

Fig. 4. Schematic representation of the structure of the 3rd cerebral ventricle wall. Selected squared region from the coronal section of the brain at the level of the ARC of the hypothalamus (left) is shown in detail in the inset (right). Neurons from hypothalamic nuclei extensively communicate with non-neuronal cells in the periventricular region surrounding the 3rd ventricle. Periventricular region is characterized by the presence of cuboidal ependymal cells, subependymal astrocytes, tanyctyes, and microglia, among other cell types (not shown). Blood capillaries are located in subependymal region (gray).
other specific hypothalamic nuclei involved in the regulation of energy homeostasis or reproduction have not been generated. Although it is challenging to isolate ependymal cells from the wall of the third ventricle as well as subependymal astrocytes or other glia cells from other hypothalamic regions, the novel laser capture microdissection technique may permit rapid one-step procurement of cells of a distinct phenotype from a small, neuroanatomically distinct region (73). Thus, considering all these factors, targeted generation of hypothalamic immortalized glial cells by novel cell-capturing and immortalization techniques should be given serious consideration.

Apart from the hypothalamus, the brain stem is also responsible for the integration of peripheral information originating from the gut to regulate appetite, particularly with regard to meal size (96). The hypothalamus has extensive connections with the brain stem, notably with the nucleus tractus solitarius (NTS), which is a heterogeneous group of neurons representing the key integrating relay in the processing of visceral sensory and gustatory information (239, 240). The NTS has its own NPY neurons that project to the PVN (220) and has a high density of NPY-binding sites, including Y1 and Y5 receptors (67, 90). The NTS synthesizes POMC-derived peptides and expresses both MC4R and leptin receptors, suggesting that it is able to integrate peripheral satiety and adiposity signals with the hypothalamus (133, 184, 222). Importantly, humoral satiety signals, such as cholecystokinin, glucagon, GLP-1, GLP-2, bombesin, amylin, somatostatin, and peptide YY, reach the AP and NTS and interact with local receptors to modulate the biochemical pathways (Fig. 5).

Glucose-sensing neurons respond to changes in glucose levels by increasing [glucose-excited (GE) neurons] or decreasing [glucose-inhibited (GI) neurons] electrical activity (33, 91, 191). The mechanisms regulating GE cells are more thoroughly understood and were first addressed within pancreatic β-cells. Glucose enters the β-cell via transmembrane glucose transport proteins, facilitating its diffusion down a concentration gradient. Glucose is then phosphorylated by glucokinase, allowing for its entry into the glycolytic pathway. Glucose metabolism in the cell leads to an increase in the ATP-to-ADP ratio, resulting in the inhibition of ATP-sensitive transmembrane potassium channels (K\textsubscript{ATP}), thereby decreasing potassium outflow and depolarizing the cell. The resulting change in membrane potential activates voltage-gated calcium channels, and the accompanying rise in cytosolic calcium leads to the vesicular secretion of insulin (69, 71). The Penicaud and Magnuson groups (121, 152) were first to put forth the idea that the glucose-sensing machinery of the brain could function like the mechanism described in the β-cell of the pancreas. Since then, it has been hypothesized that the hypothalamus senses plasma glucose levels in a similar fashion, causing changes in the expression and secretion of neuropeptides (258).

As a result, many studies have addressed the role of low-affinity glucose transporter type 2, glucokinase, and the K\textsubscript{ATP} channel subunits SUR1 and SUR2, as well as Kir6.2, in central glucose sensing (123). In GE neurons, pharmacological inhibition of glucokinase has been shown to decrease neuronal activity, while in vitro studies on primary VMH cultures have shown that selective downregulation of glucokinase leads to selective loss of glucose sensing and a decrease in cellular ATP concentration corresponding with an increase in K\textsubscript{ATP} channel activation (68, 129, 247). This supports the model that inhibition of glucokinase, in at least a proportion of GE neurons, reduces ATP production, causing K\textsubscript{ATP} channel opening and cell hyperpolarization.

In addition to the glucokinase-K\textsubscript{ATP} pathway, the AMPK pathway has been shown to have an important role in glucose sensing (104, 105, 177). Virally mediated expression of constitutively active or dominant-negative mutants of AMPK in the hypothalamus results in an increase or a decrease in food intake, respectively (138, 177). The AMPK pathway couples the changes in cellular AMP-to-ATP ratios to activation of downstream anabolic or catabolic pathways. Thus, AMPK may link glucose sensing to regulation of food intake (124, 153, 250, 251). Briefly, high glucose levels translate to a decrease in the AMP-to-ATP ratio, reducing the activity of AMPK and suppressing food intake by the activation of downstream anabolic pathways (Fig. 5).

Much less is known about GI neurons, and whether these neurons follow the same glucose-sensing strategy remains unclear (32, 93). Although it has been suggested that the metabolic model of glucose sensing applies to GI neurons, there is convincing evidence for metabolism-independent neuronal glucose sensing in GI neurons (68, 92, 130). Kang and
Glucose enters the cell down its concentration gradient via transmembrane glucose transport proteins. Glucose is then phosphorylated by glucokinase to form glucose 6-phosphate, allowing its entry into the glycolytic pathway. Glucose 6-phosphate is metabolized in the cell, leading to an increase in the ATP-to-ADP ratio, resulting in an inhibition of ATP-sensitive transmembrane potassium channels (K\textsubscript{ATP}) and decreasing potassium outflow. Resulting change in membrane potential activates voltage-gated calcium channels (VGCC), leading to calcium influx and inhibition of ATP-sensitive transmembrane potassium channels (K\textsubscript{ATP}), which can alter neuropeptide gene transcription, depending on the neuronal subtype through unknown downstream mechanisms.

The depletion of ATP caused an accompanying increase in AMPK phosphorylation and, thus, AgRP expression, suggesting the direct involvement of AMPK in modulating AgRP expression. Finally, a dominant-negative AMPK construct prevented the 2-DG-induced increase in AgRP expression. Taken together, this study indicates that changes in AMPK activity, caused by glucose availability, modulate AMPK activation and, thus, AgRP transcript levels.

Using our immortalized, clonal cell line mHypoE-38, which endogenously expresses AgRP, as a representative model, Cheng et al. (41) followed up on this study. Interestingly, they confirmed that glucose attenuates AgRP expression, but they found that the decrease in the AMP-to-ATP ratio was independent of AMPK and ATP changes. They established that iodoacetate, a GAPDH inhibitor that inhibits glycolysis at the NADH-generating step, increased AgRP mRNA expression and decreased ATP. Furthermore, AMPK inhibition, via small interfering RNA, induced AgRP expression at 10 mM glucose, while aminomidazole carboxamide ribonucleotide inhibited this induction. They concluded that glucose regulates AgRP expression by stimulating glucose uptake and AMPK activation.

The electrophysiological studies performed independently by Anand et al. (8), as well as Oomura et al. (191), confirmed the existence of glucose-sensing neurons in the hypothalamus. Since then, the identification of glucose-responsive neurons in the CNS and the elucidation of the mechanisms that underlie central nutrient sensing have become major research interests. Given their critical roles in the regulation of energy homeostasis, it is not surprising that POMC and NPY/AgRP neurons of the hypothalamic ARC have been identified as glucose sensors (118, 186, 187, 197). Anorexigenic signals, such as glucose, leptin, and insulin, regulate the activity of melanocortin neurons found in the hypothalamic ARC, the critical site of body weight regulation (reviewed in Refs. 48, 49, 111, 143). Within the ARC, orexigenic NPY/AgRP neurons, when excited, promote an increase in food intake and a decrease in energy expenditure, leading to the storage of energy. Peripheral signals, leptin and insulin, inhibit NPY/AgRP neurons, and this inhibition mediates some of the anorexigenic actions of these hormones. In direct opposition to the NPY/AgRP neurons are the POMC neurons, which release the anorexigenic neuropeptide α-MSH. When POMC neurons are stimulated by insulin and leptin, they promote a decrease in food intake and body weight. In addition to their regulation by peripheral hormones, both of these neuronal populations represent glucose-sensing neurons. Specifically, glucose has been shown to inhibit ARC NPY/AgRP neurons and excite POMC neurons (118, 197). These neurons are particularly interesting because of their proposed role as the primary neurons that integrate peripheral signals related to nutrient status and regulate secondary neurons affecting energy homeostasis.

Although substantial evidence implicates glucose in the regulation of NPY and AgRP expression in vivo, the intracellular mechanisms mediating these effects remain unclear (4, 40, 248). To perform mechanistic studies, Lee and colleagues (149) used N1E-115 and GT1-7 cell lines to better understand glucose regulation of AgRP neurons at the cellular level. Their findings agreed with those from the model that glucose sensing is mediated by changes in ATP concentration. These changes then modulate activation or inhibition of AMPK, which serves as a downstream effector molecule in the signaling pathway of glucose-regulated AgRP expression. Treatment of the GT1-7 cells with glucose decreased the AMP-to-ATP ratio, thus inhibiting AMPK activation and AgRP expression. Treatment of both cell lines with 2-DG increased expression of AgRP, decreased cellular ATP levels, and increased AMPK phosphorylation. To study the direct effects of ATP depletion on AgRP expression, they used sodium azide, which affects mitochondrial oxidative phosphorylation, to deplete cellular ATP levels. The depletion of ATP caused an accompanying increase in AgRP expression. Furthermore, they found that the AMP analog aminomidazole carboxamide ribonucleotide induced AMPK phosphorylation and, thus, AgRP expression, suggesting the direct involvement of AMPK in modulating AgRP expression. Finally, a dominant-negative AMPK construct prevented the 2-DG-induced increase in AgRP expression. Taken together, this study indicates that changes in ATP levels, caused by glucose availability, modulate AMPK activation and, thus, AgRP transcript levels.
through NADH production by GAPDH and regulation of the transcription factor COOH-terminal binding protein. The apparent discordance between the aforementioned studies may be due to the use of different cell lines. It is notable that the NIE-115 line was not derived from the hypothalamus and the GT1-7 line was derived on the basis of GnRH expression. Thus both cell lines do not endogenously express AgRP in vivo. On the contrary, the mHypoE-38 neurons were derived from the mouse hypothalamus and endogenously express AgRP and may be better representative models of AgRP neurons in vivo. It is also possible that glucose regulation of AgRP may differ among distinct hypothalamic ARC NPY/AgRP subpopulations. This further illustrates the potential use of characterized cell lines endogenously expressing neuropeptides of interest to dissect the molecular mechanisms underlying hypothalamic glucose sensing.

In an attempt to further investigate how changes in AMPK activity modulate hypothalamic neuropeptide expression in response to glucose in POMC neurons, we used the mHypoE-43/5 cell line, which endogenously expresses POMC (35). We demonstrated that these neurons respond to glucose and that glucose can regulate neuropeptide gene expression associated with AMPK activity. The mHypoE-43/5 neurons were first screened for expression of neuropeptides, neuropeptide receptors, and enzymes associated with neurotransmitter synthesis. The resulting phenotypic profile indicated that the region of origin of these neurons is most likely the ARC. Furthermore, the expression of glucose-sensing machinery, including glucose transporters 1–4, glucokinase, the subunits of K_{ATP} channels, and the subunits of voltage-dependent calcium channels, was ascertained by RT-PCR or Western blotting. The functionality of the K_{ATP} channels was determined by detecting changes in calcium mobilization within the cells in response to low (2.8 mM) or high (16.7 mM) extracellular glucose. Using this cell line, we tested the effects of glucose on the phosphorylation status of AMPK and its downstream effector molecule acetyl CoA-carboxylase due to the established role of AMPK as a nutrient sensor (124). We found that increasing concentrations of glucose caused a decline in AMPK activity and a concomitant increase in acetyl CoA-carboxylase activity. Furthermore, both glucose concentrations were found to regulate POMC transcription. Both high and low glucose concentrations resulted in a significant increase in POMC transcript levels as determined by quantitative real-time RT-PCR. These studies indicate that the mHypoA-43/5 cell line is an appropriate cell model for the investigation of the mechanisms underlying glucose sensing in the hypothalamus.

Using the mHypoE-39 cell line, we have also implicated glucose sensing in the regulation of brain insulin mRNA expression (158). These cell lines were assayed for brain insulin mRNA expression and found to express the murine insulin 1 gene. It had been previously shown that brain glucose infusion increased extracellular insulin levels in the brain without altering peripheral insulin and glucose levels, suggesting central mechanisms of action (87). Treatment with 16.7 mM glucose led to a 1.5-fold increase in insulin 2 mRNA expression, implicating glucose sensing in brain insulin production. Thus the mHypoE-39 cell line can be used as a model to dissect the mechanisms involved in the regulation of brain insulin production by glucose.

As with all in vitro studies, there are inherent limitations in their translatability to the in vivo system; thus scientists must reaffirm these results in vivo to gain an understanding of the importance of hypothalamic glucose sensing in physiological function. Few studies have examined glucose sensing in specific neuronal cell types. Parton et al. (197) disrupted glucose sensing by reducing ATP sensitivity via the expression of a mutant K_{ATP} subunit Kir6.2 specifically in POMC neurons. Although these mice exhibited aberrant glucose tolerance, their feeding behavior was normal. Also, the study linked an up-regulation in the mitochondrial uncoupling protein UCP2 to the impairment of glucose sensing by POMC neurons in mice fed a high-fat diet. On the other hand, POMC-neuron-specific deletion of the a2-subunit of AMPK in mice suppressed the glucose-mediated regulation of firing activity. The deletion of the enzyme did not alter ad libitum feeding but led to increased feeding in response to fasting, an increase in the expression of orexigenic hypothalamic peptides, and weight gain in mice (43). This indicates that AMPK function is necessary for glucose sensing in these neuronal populations but lack of AMPK activity in POMC neurons is not associated with an anorexigenic response. However, it remains unknown how AMPK allows POMC neurons to sense glucose and, thus, alter POMC neuronal excitation at the molecular level (16).

Although both studies blunted glucose sensing specifically in POMC neurons, the in vivo phenotypes observed were markedly different in the animal models used. Nevertheless, they provide valuable information regarding the roles of AMPK and K_{ATP} channels in neuronal glucose sensing. It has also been suggested that K_{ATP}-independent mechanisms are involved in GE neurons of the mouse ARC (81). This implies that there are multiple pathways, including mechanisms involving the K_{ATP} channels, through which neurons are able to sense regulation of glucokinase and regulation of AMPK or UCP2 (33, 68, 175, 197). Although whole body or neuron-specific deletion of these proteins may lead to valuable data on their physiological roles, these techniques are not without limitations. Specifically, germ cell deletion may lead to compensatory changes during in vivo development of these neurons, while neuron-specific deletion may lead to nonspecific changes in extrahypothalamic neurons expressing POMC, such as in the NTS. Nevertheless, these experimental approaches are seminal in identifying key players in the glucose-sensing pathways.

Despite the elegant studies carried out to connect glucose sensing to feeding regulation, the molecular mechanisms through which this occurs remain unclear. Despite mounting evidence, not all scientists agree that POMC neurons are GE, further highlighting the inherent heterogeneity within POMC and NPY/AgRP neuronal populations of the ARC (80, 247). It is important to understand how glucose-sensing neurons operate, as it has been shown that impaired glucose sensing in hypothalamic POMC neurons can lead to diabetes and obesity (197). To this end, hypothalamic cell lines can be used for delineating the molecular mechanisms underlying glucose sensing in neurons. Electrophysiological studies on brain slices have contributed greatly to the current state of knowledge on neuronal glucose sensing. If electrophysiological techniques are optimized for use on cell lines, our clonal neuronal models can be useful in identifying and performing electrophysiological studies on GE and GI neurons to delineate the differences.
or similarities in intracellular signaling within these neuronal subtypes in response to changes in extracellular nutrient concentrations. However, it must be acknowledged that, in culture, these neurons do not reside in their natural physiological environment that consists of extracellular matrix, neuronal connectivity, and neuronal-glial interactions. These limitations must be considered when data from cell models are extrapolated.

We have characterized a population of adult POMC-expressing cell models. These adult cell lines have been screened, using RT-PCR, for the expression of multiple receptors and peptides present in ARC POMC neurons in vivo. These include CART, insulin receptor, leptin receptor, ERα, ERβ, G protein-coupled estrogen receptor 1 (GPR30), prohormone convertase 1, and prohormone convertase 2. On the basis of these screening results, we have identified POMC-expressing adult cell lines: the mHypoA-24, mHypoA-30, mHypoA-43, and mHypoA-45 neurons. These fully differentiated adult neurons represent ARC POMC neurons and, thus, make feasible mechanistic studies. These novel cell lines will allow for further dissection of hypothalamic function on the cellular level based on models representative of the adult hypothalamus. In our opinion, they could be invaluable to address conflicting, mechanistic data regarding the molecular mechanisms underlying glucose regulation in distinct subpopulations of POMC neurons. Furthermore, they may prove useful for analysis of the molecular mechanisms linking leptin or insulin resistance to glucose sensitivity.

It is clear that central glucose sensing is implicated in a variety of important physiological functions. Furthermore, novel findings highlight the fact that there are multiple mechanisms through which cells can sense glucose, and mechanistic studies are critical to dissect the underlying pathways. To understand the cellular and molecular mechanisms by which nutrients regulate the expression of feeding-related neuropeptides, it is highly advantageous to develop representative in vitro models. A greater understanding of the molecular events that contribute to altered hypothalamic nutrient sensing will lend itself to a more complete understanding of the etiology of obesity and its accompanying diseases.

New Insights Into Reproduction Using Neuronal Cell Models

The hypothalamus plays a critical role in the regulation of reproductive function. The principal regulator of the hypothalamic-pituitary-gonadal (HPG) axis is the GnRH neuron. Situated in the preoptic area, these neurons project to the median eminence, where they secrete GnRH into the hypophysial portal system (21, 36, 56). The pulsatile release of GnRH regulates the secretion of follicle-stimulating hormone (FSH) and luteinizing hormone from pituitary gonadotropes. These gonadotropins, in turn, act on the ovaries to stimulate gametogenesis and steroidogenesis and to trigger ovulation (221).

GnRH neurons are subject to autocrine and paracrine control, as well as regulation by neurotransmitters and steroid hormones. While the positive- and negative-feedback effects of estrogen on GnRH neurons have been well documented (178, 221), it has only been in recent decades that substantial evidence has begun to emerge implicating afferent neural inputs from NPY, kisspeptin, and the novel RFRP-3 neurons in the regulation of the reproductive axis. Given the heterogeneity and complex neuroanatomy of the hypothalamus and the ensuing limitations of in vivo methods in dissecting the molecular mechanisms underlying reproductive function, cell models have gained increasing attention among reproductive scientists. Indeed, hypothalamic cell lines have contributed substantially to a deeper understanding of molecular reproductive biology.

One of the most well-studied models of GnRH neurons is the GT1 cell line developed by Mellon et al. (172) in 1990. This GnRH-expressing cell line was generated through targeted tumorigenesis of GnRH neurons in transgenic mice expressing a GnRH-SV40 T antigen hybrid gene. This method of cell line generation served as a means for maintaining highly differentiated cell types (70, 192, 252). Indeed, serial dilutions of the heterogeneous GT1 cell population gave rise to the clonal cell lines GT1-1, GT1-3, and GT1-7, which express neuron-specific, but not glial cell-specific, markers, possess neurosecretory machinery, and exhibit a rhythmic pattern of GnRH secretion (160, 172). The successful generation of a representative GnRH neuronal cell model prompted numerous studies that shed light on the intrinsic properties of the GnRH neuron and the mechanisms underlying the afferent regulation of the GnRH system. These studies have been thoroughly summarized and, thus, are not covered in the current review (20, 94, 161).

After its discovery in 1982, NPY emerged as an important regulator of reproductive function (238). Some of the earliest studies demonstrated that intracerebroventricular injections of NPY elicit significantly reduced sexual behavior in rats (45). Subsequent studies linked this phenomenon to a direct regulation of the HPG axis, particularly at the level of the GnRH neuron (23, 213, 253, 254) and specifically in the potentiation of the preovulatory GnRH-induced luteinizing hormone surge (1, 14, 125, 217). This interaction between NPY and GnRH neurons is supported by neuroanatomic studies that found ARC NPY- and NPY receptor immunoreactive fibers in close proximity to GnRH perikarya in the preoptic area (65, 66, 154, 166).

Although the actions of NPY on GnRH release are firmly established, the precise molecular events that underlie this regulation are not well characterized. Using GT1-7 neurons, which express the Y1, -2, and -4 (62), our group demonstrated that NPY triggers an increase in GnRH mRNA expression in GnRH neurons via activation of the Y1 receptor and of the downstream PKA and MAPK pathways. This study sheds light on the potential mechanisms involved in NPY regulation of the GnRH neuron and suggests that NPY stimulates GnRH secretion via an increase in GnRH production (62).

The observation that NPY exerts its effects on GnRH neurons only within the preovulatory context (217) strongly suggests that NPY neurons are themselves regulated by some combination of factors. Indeed, early studies illustrated that intracerebroventricular injection of NPY has differential effects on luteinizing hormone release in ovariectomized rats compared with ovarian steroid-primed ovariectomized rats, indicating the importance of the steroid milieu to NPY regulation of GnRH neurons (126). Similar studies utilizing more direct administration of ovarian steroid hormones confirmed this modulatory role of estrogen and progesterone on NPY action (51, 52). Furthermore, in vivo models suggest that estrogen regulates NPY mRNA expression (3, 227). However, whether this regulation occurs through a direct or an indirect mechanism is unknown. Using clonal, immortalized hypotha-
lamic cell lines developed in our laboratory, we determined that estrogen directly regulates NPY mRNA expression in an ER-dependent fashion. Employing the mHypoE-38 cell line, which expresses NPY, AgRP, ERα, and ERβ, but not POMC or CART, we specifically observed that, over a 72-h time course, NPY mRNA expression increases with the ratio of ERβ to ERα (241). In a subsequent study using the same mHypoE-38 neurons, estrogen cotreatment with inhibitors against the MAPK and phosphatidylinositol-3-kinase (PI3K) pathways effectively blocked the estrogen-mediated fluctuations in NPY mRNA expression (242).

Recently, our group followed up on these studies by characterizing two other NPY-expressing cell lines, mHypoE-42 and mHypoA-2/12. Similar to the mHypoE-38 neurons, these cell lines express NPY, AgRP, ERα, and ERβ, but not POMC. Not only did we find that estrogen significantly suppresses NPY secretion in these neurons, but we also determined that this suppression is mediated exclusively by membrane-bound ERα. Furthermore, estrogen cotreatment with inhibitors against the AMPK and PI3K pathways prevented the estrogen-mediated inhibition of NPY secretion (60). Therefore, these studies effectively map out the signal transduction pathways that are required for estrogen regulation in NPY neurons to an extent that is not achievable with classical in vivo methods.

The inverse relationship that we found between estrogen levels and NPY release in the mHypoE-42 and mHypoA-2/12 neurons is in agreement with previous in vivo findings that ovariectomized rats exhibit elevated levels of NPY mRNA (3, 227). Moreover, our findings are in line with the metabolic functions of estrogen and NPY. While estrogen has been demonstrated to exert anorexigenic effects (85, 100, 195, 198), NPY is a known orexigen (44, 231, 232). Indeed, several lines of evidence suggest that estrogen mediates its anorexigenic effects through hypothalamic NPY (25, 218). Interestingly, many authors have suggested the intriguing possibility that NPY serves as the neurochemical link between our two basic evolutionary drives to feed and to reproduce, specifically by suppressing the reproductive axis in conditions of undernutrition (128, 169). Further investigation into the molecular basis underlying the actions of NPY will allow for a deeper understanding of this neuromodulatory link. To this end, the mHypoE-42 and mHypoA-2/12 cell lines present as representative hypothalamic cell models for conducting this research.

The differential regulation of estrogen on the mHypoE-38 neurons compared with the mHypoE-42 and mHypoA-2/12 neurons can be explained by the existence of distinct subpopulations of NPY neurons in the hypothalamus (39). Given that these clonal cell lines were isolated from whole hypothalami, the precise location from which they originated remains unknown. This raises the possibility that these cell lines represent phenotypically unique NPY-expressing neurons. We speculate that the mHypoE-38 neurons function as a reproductive NPY cell model, while the mHypoE-42 and mHypoA-2/12 neurons serve a bipartite function in coordinating reproduction with nutrition. This is in agreement with previous findings in our laboratory (137, 241).

In addition to the modulatory effects of NPY, there is considerable evidence supporting the notion that the neuropeptide hormone kisspeptin also functions as an afferent regulator of GnRH neurons. Research in the last decade has shed new light on the role of kisspeptin in the reproductive axis. The discovery that human patients deficient in the kisspeptin receptor Kiss1r exhibit hypogonadotropic hypogonadism and that this same phenotype is observed in knockout mice lacking the Kiss1 or Kiss1r gene firmly established kisspeptin as a key player in reproductive development and pubertal onset (55, 57, 84, 102, 224). These findings prompted investigations into the mode of action of kisspeptin, upon which it was demonstrated that kisspeptin stimulates FSH and luteinizing hormone release (95). Not surprisingly, this regulation of gonadotropin release was later shown to occur through a direct action of kisspeptin on GnRH neurons (174). Using the immortalized GnRH-secreting neuronal cell lines GT1- and GN11, which express the kisspeptin G protein-coupled receptor 54 (GPR54), Novaira et al. (189) confirmed this direct mode of action and mapped the molecular events underlying this regulation. Specifically, it was demonstrated that kisspeptin produces a robust increase in GnRH mRNA expression and secretion. Western blot analysis and inhibitor studies showed that this stimulatory effect requires the MAPK and PI3K pathways (189). In concordance with these findings, kisspeptin projections are found in close apposition to GnRH neurons in the preoptic area of the murine hypothalamus (46, 139); furthermore, double-label immunohistochemical studies in adult mice reveal that >70% of GnRH neurons in the rostral preoptic area express GPR54 (now called Kiss1r) (106). Indeed, defining the mechanisms that underlie kisspeptin action may become important in gaining an understanding of the molecular basis for GnRH rhythmicity.

Although the direct regulation of GnRH neurons by kisspeptin has been the focus of most of the scientific literature on this subject, the possibility that kisspeptin may also act through indirect mechanisms to regulate GnRH release has also been suggested. Not only is Kiss1r expressed in regions outside those traditionally associated with GnRH neurons (106, 148), but also, kisspeptin has been shown to increase the firing rate of non-GnRH neurons within the medial preoptic area (203). To investigate this hypothesis, we again employed the NPY-expressing cell line mHypoE-38, which was shown to express Kiss1r. Treatment with kisspeptin resulted in a statistically significant 70% increase in NPY mRNA expression after 24 h and a 40% increase in NPY secretion after 1 h. Inquiring into the signaling pathways that were mediating this effect, we examined the phosphorylation status of ERK1/2 and p38 over a 1-h time course and found that they were significantly upregulated at 15 and 60 min, respectively, following kisspeptin administration. Treatment with the ERK1/2 inhibitor U-0126 and the p38 inhibitor SB-239062 blocked the kisspeptin-mediated induction of NPY expression and secretion, revealing that the ERK1/2 and p38 MAPK pathways are required to effect this change. In a separate study, mHypoE-38 neurons treated with potassium chloride secreted 1.5-fold more NPY than vehicle-treated controls. Interestingly, desalted conditioned media collected from these neurons, when applied to GT1-7 cells, caused a significant induction of GnRH mRNA expression (60), which was shown to be attributable to NPY itself, since blocking the Y1 with a specific inhibitor abolished this effect. Together, these studies suggest that kisspeptin may modulate the GnRH system through an indirect NPY network (Fig. 6). However, electrophysiological studies utilizing hypothalamic brain slices suggest the opposite. In a recent study, Fu and van den Pol (83) utilized an NPY-green fluorescent protein
transgenic mouse to determine that kisspeptin inhibits NPY neurons through an indirect GABA-mediated synaptic pathway. Nonetheless, we hypothesize that there are distinct subpopulations of NPY-expressing neurons coexisting within the hypothalamus from our findings that leptin can differentially regulate NPY secretion in unique NPY neuron lines (61), and insight from each of these studies should be complementary to understand the regulatory complexities in specific regions of the hypothalamus. There is some evidence that NPY neurons in vivo may have unique ontogenic origins and, therefore, may represent subpopulations of NPY neurons in the hypothalamus (110, 194). The stimulatory and inhibitory effects of kisspeptin on NPY neurons may represent kisspeptin action on two distinct NPY neuronal subpopulations, thus signifying two independent mechanisms by which kisspeptin may regulate NPY. It is well established that estrogen has divergent effects on the anteroventral periventricular nucleus of the hypothalamus vs. ARC kisspeptin neurons; therefore, it likely that there are regional hormonal effects that are yet to be described for a number of neuropeptidergic systems. At the same time, the value of metabolically active brain slices cannot be understated. The preservation of neural connections and the extracellular milieu provide a more physiologically relevant model through which to study neuroendocrine physiology. In fact, there is mounting evidence that glial interactions with GnRH neurons play a major role in the regulation of GnRH secretion through the production of glutamate, insulin-like growth factor I, and epidermal growth factor-like growth factors (190). Thus brain slices and cell models serve as complementary tools: while brain slices provide a more accurate means to study intercellular communication, cell lines provide insight into the intracellular signaling underlying this communication.

To gain a deeper understanding of the kisspeptin system, our laboratory has identified a putative kisspeptin cell model, the rHypoE-8 neurons, from an array of our recently generated embryonic rat hypothalamic cell lines. In addition, we are confident that we have identified an RFRP-3 cell model: the rHypoE-7 neurons (89). The existence of a factor that could inhibit the reproductive axis was first suggested by Hwan and Freeman in 1987 (117). However, it was not until over a decade later that this factor was identified in birds as gonadotropin-inhibitory hormone (GnIH) (243) and that an analogous system in the mammalian brain (initially named RFRP-3 and now more widely accepted as the mammalian GnIH) was found to exist (146). Neuroanatomic studies in hamsters, rats, and mice localized RFRP-3 neurons (the mammalian ortholog of GnIH) to the DMH and found that these neurons project to hypothalamic regions containing GnRH neurons, namely, the preoptic area. Indeed, double-label staining revealed that >40% and 70% of GnRH neurons appear to receive inputs from RFRP-3 neurons in hamsters and rats, respectively (122, 146). Furthermore, intracerebroventricular injection of RFRP-3 was shown to activate GnRH neurons (9) and inhibit luteinizing hormone, but not FSH, release (122, 146). Taken together, these studies established the role of GnIH (RFRP-3) in the negative regulatory arm of the HPG axis.

The use of representative cell models in the study of these novel neuropeptide-secreting neurons has not been documented. For this reason, many of the molecular mechanisms inherent to these neurons remain unknown. Using the kisspeptin- and RFRP-3-expressing neuronal cell lines rHypoE-8 and rHypoE-7, we have been able to perform the appropriate mechanistic studies to elucidate these details. The effects of melatonin, a pineal gland hormone important in the entrainment of light-dark cycles, were of particular interest, given the recent literature. Kisspeptin and RFRP-3 are differentially expressed in sheep during the breeding season relative to the nonbreeding season (230). In a separate study, it was demonstrated that this photoperiod variation in expression in hamsters was abolished with pinealectomy, revealing the involvement of melatonin in this regulation (210, 211). However, whether melatonin acts directly on kisspeptin and RFRP-3 neurons is uncertain. Using the rHypoE-8 and rHypoE-7 lines, we showed that melatonin induces kisspeptin mRNA expression in rHypoE-8 neurons while inhibiting RFRP-3 mRNA expression in rHypoE-7 neurons after 24 h (89). The stimulatory and inhibitory effects of kisspeptin and RFRP-3, respectively, on the reproductive axis signify that melatonin has an overall positive effect in the regulation of reproduction. Indeed, these cell models have proven useful not only in confirming the
effect of melatonin on these neuropeptides, but also in demonstrating its direct mechanism of action. Thus the use of cell line models has the potential to contribute greatly to the current understanding of reproductive function in the neuroendocrine hypothalamus, particularly in the elucidation of the intracellular signaling pathways and molecular events underlying this regulation.

Experimental Models: Limitations and Advantages

Current experimental models to investigate hypothalamic functions range widely in complexity, from cell lines and nontransformed primary hypothalamic cultures to brain slices and animal models. The selection of an appropriate experimental model should be based on the experimental aim and a careful consideration of the advantages and limitations of each model. Primary hypothalamic cultures allow for the study of neuronal properties and cellular integrative mechanisms without the confounding effects of afferent inputs and other external factors. However, the main disadvantages of using primary cultures are their short lifespan and heterogeneous nature; primary cultures consist of a mixture of neuronal and glial cell populations, often with a minimal number of healthy, peptide-secreting neurons. Brain slices, on the other hand, are advantageous, in that the local circuits and cytoarchitecture of the tissue are largely preserved, rendering them a useful tool for electrophysiological studies in characterizing electrical behavior, connectivity, and responsiveness to peptides implicated in the modulation of feeding behavior, reproduction, or other physiological functions. However, it has been challenging to identify specific neuropeptidergic neurons in brain slice preparations, unless transgenic mice expressing fluorescent protein-tagged neuropeptides are used (204, 209). It is also possible that connections between neurons might be lost in the slice preparation, with potential loss of important channels or receptors that may generate conflicting results. Additionally, not only is the generation and maintenance of such transgenic mice a complex, expensive, and time-consuming process, but the success rate is also very low. At the same time, animal models have been the major contributor to our knowledge of hypothalamic functions. However, studying a specific hypothalamic neuropeptide’s gene regulation, its mechanisms of action, and its original or mediated roles within the whole animal is challenging, time-consuming, labor-intensive, and expensive. On the contrary, the use of immortalized cell lines is comparatively advantageous, because they are easy to culture and maintain. Moreover, they are inexpensive relative to other experimental models and generate reproducible results. For example, large amounts of protein or mRNA for analysis of second messenger pathways or gene expression can be obtained easily, particularly compared with primary cultures or animal models. Furthermore, clonal cell lines are derived from a single cell type, so the likelihood of contamination by other confounding nonneuronal cell types is low. Another major advantage is that cell culture offers a controlled physichemical environment to study specific cellular and molecular mechanisms and allows for simpler experimental designs and procedures on shorter time scales. In addition, smaller quantities of reagents are required for cell culture experiments, further reducing the cost of experiments compared with in vivo research. Despite all these advantages, cell lines have several limitations that must be acknowledged to accurately interpret experimental results and avoid drawing premature conclusions.

On the basis of the expression profile of our novel hypothalamic cell models, one of their main advantages is their capacity to demonstrate the direct cause-and-effect relationship of specific neuromodulators. However, researchers must avoid overstating their conclusions, because immortalized neuronal cell models have inherent limitations due to the absence of the complex architecture and afferent cellular connections present in the intact brain. As a result, it is challenging to investigate mechanisms of interneuronal communication within the hypothalamus that are known to play important roles in feeding behavior and energy homeostasis. Inherent in all cultured cells, a major disadvantage is that the cells adapt to different culture environments, such as different nutrients or temperatures, by changing the activity of their enzymes. Also, because cell lines are derived through an immortalization process, there is a possibility that their metabolism may have shifted to growth and proliferation and away from the fully differentiated function of the original cell type (162). Another critical issue is, following a long period of continuouspassaging, cell characteristics may change and become phenotypically different from those found in the original single cell population of lower-passage cells. Therefore, in addition to minimizing passage number, it is recommended that passage number variance be kept to a minimum. Furthermore, cultured cells may not express certain proteins, receptors, or structures that mimic natural expression patterns in vivo. For example, it has been reported that the correct development of the sensory processes of olfactory neurons and terminals of other sensory neurons strictly depends on their physical interaction with the glia-like sheath cells in Caenorhabditis elegans (10). It is, therefore, possible that, in immortalized neuronal cells, the lack of input from or communication with other neurons or nonneuronal cells may limit their development and function and, therefore, their use in the study of certain types of proteins. Thus, only after careful investigation into whether the cell lines being employed accurately reflect natural physiological properties, can they be used as a complementary tool for in vivo or in situ experimentation to delineate cellular and molecular mechanisms that underlie neuroendocrine function.

Despite these limitations, the value of cell lines to the advancement of scientific knowledge should not be understated. Indeed, examples from history illustrate that cell models are reflective and revealing of natural physiology. Perhaps one of the most compelling examples comes from the tumorigenic GT1 GnRH-expressing neuronal cell lines. Prior to the 21st century, there was considerable debate concerning whether estrogen regulates GnRH neurons in a direct or an indirect fashion. In vivo evidence in rats, ewes, and primates suggested that native GnRH neurons do not express ERs (107–109, 151, 228, 234). Conversely, findings from in vitro studies utilizing the GT1-1 and GT1-7 neurons illustrated that estrogen binds to receptors on these neurons and that functional ERα and ERβ are expressed (34, 205, 212, 225). To resolve this discrepancy, Sisky et al. (229) employed single-cell multiplex RT-PCR to analyze in vivo gene expression within GnRH neurons in mice. In doing so, they found that >50% of GnRH neurons express ERα mRNA, while ~10% express ERβ mRNA. In 2008, Hu et al. (115) verified these findings in adult and embryonic rat GnRH neurons. These discoveries demonstrate how cell mod-
els can help overcome a lack of sensitivity inherent in immuno-lococolocalization studies and can provide insight where these in vivo approaches fall short.

Cell lines can be employed to aid in the understanding of the in vivo context by confirming molecular events at the cellular level and by guiding whole animal studies. It has been confirmed through several studies investigating the regulation of gene expression or receptor activation that insights gained from cell lines replicate those gained from in vivo studies. Moreover, in vivo knockout animal models have explored the importance of specific neuropeptides and their roles in regulation of feeding behavior or reproduction. Furthermore, these novel hypothalamic cell models have been instrumental in defining the molecular mechanisms involved in the regulation of neuropeptide gene expression and signal transduction. For example, these models have been used to study the direct effect of insulin, estrogen, kisspeptin, leptin, and α-MSH on the gene regulation of NPY, AgRP, preproghrelin, hypothalamic insulin, and NT that would otherwise have been challenging and expensive to perform in vivo (53, 54, 60, 79, 137, 158, 163). Importantly, in our opinion, the knowledge generated through the analysis of these cell models will lead to a more focused experimentation in the whole animal. Investigation of the fundamental units comprising the hypothalamus will lead to a clearer view of how this complex part of the brain achieves its highly regulated control of vital physiological processes.

Conclusions and Future Directions

The hypothalamus contains several nuclei and regions that regulate numerous critical physiological functions, including energy and glucose homeostasis, as well as reproduction. Each of these hypothalamic regions comprises a complex network of heterogeneous neurons; however, the regulatory mechanisms of these unique peptidergic neurons remain largely unknown. To comprehend how the hypothalamus achieves its diverse control of vital physiological functions, the cellular mechanisms underlying the function of these individual neuronal phenotypes must be understood. Using existing animal models, it is challenging to perform molecular genetic experiments, such as in vivo gene transfer for gene promoter studies, which is limited using classical in vivo studies. We suggest that these models will help overcome a lack of sensitivity inherent in immuno-lococolocalization studies and can provide insight where these in vivo approaches fall short.

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In this review, we have discussed the generation of hypothalamic cell lines from embryonic mouse and rat, as well as adult mouse primary hypothalamic cultures, and their current and future use in advancing our knowledge of central regulation of glucose homeostasis and reproduction. The neuronal cell lines generated in our laboratory represent the first in vitro hypothalamic cell models that have been extensively characterized and shown to endogenously express several neuropeptides and receptors implicated in the central regulation of homeostatic function. While the use of immortalized cell lines is complementary to the use of animal models, cell models permit a more detailed investigation of molecular mechanisms at the cellular level. Using embryonic and adult hypothalamic cell models, we will be able to investigate how embryonic neurons differ from adult neurons in the basic control mechanisms involved in neuronal function. The novel technology that we established in immortalizing adult hypothalamic neurons using CNTF-mediated neurogenesis can be used to immortalize brain cells for which region-specific models do not exist, such as the brain stem neurons, which are also involved in feeding regulation (Fig. 1), as well as glial cells, which have been found to regulate energy homeostasis via the release of pro- and anti-inflammatory cytokines (Fig. 4).

Perspectives and Significance

Advancements in technology will soon allow for single neuronal analysis in vivo. In the meantime, given the lack of representative cell models for the study of specific hypothalamic neuronal subtypes, immortalized cell lines have become indispensable in gaining a more complete understanding of the precise molecular events that underlie hypothalamic control of fundamental physiological processes. It is evident that the research directed toward understanding the central control of energy homeostasis, fluid balance, cardiovascular regulation, immune regulation, and reproductive function cannot be completed without studying the hypothalamic cellular heterogeneity that underlies all vital functions. Therefore, it is essential to develop research tools using our novel immortalization technology to study various other cell types, such as glial and endothelial cells that interact with each other and various neuronal types. Indeed, immortalized hypothalamic cell lines and in vivo models are complementary to each other, and both have been used effectively to investigate mechanisms of physiological processes. Ultimately, we postulate that these models together have great potential for use in drug discovery and therapeutic drug testing and, thereby, to improve health.

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DISCLOSURES

No conflicts of interest, financial or otherwise, are declared by the authors.

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