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Hormonal Regulation of Clonal, Immortalized Hypothalamic Neurons Expressing Neuropeptides Involved in Reproduction and Feeding

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Abstract The hypothalamus has been particularly difficult to study at the molecular level because of the inherent cellular heterogeneity and complexity of neuronal circuits within. We have generated a large number of immortalized, clonal cell lines through retroviral gene transfer of the oncogene SV40 T-Ag into primary murine hypothalamic neuronal cell cultures. A number of these neuronal cell lines express neuropeptides linked to the control of feeding behavior and reproduction, including neuropeptide Y (NPY) and neurotensin (NT). We review recent studies on the direct regulation of *NPY* gene expression by estrogen, and the leptin-mediated control of signal transduction pathways and NT transcription. These studies provide new insights into the direct control of neuropeptide synthesis by hormones and nutrients at a mechanistic level in the individual neuron, not yet possible in the whole brain. Using these novel cell models, we expect to contribute substantially to the understanding of how individual neuronal cell types control overall endocrine function, especially with regard to two of the most well-known roles of distinct peptidergic neurons; these being the control of reproduction and energy homeostasis.

Keywords Hypothalamus · Neuropeptide Y · Neurotensin · Estrogen · Leptin

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Abbreviations

SV40 T-Ag	simian virus large T antigen
GnRH	gonadotropin-releasing hormone
NPY	neuropeptide Y
NT	neurotensin
AgRP	agouti-related peptide
MCH	melanin-concentrating hormone
GHRH	growth hormone-releasing hormone
POMC	proopiomelanocortin
CNS	central nervous system

Introduction

The Hypothalamus

The neuroendocrine hypothalamus consists of a complex array of distinct neuronal phenotypes, each expressing a specific complement of neuropeptides, neurotransmitters, and receptors [1]. Many of our vital needs depend on hormonal balance or homeostasis, which is controlled in part at the hypothalamic level. Perturbation of this delicate balance can lead to detrimental effects resulting in major health problems, such as obesity, infertility, and diabetes [2]. Obesity is a major global health concern [3] and is a major risk factor for other disorders, including diabetes, hypertension, heart disease, and infertility [2]. A balance between feeding and energy expenditure is critical to maintain a healthy body weight. A complex neuronal system has evolved to maintain energy homeostasis [4]. Leptin, ghrelin, glucose, and insulin are known peripheral signals that act to regulate feeding and energy balance by modulating the expression of neuropeptides in the brain. Therefore, major efforts are currently underway to dissect the central pathways involved in the

regulation of feeding behavior and energy balance with the hope of discovering targets for the treatment of obesity. On the other hand, estrogen is critical for the maintenance of normal reproductive function, but also plays an anorexigenic role in energy homeostasis [5, 6]. Therefore, estrogen may regulate neurons involved in both feeding and reproduction. Knowledge of the control mechanisms of unique peptidergic neurons from the hypothalamus is critical before we can understand how the brain achieves its diverse central control of basic physiology. However, the cellular mechanisms involved in this process are poorly understood, mainly because of the complexity of the in vivo architecture of the hypothalamus and the lack of appropriate cell models for these studies.

Numerous studies have been undertaken to map the afferent connections between distinct hypothalamic neurons utilizing methodology such as double-label, and recently, triple-label immunocytochemistry and in situ hybridization [7–10]. These studies are useful to generate an emerging picture of the potential cellular communication within the hypothalamus, but are not comprehensive and do not address the mechanisms involved in gene regulation and cellular signaling. Historically, it has proven to be difficult to establish immortalized hypothalamic cell lines, because of the lack of naturally occurring central nervous system (CNS) tumors and the inherent difficulty of transforming or immortalizing highly differentiated neurons from primary culture [11]. Cell lines from the peripheral nervous system have been established from neuroblastomas, such as the Neuro2A cell line, and pheochromocytomas, such as the PC12 cell line; however, these models are not truly representative of differentiated CNS neurons. For instance, the murine N1E 115 neuroblastoma cell line is routinely used as a neuronal model, however, it was originally generated from a spontaneous tumor on the spinal cord [12]. Infection of primary cultures of hypothalamic tissue from embryonic day 14 with SV40 T-Ag in the early 1970s produced cell lines that were not considered fully differentiated [13]. However, a few cell models from the hypothalamus have been developed and have proven to be extremely useful toward understanding the cellular biology of specific neuroendocrine cells [14–16]. However, the number of cell models from the hypothalamus, and from the entire brain for that matter, consist of a few isolated cell types and represent an infinitesimal percentage of the neuronal phenotypes represented within the brain. For this reason, we have used retroviral transfer of SV40 T-Ag into primary hypothalamic cell culture to generate an array of immortalized cell models from the hypothalamus. The mixed populations were subcloned and defined by expression of specific neuropeptides and receptors. The clonal cell models express neuronal cell markers, exhibit neurosecretory properties, and respond appropriately to hormonal stimulation.

Cell Culture Models

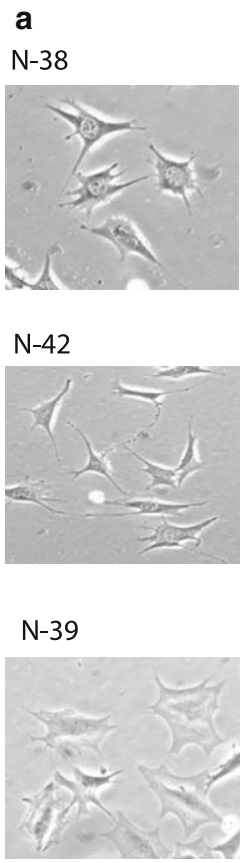
Hypothalamic GnRH Cell Models

Nontransformed primary hypothalamic cultures are difficult to maintain, have a short life-span, and represent a heterogeneous neuronal and glial cell population, often with a minimal number of healthy peptide-secreting neurons. Immortalized, clonal cell lines represent an unlimited, homogeneous population of specific neuronal cell types. Classical in vivo approaches cannot firmly establish the direct action of an agent, such as estrogen or leptin, on specific hypothalamic neurons or on neuropeptide transcription, mainly because the cell receives input from other neurons. For this reason, researchers have turned toward immortalized cell models. A directed tumorigenesis technique was used to develop murine immortal cell lines of gonadotropin-releasing hormone (GnRH)-secreting hypothalamic neurons in an attempt to produce a suitable model to study the GnRH gene. There have been two models of the in vivo GnRH neuron generated by targeted tumorigenesis in transgenic mice. In each case, the oncogene SV40 T-Ag was directed by either the rat (GT1) [14] and human (GN) [17] 5′ regulatory regions of the *GnRH* gene to specifically target the GnRH neuron for immortalization. The characteristics of the cell lines have been previously reviewed [18]. The GT1 cells have been characterized extensively and thus represent the most accepted model of the GnRH neuron available. It is interesting to note that, using the information gained from this important model system [19–21], researchers have been able to return to the animal model with renewed focus, resulting in new insights into the role of GnRH neurons in normal reproductive physiology [22–24]. This model has been used extensively by myself and others to study the cell biology of the GnRH neuron, and neuronal transcriptional control mechanisms (for some examples, see 19, 21, 25–51). On the other hand, the GN cells are not well-characterized, but are generated from a tumor excised from the olfactory placode [17]. These cells secrete relatively lower levels of GnRH and are generally considered to be a developmentally earlier version of the GT1 cells. Recently, these cells have been used in comparative studies to define the mechanisms involved in GnRH neuronal migration [52, 53]. These cells have been reviewed previously [18, 54–56] and will not be the focus herein. Instead, we turn our attention toward new cell models representing many distinct cell types from the hypothalamus.

Novel Hypothalamic Cell Models

Because the use of the GT1 cells has contributed an immense wealth of knowledge to the study of the cell biology of the GnRH neuron and neuronal function in general, it would be

logical to clone other neuroendocrine cell models from the hypothalamus to understand other unique neuroendocrine cell types. The generation of hypothalamic, immortalized neuronal cell lines is a labor-intensive undertaking, but when successful, offers a wealth of unique research opportunities. We have generated a large number of immortalized, clonal cell lines through retroviral gene transfer of the oncogene SV40 T-Ag into primary hypothalamic neuronal cell cultures from mice at embryonic days 15, 17, and 18 [57]. The cells generated by this method are unique, as compared to other immortalized cell lines, as they did not originate as a tumor, but were transformed only by the expression of SV40 T-Ag in monolayer primary culture. Detailed analysis of the cell lines indicates that they express neuronal cell markers, such as neuron-specific enolase and the 68 kDa neurofilament protein, but not glial fibrillary acidic protein, normally found in astrocytes. They also express markers of neurosecretory machinery, such as syntaxin. The cells are stable in culture, with a doubling time of 1–2 days. They can be passaged extensively, maintaining the expression of the SV40 T-Ag, critical to maintain the immortalized phenotype. Although each cell line appears to exhibit distinct cell morphology, they all appear to possess a neuronal phenotype with clearly defined perikarya and neuritis (Fig. 1a). Some of the neurites appear as short dendritic-like processes, whereas others form lengthy processes. Through electron microscopy, we have determined that the cells harbor a large number of neurosecretory granules and form connections with other neurons in culture. The cells exhibit an intracellular calcium response after depolarization by KCl [57]. Furthermore, we have found that some of the lines secrete NPY in response to a KCl challenge. A wide variety of neuronal phenotypes were generated, often with coexpression of multiple markers, confirming what has been detected in vivo through mainly immunocytochemical methodology [58]. These immortalized, clonal cell lines provide valid model systems for molecular and biochemical investigations on the regulation of specific hormones, characteristics of their respective secretory neuronal population and an unlimited source of homogenous cell material and of the neuropeptide itself. A number of these cell lines express neuropeptides linked to the control of feeding behavior and reproduction, including NPY or NT. Some of our recent studies on the estrogenic control of *NPY* gene expression and leptin regulation of NT transcription will be reviewed herein.



b

MARKER	N-38	N-42	N-39
T antigen	+	+	+
NSE	+	+	+
NPY	+	+	-
Agrp	+	+	+
POMC	-	-	-
CART	-	-	+
NT	-	+	+
Gal	-	+	-
proGlu	-	-	-
proTRH	-	-	-
AVP	-	-	
VIP	+	+	+
Ucn	-	+	+
MCH	+	+	+
orexin	-	-	-
SOM	-	+	+
PACAP	-	-	+
TH	-	+	+
DAT	-	+	+
Gad1 (Gad 67)	-	+	+
Gad 2 (Gad 65)	+	+	+
MC4R	-	+	+
ER α	w	+	+
ER β	+	+	+
AR	+	+	-
GR	+	+	+
IR	+	+	+
IGF1R	+	+	+
Ob-Rb	+	+	+
MCHR1	+	+	+
NPY-Y1	+	+	+
NPY-Y2	+	+	+
NPY-Y4	+	+	+
NPY-Y5	-	+	+
5-HT R2a	+	+	+
NTR1	-	-	+

Fig. 1 N-38 NPY, N-42 NPY, and N-39 NT phenotypic profiles. **a** Phase contrast micrographs of immortalized mouse N-38, N-42, and N-39 neurons. **b** RT-PCR analysis showed cell lines expressed neuron-specific markers, neuropeptides, and receptors related to energy homeostasis and reproduction. *T-Ag* T-antigen, *NSE* neuron-specific enolase, *NPY* neuropeptide Y, *Agrp* agouti-related peptide, *POMC* proopiomelanocortin, *CART* cocaine- and amphetamine-regulated transcript, *NT* neurotensin, *Gal* galanin, *proGlu* proglucagon, *proTRH* prothyroid-releasing hormone, *AVP* arginine vasopressin, *VIP* vasoactive intestinal peptide, *Ucn* urocortin, *MCH* melanin-concentrating hormone, *SOM* somatostatin, *PACAP* pituitary adenylate cyclase-activating polypeptide, *TH* tyrosine hydroxylase, *DAT* dopamine transporter, *GAD* glutamate decarboxylase, *MC4R*, melanocortin 4 receptor, *ER* estrogen receptor, *AR* androgen receptor, *IR* insulin receptor, *IGF1R* insulin-like growth factor-1 receptor, *ObR* leptin receptor b, *NPY-R* neuropeptide Y receptor, *5-HT R2a* serotonin receptor, *NTR1* neurotensin receptor 1. All data was generated by RT-PCR with RT- and hypothalamic tissue as controls

Characterization of Immortalized Hypothalamic Cell Models

Known hypothalamic markers were examined with particular attention to neuropeptides linked to energy homeostasis, specific releasing hormones, and enzymes responsible for neurotransmitter synthesis. A wide variety of neuronal phenotypes were generated, often with coexpression of multiple markers, confirming what has been detected in vivo through mainly immunocytochemical methodology [58]. However, this comparative analysis is fairly limited because of the detection of only two to three peptides and/or markers in individual dual-label experiments. Therefore, we suggest that the appropriate experimental results are not yet available from in vivo studies to compare expression

profiles for the extensive list of markers detected in our cell line analysis. The neurons expressing peptides linked to the regulation of energy homeostasis coexpressed peptides consistent with the neuronal profiles reported by immunocytochemistry or in situ hybridization [9]. For instance, all NPY expressing neurons also expressed agouti-related peptide (AgRP), but not proopiomelanocortin (POMC), a precursor to the anorexigenic neuropeptide alpha-melanocortin-stimulating hormone [59, 60]. We also detected the expression of cocaine- and amphetamine-regulated transcript (CART) in the POMC-expressing cell lines, and this protein is reported to be coexpressed in many POMC neurons in situ. Many of the lines expressing peptides associated with energy homeostasis also express the long form of the leptin receptor, Ob-R_b [9], suppressor of cytokine signaling (SOCS), a downstream effector molecule of Ob-R_b [61], and the insulin receptor (InR).

A number of the neuronal cell models have the potential to produce major neurotransmitters, as many of the lines expressed tyrosine hydroxylase, a marker of catecholaminergic neurons. Cells expressing tyrosine hydroxylase have the potential to produce dopamine, norepinephrine, and epinephrine, depending upon the complement of downstream catalytic enzymes. Other cell lines expressed tryptophan hydroxylase, the rate-limiting enzyme of serotonin production and an important component of melatonin biosynthesis. They also exhibited evidence for gamma-aminobutyric acid (GABA) synthesis, through glutamate decarboxylase expression. Because of the involvement of these neurotransmitters in the development of neurological disorders, such as depression, the use of the cell lines to study their regulation is fundamental for the development of satisfactory treatment options. Cell lines were also generated expressing specific releasing peptides, such as growth hormone-releasing hormone, corticotropin-releasing factor, and thyroid-releasing hormone or secreted peptides, such as proglucagon-derived peptides, galanin, urocortin, orexin, oxytocin or arginine vasopressin—many of these being linked to energy homeostasis. Furthermore, receptor profiles revealed endogenous receptors for estrogen receptor α and β, androgen, leptin, insulin, melanin-concentrating hormone (MCH), melanocortin, glucocorticoid, and serotonin in a number of the lines. The abbreviated phenotypic profiles of the three cell lines mentioned in this review are found in Fig. 1b, and the full list of marker information for all of the cell lines can be found at http://www.CELLutionsBiosystems.com. These cell lines have been used by myself and others to study a wide variety of current topics in neurobiology [57, 62–67]. With such a vast array of cell models, we are able to study many components thought to be involved in reproductive function and energy regulation at the molecular level, and there is potential to discover novel regulatory mechanisms using available sophisticated technologies.

Using Immortalized Neurons to Study Components of Reproductive Function

Reproduction

The GnRH-producing neurons of the hypothalamus integrate information from the CNS to control sexual maturation, reproductive cycles, and sexual behavior. They are regulated by a number of extracellular messages, including neurotransmitters, steroid hormones, and peptide hormones [68]. These neurons, estimated to be only 400–1,000 in total in rodents, are scattered throughout the preoptic area and anterior hypothalamus [69]. Classical in vivo approaches cannot firmly establish the *direct* action of an agent, such as estrogen, on the GnRH neuron or on GnRH transcription, mainly because the GnRH system receives input from other steroid-sensitive neurons. Therefore, a directed tumorigenesis technique was used to develop murine immortal cell lines of GnRH-secreting hypothalamic neurons in an attempt to produce a suitable model to study the GnRH gene, the GT1 cells [14]. Many studies support the validity of the GT1 cell line as a model for the hypothalamic GnRH neuron. However, it is recognized that many neurons afferent to the GnRH neuron also play a substantial role in the control of reproduction. An example of one of these afferent neurons is the NPY neuron.

Estrogen

The GnRH neuron is a well-established target of estrogen. Estrogen exerts both positive and negative feedback effects on GnRH secretion and pituitary release of LH and FSH acting as a homeostatic feedback molecule between the gonads and the brain [70]. The mechanisms by which this occurs are not yet resolved. It is now established that the GnRH neuron should be able to respond to estrogen directly, as estrogen receptors (ER) are expressed in these cells in vivo [71–73], and the *GnRH* gene promoter appears to respond to estrogen in heterologous cell lines [74, 75]. We have found that estrogen represses *GnRH* gene expression in a model of the GnRH neuron, the GT1-7 cell line [42], thereby indicating a direct negative feedback role at the level of the GnRH neuron itself. This evidence has been corroborated using a phytoestrogen, coumestrol [76]. We found that downregulation of *GnRH* gene expression requires ERα, although we cannot yet rule out the involvement of ERβ, as a heterodimer, in this process [42]. Furthermore, studies in the ER knockout mice with targeted disruption of either ERα or ERβ have demonstrated the necessity of ERα for the estrogen-mediated negative feedback of *GnRH* gene expression in the female mouse [77]. However, how the positive regulation of GnRH occurs, necessary for the preovulatory surge, is still debated. At the

level of the pituitary, where estrogen also exerts a so-called biphasic effect at the cell level, it is thought that the positive feedback appears to occur via an independent mechanism and not through an escape of negative feedback inhibition by rising levels of estrogen [78]. In the hypothalamus, it is generally believed that afferent neuronal systems play a dominant role in the switch to positive regulation of GnRH [79]. One of these cell types may indeed be the NPY neuron, which has been linked to the control of the preovulatory GnRH surge.

Neuropeptide Y

Although recent data indicate that GnRH neurons may be directly regulated by estrogen, neurotransmission of estrogen-sensitive afferent neuronal systems are necessary to stimulate GnRH neurons to induce the midcycle luteinizing hormone (LH) surge [80]. A primary candidate for this role is NPY-synthesizing neurons from the hypothalamus [80, 81]. The most recognized functions of NPY include the regulation of endocrine function, circadian rhythms, and satiety [82]. Central administration of NPY stimulates feeding and repeated doses results in an increase in body weight [83]. *NPY* gene expression and accumulation increase abruptly immediately before the preovulatory GnRH surge [84, 85]. Much insight into the exact role of NPY in reproductive physiology has been achieved through the use of antisense oligonucleotides directed against NPY mRNA, injected into the arcuate nucleus of mice or primates [85–87]. If NPY de novo synthesis is blocked before the steroid-induced preovulatory rise in GnRH, the surge release of LH causing ovulation does not occur [86]. Using this technology, NPY has also been linked to the control of basal LH secretion and all parameters of pulsatility, including number of pulses, magnitude and duration of the pulse, and the resulting levels of LH [86]. Studies in the NPY knockout mouse indicates that NPY plays an important role in the GnRH preovulatory surge, but does not appear critical for basal pulsatile secretion [88]. NPY and GnRH neurons form synaptic connections in situ [89, 90]. However, whether estrogen acts through NPY neurons to achieve GnRH control is not yet conclusive as few NPY neurons in the hypothalamus have been found to express estrogen receptors [91]. However, this is based on a very limited number of studies performed with technologies with less than optimal sensitivity. We have used two of our NPY-expressing cell lines to study the direct regulation of these neurons by estrogen [65].

NPY is expressed throughout the hypothalamus, and depending upon its specific location, may have unique functions because of differences in developmental origins or afferent connections. We have used two of our NPY-expressing cell lines, N-38 and N-42, to determine if estrogen can directly affect *NPY* gene expression. Over a 48 h time course, we found a biphasic regulation of NPY transcription in the N-38 neurons, but a continuous downregulatory effect in the N-42 neurons [65]. In the N-38 neurons, we demonstrated that there was an initial downregulation, which was followed by a strong induction of NPY transcription (Fig. 2b). Using siRNA knockdown of either ERα or ERβ, we determined that the repression of *NPY* gene expression by estrogen at 6 h was because of an ERα- and ERβ-mediated event at the level of the *NPY* gene promoter. However, estrogen-mediated induction of NPY transcription that occurs at 48 h was dependent upon ERβ alone (Fig. 3; [65]). On the other hand, using this same technology, we found that the overall estrogen-mediated repression of NPY transcription in the N-42 neurons is dependent upon both ERα and ERβ. It is interesting to note that the levels of *ERα* or *ERβ* gene expression preceding the *NPY* gene changes followed the same general pattern when the cells were exposed to estrogen, that being, ERα was generally repressed by estrogen in both cell lines, but ERβ was significantly upregulated only in the N-38 neurons (Fig. 2a, [65]). From these results, we speculate that the mechanisms by which estrogen controls the *NPY* gene may differ between the early repressive effects vs the longer-term upregulation of transcription. In general, estrogen has anorexigenic effects in animal models [5, 6]; therefore, it would be expected in the specific NPY neurons that control feeding, NPY synthesis should be repressed by estrogen. However, in reproduction where estrogen plays a dual role, there is both a tonic negative regulation of the HPG axis and an estrogen-mediated stimulatory event necessary for the preovulatory surge. These results present a novel mechanism by which estrogen may exert its distinct effects on feeding behavior and GnRH neuronal control in specific hypothalamic cell types.

Using Immortalized Neurons to Study Specific Neuropeptides Involved in Energy Homeostasis

Feeding Regulation

There is a growing list of neuropeptides and neurotransmitters that have been associated with the regulation of feeding behavior in animals [92]. All of these neuropeptides are expressed in defined cell types located in specific regions of the hypothalamus, mainly the arcuate nucleus (Arc) and paraventricular nucleus (PVN), and a vast wealth of literature on these molecules is available (for reviews, see 9, 92, 93). Orexigenic peptides include NPY, AgRP, ghrelin, MCH, galanin, and GHRH, whereas anorexigenic peptides include POMC, urocortin, and NT. Although NPY knockout animals exhibit a relatively normal phenotype [94], evidence from intracerebroventricular (ICV) injections suggest that NPY potently stimulates feeding, NPY deficiency ameliorates

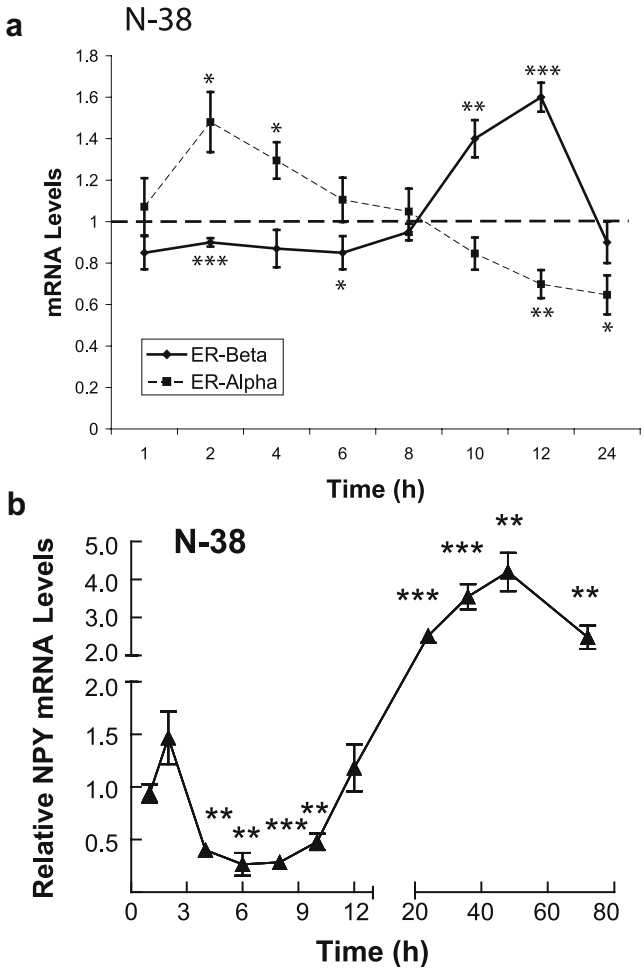
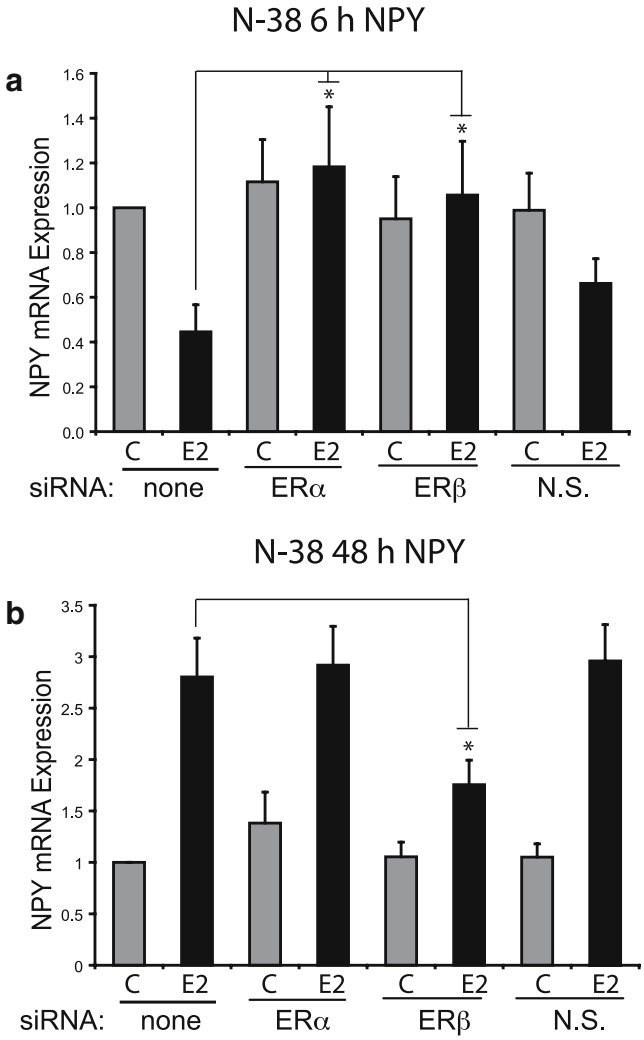


Fig. 2 Temporal regulation of ER and NPY mRNAs in NPY-expressing N-38 neurons. N-38 neurons were serum starved for 12–16 h before treatment with either 10 nM 17β-estradiol or vehicle alone over a 24 or 72 h time course. At the indicated time points, total RNA was extracted and used as a template for real-time RT-PCR with primers specifically designed to amplify ERα and ERβ (**a**) or NPY (**b**). Real-time RT-PCR products were amplified on an ABI Prism 7000. mRNA levels were quantified using the ΔCT method and normalized to the internal control (actin). All results shown are relative to the corresponding control mRNA levels (set to 1.0) at each time point and are expressed as mean±SEM (n=3 independent experiments). *P<0.05, **P<0.01, ***P<0.005. Modified from Titolo et al. [65]. Copyright 2006, The Endocrine Society

obesity, and NPY levels are increased with fasting [95]. NPY and AgRP have been shown to be largely expressed in the same neurons [60]. AgRP also stimulates feeding in mice, and transgenic overexpression of the peptide results in obesity [96]. MCH knockout mice exhibit hypophagia, increased metabolic rate, and leanness [97], whereas overexpression of MCH leads to obesity and insulin resistance [98]; and therefore moved MCH to the forefront as an important feeding molecule [99]. Although galanin and GHRH have not been studied in detail, both also increase feeding [92]. On the other hand, the major anorexigenic candidate is α-melanocyte-stimulating hormone, a peptide derived from the *POMC* gene [96]. POMC knockout mice are hyperphagic and obese. α-MSH activates the melanocortin receptors (MC3-R and MC4-R), and MC4-R knockout mice are also obese [100]. The NPY/AgRP and POMC neurons, also referred to as the melanocortin system, have been postulated to be directly antagonistic in the overall control of energy homeostasis, as AgRP has been found to be a specific antagonist of the MC-Rs [101]. Urocortin inhibits feeding, likely mediated through the corticotrophin releasing hormone-2 receptor [92]. Neurotensin, a peptide with a wide variety of neuroendocrine effects, has also been shown to be involved in feeding suppression [102], and may have a more important role than previously recognized. It is believed that many of these neuropeptides may have developed redundant functions for the control of energy homeostasis through evolution, and together likely play modulatory roles in the maintenance of body weight.

Leptin

Leptin and insulin, among other hormones, regulate feeding and energy balance by modulating the expression of neuropeptides in the hypothalamus [103]. Leptin is synthesized by adipose tissue, is transported to and acts on the hypothalamus to achieve its regulatory action. Indeed, leptin receptors are highly expressed in the hypothalamus [103], and through elegant double-label immunocytochemistry have been localized to NPY/AgRP, POMC, galanin, tyrosine hydroxylase, MCH, vasopressin, oxytocin, CRF, and NT-containing cell types [104–106]. Leptin has been postulated to signal through the JAK-STAT signal transduction pathway, yet a knockout mouse that leaves the leptin receptor intact, but specifically disrupts the ObR-STAT3 signal (leprS1138) does not affect repression of NPY by leptin [107]. This suggests that there is a STAT3-independent pathway, specifically used to control NPY neuron responsiveness to leptin. There are other examples of STAT3-independent leptin signaling through PI3K/Akt–Foxo 1 and Shp2–ERK 1/2 [108, 109], indicating that leptin stimulation may activate a number of signaling pathways, either acting independently or in concert to control diverse functionality. Knockout mice without leptin (ob/ob) or leptin receptor (db/db) are morbidly obese and infertile, and leptin is able to reverse this etiology in ob/ob mice [9]. However, most obese patients do not have leptin or leptin receptor defects, and instead are resistant to the anorectic effects of the hormone [93]. Mice with a specific deletion of brain leptin receptors (ObR(SynI)KO) are obese and infertile, but deletion of peripheral hepatocyte leptin receptors does not change the normal phenotype of the animal [110]. On the other hand, brain-specific restoration of leptin receptor signaling in mice with a whole body deletion of the leptin receptor (db/db) is sufficient to reverse



◀ **Fig. 3** *NPY* gene expression is dependent on ERα and/or ERβ in N-38 neurons. After transfection of siRNA directed toward ERα and ERβ for 36 h for optimal knockdown of the ER subtypes, N-38 neurons were treated with either 10 nM 17β-estradiol or vehicle alone for 6 or 48 h. RNA and protein were isolated from the cells. RT-PCR analysis determined that knockdown of both ERα and ERβ significantly blocked the estrogen-mediated repression of NPY at 6 h (**a**). RT-PCR analysis determined that knockdown of ERβ significantly attenuated the estrogen-mediated increase of NPY at 48 h (**b**), whereas knockdown of ERα had no significant effect. All results shown are relative to the control mRNA levels (set to 1.0) at each time point and are expressed as mean±SEM (n=3 independent experiments). *P<0.05. **c** Cell lysates from N-38 neurons were isolated and subjected to SDS-PAGE. Western blot analysis demonstrated that the siRNA knocked down ERα by 79% (SE=4.6%) and ERβ by 84% (SE=3.8%) in N-38 neurons. A representative Western blot is shown. Nonsense (*N.S.*) siRNA was used as a negative control. Modified from Titolo et al. [65]. Copyright 2006, The Endocrine Society

the obesity, diabetes, and infertility of db/db mice [111]. Therefore, major efforts are currently underway to target hypothalamic effectors of leptin action for the therapeutic treatment of obesity. The diverse actions of leptin on feeding and metabolism probably involve differential regulation of neuronal circuits in the hypothalamus and brainstem. For instance, leptin differentially engages NPY/AgRP and POMC neurons [112], and NPY and melanocortins exert opposing effects on feeding and metabolism [96]. Nonetheless, there are some conflicting reports that leptin does not change NPY release, but instead regulates GHRH, somatostatin, and α-MSH [113]. Galanin and MCH neurons have also been postulated to be targets of leptin signaling [102]. Furthermore, leptin stimulates NT synthesis in the hypothalamus, implicating that NT plays a part in leptin action as well [102, 114, 115].

Neurotensin

NT is a 13-amino acid peptide originally isolated from the bovine hypothalamus [116] and is widely distributed throughout the CNS and digestive tract [117]. In the CNS, NT controls a number of physiological processes including: feeding suppression, regulation of the circadian pacemaker, antipsychotic-like action, anti-pain, regulation of body temperature, secretory stimulation of hypothalamic releas-

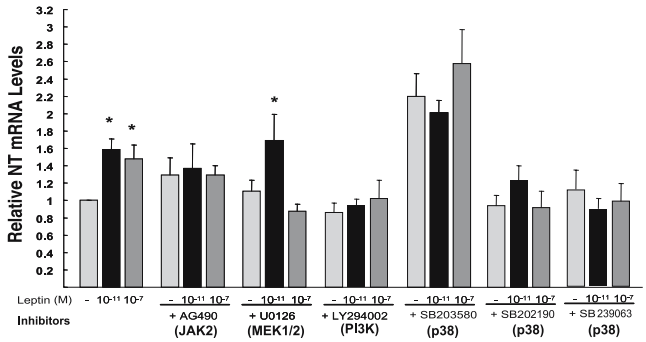


Fig. 4 Effect of pharmacological inhibitors on leptin-mediated induction of *NT* gene expression. Cells were plated in 60 mm plates to about 80% confluence, then serum-starved for 2 h. After pretreatment with or without the specified pharmacological inhibitors for 1 h, N-39 neurons were stimulated with leptin (10^{-11} or 10^{-7} M, respectively) for 4 h. The expression of neurotensin mRNA was determined by real-time PCR. Values for NT are expressed relative to γ-actin mRNA levels (mean±S. E., n=3). The *asterisk* indicates points that are significantly different (P<0.05) compared with the untreated control. Modified from Cui et al. [66]. Copyright 2006, FASEB J

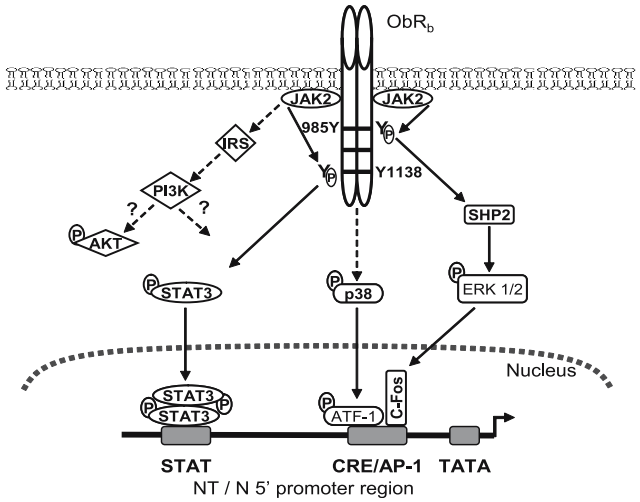


Fig. 5 Schematic diagram of signal transduction pathways induced by leptin in the clonal, hypothalamic NT cell model, N-39. In the hypothalamic cell model, N-39, we provide evidence for the activation of three specific signal transduction pathways by leptin, all dependent on JAK2 signaling. The MAPKs, ERK1/2, and p38 are induced by leptin receptor activation. STAT3 is also involved in the leptin-mediated regulation of *NT*/*N* gene expression, as we have described its binding to the mouse NT/N promoter region. We describe the involvement of the downstream activators c-*fos* and ATF-1. We speculate that the JAK/STAT and p38 MAPK pathways are predominant at physiological leptin levels, whereas the ERK1/2 MAPK may be more prominently utilized at higher leptin concentrations (from Cui et al. [66]). Copyright 2006, FASEB J

ing hormones and anterior pituitary hormones, and neuromodulation of dopamine neurotransmission [118–121]. In addition to hypothalamic POMC and NPY neurons, other potential targets of direct leptin signaling include galanin-, MCH-, thyrotropin-releasing hormone- (TRH), and NT-expressing neurons [102, 122]. NT neurons, located in the hypothalamus, are responsive to leptin [102, 115, 123]. Furthermore, evidence from in vivo studies in rats indicates that NT may modulate the central effects of leptin on feeding behavior [114, 124]. NT neurons appear to play an anorectic role downstream of leptin. Evidence of this is seen in obese leptin-deficient ob/ob mice [125] or leptin-insensitive fa/fa rats [124], where hypothalamic NT expression is decreased. On the other hand, microinjection of leptin into the PVN significantly stimulates NT synthesis in association with reduced food intake [102, 124, 126]. Furthermore, immunoneutralization with a NT antibody or a NT receptor antagonist completely reverses the effects of a leptin-induced decrease in food intake [114]. These results suggest that NT may mediate leptin action, at least in part. Previous cell models have been used to study hormonal control of *NT* gene activation, such as PC12 pheochromocytoma cells [127] and SK–N–SH neuroblastoma cells [128]; however, these cell types are not likely representative of endogenous hypothalamic NT-expressing neurons, particularly those involved in feeding control [114, 124]. We have therefore generated cell lines that express endogenous NT [57] and have used these cell lines to study the direct regulation of NT neurons by leptin [63, 66].

It is difficult to determine whether the effect of leptin on NT-expressing neurons is direct or through afferent neurons using in vivo studies. To elucidate the direct role of leptin within NT neurons, we screened two of our NT cell lines, N-39 and N-36/1, and found that they express the leptin receptor (Ob-R$_b$). The presence of this receptor indicates that NT neurons are directly influenced by leptin and may play an important role in mediating leptin-induced effects within our cell lines and potentially the hypothalamus. We analyzed leptin responses within these NT cell lines and defined the molecular mechanisms involved in leptin-mediated regulation of *NT* gene expression. We found that leptin stimulation increased *NT* gene expression approximately twofold and that this regulation occurred at the transcriptional level [63]. We were able to map the leptin-responsive region to within −381 of the *NT* gene 5′ regulatory region [63]. We found that the classic JAK-STAT signal transduction pathway was involved in this process, as STAT was able to bind to the NT promoter region [63]. Upon further examination of the signal transduction events involved in the leptin-mediated control of *NT* gene expression, we found that not only JAK-STAT is directly involved but also defined unique signaling events in these neurons. We demonstrated that the MAP kinases, ERK1/2 and p38, are also necessary for the upregulation of NT transcription by leptin in the N-39 neurons (Fig. 4; [63, 66]). Using chromatin precipitation, we found that two

Table 1 Some potential uses of neuronal cell models in neurobiology research

Research potential for neuronal cell lines
Drug discovery
Therapeutic drug testing platform
Phenotypic profiling of individual neuronal cell types
Gene expression studies
Mechanisms of action of novel neuropeptides
Neuronal function
Ion channel function
Signal transduction in neurons
Knockdown of specific proteins
Receptor cloning and characterization
Proteomic studies
Genome-wide gene expression profiling in selected neuronal cell types
Transcriptional mechanisms
Neuron-specific transcription factor analysis
Neuron–neuron interactions and communication
Brain modeling

specific downstream components of these pathways also bind the NT promoter, transcription factors ATF-1 and c-*fos* [66]. What is quite remarkable is that these two transcription factors appear to bind the NT promoter differentially depending upon the concentration of leptin used, physiological or supraphysiological, indicating unique signal transduction events at two specific levels of leptin (Fig. 5). These cell models provide a novel tool to understand the direct control of NT neurons by hormones and neuromodulators involved in the regulation of feeding and energy homeostasis, but may also be useful to understand the molecular events involved in the development of hormone resistance, a tremendous problem for obesity therapeutics.

Future Directions

Knowledge of how the brain achieves its diverse central control of basic physiology is severely limited by the virtual absence of appropriate cell models. The complexity of the hypothalamus, because of numerous cells harboring unique characteristics and identities, represents a major difficulty in the direct study of the cellular biology of individual neurons from this region of the brain. In particular, expression of specific neuropeptides, which characterize the identity of these unique neurons, are detected in relatively small populations of cells and, as evidence suggests, are distributed throughout the region. One can get an idea of the importance of specific receptor signaling in the brain using neuron-specific knockout mice models, for instance estrogen receptor isoform knockouts in NPY neurons using the Cre-lox system. However, the study of the mechanism of action of a specific neuropeptide, its gene regulation, and both its original or mediated roles and contributions within the hypothalamus is limited in situ. A way to attenuate this complexity and to investigate such questions at the molecular and cellular levels is the establishment of clonal immortalized hypothalamic neuron-like cell lines that express the cellular markers of interest. There are particular technologies that are conducive to cell lines that are difficult, if not impossible in vivo. These may include siRNA knockdown [65], nuclear run-on assays to directly demonstrate transcriptional effects [32], promoter analysis using chromatin immunoprecipitation and electrophoretic mobility shift assays [63, 66], and high-throughput DNA microarray or protein assays [129]. We have developed a novel technology to mass immortalize primary cell culture from the hypothalamus. We have been able to generate a wide phenotypic array of clonal cell lines expressing relevant neuropeptides, neurotransmitters, and receptors. We have used these cell lines to study the molecular events involved in the control of neurons associated with reproductive function and energy homeostasis. However, the scope of use for these novel cell models is broader (Table 1). The potential uses of the cell lines include: drug discovery, a therapeutic drug testing platform, phenotypic profiling of individual neuronal cell types, gene expression studies, mechanisms of action of novel neuropeptides, neuronal function, signal transduction in neurons, receptor cloning and characterization, proteomic studies, genome-wide gene expression profiling in selected neuronal cell types, neuron-specific transcription factor analysis, neuron–neuron interactions and communication, brain modeling, and ion channel function, among numerous others only limited to the researchers' imagination.

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