Babwah

Modification Form for Permit No. HR-009
Permit Holder: Babwah

Approved Personnel
(Please stroke out any personnel to be removed)

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	E. coli (DH5 alpha), E.coli (Top 10), XL1-Blue super-competent cells	
Approved Cells	Human (established), rodent (established), nonhuman primate (established), Hek 293, HTR-8/Svneo, MDA-MB-231, MDA-MB-435S, MCF-10A, PC-3, PZ-HPV-7, JEG-3, ARIP, AR42J, COS-7, Mouse MEF, B-	GT1-7 mouse neuronal cell line Galpha deficient MEFs
Approved Use of Human Source Material	Human chorionic gonadotropin - purified	
Approved GMO	SV 40 Large T antigen, Adeno E1A gene in HEK 293 cells	
Approved use of Animals		
Approved Toxin(s)	Cholera toxin (C-08052 from Sigma Canada)	

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

Classification: 2

Date of last Biohazardous Agents Registry Form: Aug 29, 2007

Signature of Permit Holder: [signature]

BioSafety Officer(s): [signature] July 30/09

Chair,Biohazards Subcommittee: _____

The GT1-7 cells will be used as a model of the GnRH neuron to study the molecular mechanisms regulating GPR54 (a receptor that regulates GnRH secretion) activity. These GnRH neurons express GPR54. The GPR54 and GnRH signaling systems are regulators of puberty and we hope that our results will lead to a better understanding of the molecular regulation of puberty in man.

Re: *Galphaq/Galpha11 and Galpha12/Galpha13 deficient MEFs* (mouse embryonic fibroblasts)

------- Original Message --------

Subject: Re: MTA for cell lines from Germany: Babwah
Date: Fri, 21 Aug 2009 20:21:10 -0400
From: Andy V Babwah <ababwah@uwo.ca>
To: Jennifer Stanley <jstanle2@uwo.ca>
References:<4A8F244E.9080805@uwo.ca>

Hello Jennifer,

Thanks very much.

These cells will be used to study the mechanisms by which the receptor, GPR54 perceives extracellular stimuli and transmit them into intracellular signals. GPR54 is a major regulator of puberty and reproductive functions. None of these studies will involve animal work.

Andy

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Review

Hypothalamic cell lines to investigate neuroendocrine control mechanisms

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ABSTRACT

The hypothalamus is the control center for most physiological processes; yet has been difficult to study due to the inherent heterogeneity of this brain region. For this reason, researchers have turned towards cell models. Primary hypothalamic cultures are difficult to maintain, are heterogeneous neuronal and glial cell populations and often contain a minimal number of viable peptide-secreting neurons. In contrast, immortalized, clonal cell lines represent an unlimited, homogeneous population of neurons that can be manipulated using a number of elegant molecular techniques. Cell line studies and in vivo experimentation are complementary and together provide a powerful tool to drive scientific discovery. This review focuses on three key neuroendocrine systems: energy homeostasis, reproduction, and circadian rhythms; and the use of hypothalamic cell lines to dissect the complex pathways utilized by individual neurons in these systems.

1. Introduction

The hypothalamus maintains the homeostasis of our internal environment and is the control center for all endocrine functions (Fig. 1). It is situated below the thalamus, posterior to the optic chiasm and surrounds the third ventricle; access to the ventricle allows it to integrate signals from circulating factors that cross the blood-brain barrier (BBB). A study by Faouzi et al. indicates that there is differential accessibility to circulating factors within the hypothalamus. They have shown evidence that one of the ventral regions of the hypothalamus, the arcuate nucleus, does not require peripheral factors to cross the BBB, but instead may have neuronal projections that extend outside of the BBB [53]. The regulatory actions of the hypothalamus can be divided into three categories: control of internal homeostasis, endocrine system regulation and autonomic nervous system regulation. Within these three categories there are numerous critical physiological functions regulated by the hypothalamus. Internal homeostasis covers the regulation of water balance, temperature, circadian rhythms and energy and glucose homeostasis [60]. The latter two processes, circadian rhythms and energy and glucose homeostasis, are two areas that are heavily studied, especially as the hypothalamus has recently been considered a key region in the pathogenesis of obesity and diabetes [62]. With regard to endocrine system regulation the hypothalamus, via the pituitary gland, regulates stress, growth, metabolism and reproduction [60]. The third category, autonomic nervous system regulation, includes the control of blood pressure, gut motility and respiration [60]. As the hypothalamus controls these numerous vital tasks, a perturbation of the delicate regulatory balance can lead to detrimental effects resulting in major health problems.

The hypothalamus contains multiple nuclei, which are comprised of a complex network of neurons. Within this complex array of neurons there are distinct neuronal phenotypes, each expressing a specific complement of neuropeptides, neurotransmitters and receptors [52]. An understanding of the control mechanisms of the unique hypothalamic, peptidergic neurons is critical before we can understand how the brain achieves its diverse control of basic physiological functions. The cellular mechanisms involved in this process are not clearly understood, mainly due to the complexity of the in vivo hypothalamic architecture. Numerous studies have been undertaken to map the afferent connections between distinct hypothalamic nuclei and neurons, utilizing methodology such as double- and triple-label immunocytochemistry, in situ hybridization and retrograde tracing [43,47,51,74,145]. 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 molecular mechanisms involved in gene regulation and cellular signaling. In order to overcome the complexity of in vivo models, numerous labs have attempted hypothalamic cell line generation. Historically, it was difficult to establish immortalized hypothalamic cell lines, due to the lack of naturally occurring tumors and the inherent difficultly of transforming or immortalizing highly differentiated neurons

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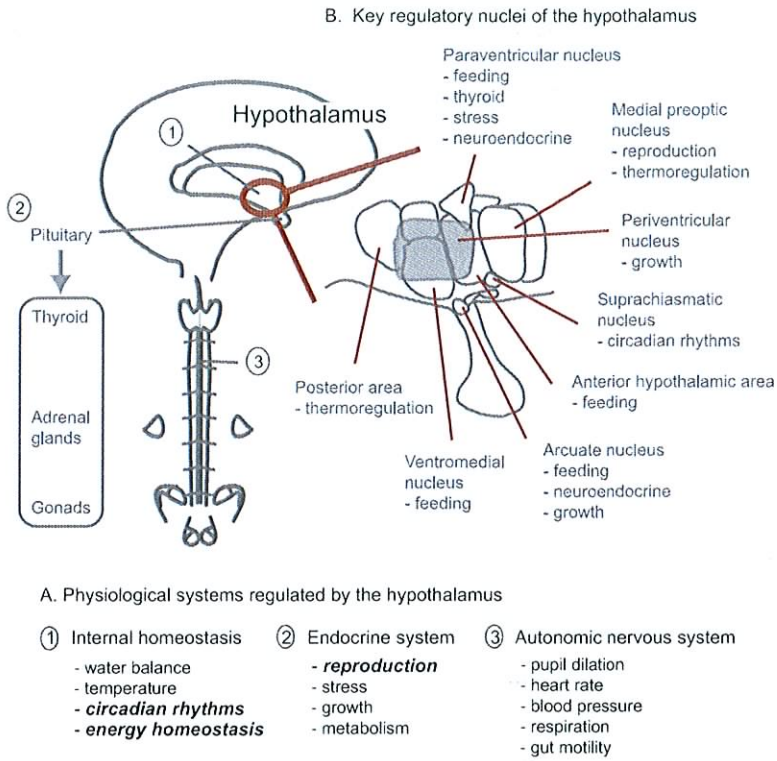

Fig. 1. Hypothalamic function. (A) The hypothalamus regulates numerous functions, all of which can be categorized under three headings: internal homeostasis, endocrine regulation and autonomic nervous system regulation. The three functions specifically discussed in this review, circadian rhythms, energy homeostasis and reproduction, are italicized. (B) Within the hypothalamus these processes are controlled by discrete nuclei, each with a different complement of phenotypically distinct neurons.

from primary culture [28]. Cell lines from the peripheral nervous system were established from neuroblastomas, such as the Neuro2A, and pheochromocytomas, such as the PC12 cell line; however, these models do not truly represent differentiated central nervous system neurons (CNS). For instance, the murine N1E 115 neuroblastoma cell line is routinely used as a CNS-derived neuronal model, although it was originally generated from a spontaneous tumor on the spinal cord [9]. Recently, cell models from the hypothalamus have been developed and have proven to be invaluable towards understanding the cellular biology of specific neuroendocrine cells. The number of cell models from the hypothalamus and from the entire brain, consist of a few isolated cell types and represent an infinitesimal percentage of the neuronal phenotypes represented within the brain. For this reason, our group and other labs have been continually working on developing new hypothalamic, neuronal cell models. In this review, we will be summarizing current hypothalamic cell lines in use and will examine how they have enriched our understanding of hypothalamic function, with a focus on three important neuroendocrine topics: energy homeostasis, reproduction and circadian rhythms.

2. Hypothalamic cell lines

Non-transformed primary hypothalamic cultures are difficult to maintain, have a short life span and represent a heterogeneous neuronal and glial cell population; often these cell populations contain a minimal number of healthy peptide-secreting neurons. On the other hand, immortalized, clonal cell lines represent an unlimited homogeneous population of specific neuronal cell types. Additionally, they offer a model with fewer uncontrolled variables than the in vivo situation and are maintained in a controlled and homogeneous condition. Classical in vivo approaches cannot firmly establish the direct action of an agent on specific hypothalamic neurons or on neuropeptide transcription, mainly because the cell receives input from other neurons. As well, little is known about the molecular mechanisms involved in intracellular signaling, promoter regulation, regulation of gene transcription, or regulation of secretion in native neurons due to the complexity and difficulty of studying molecular events in vivo. The use of cell lines provides a simpler model to begin these investigations. Since few studies have been performed in vivo, researchers are unable to state whether neuronal cell lines function identically to native neurons. For this reason, caution must be taken when extrapolating theories from the cell line to the in vivo model. As well, cell lines lack the complexity and integrated network of neuronal connections and signaling. Despite these limitations, cell line studies can be used to understand the in vivo model by having a clearer idea of further studies to pursue with, as well as confirming molecular events. From the current studies available that have looked at effects such as hormonal regulation of gene expression or receptor activation, most studies have found that the results from cell lines replicate that of in vivo studies. Cell lines also provide a good model to screen different neuronal phenotypes for the expression of specific genes or proteins; studies that are difficult in vivo due to the numerous phenotypes of neurons present in a given hypothalamic area. Because of this, limited characterization of native hypothalamic neurons has been performed, and though hypothalamic cell lines express a compilation of neuropeptides, receptors and signaling molecules, it is unconfirmed that this differs from in vivo. It was originally shown from in vivo studies that the GnRH neuron only expressed one of the estrogen receptor (ER) isoforms, ERalpha. Using the GnRH cell line, GT1-7, researchers found that both isoforms ERalpha and beta were expressed [128], after which it was confirmed that both isoforms are present in the native GnRH neuron [143]. This exemplifies how cell lines can be utilized to clarify in vivo studies and although hypothalamic cell lines express

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numerous factors not yet shown in native neurons, this may be due to a lack of in vivo studies.

For the reasons presented above, researchers have turned towards immortalized cell models for detailed molecular and mechanistic studies. In 1885 Wilhelm Roux established basic tissue culture techniques but it was not until 1940 when the first immortal cell line was developed by Earle: the L Strain cells [48,55]. Since then numerous cell lines have been developed from many different tissues; although the first attempt at immortalizing neurons was not until 1974, performed by Shaw et al. [42]. They infected primary hypothalamic cells from embryonic mice day 14 with intact simian virus 40, a DNA virus that contains the oncogene, large T antigen (SV40 T-Ag), creating an immortalized cell population labeled HT9. Unfortunately, these cells were morphologically similar to precursor cells and were not fully differentiated neurons. In 1984, Cepko et al. [27] developed retroviral shuttle vectors allowing for the introduction of DNA sequences into mammalian cells, utilizing SV40 T-Ag mediated replication. It allowed for increased efficiency of gene transfer and contained a gene conferring resistance to specific antibiotics, such as neomycin, allowing for selection of infected cells. This technology has permitted researchers to retrovirally infect primary cells with an immortalizing oncogene and selectively propagate them. This is one of the key technologies, along with the development of transgenic mice expressing oncogenes, which has allowed for the development of cell lines. In the following subsections we will discuss how these technologies were utilized to develop gonadotropin-releasing hormone (GnRH), suprachiasmatic nucleus (SCN) and other general hypothalamic cell lines.

2.1. GnRH expressing cell lines

One of the first fully differentiated hypothalamic cell lines was a GnRH expressing cell model developed by Mellon et al. in 1990 [104]. They created a transgenic mouse utilizing 5′ flanking DNA of the rat GnRH gene to target expression of SV40 T-antigen in GnRH neurons. They obtained nine transgenic mice that expressed the GnRH-SV40 T-Ag gene, none of which were fertile. Two of these mice developed anterior hypothalamic tumors. From one of the female mice, portions of the tumor were removed for cell culture, ultimately becoming the GT-1 cell line population. Following serial dilutions of the GT-1 cells, they developed three homogeneous cell populations, labeled GT1-1, GT1-3 and GT1-7. These cells expressed neuronal morphology and secreted GnRH when depolarized. These cells became one of the most highly utilized neuronal cell models for studies related to not only GnRH, but also basic neuronal function, as they represented one of the few appropriate neuronal models available.

Currently, there are now four GnRH expressing cell models: the GT1, GN, Gnv and GRT cells. The GN cells were developed utilizing a similar method as the GT1s, except they used the 5′ flanking region of the human GnRH gene [122]. Also, the T-Ag driven tumors developed before migration of the GnRH neurons from the olfactory placode to the hypothalamus was complete and thus the cells are considered to be immature GnRH neurons. One of their mice, a male mouse, contained T-Ag and GnRH expressing cells, from which two cell lines were created, the NLT and Gn11 cells. Interestingly, there were distinct phenotypic differences between the two lines, as the NLT cells expressed GnRH at levels 10-fold higher than the Gn11 cells, while the Gn11s expressed a splicing variant of the GnRH gene lacking exon 2. The last two GnRH cell lines, the Gnv and GRT cells, were developed using quite different methods.

The Gnv cells were developed by Salvi et al. [136] and were one of the first attempts to create cell lines from adult hypothalamic cultures. Isolated hypothalami from 10 to 12 weeks old rats were infected using two lentiviral vectors. The first vector expressed a tetracycline (Tet) transactivator gene driven by the GnRH promoter and the second vector expressed the v-myc oncogene and puromycin resistance gene fused with a Tet-responsive element. These vectors allowed for two things: the Tet-transactivator gene to only be expressed in GnRH neurons and the conditional activation of the v-myc oncogene with Tet treatment. They developed 12 clones of which clones 3 and 4, named Gnv-3 and Gnv-4, expressed the highest levels of GnRH and were thus selected for future studies. These cells have a phenotype of mature neurons and secrete GnRH in a pulsatile pattern.

The GRT cells, developed by Wolfe et al. [164], are the most recent hypothalamic cell lines and were also immortalized using conditional activation of an oncogene. They created transgenic mice expressing a Tet-regulated, GnRH promoter driven T-Ag hybrid gene. At 4 months of age, mice were treated with doxycycline and hypothalami were dissected for cell culture at 5 months. The cells were passaged in doxycycline containing media, which allowed for proliferation of the cells. The GRT cell line expresses and secretes GnRH, although at lower levels than the GT1-7 cells.

2.2. SCN cell lines

Motivated in a similar fashion as reproductive researchers, scientists with a circadian background desired a cell culture model of the SCN. In 1999, Earnest et al. developed the first SCN cell line [49]. They isolated SCN from embryonic rat hypothalamic, day 15 and 16, and infected them with a retrovirus expressing the adenovirus 2-adenovirus five hybrid E1A 12s sequence (12S E1A), an oncogene, and a neomycin resistance gene. The infected cells were selected using geneticin and two cell lines were subcloned: the SCN2.2 and SCN1.4. Both lines expressed neuronal markers and SCN specific peptides, such as VIP and GRP. These cell lines have become the most well studied SCN cell models.

Recently, two other SCN cell lines have been developed: the N14.5 and RS182 cells. The N14.5 cells were created using novel technology [102]. They isolated cells from the venterolateral region of the SCN, from transgenic rats expressing the temperature sensitive (ts) SV40 T-Ag. The SV40 T-Ag gene is only activated at 33 °C, thus once the primary SCN neurons were isolated and grown at 33 °C, SV40 T-Ag was activated and the cells began to proliferate. When grown at 39 °C, proliferation is arrested and the cells differentiate further. This cell line is another example of a conditionally activated SV40 T-Ag cell line. The N14.5 cell line expresses neuronal markers and the SCN marker, VIP.

The RS182 cells were also created using tsSV40 T-Ag expressing rats, except the rats were crossed with Per1 promoter driven luciferase gene expressing [85]. SCN cells were isolated from these rats at embryonic day 19 and grown at 33 °C. They established 512 cell lines of which 17 displayed stable Per1 oscillations, visualized by luciferase protein expression, indicative of intrinsic circadian gene cycling. From the 17 lines, the RS182 cells had the highest amplitude of cycling and were selected for further studies.

2.3. A Wide array of other hypothalamic neurons

The GnRH and SCN cells are only a few neuronal cell types from the hypothalamus. Thus other groups, including ours, have developed cell lines that are representative of the enormous range of cell types in the hypothalamus. Two groups have utilized retroviral gene transfer and SV40 T-Ag to immortalize rat embryonic cell lines. In 1990, Rasmussen et al. developed the RCF-8 and 12 and RCA-6 cell lines [123]. These lines are responsive to estrogen and the RCA-6 cells express NPY and IGF-1. Although these cells were developed in 1990, few studies have utilized these models and they have not been further characterized them. In 2003, Kasckow et al. [83] also retrovirally infected embryonic day 19 rat hypotha-

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lami, producing cell lines. They screened the cells and identified one line, IVB, which expressed CRH, for further studies. From their initial experiments, they have found that this cell line serves as a model for parvocellular CRH neurons.

Recognizing the need for mouse cell lines representative of other unique hypothalamic neurons, our group initially established 38 embryonic, clonal hypothalamic mouse cell lines [18]. We utilized retroviral infection of SV40 T-Ag of primary hypothalamic cell cultures from fetal mice day 15, 17 and 18 to produce a heterogeneous mix of cells, which were further subcloned into homogeneous cell populations. The cells are currently labeled as mHypoE-'clone number' to distinguish them from other newly created cell lines and for clarity, although it should be noted that in previous studies they were labeled as N-'clone number'. These cells have been well characterized and screened for over 28 neuroendocrine markers, while the number of clonal lines has now increased to over 60. The cell lines also express mature neuronal markers and exhibit neuronal morphology (Fig. 2). Each of the cell lines expresses a unique complement of receptors and neuropeptides and has a distinctive morphology. Some key neuropeptides expressed in these cells are neuropeptide Y (NPY), agouti-related peptide (AgRP), proopiomelanocortin (POMC), cocaine- and amphetamine-related transcript (CART), neurotensin (NT), melanin concentrating hormone (MCH), vasoactive intestinal peptide (VIP) and corticotopin-releasing hormone (CRH), among many others (see Table 1).

Embryonic cell lines may or may not accurately represent the adult, fully-differentiated neuron due to their unique developmental and physiological roles, and this is difficult to study without appropriate models. Therefore, our group has recently devised a novel method to immortalize neurons from the adult mouse (D.D. Belsham et al., submitted for publication). In order to retrovirally incorporate the oncogene SV40 T-Ag into the cellular genome, cells must be dividing. As such, we utilized the nerve growth factor, ciliary neurotrophic factor (CNTF), to treat primary cell culture from adult mouse hypothamali, inducing neurogenesis and cell division. Following 10 days of CNTF treatment, the cells were retrovirally infected with SV40 T-Ag and the infected cells were selected with geneticin. The cells express markers of mature neurons and exhibit neuronal morphology. We have established over 50 cell lines, labeled mHypoA-'clone number', and are currently characterizing these lines for expression of pertinent neuropeptides,

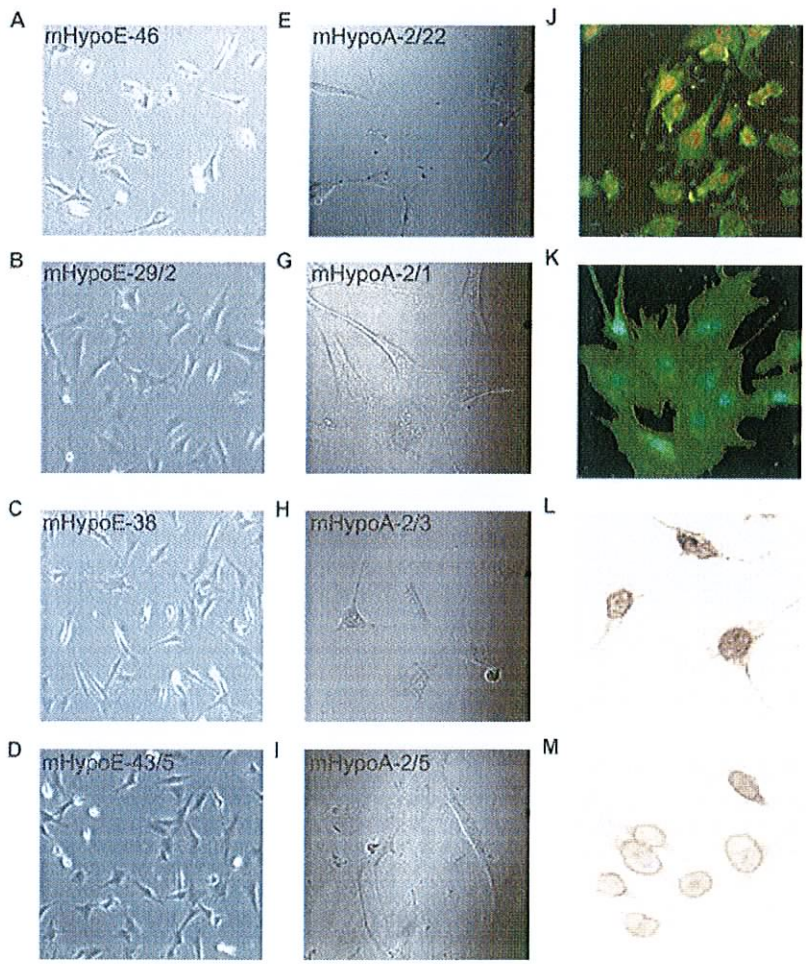

Fig. 2. Imaging of embryonic and adult hypothalamic cell lines Examples of hypothalamic cell lines, both embryonic and adult, are illustrated within this figure. (A–D) The embryonic mHypoE-46, −29/2, −38 and −43/5 were imaged using phase contrast microscopy. These four lines were used in studies described in the energy homeostasis section and the mHypoE-38 line was also used in studies described in the reproduction section. (E–I) The adult mHypoA-2/22, -2/1, -2/3 and -2/5 were imaged using confocal differential interference contrast microscopy. (J) The embryonic mHypoE-38 neurons were imaged using fluorescent confocal microscopy after immunocytochemical analysis with anti-ghrelin sera (green); nuclei were counterstained with propidium iodide (red) (originally published in [56]). (K) The adult mHypoA-2/12 neurons were imaged using fluorescent microscopy after immunocytochemical analysis with an antibody against NPY (green); nuclei were counterstained with DAPI (blue). (L and M) The mHypoE-36/1 neurons were imaged using DAB staining during immunocytochemical analysis with antibodies against neurofilament (NF) and neurotensin (NT) (originally published in [40]) (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.).

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Table 1
Genes expressed in hypothalamic cell lines.

Gene	mHypoE-29/2	mHypoE-38	mHypoE-29/4	mHypoE-39	mHypo-36/1	mHypoE-46	GT1-7	GnV3	GnV4	SCN2.2
Agouti-related peptide	+	+	+	+	+	+	+			
Androgen receptor	–	–	–	–	–	+	+			
Arginine vasopressin		+					+			+
Arginine vasopressin receptor 1a	+	+	+	+	+	+	+			
Arginine vasopressin receptor 1b	+	+	+	+	+	+	+			
Cocaine and amphetamine related transcript	–	+	..	+	+	+	+			
Cannabinoid receptor 1			–	+	..	+	+			
Ciliary neurotrophic factor receptor	+	+	–	+	+	+	+	+	+	
Corticotropin releasing hormone receptor 1	+	–	+	+	+	–	+			
Corticotropin releasing hormone receptor 2	+	–	+	+	+	+	+			
Corticotropin releasing hormone	–	+	–	+	–	+	–			–
Dopamine transporter	–	+	.	+	+	+	+			
Dipeptidylpeptidase 4	–	–	–	–	+	+	+			
Estrogen receptor α	+	+	–	+	+	+	+	+	+	
Estrogen receptor β	+	+	+	+	+	+	+	+	+	
Galanin	–	–	–	–	+	+	–			
Galanin-like peptide	–	–	+	+	–	–				
Gastrin-releasing peptide	+	–	+	+	+	..	+			–
Glucokinase	+	+	+	+		+	+			
Glial fibrillary acidic protein	–	..	–	..	–	–	–	..	–	–
Ghrelin	+	+	+	+	+	+	+			
Growth hormone secretagogue receptor	+	+	+	+	+	+	+	+	+	
Glucagon-like peptide receptor 1	–	–		+	+	–	+			
Glucagon-like peptide receptor 2	–	..	–	–	+	–	+	.	–	
Glucagon receptor	–	.	–	–	–	+	–	–	–	
Glucocorticoid receptor	+	+	+	+	+	+	+			
Glucose transporter 1	+	+	+	+	+	+	+			
Glucose transporter 2	–	..	–	+	–	–	+			
Glucose transporter 3	+	+	+	+	+	+	+			
Glucose transporter 4	+	+	+	+	+	+	+			
Gonadotropin inhibitory hormone	+	+	+	+	+	+	+			
Insulin-like growth factor 1	+	+	+	+	+	+	+			
Insulin-like growth factor 1 receptor	+	+	+	+	+	+	+			
Insulin II	–	..	–	+	..	–				
Insulin receptor	+	+	+	+	+	+	+			
Insulin receptor substrate 2	+	+	..	+	–	+	+			
KISS-1 metastasis-suppressor	–	..	–	–	+	–	–			
KISS-1 metastasis-suppressor receptor	+	+	+	+	+	+	+			
Leptin receptor	+	+	+	+	+	+	+			
Melanocortin 4 receptor	–	.	–	+	–	+	+			
Melanin concentrating hormone	+	+	+	+	+	+	+			
Melanin concentrating hormone receptor 1	+	+	+	+		+	+			
Melatonin receptor 1							+			+
Melatonin receptor 2							+			+
Neuropeptide peptide Y	–	+	+	–	–	+	–			
Neuropeptide peptide Y receptor Y1		+	+	+	+		–			
Neuropeptide peptide Y receptor Y2		+	+	+	+		+			
Neuropeptide peptide Y receptor Y4		+	+	+	+		+			
Neuropeptide peptide Y receptor Y5		..	+	+	.		–			
Neuron specific enolase	+	+	+	+	+	+	+	+	+	+
Neurotensin	+	.	+	+	+	+	+			
Neurotensin receptor 1	–	–	–	+	+	–	–			
Orexin receptor 1	+	+	+	+	+	+	+		–	
Orexin receptor 2	+	.	+	+	+	+	+	+	+	
Oxytocin		+					+			–
Proopiomelanocortin	+	–	–	–	–		+	–	–	
Proglucagon	–	..	–	+	+	–	+	–	.	
Seratonin receptor 1b	+	+	+	+	+	+	+	–	–	
Seratonin receptor 2a	+	+	+	+	+	+	–			
Seratonin Receptor 2c	..	.	–	–	–	–	+	.		
Somatostatin		+	+	+	+	+				+
Splicing factor 1	+	+	+	+	+	+	+			
Suppressor of cytokine signaling 3	+	+	+	+	+	+	+			
Spermiogenesis specific transcript on the Y	+	+	+	–	–	+	–			
Signal transducer and activator of transcription 3	+	+	+	+		+	+			
Signal transducer and activator of transcription 5A	+	+	+	+		+	+			
Signal transducer and activator of transcription 5B	+	+	+	+		+	+			
Sulfonylurea receptor 1	–		–	–		.	+			
Sulfonylurea receptor 2	–	–	–	+		+	–			
Syndecan 3	+	+	+	+	+	+	+	+	+	
T-antigen	+	+	+	+	+	+	+			

(*continued on next page*)

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Table 1 (*continued*)

Gene	mHypoE-29/2	mHypoE-38	mHypoE-29/4	mHypoE-39	mHypo-36/1	mHypoE-46	GT1-7	GnV3	GnV4	SCN2.2
Urocortin	+	–	+	+	+	+	+			
Vasoactive intestinal peptide	+	+	+	+	+	+	+			+
Vasoactive intestinal peptide receptor 1	–	–		+	+	+	-			
Vasoactive intestinal peptide receptor 2	+	+	+	+	+	+	+			

'+' indicates that the gene is expressed;'–' indicates that it is not expressed; blank entry indicates that the presence/absence is unknown.

receptors, and neurotransmitter systems. These lines will be important to understand the control mechanisms utilized by mature neurons in terms of basic physiological functions and stimulus control, and can be used for direct comparisons with neurons of embryonic origin. We continue to develop new models for neuroendocrine research and our repertoire now includes comparable models from the embryonic mouse (mHypoE, previously called N-xx), male and female adult mouse (mHypoA), embryonic rat (rHypoE), and adult mouse pituitary (mPitA). Eventually, we hope to be able to provide a representative cell model for virtually all hypothalamic research involving specific neuropeptide-expressing neurons from our mixed cell populations.

3. Energy homeostasis

The hypothalamus was first considered important for the regulation of feeding after lesions in the ventromedial hypothalamus and lateral hypothalamus led to hyperphagia and obesity or aphagia and starvation, respectively [135]. Based on this work, the hypothalamus is known to be the primary center for food intake

Table 2
Key studies performed using hypothalamic cell lines.

	Study	Key findings	Cell line(s) used
Energy homeostasis	Lee et al. [90]	Glucose decreases AgRP by increasing ATP and inhibiting AMPK	GT1-7
	Cheng et al. [34]	Glucose regulates AgRP through NADH production	mHypoE-38
	Li et al. [94]	GLUT2 overexpression decreases AgRP through increased ATP and inhibition of AMPK	GT1-7
	Mayer et al.	Insulin represses NPY and AgRP through MAPK MEK/ERK pathway	mHypoE-46
	Frago et al. [58]	GHRH-6 regulates IGF-1 and NPY through an PI3K-Akt independent pathway	RCA-6
	Anderson et al. [8]	Increased intracellular calcium activates CAMKK2, activating AMPK and increasing NPY	mHypoE-38
	Fick et al. [56]	Insulin regulates ghrelin through the PI3K-Akt and MAPK MEK/Erk pathways	mHypoE-38
	Cui et al. [40]	Leptin regulates neurotensin through JAK-STAT3 pathway	mHypoE-36, -39
	Miroshamsi et al. [110]	Leptin and insulin activate K_{ATP} channels through PI3K	GT1-7
	Ning et al. [115]	Leptin inhibits PTEN, increasing PIP3 and decreasing F-actin	GT1-7, mHypoE-29/4
	Kaszubska et al. [84]	PTP1B negatively regulates leptin signaling	GT1-7
	Mayer et al.	Prolonged insulin exposure induces neuronal insulin resistance by degrading IR and IRS1, and serine phosphorylating IRS1	mHypoE-46
	Fox et al. [57]	Leptin activates PC1/3 transcription via Nhlh2 and STAT3	mHypoE-29/2
Reproduction	Roy et al. [128]	Estrogen decreases GnRH gene expression through ERα	GT1-7
	Pak et al. [118]	Estrogen provides negative feedback on GnRH promoter activity	GT1-7
	Shakil et al. [139]	Androgen decreases GnRH expression via nuclear ARs and increases GnRH secretion via membrane ARs	GT1-7
	Krsmanovic et al. [89]	Autocrine regulation of GnRH promotes switch from basal to surge -like GnRH secretion	GT1-7
	Quaynor et al. [121]	Kisspeptin increases GnRH secretion in an autocrine fashion	GT1-7
	Bowe et al. [21]	NPY stimulates GnRH secretion	GT1-7
	Roy et al. [129]	Melatonin decreases GnRH secretion and gene expression	GT1-7
	Igaz et al. [78]	Insulin increases GnRH expression	GT1-7
	Yu et al. [167]	cAMP and PKC induce GnRH secretion	GT1-7
	Titolo et al. [150]	Estrogen differentially regulates NPY depending on ratio of ERα and ERβ	mHypoE-38, -42
	Jacobi et al. [80]	Estrogen increases kisspeptin and GRP54	GT1-7
	Luque et al. [97]	NPY stimulates kisspeptin expression	mHypoE-6
	Belsham et al. [15]	NMDA and NO repress GnRH expression	GT1-7
Circadian rhythms	Balsalobre et al. [11]	Serum shock synchronizes cultured rat fibroblasts	Rat-1 fibroblasts
	Balsalobre et al. [12]	Glucocorticoids synchronize cultured rat fibroblasts	Rat-1 fibroblasts
	Balsalobre et al. [13]	cAMP, Ca^{2+}, and protein kinase C (PKC) affect rhythmicity and synchronization	Rat-1 fibroblasts
	Izumo et al. [79]	Temperature compensation and real time imaging of circadian rhythms	Rat-1 fibroblasts
	Welsh et al. [158]	Peripheral cells in culture cycle over 24 h, but lose synchronicity	Rat-1 fibroblasts
	Akashi et al. [2]	MAP kinase cascade is involved in resetting of the clock	NIH-3T3 fibroblasts
	Hirayama et al. [73]	Mutant BMAL1 protein could not restore rhythmicity when not acetylated by CLOCK	BMAL1 mutant MEFs
	Earnest et al. [49]	Establishment of adenoviral E1A immortalized SCN2.2 cell lines	SCN2.2
	Earnest et al. [50]	SCN2.2 neurons restore behavioral rhythms when transplanted into SCN-lesioned rats	SCN2.2
	Allen et al. [4]	SCN2.2 neurons synchronize rat-1 fibroblasts in culture	SCN2.2
	Allen et al. [6]	SCN and peripheral clocks respond to different entraining stimuli	SCN2.2, rat-1 fibroblasts
	Chappell et al. [33]	Altered circadian function affects GnRH secretion	GT1-7
	Roy et al. [129]	GT1-7 neurons express functional melatonin receptors and melatonin alters GnRH gene expression	GT1-7
	Gillespie et al. [65]	GT1-7 neurons express functional clock genes	GT1-7

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and body weight regulation. Within the hypothalamus there are specific areas that are involved with energy homeostasis, namely the arcuate nucleus (ARC) and the paraventricular nucleus (PVN) [82]. These regions of the hypothalamus are comprised of orexigenic and anorexigenic signals. There are numerous orexigenic and anorexigenic neuropeptides that are involved in the regulation of feeding. Some of the peptides expressed by these neuropeptidergic neurons include: NPY, AgRP, MCH, galanin, Orexin, alpha-MSH, CRH, CART, and NT [92,138]. Energy homeostasis is maintained through regulation of these neuropeptidergic neurons by peripheral signals. These signals include hormones such as leptin and insulin, and nutrients such as glucose and free fatty acids [162]; in concert they act to either repress or stimulate feeding neurons, altering secretion and gene expression. In the following subsections, we will look at how hypothalamic cell lines have been used to study the regulation of neuropeptide gene expression, peripheral hormone signaling and the regulation of intracellular signaling molecules.

3.1. Neuropeptide regulation

One of the key steps in altering feeding responses is the change in neuropeptide expression. Nutrients, like glucose, and peripheral hormones, like leptin and insulin, act in the hypothalamus to alter the expression of feeding-related neuropeptides. In vivo studies using knockout animals have explored the importance of individual neuropeptides and the role they play in feeding regulation. As well, in vivo studies have looked at the overall response of the hypothalamus to specific hormones and nutrient signals, but it is in the in vitro model where researchers have begun to define the molecular mechanisms involved in the regulation of neuropeptide gene expression.

Neuropeptide Y and agouti-related peptide are two key potent orexigenic peptides, as indicated by the adult NPY/AgRP neuron knockout mice [68,98], which exhibit extreme aphagia and eventual starvation. In vitro studies have recently been investigating the intracellular mechanisms involved in the regulation of these neuropeptides. Glucose was shown to regulate NPY and AgRP expression in vivo, although the intracellular mechanisms were not known [32,156]. Lee et al. used the GT1-7 cells to study how glucose regulates NPY/AgRP [90]. GT1-7 treatment with glucose decreased AgRP mRNA expression and increased ATP levels. In response to the rising ATP levels, they found that phospho-AMPK, an intracellular fuel sensor, decreased. An inhibitor of glucose metabolism, 2-deoxy-D-glucose (2DG), decreased ATP while increasing AgRP mRNA and phospho-AMPK. To determine if ATP was directly involved with AgRP regulation, they depleted cellular ATP using sodium azide, which affects mitochondrial oxidation. The depletion of ATP led to an increase in AgRP. Next they studied if AMPK was directly involved and found that an AMP analog, AICAR, increased phospho-AMPK, as AMPK is activated by an increase in the ratio of AMP to ATP, and in turn, AgRP expression. Lastly they used a dominant-negative AMPK construct and found that it prevented the 2-DG induced increase in AgRP. This study indicates that glucose levels affect AgRP expression by changes in ATP levels, which lead to activation of inhibition of AMPK. Cheng et al. [34] followed up on this study using the N-38 cells (now known as mHypoE-38) which endogenously express NPY and AgRP. They confirmed that glucose inhibits AgRP expression and decreases cellular ATP levels. Although they found that ATP and AMPK were not involved in the regulation of AgRP. Instead they found that a GAPDH inhibitor, iodoacetate, which decreases the levels of NADH, increased AgRP mRNA and decreased ATP. As well, inhibition of AMPK with RNA interference, induced AgRP mRNA at 10 mM glucose and AICAR inhibited AgRP. They proposed that glucose regulates AgRP through the production of NADH by GAPDH and regulation of the C-terminal binding protein. These two studies indicate that glucose may regulate AgRP dissimilarly in different neuronal populations, supporting the need for studies at the individual neuronal level. One of the key steps in glucose sensing is the uptake of glucose into cells. Li et al. used the GT1-7 cells to study the role of the glucose transporter 2 (GLUT2) in the regulation of AgRP by glucose [94]. They found that GLUT2 overexpression increased ATP levels leading to inhibition of AMPK and a decrease in AgRP mRNA expression. As well, overexpression of GLUT2 blocked the 2DG-mediated increase in AgRP mRNA levels. This study further illustrates that neuropeptides, such as AgRP, can be regulated by glucose. Taken together with the previous two studies, these studies indicate that researchers are just beginning to understand the mechanisms utilized by glucose to regulate feeding-related neurons and that neuronal cell lines provide an important model in which this may be investigated.

Along with glucose, peripheral hormones are known to regulate NPY and AgRP gene expression. One of these key hormones is insulin. In order to understand how the mechanisms through which insulin regulates NPY and AgRP, our lab has used a cell line that expresses these peptides, mHypoE-46 (C.M. Mayer and D.D. Belsham, submitted for publication). Insulin treatment decreased NPY and AgRP mRNA levels and induced phosphorylation of Akt and Erk1/2. Using inhibitor analysis we determined that the MAPK MEK/Erk pathway is involved in insulin regulation of NPY and AgRP mRNA expression. Lin et al. [95] studied the effects of another peripheral hormone enterostatin, an anorexigenic peptide found in the pancreas, gastric mucosa and specific brain regions, on AgRP expression. They found that enterostatin decreased AgRP mRNA levels in GT1-7 cells. This data was presented in conjunction with in vivo data and used to confirm the direct actions of the peptide on neurons. Ghrelin is a peripheral hormone produced by the stomach, thought to counteract the anorexigenic effects of leptin. Using a ghrelin receptor agonist and the RCA-6, NPY expressing cell line, Frago et al. [58] studied the actions of the agonist on NPY and IGF1 expression. They found that the agonist, growth hormone-releasing peptide-6 (GHRH-6) increased insulin-like growth factor 1 (IGF-1) and NPY mRNA expression and induced phosphorylation of Akt. IGF-1 treatment also increased NPY mRNA expression. Interestingly, inhibition of the PI3 K-Akt pathway using LY294002 did not affect GHRH-6 regulation of IGF-1 and NPY. This study indicates that the PI3 K-Akt pathway does not mediate the effects of GHRH-6 on IGF-1 and NPY.

Cell lines have also been used to study the effects of intracellular signaling molecules on NPY regulation. In conjunction with in vivo studies, Anderson et al. [8] used the N-38 (mHypoE-38) cell line to provide more direct evidence linking CAMKK2, a serine/threonine protein kinase, to pathways regulating NPY. Ionomycin treatment, which increases intracellular calcium, activates CAMKK2 and induces phosphorylation of AMPK in the N-38 cells. Inhibition of CAMKK2 blocked the ionomycin-induced increase in phospho-AMPK. As well, ionomycin increased NPY mRNA expression and the CAMKK2 inhibitor attenuated this increase. This study indicated that intracellular calcium increases activate CAMKK2, leading to phosphorylation of AMPK and increased NPY mRNA.

Cell lines have allowed for the analysis of the NPY, AgRP and GnRH promoters. There are excellent reviews on GnRH promoter analysis [19,66,114], and therefore this will not be further covered in this review. However, relatively little has been reported about the regulation of the NPY promoter [7,96,108,109,159], and these studies were only performed in heterologous cell models. It is known from bilateral neural transection experiments and antisense data that the NPY neurons responsible for the reproductive and orexigenic effects of NPY lie within defined regions of the hypothalamus, including the arcuate nucleus [81,133,134], which clearly are not represented by any of the tumor-derived cell lines

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previously used for NPY studies. Using the GT1-7 cell line, our lab transiently transfected NPY 5′ flanking gene luciferase reporter constructs and found a repressor region between −867 and −1078 [103]. Three protein binding regions were determined with DNase I footprint analysis and the region between −943 and −922 were further analyzed using electrophoretic mobility shift assays (EMSA). This revealed that four different transcription factor-DNA complexes formed with GT1-7 nuclear proteins and that two of these proteins were the Oct-1 and Pbx-1 transcription factors. We are now using the cell lines derived in our laboratory expressing endogenous NPY from appropriate hypothalamic nuclei to study the direct regulation of the NPY promoter by hormones and to map these effects to distinct regions of the NPY gene 5′ regulatory region. This type of analysis has already been performed on the AgRP gene in the N-38 cell line, in which a novel SNP was found allowing functional dimorphism [10]. As exemplified above, cell lines provide a useful model in which to examine 5′ regulatory regions and to identify transcription factor binding regions involved in the regulation of a gene of interest. As well, using phenotypically different cell lines one can begin to compare cell type specific transcription factors involved in differential expression of neuropeptides.

Ghrelin, a potent orexigenic hormone, is secreted by the stomach and acts in the hypothalamus to stimulate NPY/AgRP neurons. Ghrelin is also expressed in the brain, possibly having a more local action. Our lab found that one of our hypothalamic cell lines, mHypoE-38, expressed preproghrelin [56]. This cell line was used to determine if peripheral hormones, like insulin, could regulate brain ghrelin. We found that insulin decreased preproghrelin and phosphorylated Akt and Erk1/2. The MAPK-MEK/Erk1/2 pathway inhibitor, PD98059, attenuated the insulin-mediated decrease in preproghrelin, while the PI3 K inhibitor, LY294002, upregulated gene expression. This study indicated that insulin directly regulates brain ghrelin and the MAPK MEK/Erk and PI3 K-Akt pathways are involved in the regulation of preproghrelin.

Along with study of orexigenic peptides, hypothalamic cell lines have also been used to study the regulation of specific anorexigenic factors: in particular, neurotensin (NT) and insulin. Neurotensin is expressed in the CNS and digestive tract and is involved in a number of physiological processes, including feeding. Our group utilized the N-39 and N-36/1 cells (mHypoE-39 and mHypoE-36/1) to analyze the effects of leptin, insulin and alpha-melanocyte stimulating hormone (alpha-MSH) on NT gene expression [40]. Treatment of the two cell lines with leptin, insulin and alpha-MSH increased NT gene expression. Promoter analysis revealed a leptin responsive region located in the NT 5′ flanking region between −250 and −391. This region contained STAT3 responsive elements and chromatin immunoprecipitation (ChIP) showed binding of STAT3 to this region. In order to determine if STAT3 was involved in leptin-mediated NT regulation, the cells were transfected with dominant negative STAT3 constructs. The dominant negative construct attenuated leptin-induced increases in NT mRNA. This study found that leptin, insulin and alpha-MSH directly regulate NT gene expression and that leptin does so through the STAT3 transcription factor.

Insulin is mainly produced in pancreatic beta cells, although there is also some evidence for expression in the rodent brain. Two of our cell lines, mHypoE-39 and mHypoE-46, express the rodent Ins2 gene, which is highly homologous to the single human insulin gene [99]. We found that mouse and rat Ins2 5′ flanking gene reporter constructs were active in these cell lines, while the human construct was not. Treatment of the mHypoE-39 with glucose increased Ins2 mRNA levels, while exendin four (a GLP-1 R agonist) decreased Ins2 mRNA. As well, we found that exendin four increased cAMP levels indicating that the GLP-1 receptor is active in our cell lines. Through this study we show that rodent Ins2 is expressed in neuronal cells and can be potentially regulated by central or peripheral hormones.

3.2. Peripheral hormone signaling

Although determining the regulation of neuropeptides by hormones and nutrient signals is important, an understanding of the mechanisms through which these hormones and nutrients signal in neurons is critical to gaining an insight into the pathogenesis of diseases such as diabetes and obesity. For these mechanistic studies, hypothalamic cell lines will play an invaluable role. Over the last few years, researchers have begun utilizing cell lines to work out the signaling pathways employed by insulin and leptin. Leptin and insulin are known to activate the classic signaling pathways PI3 K-Akt and MAPK MEK/Erk1/2 in neurons. Mirshamsi et al. [110] further studied the linkage between the PI3 K-Akt pathway and other intracellular signaling mediators. Using the GT1-7 cell line they confirmed that insulin and leptin phosphorylate Akt, STAT3, Erk1/2 and GSK3. The PI3 K inhibitor LY294002 inhibited leptin- and insulin-mediated Akt and Erk1/2 phosphorylation. Interestingly, they found that leptin and insulin increased activation of the K_{ATP} channel, as well as increasing the phosphatidylinositol (3,4,5)-trisphosphate (PIP_3), the downstream product of PI3 K. Next they investigated the cellular mediators involved in the activation of the K_{ATP} channel. They found that leptin induces reorganization of actin filaments, causing an increase in G-actin and a decrease in F-actin. This effect was dependent on PI3 K, as the PI3 K inhibitors, LY294002 or wortmannin, attenuated leptin-mediated actin reorganization. From this study, they proposed that leptin and insulin activate the K_{ATP} channel through PI3 K and that leptin may utilize actin reorganization to mediate this action. This study was followed up by the same lab, in which they analyzed the role of the endogenous PI3 K pathway antagonist, PTEN, in leptin and insulin signal transduction [115]. They used the GT1-7 cell line along with the leptin sensitive N-29/4 (mHypoE-29/4) cell line for these studies. They found that decreased levels of PTEN, through siRNA knock down, led to increased PIP3 levels. As well, increasing PTEN levels with protein expression constructs did not alter F-actin levels alone, but prevented a leptin-mediated decrease in F-actin. The authors hypothesized that a decrease in PTEN leads to an increase in PIP3 via PI3 K, which causes a decrease in F-actin. Leptin did not alter PI3 K activity, whereas insulin increased it and PIP3 levels, but did not affect F-actin. From this they stated that changes in F-actin were not dependent upon activation of PI3 K. This study then went on to show that leptin phosphorylates PTEN leading to its inactivation, which causes an increase in PIP3 levels and a decrease in F-actin.

Our lab explored the signaling pathways activated by leptin in a NT expressing cell line, N-39 (mHypoE-39) [41]. We found that leptin increased phospho-STAT3, -Erk1/2, -p38 and -ATF1. To determine if these pathways are utilized by leptin to affect downstream NT gene expression, the N-39 cells were treated with leptin and specific pathway inhibitors. The p38 inhibitors, SB203580, SB202190 and SB239063 all attenuated the effects of leptin on NT. Applying EMSA and ChIP we determined that leptin induces binding of ATF-1 and c-fos to the NT promoter, both downstream signaling proteins of the MAPK p38 pathway.

The negative regulator of insulin signaling, protein tyrosine phosphatase 1B (PTP1B), is implicated in the regulation of feeding. Kaszubska et al. [84] used the GT1-7 cell line to study if it affects leptin signaling in neurons. Leptin increased phospho-STAT3 and activated a STAT3 responsive luciferase reporter construct. Over-expression of PTP1B attenuated these leptin actions. Microarray analysis revealed that the over-expression of PTP1B decreased the number of genes upregulated by leptin. This study indicated that PTP1B does negatively regulate leptin signaling in neurons.

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Glucose signaling is active in hypothalamic neurons, although the cell types that are glucose-responsive are not yet fully defined. Exploiting an anorexigenic-POMC expressing cell line, N-43/5 (mHypoE-43/5), our lab determined if POMC neurons are glucose-responsive [26]. Glucose depolarized the cells and caused an increase in cellular calcium levels, indicative of functional glucose-sensing machinery. As well, glucose altered intracellular signaling proteins, noted by decreased phospho-AMPK and -ACC levels. We also analyzed if changes in glucose levels affected the response of the N-43/5 cells to leptin and insulin. In low glucose conditions, leptin and insulin decreased phospho-AMPK, an effect that was absent in high glucose. This study indicates that the POMC expressing cell line, N-43/5, is responsive to glucose and that changes in glucose levels alter the cellular response to other hormones.

Along with determining hormonal and nutrient signal transduction pathways, we found that hypothalamic cell lines could be utilized to probe the intracellular changes in pathological states. Using the mHypoE-46 cell line we studied the effects of hyperinsulinemia on neurons and analyzed the mechanisms involved in the development of cellular insulin resistance (C.M. Mayer and D.D. Belsham, submitted for publication). Prolonged exposure to insulin caused cellular insulin resistance, noted by attenuation of PI3 K-Atk pathway activation by insulin. Insulin induced phospho-Akt and -S6 K, as well as IRS1 serine phosphorylation. As well, long term treatment with insulin decreased IRS1 and insulin receptor (IR) protein levels. In order to determine the pathways involved with the decrease in insulin-mediated Akt phosphorylation, we used inhibitors for lysosomal (3-methyladenine) and proteasomal (epoximicin) degradation pathways, as well as an mTor-S6 K pathway inhibitor (rapamycin). We found that prolonged exposure to insulin decreases IR levels through a lysosomal pathway and IRS1 levels through a proteasomal degradation pathway. As well, insulin increased IRS1 serine phosphorylation through the mTor-S6 K pathway. Phosphorylation of IRS1 on serine residues is known to decrease insulin signal transduction. Interestingly, treatment with any one of the inhibitors in the presence of high insulin concentrations restored insulin signaling. This study indicates that prolonged insulin exposure causes cellular insulin resistance through lysosomal degradation of IRs, proteasomal degradation of IRS1 and serine phosphorylation of IRS1.

3.3. Regulation of intracellular signaling molecules

This last subsection of feeding looks at how hypothalamic cell lines have been used to study the regulation of intracellular signaling molecules. Four current studies on this topic have been reported. In the first study Fox et al. [57] used the POMC expressing N-29/2 (mHypoE-29/2) cell line to analyze the regulation of proconvertase 1/3 (PC1/3) expression. PC1/3 is required in POMC neurons in order to process the prohormone, POMC, into active peptides such as alpha-MSH and beta-endorphin. They found that leptin stimulated PC1/3 promoter transcription, using a 5′ flanking PC1/3 gene reporter construct, and required the presence of two transcription factors, Nhlh2 and STAT3. A ChIP assay revealed that Nhlh2 binds to the PC1/3 5′ flanking region. Using site directed mutagenesis, they found that STAT3 sites within the PC1/3 5′ flanking area were required for leptin activation of PC1/3 transcription. Lastly, a modified ChIP assay was used to determine that STAT3 and Nhlh2 heterodimerize and interact with the PC1/3 promoter. Thus, leptin activates PC1/3 transcription via the transcription factors Nhlh2 and STAT3.

Exploiting the N-38 (mHypoE-38) cell line in combination with a novel protein–protein interaction determining method, MAPPIT, Wauman et al. [157] analyzed the interaction between the leptin receptor (ObR) and IRS4. IRS4 is a member of the IRS family of proteins, which are required by insulin for signal transduction and may also be involved with leptin signaling, and is highly expressed in the hypothalamus. IRS4 was found to interact with the ObR in N-38 cells and phosphorylation of the ObR at tyrosine 1077 is required for this interaction. As well, the authors found that IRS4 interacted with other intracellular signaling molecules: the p85 subunit of PI3 K, phospholipase C and SOCS2, 6 and 7. This study indicates that IRS4 may serve a function in leptin signaling.

In the third study, Brown et al. [23] analyzed the effects of adipokines on SOCS3 expression. Adipokines are produced in adipose tissue, like leptin, but are also secreted in certain regions of the brain. The authors examined the central role of two adipokines, resistin (rstn) and fasting-induced adipose factor (FIAF), using a hypothalamic cell line that expresses the two factors, N-1 (mHypoE-1). They found that resistin treatment decreased both FIAF and SOCS3 mRNA levels and that over-expression of rstn in the N-1 cells had a similar effect. Conversely, decreased in FIAF levels via siRNA did not affect rstn or SOCS3 levels. This study indicates that resistin has a novel paracrine/autocrine effect upon FIAF and SOCS3 in neurons.

The last study used the GT1-7 cell model to evaluate the effect of leptin and alpha-MSH upon melanocortin four receptor (MC4R) expression [67]. The MC4R is part of the anorexigenic melanocortin pathway and is activated by alpha-MSH. Gout et al. found that leptin and alpha-MSH act directly on neurons to increase MC4R mRNA expression. This study took advantage of the in vitro model to reinforce the direct actions of leptin and alpha-MSH on MC4R expression, confirming experiments performed in in vivo studies.

The studies above exemplify how appropriate cell models can be used to dissect the intricate molecular events utilized by individual neurons to control basic aspects of physiology by sensing central and peripheral signals. The generation of the cell lines with a clonal, homogeneous population of neurons allows the use of technologies not yet possible in the whole brain. These discoveries will allow for more detailed and directed studies in the whole animal and with perseverance, a confirmation of the cellular events determined in vitro in the in vivo situation.

4. Reproduction

The reproductive system is regulated by a complex interaction of neuropeptides and peripheral hormones acting upon the hypothalamic–pituitary–gonadal (HPG) axis. Situated at the peak of the HPG axis are the gonadotropin-releasing hormone (GnRH) neurons. GnRH is secreted by these neurons and acts upon pituitary gonadotropes inducing the secretion of the gonadotropic hormones LH and FSH into portal circulation where they ultimately act on the ovaries to stimulate ovulation. Estrogen produced in the ovaries regulates reproduction by controlling GnRH synthesis and secretion by acting through both positive and negative feedback mechanisms on the HPG axis.

GnRH neurons represent a small population estimated at 400–1000 neurons, which are found scattered throughout the preoptic and anterior hypothalamus [137]. GnRH neurons are regulated through autocrine mechanisms and by many extracellular signals including neurotransmitters, steroid hormones and peptide hormones. In addition, they receive paracrine inputs from many different neuronal phenotypes including steroid-sensitive neurons such as the neuropeptide Y (NPY) and kisspeptin neurons.

GnRH controls reproduction through secretory actions on pituitary gonadotrophes. GnRH is secreted in rhythmic pulses which are required for reproductive maturation and homeostasis [59], and surges, which are responsible for inducing ovulation [93]. While the function of GnRH in reproduction is well documented, the complex regulation of GnRH expression and secretion are not fully understood, mainly due to the difficulty of studying these

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mechanisms using in vivo models. Over the past 18 years and after approximately 300 publications researchers have used the immortalized GnRH cell lines, GT1, GN11 and Gnv3 to advance our understanding of GnRH regulation to a degree that would not have been attainable solely using classical in vivo approaches. The GT1 cell model has proven to be an excellent model to study the regulation of GnRH, as GT1 neurons mimic GnRH secretion in vivo. GT1 neurons not only basally secrete GnRH in a rhythmic pulsitile manner [160], but also acutely secrete increased levels of GnRH in response to depolarization [104]. The immature GnRH cell line, GN11, was found to express [122] and modestly secrete GnRH [168]. The recently developed conditionally immortalized GnRH cell line, Gnv3, was found to secrete GnRH in a pulsitile manner and secretion is acutely increased following NMDA stimulation [136]. In the following subsections we will look at the role these cell lines have played in studying GnRH neuronal regulation.

4.1. Hormonal regulation of GnRH

Hormonal regulation of GnRH synthesis and secretion through negative and positive feedback mechanisms is crucial for the maintenance of normal reproductive function. Estrogen regulates normal tonic GnRH secretion through negative feedback mechanisms [59], while inducing the preovulatory LH surge by positive feedback mechanisms [93]. Over the past 30 years scientists have debated whether estrogen regulates GnRH by acting directly on GnRH neurons itself or indirectly through estrogen-responsive interneurons. The latter view was supported by several immunocytochemical studies that showed GnRH neurons of several species in vivo lack estrogen receptors (ERs) [70–72,91,142,148]. However, because the brain contains few GnRH neurons that are scattered throughout several nuclei, classical immunocytochemical approaches are likely not sensitive enough to detect expression of ERs. As a way of overcoming this inherent in vivo obstacle, several groups utilized the clonal GT1 neuronal cell line to investigate whether functional ERs are expressed in GnRH neurons. Indeed, several groups demonstrated that estrogen binds to receptors in GT1-1 cells [119] and detected functional ERα [25,128,140] and ERβ [128] receptors in GT1-7 cells. In light of these findings, Skynner et al. used the advanced technique of single-cell multiplex RT-PCR to demonstrate for the first time that GnRH neurons in vivo express ERβ mRNA [144]. More recently Hu et al. showed that both fetal and adult GnRH neurons in vivo express both ERα and ERβ mRNA [75].

Confirmation that ERs are expressed in GnRH neurons prompted groups to begin to investigate the direct actions of estrogen on GnRH and the mechanisms mediating these effects by using the GT1 cell lines. 17β-estradiol was shown to repress GnRH mRNA expression [21,128], an effect that was mimicked by HPTE, an ERα agonist/ERβ antagonist [128]. This effect was blocked by the non-selective ER antagonist ICI 182,780 [21,128], but not by the selective ERβ antagonist, R,R-THC [21], suggesting that ERα mediates 17β-estradiol-induced repression of GnRH mRNA expression. The phytoestrogen, coumestrol, was found to decrease GnRH mRNA expression in GT1-7 cells, an effect that is likely mediated by ERβ, as R,R-THC blocked the effect of coumestrol [21]. Investigation of ERβs role in regulating the GnRH promoter found that GT1-7 cells transfected with splice variants of ERβ increased promoter activity in a ligand-independent manner. When these transfected cells were treated 17β-estradiol the increased promoter activity was attenuated, suggesting that ERβ acts as a transcription factor for the GnRH promoter and that estrogen provides negative feedback on GnRH promoter activity [118]. 17β-estradiol was also found to rapidly inhibit cyclic adenosine monophosphate (cAMP) production and GnRH secretion, which suggested the involvement of a G_i-coupled membrane ER [112].

The effects of 17β-estradiol on the electrophysiological properties of GnRH neurons have been studied using GT1-7 cells. 17β-estradiol was found to augment Ca^{2+} activated potassium channels [116] and modulate potassium currents [54] through ERs. More specifically, the effect on Ca^{2+} activated potassium channels was mediated by ERβ, as the ERβ agonist, 2,2-bis(4-hydroxyphenyl)-propionitrile (DPN) mimicked the effect while a selective ERα agonist 1,3,5-tris(4-hydroxyphenyl)-4-propyl-1H-pyrazole (PPT) did not and the effect was blocked by ERβ, but not ERα, knockdown by RNA interference [116].

Aside from estrogen the effects of other steroid hormones on GnRH expression have also been investigated using cell lines. The sex steroid precursor dehydroepiandrosterone (DHEA) was also found to inhibit GnRH mRNA expression, an effect that could not be attributed to the metabolism into 17β-estradiol since the enzyme aromatase is not present in GT1 neurons [39]. When treated with the androgen 5α-dihydrotestosterone (DHT), GnRH mRNA expression was repressed in GT1-7 cells, an effect that was mediated by androgen receptors (AR) [16]. Further experiments revealed that DHT and testosterone decreases GnRH mRNA expression while the membrane-impermeable BSA-conjugated testosterone (T-3-BSA) did not, suggesting that DHT and testosterone act through nuclear ARs to regulate gene expression [139]. Conversely, GnRH secretion and intracellular calcium were rapidly increased by DHT, testosterone and T-3-BSA, suggesting that membrane androgen receptors mediate these effects. DHT also blocked the forskolin-induced increase in cAMP production via membrane androgen receptors coupled to the inhibitory G-protein, G_i [139].

4.2. Autocrine, peptide and second messenger regulation of GnRH

GnRH neurons express functional GnRH receptors [88] suggesting the possibility of autocrine regulation. GnRH was found to induce the expression of the immediate early gene, c-FOS, through a protein kinase C (PKC) mechanism in GT1-7 cells [29]. Using the GnRH agonist, buserelin, basal GnRH secretion, promoter activity, and mRNA levels were decreased in GT1-1 cells [35]. Furthermore, GnRH was found to increase mobilization of intracellular calcium and to decrease GnRH secretion frequency, but, increase pulse amplitude [88]. These findings suggest that autocrine regulation of GnRH promotes a switch from basal to surge-like release of GnRH.

Paracrine regulation of GnRH by afferent neurons including, neuropeptide Y (NPY) and kisspeptin neurons has been suggested based on neuroanatomical and pharmacological studies. Neuroanatomical studies provide evidence that NPY-producing [154] and kisspeptin-producing [37,120] neurons project to or have terminals in close proximity to the preoptic GnRH neurons, respectively. Pharmacological studies demonstrate that NPY [14,38,86,132,165] and kisspeptin [101,105] increase GnRH secretion in vivo. Although these studies suggest that NPY and kisspeptin are capable of acting directly on GnRH neurons, indirect actions through other afferent neurons cannot be ruled out. Utilizing GT1-7 neurons, Besecke et al. demonstrated that NPY increases secretion of GnRH through direct actions on GnRH neurons, likely through the Y1 NPY receptor and intracellular calcium mobilization [20]. The GT1 cell line expresses the kisspeptin receptor, G-coupled protein 54 (GPR54) [80,121]. Recently kisspeptin-10 has been shown to rapidly increase GnRH secretion [80,121] and more specifically increase both the frequency and amplitude of GnRH secretory pulses from GT1-7 neurons [121]. Furthermore, kisspeptin-10 was shown to increase GnRH mRNA expression [80].

Many other peptides have been demonstrated to regulate GnRH secretion, mRNA expression and promoter activity in GT1 neurons including melatonin, retinoic acid, insulin and prolactin. Melatonin

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was found to decrease GnRH secretion [131] and decrease GnRH mRNA expression in a 24-cyclical manner [130] and is described below in the circadian section of this review. Retinoic acid was found to increase GnRH secretion, mRNA expression and promoter activity [36]. Insulin has been shown to induce increases in GnRH mRNA expression, c-FOS mRNA expression and activate phosphoinositide 3-kinase (PI3 K) and ERK1/2 in GnV3 cells [78]. Prolactin was found to decrease GnRH secretion in GT1 cells [106].

Specific second messenger pathways have been found to be involved in the regulation of GnRH secretion and mRNA expression. Activation of calcium, cAMP [167], PKC (protein kinase C) [161,167], and PKA (protein kinase A) [24,161] pathways were found to induce GnRH secretion from GT1 neurons. Interestingly, the activation of calcium, cAMP, and PKC decreased GnRH mRNA expression [167].

The regulation of GnRH gene expression and secretion, as illustrated above, is highly complex and occurs through several avenues. Autocrine signals promote the switch from basal to surge-like secretion of GnRH inducing ovulation, while paracrine signals such as melatonin and insulin control reproductive function according to season/photoperiod and nutritional status. Reproductive function during pregnancy, postpartum, and lactation is regulated in part by prolactin effects on GnRH. Although regulation of normal reproductive function is complex, studies from clonal cell lines have begun to characterize the key signaling mechanisms.

4.3. Regulation of the GnRH afferents NPY and kisspeptin

Neuropeptides are integral to the regulation of reproduction in part by mediating indirect effects of estrogen on GnRH neurons through paracrine signaling. In vivo studies demonstrate that estrogen affects NPY expression [1,141]. However, because it was uncertain whether these effects were direct or indirect, by using immortalized neuronal cell lines developed in our laboratory we were able to investigate the direct actions of estrogen on the NPY neuron. Using N-38 (mHypoE-38) and N-42 (mHypoE-42), neuronal cells lines that express NPY, ERα, and ERβ we investigated the effects of 17β-estradiol on the regulation of ERs and NPY mRNA expression. In N-38's, 17β-estradiol regulated ERα mRNA and protein expression in a biphasic manner, beginning with an initial increase, but was later repressed by 17β-estradiol, while ERβ slowly increased expression over time (Fig. 3A) [150]. NPY mRNA expression was initially repressed and overtime was greatly increased by 17β-estradiol (Fig. 3B). These results raise the intriguing possibility that NPY mRNA expression in N-38 is dependent on the ERα/ERβ ratio and that the increase in ERβ levels is an important factor in the increase in NPY mRNA expression. Interestingly, the effects of 17β-estradiol on ERα and ERβ expression in N-42 neurons were strikingly different, as the expression of both receptors was steadily repressed. Additionally, the estrogen-mediated repression of NPY in N-38 was mapped to the 5′ regulator region. Finally, by using small-interfering RNA knockdown of each ER subtype the repression of NPY mRNA gene expression by 17β-estradiol in NPY expressing N-38 neurons was found to be mediated by both ERs, while the induction was solely through ERβ. Further investigations into the signaling mechanisms activated by 17β-estradiol in N-38, NPY neurons, demonstrated that Akt and ERK1/2 are rapidly activated by 17β-estradiol (Fig. 3C and D) and that the PI3 K and MAPK pathways are both involved in the activation of these signaling molecules. Furthermore, the selective ERα agonist, PPT, activated Akt, ERK1/2 and CREB (Fig. 3C and D). Importantly, activation of the PI3 K and MAPK pathways were found to mediate the 17β-estradiol-induced repression and induction of NPY mRNA levels (Fig. 3E) [151]. This indicates that early membrane signaling events may potentiate or amplify the long-term transcriptional response.

The differing effects observed in the above study between the mHypoE-42 and mHypoE-38 clonal cell populations can be attributed to the differing phenotypes of the two cell lines, indicating that they are different neuronal subtypes. In vivo, it is unknown how many subtypes of NPY neurons exist, nor for any other neuropeptide neuronal phenotype. Each clonal cell line developed may represent a different subtype of a specific neuropeptide and may thus allow for studies investigating how each neuronal subtype differs in both the mechanisms through which it is regulated and the mechanisms through which it can signal to and regulate other neurons. GT1-7 neurons, although classically known for their GnRH-secreting characteristic, also basally secrete kisspeptin [121]. Not only does kisspeptin increase GnRH secretion in GT1-7 cells, but, GnRH decreases kisspeptin secretion, suggesting that autocrine regulation of these systems in the GT1-7 cell involves negative feedback mechanisms [121]. The effect of 17β-estradiol on Kiss and GPR54 mRNA levels in GT1-7 cells was investigated and Jocabi et al. found that long term exposure (24 h) to 17β-estradiol increased mRNA expression levels of both genes [80]. Because NPY has been implicated as a very important metabolic regulator of reproduction Luque et al. investigated the potential role of NPY in kisspeptin regulation. Initial studies using NPY knockout mice demonstrated that in the absence of NPY, kisspeptin mRNA expression was attenuated, suggesting a regulatory role, either directly or indirectly, for NPY on kisspeptin [97]. By using the hypothalamic cell line, N-6 (mHypoE-6), the potential direct effects of NPY on kisspeptin expression were investigated. N-6 cells were confirmed to express NPY receptors, Y1-Y6, GPR54, and kisspeptin and were found to express higher levels of kisspeptin mRNA when treated with NPY [97].

The use of immortalized, clonal cell lines has been instrumental towards our current understanding of GnRH neuronal function. These studies have allowed a detailed study of the molecular events in the whole animal. An excellent example of this is the finding that the GnRH neuron was controlled by the glutamate, nitric oxide, cGMP signaling pathway in the GT1-7 neurons [15,17]. A number of years later, it was confirmed that the specific knockout of neuronal nitric oxide synthase indeed had major effects on the reproductive axis and this could be traced to a direct action at the level of GnRH synthesis [69]. Similarly, using the information gained from this important model system [15,100,146], 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 [22,87,113,117].

5. Circadian rhythms

The rhythmic nature of our environment dictates that there will be a changing availability of resources at any given time. In this dynamic setting it is essential for organisms to maintain cellular and behavioral homeostatic efficiency. To maintain this optimal efficiency, an innate cellular rhythm generating machinery has evolved that enables organisms to adapt to these cyclic environmental changes by preparing cells for periodic stimuli during the 24-h day. The rhythm generator exists in even the simplest cyanobacteria and has become more complex as the changing needs of the organisms have evolved. As evolution progressed and multicellular eukaryotic organisms could no longer rely on direct cellular photic stimulation, organized neural and humoral machinery developed. In higher animals, such as mammals, these signals are coordinated by a core circadian oscillator or master clock. This master clock is situated within the mammalian suprachiasmatic nucleus (SCN) of the hypothalamus. Signals from the SCN lead to rhythm entrainment within other cells allowing for a healthy response to external stimuli through smooth operation of the circadian system.

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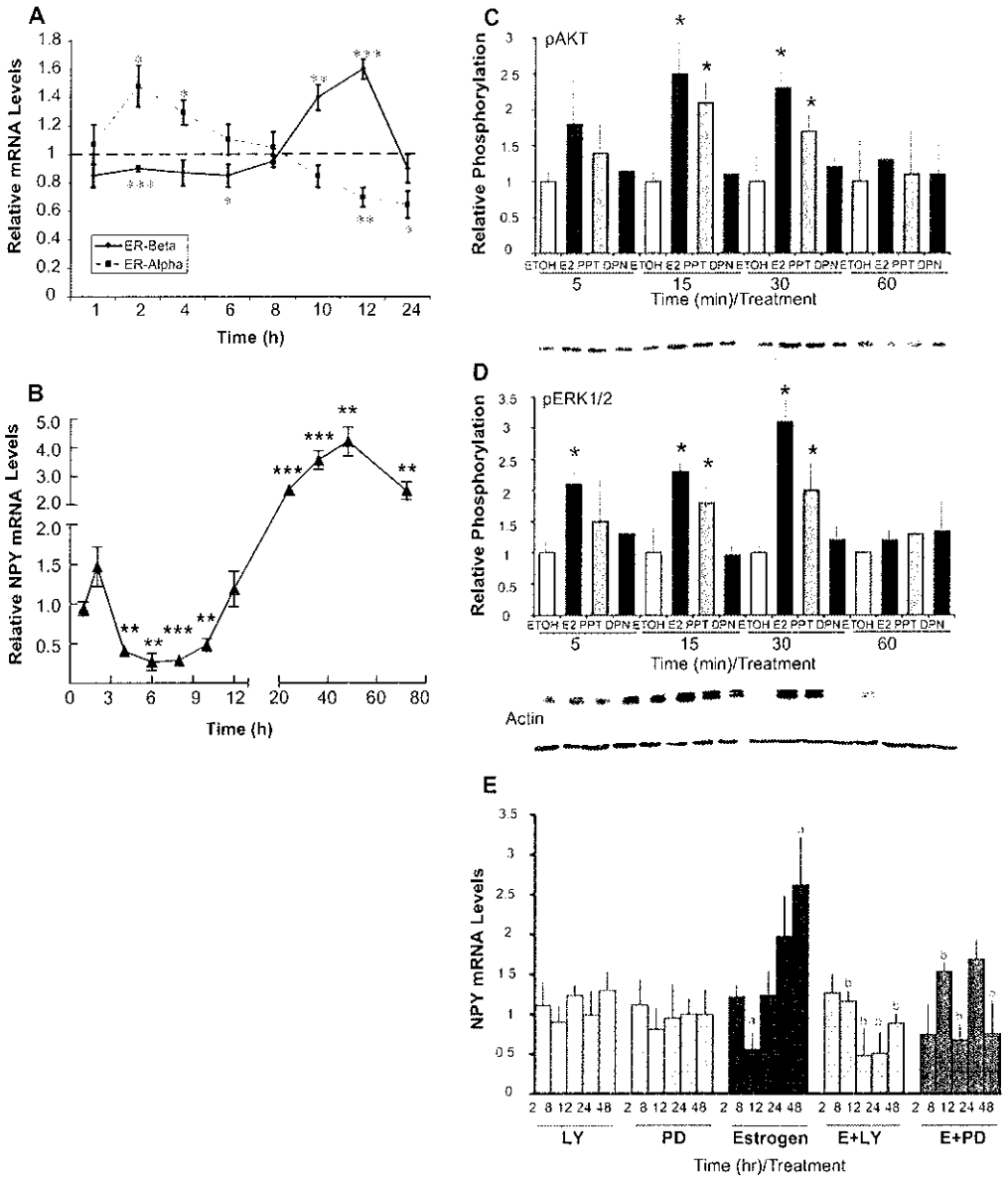

Fig. 3. Effect of 17β-estradiol on ERα, ERβ, and NPY mRNA expression in mHypoE38 (N-38) neurons and the role of the PI3K and MAPK signaling pathways. (A) ERα and ERβ mRNA expression pattern in mHypoE38 (N-38) neurons treated with 10 nM 17β-estradiol over a 24-h time course as determined by real time RT-PCR (originally published in [150]). (B) Biphasic NPY mRNA expression pattern in mHypoE38 neurons treated with 10 nM 17β-estradiol over a 72-h time course as determined by real time RT-PCR (originally published in [150]). (C) Activation of Akt kinase. (D) Activation of ERK1/2 kinase following treatment of vehicle (ETOH), 17β-estradiol (E2), the selective ERα agonist; PPT, and the selective ERβ agonist; DPN over a 60-min time course as determined by Western blot analysis (originally published in [151]). (E) Inhibition of the 17β-estradiol-induced changes in NPY mRNA expression by the selective PI3-kinase inhibitor; LY294002 (LY) and the MAP kinase inhibitor; PD98059 (PD) as determined by real time RT-PCR (originally published in [151]).

The circadian system is distinguished by positive and negative transcription/translation cycles within the cell and posttranslational signal transduction cascades (Fig. 4A). Approximately, 10% of the transcriptome is controlled by the circadian rhythm generator [107,147]. While this number does not initially seem significant, the transcripts controlled in a rhythmic fashion are the rate-limiting enzymes for virtually every process within the cell. Additionally, in an elegant study on the hepatic proteome, Reddy et al. showed that over 20% of proteins within the cell are cyclic [125]. Interestingly, nearly half of these proteins lacked an oscillating transcript suggesting that post-translational processing occurs in a circadian and rhythmic fashion [124]. That we have elucidated this much about the nature of circadian biology is due largely to studies at the cellular level.

5.1. Early cell-based circadian research

During the 1970s and through the 1980s avian pinealocytes were extracted and cultured to study the mechanics of the circadian system within vertebrates. Dissociated pinealocyte cultures demonstrated that individual cells could exhibit oscillatory properties, express photoreceptors and synthesize melatonin [44–46]. However, the pinealocyte model has yet to be efficiently transformed and so interventions at the molecular level in the pinealocyte have yet to be performed. Much of the current knowledge of the biochemical circadian system owes its ancestry to initial studies in *Neurospora crassa* and *Drosophila melanogaster*. The molecular clock was first characterized within bread mould and the fruit fly in the early 1980s. Classical and molecular genetic research

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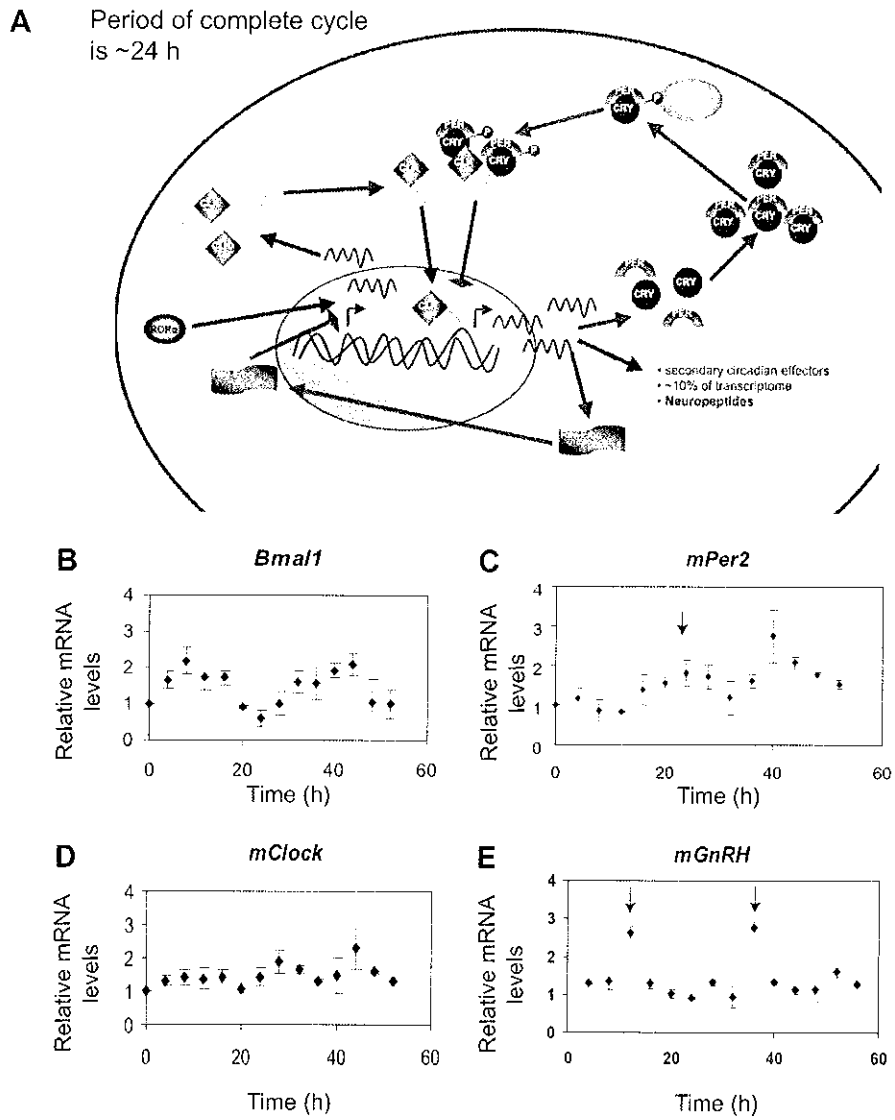

Fig. 4. Circadian regulation within hypothalamic neuronal models. (A) Schematic diagram of the basic transcriptional/translational molecular clock. Bmal1 and Clock are transcribed and translated. They heterodimerize and translocate to the nucleus where they initiate the transcription of the period (per) and cryptochrome (cry) genes, Rev-Erbα, secondary circadian effectors and approximately 10% of the transcriptome. Per and Cry proteins heterodimerize and build up in the cytoplasm where they are phosphorylated by casein kinase 1ε (CK1ε). They then inhibit the Bmal1/Clock heterodimer, thereby inhibiting their own transcription. Rev-Erbα also inhibits Bmal1 transcription. Eventually levels of Per, Cry and Rev-Erbα diminish and the cycle starts again, approximately 24 h later. (B–E) Gene expression profiles that demonstrate a circadian rhythmicity of clock gene and GnRH mRNA levels within the GT1-7 neurons (originally published in [63]).

yielded the first mammalian homolog of the fly system in 1997, the protein CLOCK. Further genomic research revealed many more mammalian homologs indicating that a mammalian model was required for continued research into mammalian circadian rhythmicity. As a result, mammalian retinal cells and SCN explants were cultured, as it was recognized that these areas exhibited innate oscillatory properties and had measurable endocrine outputs such as arginine vasopressin. But primary retinal and neuronal cultures are heterogeneous and only suitable for single interventions that often lack a genetic basis unless the primary culture came from a knock out or transgenic mouse model. With the advent of cell line development this has rapidly changed the understanding of the mammalian circadian system.

5.2. Clocks in peripheral cell lines

Initially circadian studies in cell lines were not thought to be advantageous as the circadian clock did not appear to cycle. However, in 1998 it was discovered that immortalized rat-1 fibroblasts could be synchronized with a serum bolus [11]. After synchronization, the fibroblasts demonstrate predictable patterns of clock gene transcription for up to three 22.5 h periods. From this study it was ascertained that a serum shock could mimic light-induced immediate early gene expression in a non-photoreceptive cell model. Balsalobre et al. then went on to prove that glucocorticoids could reset and synchronize rat-1 cultures, leading to the discovery that unlike central oscillators which are responsive to phase shifting at certain periods, peripheral oscillators retain the ability to be phase reset throughout different periods of the day [12]. Further, this group identified numerous signaling pathways including those for cAMP, Ca^{2+}, and protein kinase C (PKC) as being capable of eliciting changes in rat-1 fibroblast circadian gene expression and rhythmicity [13]. Two separate groups stably transfected the rat-1 fibroblast cell lines with reporter constructs for real time imaging. Through this technique it was successfully proven that the rhythms within the rat-1 lines are indeed true circadian rhythms,

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as they exhibit robust temperature compensation [79] and that individual peripheral cells in culture do cycle over 24 h, but lose their synchrony with their neighbours through lack of cell coupling [158]. This model was then expanded using the murine NIH-3T3 fibroblasts where it was discovered that the MAP kinase signal transduction pathway was also involved in the setting of the peripheral circadian clock in response to a potent PKC activator 12-O-tetradecanoylphorbol-13-acetate (TPA) administration and/or serum bolus [2]. Further work with the NIH-3T3 fibroblasts elaborated on the mechanisms of nucleocytoplasmic shuttling of BMAL1 [149], circadian temperature compensation [152], the importance of casein kinase 1 epsilon (CK1ε) to the stability of the period proteins [3] and that prostaglandin E2 could be used as a zeitgeber in cell culture studies [153]. Additional fibroblast-based studies involving the use of mouse embryonic fibroblast (MEF) cells has allowed for elucidation of alterations in the circadian system within transgenic mouse models, an innovation that removes the necessity for plasmid transfection or transduction. Notably, Hirayama et al. used MEF cells from BMAL1 knock out mice to prove that a mutant BMAL1 protein could not restore rhythmicity when it is unable to be acetylated by CLOCK [73].

Relatively little has been elucidated in the field of circadian rhythms in other tissue specific peripheral immortalized lines. In our lab, Chalmers et al. used the MOVAS-1 murine aortic smooth muscle cell line to clarify the expression of circadian rhythms in vasculature remodeling genes and the similarity in the circadian expression of these genes in vivo [31]. We also went on to characterize the expression of neuroendocrine genes in the heart and further elucidated the circadian profiles of proopiomelanocortin gene expression in the healthy and hypertrophied heart and MOVAS-1 cells [30].

Human cell lines have been used in cancer studies to elaborate on the link between circadian rhythmicity and cell cycle progression [155]. These cell lines include the HEK293 kidney cells, HeLa cervical cancer cells and the NHF-1 fibroblastic cells. Further, a group has recently established a stably transfected human retinal pigment epithelial (hTERT) cell line that expresses luciferase under the control of the BMAL1 promoter to elaborate on the nature of retinal circadian rhythms and photoresponsiveness [166].

5.3. Clocks in SCN neuronal cell lines

In addition to the important innovations accomplished through the use of peripheral cell lines, in 1999 Earnest et al. successfully established embryonic rat SCN cell lines, named the SCN 1.4 and 2.2 cells, that exhibit the functional characteristics of SCN neurons and exist in immortalized culture [49]. These cells are a heterogeneous population of cells and exhibit pacemaker potential and rhythmic expression of clock gene mRNAs, 2-deoxyglucose (2-DG) uptake and bone-derived neurotropic factor (BDNF) expression, much like the intact SCN [50]. These cells were further characterized and found to express a broad range of clock genes, and circadian regulatory pathways [76]. Additionally, the SCN2.2 cells were able to be phase shifted with a glutamatergic stimulus, similar to the SCN in vivo [77].

Of key importance, the SCN2.2 neurons were also able to restore behavioral rhythms when transplanted into arrhythmic, SCN-lesioned rats; an effect that was not reproducible with immortalized mesencephalic or fibroblastic cells [50]. This finding represents an incredibly important innovation in cell line usage. While the SCN2.2 neurons are immortalized, they have retained enough of their neuronal SCN phenotype to function appropriately in the in vivo context. This marks a fundamental keystone in the potential usage of immortalized cell lines in the treatment of in vivo disorders.

Further work with the SCN2.2 cells has highlighted valuable information about the study of circadian rhythms. The SCN2.2 cells were able to synchronize rat-1 fibroblasts in co-culture through diffusible signals [4]. While in co-culture, the SCN2.2 cells conferred rhythms within the rat-1 fibroblasts in their metabolic and clock gene expression with a 4 h delay, similar to the phase-shift delay seen between the SCN and periphery in vivo. Interestingly, only co-culture with the SCN2.2 was able to confer metabolic rhythmicity in the rat-1 fibroblasts; whereas serum shock was only able to synchronize gene expression. This effect suggests that diffusible signals from the SCN are required for metabolic cyclicity, whereas serum bolus only confers a synchronization of the molecular clock. The SCN2.2 cells were then assayed with real time analysis of a human c-fos reporter gene to catalog their responses to serum and potassium chloride (KCl). It was determined that the SCN2.2 cells exhibited a similar pattern of c-fos reactivity as the SCN in vivo [5]. Allen et al. then proceeded to assay the importance of CLOCK in the SCN2.2 and rat-1 fibroblast lines by transfecting the cell lines with anti-sense RNA for CLOCK. Subsequently, they found that disruption of CLOCK in the SCN2.2 neurons altered cyclicity of period gene expression and 2-DG uptake. They also reiterated these findings in the rat-1 cell lines, but then surprisingly found that even with antisense inhibition of CLOCK the rat-1 cell lines could be synchronized to cycle with a serum shock indicating that the SCN and periphery have different entrainable stimuli [6].

Other groups have elucidated a variety of important circadian findings that bolster the applicability of the SCN2.2 cells as a circadian model including: the circadian expression of nicotinamide adenine dinucleotides within the SCN2.2 cells [163], the functionality of melatonin receptors within the SCN2.2 cells [127], a rhythmicity of PKC with melatonin administration [126], the importance of voltage-dependent calcium channels in SCN and SCN2.2 rhythmicity [111] and a temporal desensitization of MT2 receptors with melatonin administration that allows a cyclic sensitivity of the SCN to melatonin [61]. All of these studies have helped to reiterate findings found within the explanted SCN, however, now exist in a model that has vastly greater potential for genetic and molecular intervention.

Recently, two additional SCN cell lines have been generated: the N14.5 cells, which are SV40-temperature sensitive ventrolateral SCN neurons developed for glutamatergic/photic entrainment studies [102]; and the per1-luciferase expressing RS182 cells [85]. Further circadian research with these new models is forthcoming and will likely shed further light on the field of circadian rhythms.

5.4. Circadian studies in Non-SCN neuronal cell lines

Owing to the relative shortage of neuronal cell lines for this type of work, there is a limited amount of data about the cyclicity of the circadian clock within non-SCN neuronal cell lines. Much of the existing data from non-SCN neuronal cell lines was elucidated using the immortalized GnRH-expressing GT1-7 cells. Chappell et al elucidated that GnRH secretion is altered by perturbation of the molecular clock. Over-expression of a dominant-negative Clock-Delta19 protein reduced mean pulse frequency of GnRH secretion. Further, over expression of mCry1 increased pulse amplitude of GnRH secretion, but did not affect overall frequency [33]. Research from our lab showed that the GT1-7 neurons contain functional melatonin receptors on the GT1-7 GnRH neurons and we clarified the actions of melatonin on the gene expression of GnRH over 24 h. We mapped the regions of melatonin responsiveness on the GnRH promoter and showed the first evidence of direct melatonin action on GnRH neurons [64,130]. We further elucidated the signaling cascades of melatonin within these pulsatile neuronal models. We were able to determine that melatonin signaling acts through multiple pathways including inhibition of the forskolin-

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induced increase in cAMP and activation of PKC, MAPK and the immediate early genes. We also determined that these pathways are involved in the melatonin-mediated decrease in GnRH secretion [131]. Finally, we went on to fully characterize the circadian gene expression and protein profiles within the GT1-7 neurons. The GT1-7 neurons express clock, BMAL1, timeless (tim), period1 (per1), period2 (per2), cryptochrome1 (cry1) and cryptochrome2 (cry2). Of these transcripts BMAL1, per1, per2 and GnRH mRNA expression was assayed over 54 h and the transcripts were found to cycle over 24 h (Fig. 4B–E). Accordingly, the protein levels of BMAL1 oscillated as well [63].

Further studies from our laboratory have exploited the novel generation of an array of clonal hypothalamic neuronal models [18]. We have successfully characterized the circadian profiles within multiple cell lines including the mHypoE-44, -42, -36/1, -36/2 and -39 neuronal models and have found that they represent an excellent model for neuronal circadian research outside the SCN. These cell lines express phenotypically distinct expression profiles of neuropeptides, receptors and signaling molecules. In addition to characterizing the clock gene profiles of these neurons – clock, BMAL1, timeless (tim), period1 (per1), period2 (per2), cryptochrome1 (cry1) and cryptochrome2 (cry2) – we have also generated a circadian gene expression profile for a number of important neuropeptides including the orexigenic: neuropeptide Y (NPY), preproghrelin and agouti-related peptide (AgRP); and the anorexigenic corticotropin-releasing hormone (Crh), neurotensin (NT) and neuromedin U (NMU) (L.J. Fick and D.D. Belsham, unpublished data). Interestingly, there appears to be inductive, but not cyclic, expression in NPY and NT gene expression following serum shock, but AgRP cycles over 24 h. Interestingly, both Crh and NMU exhibit ultradian (<24 h) rhythms. Preproghrelin oscillates, but without a fixed period. These are the first models to demonstrate direct rhythmicity within a single neuronal phenotype. We further elucidated the role of nutrient signals on the circadian system and discovered that palmitate, a 16-carbon saturated fatty acid, blunted clock cyclicity within the mHypoE-44 neurons and elevated orexigenic neuropeptide gene expression. Additionally, fructose administration reduced NMU gene expression, indicating a role for sugars in the abrogation of anorexigenic neuropeptide signaling. Further research into the mechanisms of these findings is currently under way. The use of cell lines will ultimately allow a characterization of the molecular mechanisms controlling circadian neuronal gene expression, whether the classic clock genes are directly involved in this process, and how these rhythmic patterns of expression can be disturbed by peripheral and central signals, perhaps leading to circadian disruption and disease.

6. Conclusion

Elucidating the functions of the hypothalamus is essential to generating an understanding of how the brain orchestrates many important processes vital to life. The hypothalamus is the seat of neuroendocrine control and as such is responsible for the regulation of feeding, reproduction and the coordination of circadian rhythmicity (Fig. 5). However, the location and heterogeneic nature of the hypothalamus precludes easy determination of its mechanisms within in vivo situation. This state of affairs has been rapidly altered with the advent of hypothalamic cell lines. Clonal and nucleus specific hypothalamic cell lines have permitted the clarification of many neuronal mechanisms that until now had been virtually impossible to determine. As discussed in this review, the knowledge gained from using cell lines representative of a single neuronal phenotype include analyses of neuropeptide gene expression, genetic intervention using RNAi and plasmid transfection, secretion, signal transduction, 5′ regulatory region control, ion channel function, peripheral hormone and factor responsiveness, and global microarray and proteomic data. While technology closes the gap between current in vivo techniques and future assays which will allow for single cellular analysis in situ, in the interim, cell lines have proven themselves invaluable as the workhorses of molecular biology within the hypothalamus and throughout the entire organism.

This review has highlighted the roles of hypothalamic cell lines in advancing the fields of feeding, reproduction and circadian rhythms (Table 2). While these three fields are the most heavily studied and have an essential impact on the health sector, further research with hypothalamic cell lines should yield novel information about these and the many other vital and ubiquitous functions of the hypothalamus. Future studies made possible by the advent of hypothalamic cell lines may include elaboration on gene expres-

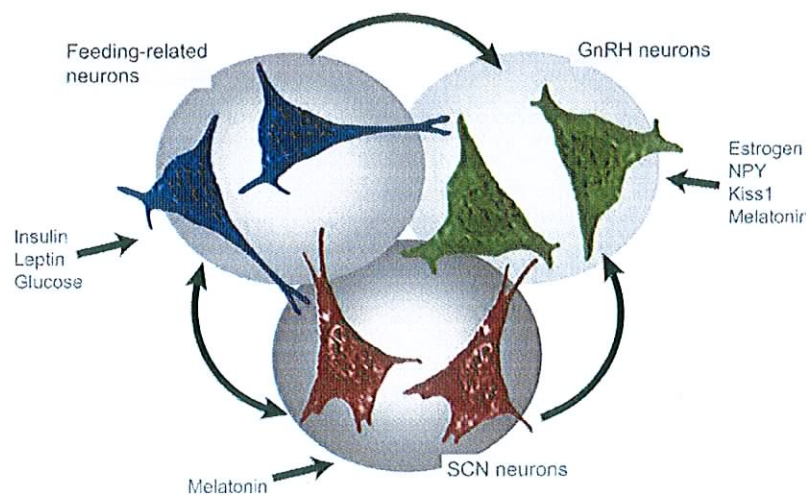

Fig. 5. Summary of hypothalamic neuronal interaction. The hypothalamus contains a complex interacting network of neurons. This network includes feeding-related, SCN and GnRH neurons. Feeding-related neurons can integrate peripheral signals, including insulin, leptin and glucose, as well as communicate to GnRH and SCN neurons. The SCN neurons receive input from melatonin and other neurons, such as the feeding-related neurons, and communicate to both the feeding-related and GnRH neurons. In contrast GnRH neurons only receive input from other hypothalamic neurons, including the feeding-related and SCN neurons. As well, they integrate signals from neuropeptides and hormones, including NPY, Estrogen, Kiss1 and melatonin. This figure indicates the integration between the neurons within these three systems and shows the relation between the hypothalamic areas regulating energy homeostasis, circadian rhythms and reproduction.

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sion, signal transduction in neurons, drug discovery, receptor cloning and characterization, ion channel function, phenotypic profiling of individual neuronal cell types, neuron–neuron interactions and communication, and numerous others. Cell line studies and in vivo research are complementary to each other; and, as demonstrated within this review, both models have been used effectively in concert to elucidate the mechanisms of our physiology. Cell line usage is an invaluable asset that can expand upon the molecular basis of knowledge gathered in the in vivo situation. Cell lines can be used for hypothesis clarification or as a preliminary tool upon which to base hypothesis. Together cell lines and in vivo research represent different, but equally important methods for achieving the same goal – knowledge of our physiology and an understanding of how we can use that information to advance health and treat disease.

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Modification Form for Permit BIO-LHRI-0047

Permit Holder: Andy Babwah

Approved Personnel
(Please stroke out any personnel to be removed)

Additional Personnel
(Please list additional personnel here)

	Please stroke out any approved Biohazards to be removed below	Write additional Biohazards for approval below. *
Approved Microorganisms	E. coli (DH5 alpha), E coli (Top 10), XL1-Blue super-competent cells	
Approved Cells	Human (established), rodent (established), nonhuman primate (established), Hek 293, HTR-8/Svneo, NDA-MB-231, MDA-MB-435S, MCF-10A, PC-3, PZ-HPV-7, JEG-3, ARIP, AR42J, COS-7	MOUSE MEF, B-ARRESTIN 1/2 KNOCK-OUT CELL LINES
Approved Use of Human Source Material	Human chorionic gonadotropin - purified	
Approved GMO	SV 40 Large T antigen, Adeno E1A gene in HEK 293 cells	
Approved use of Animals		
Approved Toxin(s)	Cholera toxin (C-8052 from Sigma Canada)	

* *PLEASE ATTACH A MATERIAL SAFETY DATA SHEET OR EQUIVALENT FOR NEW BIOHAZARDS.*
** *PLEASE ATTACH A BRIEF DESCRIPTION OF THE WORK THAT EXPLAINS THE BIOHAZARDS USED AND HOW THEY WILL BE USED.*

Classification: 2

Date of last Biohazardous Agents Registry Form: Aug 29, 2007

Signature of Permit Holder: [signature]

BioSafety Officer(s): [signature] Jan 30/09

Chair,Biohazards Subcommittee: [signature]

(53420)

They are 5 lines derived from mouse embryonic fibroblasts (MEFs). Two lines come from wild type animals and one from a beta-arrestin-1 knockout, one from a beta-arrestin-2 knockout and one from a beta-arrestin-1/2 double knockout animal. Beta-arrestins are small proetins expressed in just about all cell types. The lines are coming from Duke University, USA from the lab of Dr.Robert Lefkowitz. Hope this helps.

> > > >

> > > > Andy

BIO-LHRI-0047

THE UNIVERSITY OF WESTERN ONTARIO
BIOHAZARDOUS AGENTS REGISTRY FORM
Revised Biohazards Subcommittee: January, 2007

This form must be completed by each Principal Investigator holding a grant administered by the University of Western Ontario where the use of biohazardous infectious agents are described in the experimental work proposed. The form must also be completed if animal work is proposed involving the use of biohazardous agents or animal carrying zoonotic agents infectious to humans. Containment Levels will be required in accordance with Laboratory Biosafety Guidelines, 3rd edition, 2004, Health Canada (HC) or Containment Standards for Veterinary Facilities, 1st edition 1996, Canadian Food Inspection Agency (CFIA).

Completed forms are to be returned to Occupational Health and Safety (Stevenson-Lawson Building, Room 60) for forward to the Biohazard Subcommittee. For questions regarding this form, please contact the Biosafety Coordinator at extension 81135. If there are changes to the information on this form (excluding grant title and funding agencies) modifications must be completed and sent to Occupational Health and Safety. See website: www.uwo.ca/humanresources

PRINCIPAL INVESTIGATOR Andy Videsh Babwah
SIGNATURE _____ July 19, 2007
DEPARTMENT Obstetrics and Gynaecology
ADDRESS Victoria Research Laboratories, A4-140
PHONE NUMBER 519-685-8500 extension 55485
EMAIL ababwah@uwo.ca

Location of experimental work to be carried out: Building(s) Victoria Research Laboratories
Room(s) all 4th floor rooms
*For work being performed at Institutions affiliated with the University of Western Ontario, the Safety Officer for the Institution where experiments will take place must sign the form prior to it being sent to Occupational Health and Safety (See Section 12.0, Approvals). For research being done at Lawson Health Research Institute, London Regional Cancer Centre, Child and Parent Research Institute or Robarts Research Institute, University Biosafety Committee members can also sign as the Safety Officer.

TITLE OF GRANT(S):
1. GnRH-RI activity during human placentation (CIHR)
2. Molecular and Functional analysis of nuclear membrane localized GnRH-RI (NSERC Discovery)
3. Systemic evaluation of assisted reproduction (CFI New Opportunities)
4. GnRH-regulated gene expression in the human placenta (Children's Health Research Institute)

PLEASE ATTACH A BRIEF DESCRIPTION OF YOUR WORK, SUCH A THE RESEARCH GRANT SUMMARY(S) THAT EXPLAINS THE BIOHAZARDS USED. PROJECTS SUBMITTED WITHOUT A SUMMARY WILL NOT BE REVIEWED.

FUNDING AGENCY/AGENCIES: CIHR, NSERC, CFI, Children's Health Research Institute
Names of all personnel working under Principal Investigators supervision in this location:
i) Natasha Camuso
ii) Michelle Re
iii) Cindy Pape
iv) Macarena Pampillo
v) Caroline Kahiri
vi) Craig Cavanagh
vii) Adel Aziziyeh
viii) Timothy Li

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

1.0 Microorganisms

1.1 Does your work involve the use of microorganisms or biological agents of plant or animal origin (including but not limited to viruses, prions, parasites, bacteria)? X YES ف NO
If no, please proceed to Section 2.0

1.2 Please complete the table below:

Name of Biological agent(s)	Is it known to be a human pathogen? YES/NO	Is it known to be an animal pathogen? YES/NO	Is it known to be a zoonotic agent YES/NO	Maximum quantity to be cultured at one time?
E. coli (DH5 alpha)	ف Yes X No	ف Yes X No	ف Yes X No	1 litre
E. coli (Top10)	ف Yes X No	ف Yes X No	ف Yes X No	1 litre
XL1-Blue super-competent cells	ف Yes X No	ف Yes X No	ف Yes X No	1 litre

1.3 For above named organism(s) or biological agent(s) circle HC or CFIA Containment Level required. 1 (2) 3

1.4 Source of microrganism(s) or biological agent(s)?commercially available (Invitrogen, Stratagene)

2.0 Cell Culture

2.1 Does your work involve the use of cell cultures? XYES فNO
If no, please proceed to Section 3.0

2.2 Please indicate the type of primary cells (ie. derived from fresh tissue) that will be grown in culture in the table below

Cell Type	Is this cell type used in your work?	Source of Primary cell Culture Tissue
Human	ف Yes X No	
Rodent	ف Yes X No	
Non-human primate	ف Yes X No	
Other (specify)	ف Yes X No	

2.3 Please indicate the type of established cells that will be grown in culture in the table below.

Cell Type	Is this cell type used in your wor	Specific cell line(s)	Supplier / Source
Human	X Yes ف No	HEK 293	ATCC (original supplier)
Human	X Yes ف No	HTR-8/Svneo	Dr Lala, UWO (Anatomy & Cell Biol)
Human	X Yes ف No	MDA-MB-231	ATCC (original supplier)
Human	X Yes ف No	MDA-MB-435S	ATCC (original supplier)
Human	X Yes ف No	MCF-10A	ATCC (original supplier)
Human	X Yes ف No	PC-3	ATCC (original supplier)
Human	X Yes ف No	PZ-HPV-7	ATCC (original supplier)
Human	X Yes ف No	JEG-3	Dr Yang, UWO (Ob&Gyn)
Rodent	X Yes ف No	ARIP	Dr Pin, UWO (Phys & Pharm)
Rodent	X Yes ف No	AR42J	Dr Pin, UWO (Phys & Pharm)
Non-human primate	X Yes ف No	COS-7	ATCC (original supplier)
Other (specify)	ف Yes X No		

2.4 For above named cell types(s) circle HC or CFIA containment level required 1 (2) 3

' DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED'

/

3.0 Use of Human Source Materials

3.1 Does your work involve the use of human source materials? X YES ☐NO
(human corionic gonadotropin – purified, tested for human pathogens (eg: HIV))
If no, please proceed to Section 4.0

3.2 Indicate if the following will be used in the laboratory

- Human blood (whole) or other bodily fluids ☐ YES X NO If YES, Specify _____
- Human blood (fraction) or other bodily fluids ☐ YES X NO If YES, Specify _____
- Human organs (unpreserved) ☐ YES X NO If YES, Specify_____
- Human tissues (unpreserved) ☐ YES X NO If YES, Specify_____

3.3 Is human source known to be infected with and infectious agent ☐ YES X NO
If YES , please name infectious agent _____

3.4 For above named materials circle HC or CFIA containment level required. 1 (2) 3

4.0 Genetically Modified Organisms and Cell lines

4.1 Will genetic modifications be made to the microorganisms, biological agents or cells described in Sections 1.0 and 2.0? X YES ☐ NO
If no, please proceed to Section 5.0

4.2 Will genetic sequences from the following be involved:

- HIV ☐ YES X NO
  if YES specify_____
- HTLV 1 or 2 or genes from any CDC class 1 pathogens ☐ YES X NO
  if YES specify_____
- Other human or animal pathogen and or their toxins ☐ YES X NO
  if YES specify_____

4.3 Will intact genetic sequences be used from

- SV 40 Large T antigen X YES ☐ NO If YES specify: present in COS-7 cells and HTR-8/Svneo cells
- Known oncogenes X YES ☐ NO If YES specify: Adeno E1A gene in HEK 293 cells

4.4 Will a live vector(s) (viral or bacterial) be used for gene transduction ☐ YES X NO
If YES name virus_____.

4.5 List specific vector(s) to be used:_____

4.6 Will virus be replication defective ☐ YES ☐ NO N/A

4.7 Will virus be infectious to humans or animals ☐ YES ☐ NO N/A

4.8 Will this be expected to increase the Containment Level required ☐ YES X NO

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*

5.0 Human Gene Therapy Trials

5.1 Will human clinical trials using the viral vector in 4.0 be conducted? ☐ YES X NO
If no, please proceed to Section 6.0
If YES attach a full description of the make-up of the virus.

5.2 Will virus be able to replicate in the host? ☐ YES ☐ NO

5.3 How will the virus be administered? _____

5.4 Please give the Health Care Facility where the clinical trial will be conducted:_____

5.5 Has human ethics approval been obtained? ☐ YES ☐ NO

6.0 Animal Experiments

6.1 Will any of the agents listed be used in live animals? ☐ YES X NO
If no, please proceed to section 7.0

6.2 Name of animal species to be used_____

6.3 AUS protocol # _____

6.4 If using murine cell lines, have they been tested for murine pathogens? ☐ YES ☐ NO

7.0 Use of Animal species with Zoonotic Hazards

7.1 Will any of the following animals or their organs, tissues, lavages or other bodily fluids including blood be used:

- Pound source dogs ☐ YES X NO
- Pound source cats ☐ YES X NO
- Sheep or goats ☐ YES X NO
- Non- Human Primates ☐ YES X NO If YES specify species _____
- Wild caught animals ☐ YES X NO If YES specify species _____
  colony # _____

8.0 Biological Toxins

8.1 Will toxins of biological origin be used? X YES ☐ NO
If no, please proceed to Section 9.0

8.2 If YES, please name the toxin: Cholera toxin (C-8052 from Sigma Canada)

8.3 What is the LD50 (specify species) of the toxin: 250 ug/kg in mice

*** DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED***

9.0 Import Requirements

9.1 Will the agent be imported? X YES ☐ NO
If no, please proceed to Section 10.0
If yes, country of origin: USA

9.2 Has an Import Permit been obtained from HC for human pathogens? X YES ☐ NO
Cholera toxin is ordered from a Canadian company (Sigma-Aldrich Canada). This supplier takes the responsibility for obtaining the import permit in this case.

9.3 Has an import permit been obtained from CFIA for animal pathogens? ☐ YES X NO
Cholera toxin is not classified as an animal pathogen

9.4 Has the import permit been sent to OHS? ☐ YES X NO
If yes, Permit # _____ Sigma-Aldrich Canada is still processing this permit.

10.0 Training Requirements for Personnel named on Form

All personnel named on the above form who will be using any of the above named agents are required to attend the following training courses given by OHS

- Biosafety
- Laboratory and Environmental/Waste Management Safety
- WHMIS

As the Principal Investigator, I have ensured that all of the personnel named on the form who will be using any of the biohazardous agents in Sections 1.0 to 9.0 have been trained.

SIGNATURE _____ July 17, 2007

11.0 Containment Levels

11.1 For the work described in sections 1.0 to 9.0, please circle the highest HC or CFIA Containment Level required. 1 (2) 3

11.2 Has the facility been certified by OHS for this level of containment? X YES ☐ NO

11.3 If yes, please give the date and permit number: _____

12.0 Approvals

UWO Biohazard Subcommittee

Signature _____ Date Aug 7, 2007.

Safety Officer for Institution where experiments will take place

Signature _____ Date 25 Aug '07

Safety Officer for University of Western Ontario (if different than above)

Signature _____ Date Aug 28/07

* DESCRIPTION MUST BE ATTACHED TO THIS FORM OR PROJECT WILL NOT BE REVIEWED*