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**Regulation of gonadotropin releasing hormone gene expression in
a mouse hypothalamic neuronal GT1 cell line**

Yeo, Tracy Teck Si, Ph.D.

City University of New York, 1994

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Regulation of Gonadotropin Releasing Hormone Gene Expression in a Mouse Hypothalamic Neuronal GT1 Cell Line

by

Tracy Teck Si Yeo

A dissertation submitted to the Graduate Faculty in Biomedical Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy, the City University of New York

1994

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This manuscript has been read and accepted by the Graduate Faculty in Biomedical Sciences in satisfaction of the dissertation requirement for the degree of Doctor of Philosophy.

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Abstract

Regulation of Gonadotropin Releasing Hormone Gene Expression in a Mouse Hypothalamic Neuronal GT1 Cell Line

by

Tracy Teck Si Yeo

Advisor: Professor James L. Roberts

Gonadotropin releasing hormone (GnRH) is the primary neuropeptide regulating vertebrate reproductive functions. Numerous factors are known to regulate GnRH release through direct or indirect neuronal synaptic inputs or by surrounding non-neuronal cells. However, the low abundance and sparse distribution of GnRH neurons have limited the understanding of the regulation of GnRH biosynthesis by these factors. The complexity of neuronal inputs and cellular compositions surrounding the GnRH neurons have also made it difficult to determine whether the effects of these factors on the GnRH system are through direct action on the GnRH neurons or are secondary, through interneurons or surrounding non-neuronal cells. During my graduate study with Dr. James L. Roberts, we have taken the advantage of the recent production of a mouse hypothalamic neuronal GnRH-secreting cell line, the GT1 cells, to explore the cellular and molecular mechanisms by which the GnRH neuronal functions are regulated.

What I present here in this dissertation thesis contains the bulk of experimental data generated in my graduate study on establishing the GT1 system and assay methods for the elucidation of GnRH gene expression in

response to hormonal regulation. I have characterized the basic features of the GnRH gene transcripts and defined assay methods to elucidate the hormonal regulation of GnRH gene expression along its mRNA biosynthetic pathway which can act in concert to regulate the final levels of mRNA templates to be translated into GnRH prohormone. I have then studied the effects of the activation of two major intracellular second messenger pathways, cAMP/protein kinase A (PKA) and diacylglycerol/protein kinase C (PKC), by which the effects of numerous neurotransmitters and hormones are mediated, on GnRH gene expression. The establishment of this system will provide useful information on advancing our understanding on the neuroendocrine control of the GnRH system.

Format of Thesis

This thesis was prepared in accordance with the guideline of the City University of New York. Chapter I gives an overview of the background related to this thesis. To avoid redundancy, materials and experimental methods utilized in this thesis study have been consolidated and described in Chapter II. Chapters III, IV, and V describe my thesis work on the establishment of assay methods, the characterization of GnRH gene transcripts, and the regulation of GnRH gene expression by PKA and PKC pathways in the GT1 cells. Each of these three chapters contains individual introduction, results and discussion sections. Chapter VI describes the general aspects and significance of my thesis work as the general discussion section.

Acknowledgments

At this moment that I am completing my dissertation thesis, I would like to thank all the people who inspired, cherished, encouraged, and helped me to pursue a career in science at various stages of my study.

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Introduction

Gonadotropin releasing hormone (GnRH) is the primary neuropeptide regulating vertebrate reproductive functions. It is synthesized in a subpopulation of hypothalamic neurons (GnRH neurons) which largely project to the median eminence. The release of GnRH peptide into the portal blood vessels connecting to the anterior pituitary stimulates the secretion of pituitary hormones which have subsequent effects on the gonads. It has also been proposed that the release of GnRH into other brain areas through extrahypothalamic GnRH neuronal projections may be associated with emotional or behavioral changes. Numerous factors are known to regulate GnRH release through direct or indirect neuronal synaptic inputs or by surrounding non-neuronal cells. However, the low abundance and sparse distribution of GnRH neurons have limited the understanding of the regulation of GnRH biosynthesis by these factors. The complexity of neuronal inputs and cellular compositions surrounding the GnRH neurons have also made it difficult to determine whether the effects of these factors on the GnRH system are through direct action on the GnRH neurons or are secondary, through interneurons or surrounding non-neuronal cells.

During my graduate study with Dr. James L. Roberts, we have taken the advantage of the recent production of a mouse hypothalamic neuronal GnRH-secreting cell line, the GT1 cells, to explore the cellular and molecular mechanisms by which the GnRH neuronal functions are regulated. We have characterized the basic features of the GnRH gene transcripts and defined assay methods to elucidate the hormonal regulation of GnRH gene expression along its mRNA biosynthetic pathway which can act in concert to regulate the final levels of mRNA templates to be translated into GnRH prohormone. We have then

studied the effects of the activation of two major intracellular second messenger pathways, cAMP/protein kinase A (PKA) and diacylglycerol/protein kinase C (PKC), by which the effects of numerous neurotransmitters and hormones are mediated, on GnRH gene expression.

What I present here in this dissertation thesis contains the bulk of experimental data generated in my graduate study on establishing the GT1 system and assay methods for the elucidation of GnRH gene expression in response to hormonal regulation. Chapter I gives an overview of the background related to this thesis; Chapter II describes the materials and experimental methods utilized in this study; Chapter III, IV, and V are submitted or to be submitted manuscripts containing our work on the establishment of assay methods, the characterization of GnRH gene transcripts, and the regulation of GnRH gene expression by PKA and PKC pathways in the GT1 cells. Each of these three chapters contains individual introduction, results and discussion sections. In Chapter VI I have discussed the general aspects and significance of my thesis work. The establishment of this system will provide useful information on advancing our understanding on the neuroendocrine control of the GnRH system.

Chapter I

Background and Significance

I. Overview of the GnRH system

- A. Physiological effects of GnRH**
- B. GnRH peptide and gene structure**
- C. Anatomy of the GnRH system**
- D. Origin of GnRH neurons**

II. Regulation of GnRH release and gene expression in the animals

- A. Sex steroids**
- B. Catecholamines**
- C. Opiates**
- D. GnRH**
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III. Second messengers and GnRH gene expression

- A. Overview of the major second messenger pathways**
- B. The cAMP pathway (PKA pathway)**
- C. The DG/IP₃ pathway (PKC pathway)**
- D. Proto-oncogenes and GnRH neuronal activation**
- E. The role of second messengers in GnRH regulation**

IV. Hypothalamic neuronal GnRH-expressing GT1 cell line

- A. Production and characteristics**
- B. Regulation of GnRH secretion and gene expression**

I. Overview of the GnRH system

A. Physiological effects of GnRH

Mammalian reproductive functions are controlled by the complex cooperative endocrine interactions between the hypothalamus, anterior pituitary, and the gonads. Gonadotropin releasing hormone (GnRH), a decapeptide synthesized in the GnRH neurons in the preoptic area-hypothalamus, is the key molecule controlling these functions (for review, see Silverman, 1988). GnRH peptide is released into the portal capillaries in a pulsatile manner through its neuronal synaptic terminals in the median eminence and delivered to its target cells (gonadotropes) in the anterior pituitary via the hypophyseal portal veins. There it stimulates the secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into circulating blood which have subsequent effects on the gonads. It has been proposed that GnRH can also work as a neurotransmitter and may be involved in reproductive behavior, presumably through its extrahypothalamic projections (for review, see Schwanzel-Fukuda et al, 1992). The expression of GnRH peptide and mRNA has been detected in numerous tissues and is thought to be involved in paracrine control, immune and reproductive functions (Pagesy et al, 1992; Azad et al, 1993, Dong et al, in press).

The pulsatile nature of GnRH release from the median eminence into the portal blood to the pituitary, which in turn elicits the episodic secretion of luteinizing hormone (LH) and follicle-stimulating hormone (FSH) into the circulation, is very important for the control of sexual maturation and reproductive functions. It is believed that this pulsatile release of GnRH is regulated through the "pulse generators" presumably in the arcuate region of the medial basal hypothalamus. It has also been suggested that direct and indirect

communications between GnRH nerve terminals in the median eminence can also modulate the pulsatility (for review, see Dyer et al, 1989).

One of the most important and widely studied GnRH physiological functions is the stimulation of ovulation by dramatic increase of plasma LH levels (LH surge). During most of the rat estrous cycle, GnRH is released at low levels in a pulsatile manner. On the afternoon of proestrous, a large increase of GnRH release, both in frequency and amplitude, is generated which triggers the LH surge and the subsequent induction of ovulation (Levine et al, 1982; Terasawa et al, 1987). The neuronal control of the LH surge by the hypothalamic GnRH neurons has been extensively studied. Numerous factors have been identified to have effects on GnRH secretion and biosynthesis, both during the LH surge as well as during pulsatile GnRH release, which subsequently control the release of LH and its physiological functions (for review, see Weiner et al, 1988; Schwanzel-Fukuda et al, 1992). However, the cellular and molecular mechanisms mediating this control of the GnRH neurons are not yet well elucidated.

B. GnRH peptide and gene structures

GnRH is an ancient peptide whose structure can even be traced back to a yeast pheromone, the yeast alpha-mating factor. There are at least seven forms of GnRH decapeptide identified in vertebrates of which only one form of GnRH decapeptide has been found in mammals. Six other forms of GnRH peptide which are distinct from the mammalian GnRH decapeptide, including two forms in the chicken and four forms in the fish, have been found repeatedly in other species. Nevertheless, there is still at least 50% peptide sequence identity between all seven forms identified within vertebrates (For review, see Sherwood, 1987; Sherwood et al, 1993).

The gene encoding the GnRH peptide has been cloned in several mammalian species, including human (Seeburg et al, 1984; Adelman et al, 1986; Radovick et al, 1990), rat (Adelman et al, 1986; Bond et al, 1989), and mouse (Mason et al, 1986). The genomic sequence shows that it contains four exons separated by three large introns (Adelman et al, 1986). The amino acid sequence derived from the GnRH cDNA sequence indicates that it encodes a prohormone precursor which can be further processed to produce the GnRH decapeptide and the GnRH-associated peptide GAP (Adelman et al, 1987).

In addition to the expression in the hypothalamus, GnRH and GnRH-like immunoreactivity have also been detected in various extrahypothalamic tissues (Bennett-Clarke et al, 1982). The in situ synthesis of GnRH was demonstrated by the presence of GnRH mRNA in various tissues, including the placenta (Seeburg et al, 1984), peripheral T-lymphocytes (Azad et al, 1993), spleen lymphocytes (Azad et al, 1991), pituitary (Pagesy et al, 1992; Dong et al, in press), ovary (Oikawa et al, 1990; Dong et al, in press), cerebral cortex, testes, and mammary gland (Dong et al, in press). They are thought to have effects in the central and sympathetic nervous systems, the immune system, and the reproductive organs; however, the functions of GnRH other than its effects on pituitary gonadotropin release and biosynthesis are not well understood to date.

C. Anatomy of the GnRH system

The distribution of the GnRH neurons in the brain varies among species. In general, cell bodies of the GnRH immunoreactive cells are found to be distributed in the basal forebrain regions in mammals (for review, see King et al, 1984; Silverman, 1988). In the rat, GnRH neuronal cell bodies are located

largely in the rostral forebrain regions, including septal, preoptic and anterior hypothalamic nuclei, but not in the arcuate nucleus. In higher mammals including monkeys and humans, GnRH neurons are found throughout the medial basal hypothalamus and extend as far as the median eminence, infundibular complex, and the mammillary complex (King et al, 1984; Silverman et al, 1988). GnRH neuronal processes are found to extend into two major areas: the median eminence (ME), and the organum vasculosum laminae terminalis (OVLT) (Jennes et al, 1983; King et al, 1984; Merchenthaler et al, 1989). GnRH neuronal processes have also been found to extend into the midbrain, the limbic system, the olfactory-related structures, and into the neural lobe of the pituitary (for review, see Silverman, 1988). The ME projection is considered to be the key player regulating the hypothalamic-anterior pituitary-gonadal axis because of its close anatomical correlation and physiological connection with the anterior pituitary. The functions of GnRH projections into other areas are less well-defined, although much experimental evidence suggests a possible correlation with reproductive behaviors.

Many aminergic and peptidergic neurons are reported to have synaptic contacts with the GnRH neurons in different mammalian species, including opioid (Thind et al, 1988), serotonin (Thind et al, 1988), catecholamine: norepinephrine and dopamine (Jennes et al, 1983; Leranth et al, 1988; Chen et al, 1989), corticotropin-releasing factor (MacLusky et al, 1988), GABA (Leranth et al, 1985b), substance P (Tsuruo et al, 1991), neuropeptide Y (Tsuruo et al, 1990), vasopressin (Thind et al, 1991), and the GnRH containing neurons themselves (Pelletier et al, 1987; Leranth et al, 1985a). Although sex steroid hormones, particularly estrogen and progesterone, are very important for the regulation of GnRH physiological functions, it is clear that GnRH neurons do not contain

estrogen receptors (Shivers et al, 1983) nor progesterone receptors (Fox et al, 1990). This suggests that the actions of these sex steroids on GnRH may be an indirect effect via other neuronal or non-neuronal cells interacting with the GnRH neurons.

D. Origins of GnRH neurons

Based on its anatomical location and distribution, it was originally assumed that GnRH neurons originated from the surface of the ventricles, as has been found in other hypothalamic neuroendocrine cells. It has been reported that the neurophysin-containing neurons (vasopressin and oxytocin) originate from the ependymal cells of the third ventricle (Silverman et al, 1980). The first neurophysin immunoreactive neurons were detected around embryonic day 13.5 in the presumptive supraoptic nuclei (SON). At the same time, a lot of immunoreactive cells were also found in the region between the third ventricle and the SON, apparently still migrating from the ependymal wall of the third ventricle into SON (Silverman et al, 1980). However, this is certainly not the case for GnRH neurons. It was discovered that before the GnRH-immunoreactive cells are detected in the brain during prenatal development, a large population of GnRH-expressing cells can already be detected in the olfactory placode (Schwanzel-Fukuda et al, 1989). Using a combination of GnRH immunocytochemistry and tritiated autoradiography to "birth-date" neurons (Schwanzel-Fukuda et al, 1989), and a combination of immunocytochemistry and in situ hybridization (Wray et al, 1989a), it was demonstrated that the GnRH neurons are derived from progenitor cells from the medial olfactory placode around embryonic day 11.5 and migrate into the forebrain soon after that and target into the septal-preoptic area and the hypothalamus (for review, see Schwanzel-Fukuda et al, 1990; Schwanzel-Fukuda et al, 1991; Zheng et al,

1992). This peripheral origin is rather specific for the GnRH neurons because in an extensive immunocytochemical study of other hypothalamic hormones in embryonic mice, including thyrotropin releasing hormone (TRH), corticotropin releasing hormone (CRH), oxytocin, vasopressin, neuropeptide Y (NPY), and somatostatin, no immunoreactive staining of any of the above hypothalamic hormones in the sections along the GnRH neuronal migration route throughout the nasal and brain regions was detected (Schwanzel-Fukuda et al, 1991).

II. Regulation of GnRH release and gene expression in the animals

A. Sex steroids

Estrogen and progesterone are the most important physiological modulators to regulate the preovulatory LH surge. Both steroids have been shown to have positive and negative regulatory actions on reproductive functions at the hypothalamic level (for review, see Brann et al, 1991a). In ovariectomized rats, the injection or implant of estradiol is effective to induce partial LH surge and restore some reproductive functions. The negative regulatory role of estrogen has been demonstrated as that ovariectomy results in the elevation of LH secretion. The effects of estrogen on GnRH gene expression have been studied by several different laboratories and the results are, however, controversial. Estrogen has been reported to be stimulatory (Roberts et al, 1989; Kim et al, 1989; Rothfeld et al, 1989; Park et al, 1990), inhibitory (Zoeller et al, 1988; Toranzo et al, 1989; Wray et al, 1989b), or have no effect (Malik et al, 1991) on GnRH gene expression. The removal of gonadal steroid effects by ovariectomy or castration has also been reported to decrease (Roberts et al, 1989), increase (Toranzo et al, 1989), or have no effect (Kelly et al, 1989) on GnRH mRNA levels.

The possible explanation for this controversy is that estrogen may only exert its positive or negative effects on a certain population of GnRH neurons. Quantitative RNA assays (Blot assay, Northern blot, RNase protection assay) measure the sum of changes in GnRH gene expression from all GnRH neurons included in the study: changes in a certain percentage of neurons may be diluted by other neurons which have no change or exert opposite changes. Nevertheless, although in situ hybridization can detect the changes at single cell level, the hybridization efficiency, assay sensitivity, and regions being measured

can all play vital role in determining whether an appropriate measurement is being made.

Even though estrogen is thought to be the primary effector to induce the LH surge, it is evident that progesterone can facilitate the amplitude and the precise timing of the LH surge. It has also been demonstrated that progesterone can increase GnRH content, release, and its mRNA levels (for review, see Ramirez et al, 1985). However, long-term administration of progesterone also have inhibitory effects on the GnRH system (for review, see Brann et al, 1991).

B. Catecholamines: norepinephrine and dopamine

As described previously, it has been reported that the GnRH neurons do not concentrate estrogen in the cell nuclei (Shivers et al, 1983), nor have the estrogen receptors (Langub et al, 1991) or progesterone receptors (Fox et al, 1990). Thus the estrogen and progesterone effects on the GnRH neurons are most likely through the steroid-responsive interneurons which have synaptic contacts with the GnRH neurons. Many neurons, including the catecholamine, GABA, opioids, Neuropeptide Y neurons, are reported to be estrogen responsive and thus become the possible interneurons to mediate estrogen effects on the GnRH neurons.

Various reports have demonstrated that tyrosine hydroxylase (DH) and/or dopamine beta hydroxylase (DBH) immunopositive nerve terminals have synaptic contacts with GnRH neurons (Jennes et al, 1983; Leranth et al, 1988; Chen et al, 1989). This strongly suggests that the effects of catecholamines on GnRH neurons are mediated through direct synaptic action. It has been reported that the adrenergic neuronal system has stimulatory effect on GnRH release (Ojeda

et al, 1982; Hiney et al, 1991), primarily through the α -1 receptors (Berria et al, 1991; Gearing et al, 1991; Romeo et al, 1991) although the β -receptors are also reported to have moderate stimulatory effects (Romeo et al, 1991).

The effects of dopamine on GnRH release are controversial. It has been reported that dopamine can stimulate GnRH release from hypothalamic tissue fragments, but in *in vivo* studies, dopamine exerted an inhibitory effect on GnRH physiology (for review, see Weiner et al, 1988). The administration of apomorphine, a dopamine receptor agonist, decreased LH levels in ovariectomized rats (Jennes et al, 1983). It is of interest to elucidate whether these controversial results are due to the binding of different types of dopamine receptors of GnRH neurons or are mediated through activation of differential interneurons.

C. Opiates

It has been reported that opiates can suppress LH release, while the opiate receptor antagonist naloxone has stimulatory effects (Ieiri et al, 1980). This action is thought to be both on the level of the hypothalamus and the anterior pituitary. Some sexual behaviors of the female rat which are modulated by GnRH release can be inhibited by β -endorphin (Sirinathsinghji et al, 1983); LH release from cultured pituitary cells can be inhibited by morphine and endogenous opiate administration (Blank et al, 1986). The opiate inhibitory effects on GnRH release were thought to be mediated through inhibition of noradrenergic neurons which have stimulatory effects on the GnRH neurons. Recently, the observation of the synaptic contacts between GnRH and the β -endorphin-containing neurons has revealed another possibility that this inhibitory effects may be directly on GnRH neurons (Thind et al, 1988).

D. GnRH

A lot of evidence supports the possible auto-regulation or even autotfeedback mechanism by GnRH *per se* on the GnRH neuronal system (Valenca et al, 1987; Pelletier et al, 1987; Thind et al, 1988;). The administration of GnRH agonists into the third ventricle suppresses both the pulse amplitude and the frequency of basal GnRH release (Sarkar et al, 1987). The preovulatory LH surge was also suppressed by this manipulation and can be prevented by co-injection of GnRH antagonists (DePaolo et al, 1987). Although there is no strong evidence showing the involvement of endogenous GnRH on its secretion as part of the physiological control (Sarkar et al, 1987; Naylor et al, 1989), it has been shown that GnRH analogs inhibit GnRH secretion in vitro (Bourguignon et al, 1987). Using median eminence tissue from male intact rats, GnRH antagonists increased secretion of GnRH (Valenca et al, 1987). These data suggest that GnRH is somehow regulating secretion by GnRH neurons either through direct or indirect control.

E. Others

Reproductive functions can be suppressed by prolonged and repeated stress, part of this effect is proposed to be mediated through CRF control. This can be mediated through the stimulation of opioids or dopamine release, which have inhibitory effects on GnRH release; the suppression of those neurons which have stimulatory effects on GnRH release; or simply by direct excitation of the GnRH neurons (MacLusky et al, 1988). CRF administration was reported to have an inhibitory effect on LH release. This effect is found not to be modulated through β -endorphin, as shown by co-injection with the opiate receptor antagonist naloxone (Rivier et al, 1984). Direct synaptic connections between

CRF-containing axons and the GnRH neurons in the medial preoptic area have also been observed (MacLusky, 1988). These data suggest that CRF may be involved in the stress related inhibition of reproductive function by direct action on the GnRH neurons. In addition to CRF, it has also been reported that glucocorticoids can inhibit GnRH release or biosynthesis (Dubey et al, 1985) at the hypothalamic level (Brann et al, 1991b).

Many neurotransmitters and hormones other than the stress hormones have also been reported to play an important role in mediating GnRH functions: neuropeptide Y can stimulate GnRH release; the GABA system is thought to play a inhibitory role in mediating GnRH release (for review, see Weiner et al, 1988; Silverman, 1988). The administration of excitatory amino acids to stimulate different receptor subtypes including NMDA, kainate, and quinolinate receptors, results in a significant increase of GnRH secretion (Bouguignon et al, 1989). NMDA and kainic acid have also been observed to cause a rapid increase in GnRH mRNA levels (Peterson et al, 1991; Liaw et al, 1993; Gore et al, submitted).

The effect of some factors on GnRH neurons can also be mediated through indirect control. Some growth factors, including TGF- α and EGF, are reported to stimulate GnRH release, possibly mediated through the release of prostaglandin from glial cells (Ojeda et al, 1992). Whether this effect on the GnRH neurons is a glia-neuronal interaction or is mediated through interneurons is not yet clear. However, it is now well accepted that those substances which exert direct control on GnRH neurons are most likely to be released from those neurons which have synaptic contact with the GnRH neurons. Determination of those factors which can directly affect GnRH release is of importance because

this information will help to better understand the complex regulatory pathways of vertebrate reproduction at the hypothalamic level.

III. Second messengers and GnRH gene expression

A. Overview of the major second messenger pathways

The mechanisms by which aminergic and peptidergic hormones exert their specific functions on target cells are mostly mediated through binding to their membrane receptors which trigger the subsequent cellular responses. Numerous hormone membrane receptors have been identified and categorized into several different groups: the ligand-gated ion channels which can change the flow of specific ions through the cell membrane to alter the electric potential across the membrane; the ligand-triggered catalytic receptors, including several protein kinases and phosphatases, and guanylate cyclases; the G-protein coupled receptors which can indirectly act on several ion channels to change membrane potential or on membrane-bound enzymes to generate specific intracellular messengers. Two major second messenger pathways have been well characterized: the cyclic adenosine monophosphate (cAMP) which is generated by the activation of adenylate cyclase, and the diacylglycerol/phosphoinositol (DG/IP₃) which is generated by phosphoinositide-specific phospholipases. The activation/inhibition of these two second messenger pathways are found in many systems to regulate neuropeptide biosynthesis and gene expression (for review, see Karin, 1991), including GnRH (Ojeda et al, 1986; Lee et al, 1990).

B. The cAMP pathway (PKA pathway)

The elevation of intracellular cAMP levels by the stimulation of adenylate cyclase activity can directly regulate the activity of the cAMP-dependent protein kinase (PKA; for review, see Taylor et al, 1990; McKnight, 1991; Borrelli et al, 1992). In the absence of cAMP, PKA is in a tetramer form containing two inactive catalytic subunits (C-subunit) bound to two regulatory subunits (R-

subunit). The binding of two cAMP molecules to the two R-subunits causes the release of two active forms of the C-subunits to phosphorylate other cytoplasmic and nuclear proteins, resulting in a cascade of cellular responses.

The PKA pathway has been found to play an important role in regulating the expression of numerous genes at both the transcriptional and post-transcriptional levels. Several cAMP-inducible elements have been identified through promoter analysis. One of those, designated CRE (cAMP-response element), is found in many gene promoters to mediate cAMP-responsiveness. Numerous nuclear proteins have been identified which bind to the CRE element. They all share the same DNA binding motif (basic domain and leucine zipper) and are divided into several subfamilies based on regions of homology (for review, see Borrelli et al, 1992).

One of these CRE-binding proteins which was the first to be cloned and draws the most attention is the cAMP-responsive element binding protein, CREB (Montminy et al, 1987; Hoeffler et al, 1988; Sheng et al, 1991). Upon the elevation of intracellular cAMP levels, the activated PKA C-subunit can translocate into the nucleus and phosphorylate CREB which in turn acts as a transcription activator (Lamph et al, 1990; McKnight, 1991). At least two other CRE-binding factors which have sequence homology to the CREB protein, CREM (Foulkes et al, 1991) and CREB-2 (Karpinski et al, 1992), are found to inhibit this cAMP-induced transcription by antagonizing CREB function. It has also been found that some of these CRE-binding proteins can form heterodimers with factors from other groups of transcription factors (Hoeffler et al, 1990), such as protein factors from the FOS/JUN family, and bind to CRE or other DNA elements to function as transcriptional activators or inhibitors (for review, see Ziff,

1990; Lee, 1991; Borrelli et al, 1992). This interaction is thought to be important for the convergence of different intracellular signal transduction pathways to regulate gene expression at the transcriptional level.

In addition to the CRE element, several other DNA sequences have been identified which respond to the elevation of intracellular cAMP levels and either have stimulatory or inhibitory effects on gene transcription (Imagawa et al, 1987; Bernath et al, 1990). However, the mechanisms by which these elements respond to cAMP signals and the characteristics of the binding factors are less well-understood (for review, see Borrelli et al, 1992).

The elevation of intracellular cAMP levels to activate the PKA pathway can also regulate gene expression at the post-transcriptional level. The alteration of mRNA stability by PKA activation has been observed in several mRNA species, including tyrosine hydroxylase (Fossom et al, 1992) and the type 1 plasminogen activator inhibitor (Heaton et al, 1992) mRNA. Nevertheless, it has been reported that activation of the PKA pathway results in increased mRNA levels of the protein kinase A II β regulatory subunit (RII β) and surprisingly, no significant changes in the rate of RII β gene transcription nor mRNA stability were observed. Analysis of the nuclear RII β RNA precursors reveals that changes in the accumulation of precursor RNA in the nucleus is the major mechanism responsible for the changes in cytoplasmic mRNA levels in response to cAMP signals (Gross et al, 1990). To conclude, it appears that activation of the PKA pathway can regulate gene expression at several levels, including gene transcription, nuclear RNA processing and cytoplasmic mRNA stability. This may account for the complexity and multiplicity of PKA-mediated gene regulation.

C. The DG/IP₃ pathway (PKC pathway)

The intracellular signal transduction mechanisms mediating the actions of hormone receptors coupled to the DG/IP₃ pathway is by the activation of phosphoinositide-specific phospholipases to trigger the hydrolysis of membrane associated lipid phosphatidylinositol 4,5-bisphosphate (PIP₂) to produce two second messengers, diacylglycerol (DG) and inositol triphosphate (IP₃), which have subsequent cellular functions (for review, see Berridge, 1987). The binding of DG to the calcium-dependent protein kinase C (PKC) increases the affinity of PKC for calcium so that PKC becomes activated at the usually low cytoplasmic calcium concentrations. Meanwhile, the production of IP₃ also causes the elevation of cytosolic Ca⁺⁺ levels by releasing Ca⁺⁺ from intracellular storage pools and thus promotes the activation of PKC. There are several PKC subtypes identified to be Ca⁺⁺-independent, whose activation can be triggered by binding of phospholipids (for review, see Kikkawa et al, 1989; Kiley et al, 1990). The increase of intracellular Ca⁺⁺ levels can also activate another protein kinase, the Ca⁺⁺/calmodulin-dependent kinase (CaM kinase), which can also exert multiple actions.

The PKC pathway plays an important role in mediating cellular functions and proliferation (for review, see Nishizuka, 1986; Kikkawa et al, 1986) and has been identified in several systems to regulate gene expression, including several proto-oncogenes and cellular genes (for review, see Herschman, 1991). The mechanisms by which PKC acts on regulating gene expression have been extensively studied. The activation of the PKC pathway by tumor promoter phorbol esters, which mimic the action of DG to activate PKC, can induce the expression of the c-fos and c-jun genes. The products of these genes, c-FOS and c-JUN proteins, can form dimers and induce activities of promoters

containing AP-1 (FOS/JUN) enhancer elements (Angel et al, 1987; Curran et al, 1988; Chiu et al, 1988). The FOS/JUN proteins belong to a large family of DNA-binding proteins which can interact with factors from the same family or members from other families and exert different transcriptional regulatory functions (for review, see Hoeffler et al, 1990). For example, JUN-B, an immediate member of the JUN family, does not act as a transcriptional activator on promoters containing the AP-1 element. In contrast, the stimulatory effect of c-JUN on this element is inhibited by JUN-B (Schutte et al, 1989; Chiu et al, 1989). Nevertheless, if a promoter contains multimeric AP-1 elements, JUN-B can effectively activate its transcription (Chiu et al, 1989). It has also been reported that glucocorticoid receptor can interact with FOS protein (and probably with other members of the family), to regulate gene transcription (Mordacq et al, 1989; Kerppola et al, 1993). Furthermore, phosphorylations and/or modifications at different sites of the FOS/JUN proteins can change their functions from transcription activators to transcription repressors (Gius et al, 1990; Ofir et al, 1990).

The transcriptional effects of the activation of PKC pathway can also be mediated at DNA sequences other than the AP-1 element. It has been found that phorbol esters can down-regulate globin gene expression through DNA sequences located in its 3' flanking region where no consensus AP-1 element was found (Lumelsky et al, 1991). Phorbol ester administration also decreases the transcription of atrial natriuretic factor gene through a novel element which is thought to mediate tissue-specific expression (McBride et al, 1993). Activation of the PKC pathway can also regulate tyrosine hydroxylase (Vyas et al, 1990) and globin (Lumelsky et al, 1991) gene expression at the post-transcriptional level.

However, the mechanisms by which PKC exerts these functions are not well understood.

D. Proto-oncogenes and GnRH neuronal activation

The expression of proto-oncogenes is found to be very rapidly stimulated in many neuronal tissues and cells in response to exogenous stimulation, including membrane depolarization and trans-synaptic activation (for review, see Sagar et al, 1988; Sheng et al 1990). Many of the products of these proto-oncogenes have specific DNA binding activities and can potentially regulate the expression of many genes. The induction of *c-fos* gene can be mediated through several second messenger pathways and it has been found that it can also be induced by membrane depolarization and trans-synaptic activation in neuronal systems (Mehmet et al, 1988; Sheng et al, 1990). Therefore, the level of *c-fos* expression can be used as an indication of neuronal activity in response to exogenous stimulation. Since the basal levels of c-FOS protein are normally very low in neuronal cells, c-FOS immunoreactivity can thus be used to mark those neurons which are being activated (for review, see Hoffman et al, 1993).

Fos immunoreactivity was not detected in GnRH neurons during most of the estrous cycle when they exert basal pulsatile secretory property. However, on the afternoon of proestrus at the time of the LH surge (Lee W-S et al, 1990) and after steroid treatment to induce the LH surge (Hoffman et al, 1990), robust *Fos* immunoreactivity is detected in the GnRH neurons. The number of GnRH neurons expressing FOS is dramatically reduced when the LH surge is blocked by pentobarbital (Hoffman et al, 1993). These data strongly suggest a correlation between GnRH neuronal activity and *c-fos* gene expression since the LH surge is induced by a large release of GnRH peptide from GnRH neurons, presumably

through the synchronization of neuronal firing. Thus the measurement of c-fos mRNA or c-FOS protein levels in GnRH neurons can be a useful tool for an indication of activation of GnRH neurons in response to trans-synaptic stimulation (Hoffman et al, 1990; Hoffman et al, 1993). It has been reported that not all GnRH neurons projecting to the median eminence are activated during the LH surge, in particular those GnRH neurons located above the anterior commissure and those which are rostral to the OVLT, as indicated by c-FOS immunoreactivity (Hoffman et al, 1993). The finding that they can be activated after electrical stimulation suggests that these GnRH neurons may be activated under other physiological conditions (Hoffman et al, 1993). However, the functions of these GnRH neurons remain to be elucidated.

E. The role of second messengers in GnRH regulation

As described in the previous sections, many neurotransmitters and hormones are reported to exert effects on GnRH release and gene expression. Some of these effects are through direct trans-synaptic actions of these factors to activate the receptor-mediated second messenger mechanisms and trigger the subsequent cellular functions. It is known that the binding of ligands to the β -adrenergic, dopaminergic, opiate, or CRF receptors can either increase or decrease intracellular cAMP levels. The activation of alpha-1-adrenergic, NPY, and GnRH receptors can result in the activation of the DG/IP₃ pathway. In addition, the activation of neurotransmitter receptors for GABA and excitatory amino acids can also result changes in membrane electric potentials.

Indeed, direct activation of several second messenger pathways has been found to be important for the regulation of GnRH release and gene expression (for review, see Negro-Vilar et al, 1987). Both intracellular and extracellular

calcium levels are important for the stimulatory effect of prostaglandin E₂ on GnRH release (Ojeda et al, 1985a,b). The PKA pathway has been implicated in mediating progesterone effects on GnRH regulation (Ramirez et al, 1985). Activation of the PKA pathway by increasing intracellular cAMP levels increases LH release in vivo (Ojeda et al, 1974), GnRH secretion in vitro (Kim et al, 1985; Ojeda et al, 1985c; Ojeda et al, 1986; Negro-Vilar et al, 1986), and GnRH mRNA levels (Lee et al, 1990). Activation of the PKC pathway results in increased GnRH secretion from the median eminence in vitro (Valenca et al, 1985; Ojeda et al, 1986; Valenca et al, 1988; Wetsel et al, 1989) and mRNA levels in rat hypothalamus (Lee et al, 1990).

IV. Hypothalamic neuronal GnRH-expressing GT1 cell line

A. Production and characteristics

As described above, while the physiological functions of those factors regulating GnRH release have been extensively studied, the cellular and molecular mechanism of their action remains difficult to elucidate using the present *in vivo* whole animal and the *in vitro* median eminence or hypothalamic neuron culture models. The GnRH neurons are not tightly organized in groups of nuclei, like other secretory neurons. They are rather sparsely localized in the forebrain with low abundance. As previously described, many sensitive assays have been developed to overcome the difficulty of measuring the level of GnRH RNA transcripts in hypothalamic tissues, including quantitative *in situ*-hybridization (Peterson et al, 1991) and solution hybridization/RNase protection assay (Jakubowski et al, 1992). However, the complex connections between GnRH and neighboring neurons still make it very difficult to determine whether the observed effects are via direct synaptic control on GnRH neurons or are indirect effects. Nevertheless, in order to conduct detailed biochemical and molecular analyses to better understand the cellular and molecular mechanisms mediating GnRH secretion, processing, and biosynthesis under basal and stimulated conditions, a source of large quantities of homogeneous population of GnRH neurons will be needed.

Simian virus 40 (SV40) large T antigen (TAg) was first discovered to be responsible for its viral transformation ability and was later characterized to have many other functions, such as stimulation of DNA replication, activation and repression of viral gene transcription, and tumor induction (McCormick et al, 1979). Although the mechanisms by which TAg transforms normal cells into tumor cells are not yet well understood, it presumably interacts with cellular

growth-regulating factors to interfere with normal growth (Ludlow et al, 1990). This extremely potent oncogene has been used to develop tumor cell lines from various tissues for research use, particularly those which rarely have naturally occurring tumors. By genetically targeted tumorigenesis using a hybrid DNA construct containing the promoter regions which are essential to exert the specific expression pattern of a certain gene in the desired tissue and fused to the coding regions of the oncogenic protein SV40 T antigen, one can produce targeted tumors in many tissues using transgenic mice technology (Hanahan, 1989).

Two hypothalamic GnRH-expressing cell lines were produced using this method, thus creating systems which circumvent many limitations of using animal tissues as described above. These are the GT1 cells, produced by a 2.3-kb rat GnRH promoter-driven SV40 T antigen gene (Mellon et al, 1990), and the GN cells, produced by a 1.1 kb human GnRH promoter-driven SV40 T antigen gene (Radovick et al, 1991). The GT1 cells have been extensively characterized (for review, see Weiner et al, 1992) and have been used in this laboratory as an *in vitro* model for studying the regulation of GnRH release and gene expression. They extend neurites and exhibit several features resembling neuronal cells. The mRNA of various neuronal markers, including a neuro-specific enolase, the neurofilament protein, and several proteins associated with neurosecretory and synaptic membrane functions, including synaptic membrane-associated protein VAMP-2, synaptosomal protein SNAP-25, neuroendocrine secretory vesicle protein chromogranin B, and the GnRH/GAP prohormone were also detected in the GT1 cells (Mellon et al, 1990). GnRH-like immunoreactivity was found in the secretory vesicles in the GT1 cells (Liposits et al, 1991) and demonstrated to be the GnRH peptide (Wetsel et al, 1991). It has been reported that the

intra-hypothalamic injection of GT1 cells into the hypogonadal (hpg) mice was sufficient to result in reversal of hypogonadism, presumably through the secretion of biologically active GnRH peptide from these cells (Silverman et al, 1992). It has also been observed that these GT1 implants can respond to NMDA challenge resulting in stimulation of LH secretion (Miller et al, 1993)

B. Regulation of GnRH secretion and gene expression in the GT1 cells

It has been observed that the GT1 cells have intrinsic pulsatile secretory activity under basal condition (Martinez de la Escalera et al, 1992; Wetsel et al, 1992; Krsmanovic et al, 1992). GnRH release can also be stimulated in response to various exogenous stimuli, including depolarization (Mellon et al, 1990), the administration of pharmacological compounds to stimulate intracellular second messenger pathways (Bruder et al, 1992; Wetsel et al, 1993; Yu et al, submitted), and numerous neurotransmitters and hormones, including activin (Gonzalez-Manchon et al, 1991), endothelin (Krsmanovic et al, 1991), dopamine (Martinez de la Escalera et al, 1992), and norepinephrine (Weiner et al, 1992; Findell et al, 1993). In addition, the GT1 cells are also the first neuronal cell line identified to express GABA-A receptor subunits (Hales et al, 1992), suggesting a potential GABA effect. The presence of GnRH receptor in the GT1 cells (Krsmanovic et al, 1993), together with the findings that GnRH agonists and antagonists clearly affect GnRH secretion in both perfusion (Krsmanovic et al, 1993) and static incubation (Yu et al, submitted) systems indicate that GnRH may also exert ultrashort loop feedback effects in the GT1 cells.

As described earlier in this chapter, many aminergic and peptidergic neurotransmitter/hormones are known to have effects on GnRH release and gene expression in the *in vivo* GnRH system. The evidence for direct synaptic

contacts of these aminergic and peptidergic neurons with the GnRH neurons and the fact that some of these neurotransmitters/hormones also exert effects on GnRH secretion in the GT1 cells strongly suggest that these are the factors which can directly regulate GnRH functions in the animal. The effects of these factors are presumably mediated through direct binding to membrane receptors and/or ion channels coupled with various intracellular second messenger systems to trigger subsequent cellular and molecular events on GnRH release and gene expression. Thus, the understanding of the effects and mechanisms of the stimulation of each individual second messenger pathway on GnRH release and its gene expression will help to obtain the fundamental knowledge of the cellular and molecular components responsible for GnRH regulation.

It has been reported that the stimulation of diacylglycerol/protein kinase C pathways with phorbol esters in the GT1 cells acutely increased GnRH release, and this stimulation declined to baseline by 24 hours (Bruder et al, 1992). Similar phenomenon were observed in GT1 cells treated with forskolin, phorbol ester, or ionomycin to stimulate the intracellular cAMP/protein kinase A (PKA), diacylglycerol/protein kinase C (PKC) and Calcium/calmodulin-dependent kinase ($\text{Ca}^{++}/\text{CaM}$) pathways in the GT1 cells, respectively (Wetsel et al, 1993; Yu et al, submitted). Time course analysis shows that the attenuation of secretion is in parallel with the decrease in mRNA levels (Bruder et al, 1992; Wetsel et al, 1993; Yu, submitted). It has also been suggested that this decrease in GnRH mRNA levels is in part due to inhibited transcription (Wetsel et al, 1993). The mechanisms responsible for this decrease will be discussed in detail in the following chapters of this dissertation thesis.

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Chapter II

Materials and Methods

Materials

DRB (5,6-dichloro-1- β -ribofuranosylbenzimidazole), 8-BrcAMP (8-bromo-adenosine 3':5'-cyclic monophosphate, sodium salt), nucleoside 5' diphosphokinase, RNase A, and RNase T1 were purchased from SIGMA. PMA (phorbol, 12-myristate, 13-acetate), forskolin, actinomycin D, and cycloheximide were purchased from Calbiochem. DNase I was purchased from Worthington Biochemical. Proteinase K was purchased from Boehringer Mannheim GmbH. Restriction enzymes were purchased from New England Biolab. RNA polymerase T3, T7, and Sp6 were purchased from Stratagene or Promega. RNasin, Taq polymerase, and polymerase buffer were purchased from Promega. Dulbecco's Modified Eagle's Medium powder was purchased from Gibco. Fetal calf serum was purchased from Gemini Bioproducts. Penicillin/Streptomycin solution was purchased from JRH Biosciences.

Cell culture

Two sublines derived from a mouse hypothalamic neuronal GnRH-secreting tumor (Mellon et al, 1990), the GT1-3 and GT1-7 cells, were utilized for the present studies. Cells were grown in DMEM supplemented with 10% fetal bovine serum and antibiotics (100 units/ml penicillin and 100 MCG/ml streptomycin) and maintained at 37C with 5% CO₂. Cells were subcultured into either individual tissue culture dishes or multi-well plates 2-3 days before experiments and allowed to grow to about 60-70% confluency. Unless otherwise noted, triplicate dishes were used for all the experiments.

RNA Isolation--RNA isolation was based on the method described by Blum (Blum, 1989) and Jakubowski (Jakubowski et al, 1992; Jakubowski et al, submitted) with slight modification. Cells were lysed in 500 μ l of lysis buffer (10 mM Tris-HCl pH 7.5, 1.5 mM MgCl₂, 0.3 M sucrose, 0.5% NP-40, 0.25% sodium deoxycholate) and carefully overlaid onto 350 μ l of cushion buffer (10 mM Tris-HCl pH 7.5, 1.5 mM MgCl₂, 0.4 M sucrose). Cytoplasmic and nuclear portions were separated by centrifugation. The cytoplasmic portion was treated with proteinase K followed by phenol/chloroform and chloroform extraction and ethanol precipitation at -20C. The nuclear portion was resuspended in 300 μ l of DNase buffer (10 mM Tris-HCl pH 7.4, 0.5 M NaCl, 5 mM MgCl₂, 0.1 mM CaCl₂) containing DNase I (40 units) and RNasin (20 units) and incubated at 37C for 10 minutes to break large DNA fragments followed by proteinase K digestion, phenol/chloroform extraction, and ethanol precipitation. Following precipitation, RNA samples were centrifuged at 4C, and washed with 70% ethanol. The pellets were resuspended in 1X TE, quantitated by absorbance at 260 nm, and aliquoted into 20 μ l final volume with hybridization buffer (4 M guanidine monothiocyanate, 0.1 M EDTA pH 7.5). Nuclear RNA used for reverse transcription assay was treated with DNase for an additional 30-45 minutes followed by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation as described by Jakubowski and Roberts (Jakubowski et al, submitted).

Determination of RNA processing pattern by reverse transcription-polymerase chain reaction (RT-PCR)

Reverse transcription coupled with polymerase chain reaction was based on previously described methods (Jakubowski et al, submitted). Nuclear RNA isolated from GT1-3 cells was reverse transcribed into first strand cDNA by M-MLV reverse transcriptase using oligo dT as primer. The reaction mix was

brought up to final volume 100 μ l after the reaction. 2.5 μ l of cDNA and 0.5 μ g of oligodeoxy-nucleotides primer pairs against various regions in the GnRH gene, as illustrated in Figure 1 (Chapter III), were used for each PCR reaction. Sequences of the primers are as follow:

a, GAAGCTTGGTATCCCTTTGGCTTTCACATCC; b, TACCTCTGCAGTTTCTGTGAAGCC; c, TATCTAGAAGGCTGCTCCAGCCAGC; d (KW025), TCAAGCTTGTCCAAAGTGCAAGGAGG; e , CAGGATCCACAGACAAGTCTTGGTG; f , GGTCTGACAGTGCATTCGAAGTTCTGGGGTTCT; g , GTCTGCAGTGGACATGCTGTGAGGT; h , ATGAATTCACAACACATTACTTTATTATG.

The PCR reactions were performed using the following program: denature at 94C for 4 minutes, then 30 cycles of the following steps: denature at 94C for 30 seconds, anneal at 55C for 1 minute, prime at 72C for 2 minutes; after the final cycle the PCR products were refrigerated at 4C and run in 2% agarose gels. In order to obtain better resolution, 1 μ l of alpha-³⁵S-dATP was used in part of the assays to obtain radiolabelled PCR products and run in composite gels containing 0.5% agarose and 2% acrylamide.

Preparation of DNA constructs—A DNA fragment spanning mouse GnRH intron A/exon 2/intron B (A2B probe) was generated by PCR using oligodeoxy-nucleotide primer pairs based on the published genomic sequence (Mason et al, 1986) with DNA primers (sequences are described as above) B and D and subcloned into bluescript (SK) vector using the PstI and HindIII restriction sites contained within the primers. A DNA template spanning mouse GnRH intron B/exon 3/intron C (B3C probe) was obtained using the same strategy with oligodeoxynucleotide primer pairs E and G. DNA template for rat GnRH 5'/exon 1 (rat 5' probe), generously provided by Dr. Ke Wen Dong, was generated by PCR using oligodeoxynucleotide primers complementary to regions around the

first EcoR1 site 5' to exon 1 and the Hind III site in exon 1 which does not cross react with the mouse gene, and subcloned into bluescript vector. Cyclophilin template, generated by Dr. James Douglass, represents the first 111 bp PstI and XmnI fragment of the coding region of the 680 bp 1b15 cDNA clone, and c-fos template was generated by Dr. Dominique Lorang. All DNA constructs were banded by CsCl₂ gradient and sequenced by double strand DNA sequencing. For *in vitro* transcription, A2B and B3C DNAs were cut by SpeI or HindIII to create templates for antisense or sense RNA probes, respectively; rat 5' DNA was cut with EcoR1 or Hind III and cyclophilin with EcoRI or BamHI for antisense and sense probes, respectively. DNA templates were gel-purified by GeneClean kit (Bio 101), resuspended in TE, and refrigerated before use. The mouse GnRH promoter/ luciferase construct, provided by Dr. Ke Wen Dong, was made by subcloning a 3.4 kb mouse GnRH promoter/exon 1 fragment (-3446 to +23, generously provided by Dr. A. Mason), cut with Hind III and Xho 1, into a luciferase/pxp2 vector (generously provided by Dr. J. Licht).

Solution hybridization/ribonuclease protection assay (RNase protection assay)

The protocol for RNase protection assay is based on previously described methods (Blum, 1989; Jakubowski et al, 1992). ³²P-labeled antisense RNA was transcribed using an *in vitro* transcription kit (Promega) and ³²P- α -UTP followed by 30 minutes of DNase digestion and then ethanol precipitation. RNA probes were resuspended in hybridization buffer and the specific activity was determined by spotting a 1 μ l aliquot onto DE81 paper followed by 0.5 M NaPi wash and counting. Sense standard RNAs were made following a similar protocol with low-specific activity radiolabel with an additional ethanol precipitation and quantitated by absorbance at 260 nm. Aliquots of standard RNA were stored at -80C until

use. Yeast tRNA was added into the tubes with the standard RNA to make their final RNA concentration comparable to those of the samples. 1 ng riboprobe in a 5 μ l final volume was added to each standard and sample tube. Samples were denatured at 56C for 10 minutes and hybridized at 30C overnight (about 12-16 hours). Samples were then digested with 300 μ l buffer with RNase (10mM Tris pH7.5, 5mM EDTA, 0.3M NaCl, 40 mg/ml of RNase A and 2 mg/ml of RNase T₁) at 30C for 1 hour followed by proteinase K digestion, phenol/chloroform extraction and ethanol precipitation. Samples were centrifuged, washed with 70% ethanol, and pellets were resuspended in 1.5X Ficoll loading dye and run onto either 4% (nuclear RNA samples) or 6% (cytoplasmic RNA samples) acrylamide gels. The amount of mouse GnRH, cyclophilin, and c-fos RNA fragments protected was quantitated by either exposing the gels against X-ray film, cutting out the bands, and counting in a scintillation counter; or exposing the gels against a phosphorimager screen (Molecular Dynamics, Sunnyvale, CA). The amount of RNA in each protected band was calculated by normalizing the amount of radioactivity (cpm by the X-ray film method or relative intensity unit by phosphorimager) to the standard curve generated by a regression program.

Nuclear run-on assay

Nuclear run-on assay was performed according to procedures described by Blum (Blum, 1989). DNA filters were made by loading 1 mg of desired DNA fragment onto nitrocellulose filters and fixed by baking at 80C under vacuum for 2 hours. GT1 cell nuclei were isolated and the *in vitro* transcription was carried out with 300 μ Ci of ³²P-labeled UTP to allow the DNA-bound RNA polymerases to continue transcription. The ³²P-labeled RNA was hybridized to DNA filters containing both GnRH A2B fragment to compare the levels of GnRH gene transcription under basal condition or PMA administration, and pBR 322 DNA to

determine the background. The hybridization was carried out at 42C for four days, then filters were washed, dried, and exposed against the phosphorplates. The intensity of the signal was read through the phosphorimager and expressed as relative intensity units.

Transfection assay

5 µg of banded mouse GnRH promoter/luciferase DNA was transfected into the GT1 cells by the calcium phosphate precipitation method (Ausubel et al, 1989) with slight modification. The final calcium concentration added was 15.6 mM. After overnight (12 to 16 hours) incubation, cells were washed with DMEM 3 times to remove excess calcium from the cells. Either vehicle or drugs was then added to the dish with DMEM supplemented with 10% fetal calf serum for 20 to 24 hours. Cells were washed three times with PBS and lysed in lysis buffer, spun down at 2500 rpm at 4C for 10 minutes, and part of the supernatant was added into the assay buffer. Luciferin was added right before measurement. Luciferase activity was measured using a luminometer (Lumat LB9501) and expressed as relative light units (RLU). Protein content of the cellular extracts was determined by the Bradford method (Bio-Rad) for standardization of the luciferase activity.

Treatment of the GT1 cells

Triplicate GT1-3 and GT1-7 cells were grown to 60-80% confluency in 6-well dishes and were changed into DMEM one hour prior to the treatments. Actinomycin D, cycloheximide, and 8-BrcAMP were dissolved in sterile water and DRB, PMA, and forskolin were dissolved in DMSO, and diluted to 100X with culture media. Cells were harvested at different time points and RNA was isolated for RNase protection assay.

Data analysis

Statview computer program was used for all the data analysis in this study. Levels of specific nuclear and cytoplasmic RNA transcripts were converted from standard curve by a linear regression analysis and expressed as pg protected fragments/well or pg protected fragments/ μ g RNA. Data were analyzed with one-way or two-way analysis of variance (ANOVA) followed by Fisher's PLSD post-hoc analysis. The level of significance was set at $p < 0.05$.

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Chapter III

Characterization of Gonadotropin Releasing Hormone Gene Transcripts in a Mouse Hypothalamic Neuronal GT1 Cell Line

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Abstract

We have characterized the basic features of nuclear and cytoplasmic RNA transcripts derived from the gonadotropin releasing hormone (GnRH) gene in a mouse hypothalamic neuronal GT1 cell line. Analysis of nuclear GnRH RNA precursors present in the GT1 cells by reverse transcription coupled with polymerase chain reaction (RT-PCR) and by solution hybridization/ribonuclease protection assay (RNase protection assay) shows that there is no absolute order of intron excision, suggesting the existence of multiple processing pathways. Quantitative RNA analysis shows that GnRH primary transcript comprises more than 90% of the total nuclear GnRH RNA precursors, therefore, probes containing the intervening sequences of the GnRH gene will detect largely the primary transcript in the GT1 cells. We have thus measured the decay rates of GnRH nuclear primary transcript and cytoplasmic mRNA in the presence of either of the RNA synthesis inhibitors actinomycin D (Act D) or DRB (5,6-dichloro-1- β -ribofuranosyl-benzimidazole) and calculated the half-lives. We found that GnRH primary transcript is relatively short-lived (half-life about 18 minutes), while mature mRNA in the cytoplasm appears to be very stable (half-life >24 hours). In addition, decay from steady-state kinetics measurements confirmed that GnRH mRNA has a relatively long cytoplasmic half-life. To conclude, we have defined a model system for the analysis of GnRH nuclear and cytoplasmic RNA transcripts in GT1 cells which will provide useful tools for further understanding of the regulation of GnRH gene expression along its RNA synthetic pathway in response to hormonal treatment.

Introduction

Gonadotropin releasing hormone (GnRH) is a hypothalamic decapeptide which regulates vertebrate reproductive functions (Silverman, 1988). Numerous substances have been identified to regulate GnRH physiological functions by modulating GnRH secretion (for review, see Weiner et al, 1988). The cDNA for GnRH gene was first isolated from a human placental cDNA library (Seeburg et al, 1984) and subsequently cloned in human (Adelman et al, 1986; Radovick et al, 1990), rat (Adelman et al, 1986; Adelman et al, 1987; Bond et al, 1989) and mouse (Mason et al, 1986). The availability of cDNA probes for GnRH has enabled one to assess the pre-translational regulation of this key neuroendocrine peptide. The changes in GnRH mRNA levels in the hypothalamus in response to several substances have been addressed (Roberts et al, 1989; Park et al, 1990; Lee et al, 1990; Peterson et al, 1991; Jakubowski et al, 1991; Bergen et al, 1991; Weesner et al, 1992; Marks et al, 1993). However, it remains to be elucidated whether the reported changes in GnRH mRNA levels are nuclear (e.g. transcription, processing) or through cytoplasmic events (e.g. stability).

The measurement of nuclear RNA precursors of a specific gene has been utilized in many systems to elucidate the nuclear site of hormonal action (Chrapkiewicz et al, 1982; Granner et al, 1983; Narayan et al, 1985; Herman et al, 1991; Gaspar et al, 1991; Giddings et al, 1991; Strauss et al, 1993). Probes specific for the intervening sequences of a given gene in combination with either *in situ* hybridization or RNase protection assay to measure nuclear primary transcript levels have been widely used for studying the changes in gene transcription *in vivo* (Freneau et al, 1986; Freneau et al, 1989; Lipson et al, 1989; Blum, 1989; Herman et al, 1991; Herman et al, 1992). In order to identify

the intervening sequences which are present only in the GnRH primary transcript, we have characterized the processing pathways of GnRH nuclear precursor RNA in rat brain (Jakubowski et al, submitted). We found that the second intron (intron B) was the first to be spliced out in the process of producing mature RNA. Thus, we designed a probe against this intervening sequence that accurately reflects the level of GnRH primary transcript without detecting the processing intermediates and studied the changes of GnRH primary transcript in response to several conditions (Jakubowski et al, submitted; Gore et al, submitted). This assay system allows us to directly address the transcriptional effect of GnRH gene regulators.

The use of animal models and tissues has advanced our understanding of the regulation of GnRH secretion and gene expression and the intron-specific probes have enabled us to study its regulation at the level of gene transcription. However, the low abundance and sparse distribution of GnRH neurons, combined with the complexity of neuronal inputs and cellular compositions surrounding the GnRH neurons, have limited the use of the *in vivo* GnRH neuronal system for delineating the cellular and molecular mechanisms involved in the regulation of GnRH gene expression. The production of an immortalized mouse hypothalamic GnRH-secreting neuronal cell line, the GT1 cells (Mellon et al, 1990), has provided a system where these questions can be directly addressed on a pure population of GnRH neuronal cells. Using a similar approach as we have developed in the rat GnRH model, we have developed probes for the measurement of the mouse GnRH primary transcript, processing intermediates, and cytoplasmic mRNA levels. Moreover, we have taken advantage of the cell line model to analyze the basic characteristics of the nuclear and cytoplasmic GnRH transcripts and established an assay system by

which the transcriptional and post-transcriptional control of GnRH gene expression can be directly addressed.

Results

Analysis of GnRH nuclear RNA precursors in the GT1 cells by RT-PCR

The GnRH nuclear RNA precursors (heteronuclear RNA transcripts) present in the GT1 cells were identified by reverse transcription coupled with polymerase chain reaction (RT-PCR). Pairs of oligodeoxynucleotide primers complementary to various regions in the GnRH genomic sequence which correspond to individual introns or exons were used in PCR reaction to amplify first-strand cDNA reverse-transcribed from nuclear RNA isolated from GT1-3 cells. The presence of nuclear GnRH RNA precursors can be identified using primers spanning either intron-exon or intron-intron junctions as illustrated in Figure 1a. The use of intron probe in at least one of the pairs circumvents the interference of fully processed mature RNA (nuclear mRNA). Primer pairs were designed (as shown in Lanes 1-5) to detect the primary transcript and the combination of all possible processing intermediates present in the GT1 cells. The first intron to be spliced out of the GnRH primary transcript can be determined by comparing the relative splicing orders of adjacent introns. That is, if intron A is the first intron to be spliced out, we should not expect to detect any nuclear GnRH precursor containing intron A except the primary transcript. Therefore, only the processing intermediates in Lane 2 (330 bp) but not in Lane 1 (450 bp) or Lane 3 (280 bp) would be detected. If intron B is spliced out first, we should expect to detect those processing intermediates in Lane 1 (450 bp), Lane 3 (280 bp), Lane 4 (300 bp), but not in Lane 2 (330 bp) or Lane 5 (360 bp). If intron C is spliced out first, we should not expect to detect those processing intermediates in Lane 1 (450 bp), Lane 4 (300 bp). All possible GnRH RNA precursors using this set of primers were detected in GT1-3 cells (Figure 1b), including the primary transcript (A-2-B-3-C, lane 1, upper band) and several

processing intermediates with at least one of the introns excised: the transcripts containing intron B but no intron A (1-2-B , lane 2, lower band), containing intron A but no intron B (A-2-3 , lane 3, lower band), containing intron B but no intron C (B-3-4, lane 5, lower band), and the transcript containing intron C but no intron B (2-3-C, lane 4, lower band).

Analysis of GnRH heteronuclear RNA transcripts in the GT1 cells by RNase protection assay

The presence of multiple GnRH nuclear RNA precursors in the GT1-3 and GT1-7 cells was also determined by solution hybridization/ribonuclease protection assay (RNase protection assay) using intron/exon/intron probes which can identify multiple GnRH RNA species (Blum, 1989). Two DNA fragments spanning either intron A/exon 2/intron B or intron B/exon 3/intron C of the genomic sequence of the mouse GnRH gene were synthesized by PCR and subcloned into vectors as the templates for *in vitro* transcription of RNA probes. Nuclear RNA precursors, including the primary transcript, several processing intermediates and nuclear mRNA containing regions spanning the intron A/exon 2/intron B junction of the GnRH gene will be protected by the intron A/exon 2/intron B probe (A2B probe) and be identified by different sizes when electrophoresed through acrylamide gels, as illustrated in Figure 2a. This includes RNA transcripts containing intron A/exon 2/intron B, exon 2/intron B, intron A/exon 2, and exon 2. The same approach can also be applied to the measurement of RNA transcripts containing regions spanning intron B/exon 3/intron C junction with the intron B/exon 3/intron C probe (B3C probe) which will detect the presence of the following RNA species: intron B/exon 3/intron C, exon 3/intron C, intron B/exon 3, and exon 3. As shown in Figure 2b, the A2B probe identified four nuclear species in GT1-7 cells with sizes similar to those predicted

for the putative GnRH RNA precursors, indicating the presence of different RNA species containing both intron A and intron B, containing only one of the introns, and without either intron. B3C probe gives two major bands corresponding to the full-length (intron B/exon 3/intron C, 297 bp), exon 3 (96 bp), and three minor bands with sizes ranging from 170-240 bp. When compared to the protection pattern produced by B3C probe alone, one of the bands corresponds to the background produced by the B3C probe (data not shown) while the other two correspond to the correct sizes of the predicted precursor RNA containing only intron B (172 bp) or intron C (221 bp). None of the intron-containing species was detected in the cytoplasm, indicating that all of these GnRH RNA precursors are nuclear species. Similar results were observed in the GT1-3 cells (data not shown).

Quantitation of nuclear and cytoplasmic GnRH RNA transcripts in GT1 cells by RNase protection assay

Nuclear and cytoplasmic RNA isolated from GT1-7 cells was used for the measurement of the levels of nuclear and cytoplasmic GnRH RNA transcripts. The amount of each protected fragment was derived from a linear standard curve run concurrently with the samples. As shown in Table 1, RNA transcripts containing intron A/exon 2/intron B (383 bp), exon 2/intron B (297 bp), intron A/exon 2 (230 bp), and exon 2 (144 bp) comprise 11%, <1%, <1%, and 88% of the nuclear transcripts, respectively, and RNA transcripts containing intron B/exon 3/intron C (297 bp), exon 3/intron C (221 bp), intron B/exon 3 (172 bp), and exon 3 (96 bp) comprise 13%, <1%, <1%, and 86% of the nuclear transcripts, respectively. Since the levels of processing intermediates in both assays using either probe do not comprise more than 10% of the total heteronuclear transcripts containing at least one intron, the full-length protected

fragment from either probe can thus reflect accurately the level of GnRH primary transcript. Similarly, the protected fragments containing only one exon are predominantly the nuclear mature mRNA. In the GT1 cells they comprise less than 5% of the total GnRH transcripts, leaving the cytoplasmic GnRH mRNA the predominant RNA species.

Half-life of GnRH primary transcript in GT1 cells determined by the RNA synthesis inhibitor Act D and DRB

To determine whether the RNA precursors we have observed in the steady-state measurement can undergo further processing and to measure the half-life of GnRH primary transcript for further understanding of the kinetics of GnRH RNA synthesis/turnover, the decay of GnRH primary transcript was measured in GT1 cells by the administration of either of two different RNA synthesis inhibitors Act D or DRB. GT1-7 cultures were treated with either Act D (5 $\mu\text{g/ml}$) or DRB (100 $\mu\text{g/ml}$) and harvested at various time points after drug treatment. Nuclear RNA was isolated and the levels of GnRH primary transcript were measured with either A2B or B3C probes. There was a rapid time-dependent decrease in the level of primary transcript followed by the disappearance of RNA species containing only one intron (processing intermediates), indicating that these RNA species can undergo further processing (data not shown). The half-life of a specific RNA can be obtained by plotting the log of the amount of protected RNA fragment against time of drug treatment (Kessler, 1993). Using this calculation, the measurement by either A2B (Figure 3a) or B3C (Figure 3b) probe resulted similar half-life for GnRH primary transcript (about 18 minutes) when either Act D or DRB was used. Similar results were observed in the GT1-3 cells (data not shown).

Half-life of GnRH, rat GnRH/T antigen and cyclophilin mRNA in the GT1 cells as determined by RNA synthesis inhibition

The decay of cytoplasmic GnRH mRNA in the GT1 cells was measured in the presence of two different RNA synthesis inhibitors Act D and DRB in order to determine its half-life. In addition, two other mRNAs, the endogenous rat GnRH promoter driven SV40 T antigen (rat GnRH/TAg, Mellon et al, 1990) mRNA, and the cyclophilin mRNA, a non-regulated transcript, were also measured for comparison. GT1-3 and GT1-7 cultures were treated with actinomycin D (5 $\mu\text{g/ml}$) or DRB (100 $\mu\text{g/ml}$) and harvested at various time points after drug treatment. The loss of GnRH mRNA levels was very slow in the presence of actinomycin D (data not shown) or DRB (Figure 4a). In contrast, there appeared to be moderate changes on cyclophilin mRNA levels (Figure 4a) and relatively rapid changes in the levels of the rat GnRH/TAg mRNA (Figure 4b) in the presence of these two inhibitors. The half-lives of these three mRNAs were calculated by plotting the log of the levels of mRNA against time and in the case of GnRH and cyclophilin mRNA, we extrapolated the line out to 50% decay. The half-life of GnRH mRNA is about 72 hours, and the half-lives of cyclophilin and the rat GnRH/TAg mRNA are about 30 and 2 hours, respectively.

Half-life of GnRH mRNA in the GT1 cells determined by steady state kinetics

The cytoplasmic turnover rate (half-life) of a given mRNA can also be estimated at steady-state from primary transcript turnover under the assumption that all reactions follow first order kinetics, splicing is the major mechanism for primary transcript "degradation", and subsequent nuclear processing steps are rapid relative to primary transcript processing. Thus, when the turnover rate of the primary transcript of a given gene and the steady-state concentrations of its

primary transcript and mRNA are known, the theoretical half-life of a given mRNA can be calculated through equations modified from those originally described by Pikielny and Rosbash (Pikielny et al, 1985). The rate of turnover is equal to steady-state concentrations times the rate constant:

$$T_p = P \times K_p \quad (\text{equation 1})$$

$$T_m = M \times K_m \quad (\text{equation 2})$$

T_p , T_m : turnover rate of primary transcript and mRNA, respectively;

P , M : steady-state levels of primary transcript and mRNA, respectively;

K_p , K_m : turnover rate constant of primary transcript and mRNA, respectively.

By definition, the rate of turnover can also be calculated from equation 3 when the steady-state concentration of GnRH primary transcript (P) and its half-life ($tp_{1/2}$) is known:

$$T_p = (P/2)/tp_{1/2} \quad (\text{equation 3})$$

The concentration of GnRH primary transcript is approximately 100 molecules per cell ($P=100$, Table 1) and its half-life is 18 minutes as calculated by Act D or DRB treatment ($tp_{1/2} = 18 \text{ min} = 0.3 \text{ hr}$, Figure 3), therefore, its turnover rate is 170 molecules/hr. Since $T_p = P \times K_p$ as we have defined in equation 1, the rate constant of GnRH primary transcript $K_p = 1.7$. Since at steady state the turnover rate of the primary transcript should equal the synthetic rate of its mRNA, which is also equal to the rate of mRNA turnover. The following equation is thus obtained when equation 1 and 2 are substituted into the equation $T_p = T_m$:

$$P \times K_p = M \times K_m \quad (\text{equation 4})$$

The concentration of GnRH mRNA (M) is known to be approximately 13,400 molecules per cell (Table 1), therefore, the turnover rate constant of GnRH mRNA $K_m = 0.013$. The half-life of GnRH mRNA can thus be calculated by the following equation similar to that described in equation 3:

$$T_m = (M/2)/tm_{1/2}; \quad (\text{equation 5})$$

$tm_{1/2}$: half-life of the mRNA.

Thus, the estimated half-life of GnRH mRNA derived from this equation is 39.4 hours.

Discussion

The release of the key neuroendocrine peptide gonadotropin releasing hormone (GnRH) can be regulated by numerous substances through direct or indirect synaptic inputs or by surrounding non-neuronal cells for the appropriate control of reproductive functions. It is believed that in addition to the effects on release, the regulation of GnRH can also happen at the level of its biosynthesis, resulting a slower but long-term effects on its physiological functions in response to different physiological or environmental changes. In order to understand the regulation of GnRH gene expression along its biosynthetic pathway, we have analyzed the processing patterns of the GnRH nuclear RNA precursors and their relative abundance having developed probes for the detection of the mouse GnRH primary transcript. These intron/exon/intron probes can provide useful tools for studying the regulation of GnRH gene expression at three different levels in one single assay since they can detect multiple GnRH transcripts, including primary transcript, processing intermediates, and mature RNA. The use of an immortalized mouse hypothalamic neuronal GT1 cell line has enabled us for the first time to measure the half-lives of GnRH primary transcript and cytoplasmic mRNA and provided a model for future elucidation of the changes of GnRH nuclear and cytoplasmic RNA transcripts in response to hormonal manipulation.

It is generally accepted that the levels of primary transcript are a reflection of the rate of RNA synthesis based on the assumption that nuclear RNA species are short-lived and therefore can rapidly reflect the changes in the rate of transcription. This concept is supported by the observations that the measured half-lives of several RNA precursors are indeed very short (Weil et al, 1990;

Kessler et al, 1993), and the levels of nuclear RNA precursors have close temporal relations to the changes in phosphoenolpyruvate carboxykinase (Granner et al, 1983) and proopiomelanocortin (Levin et al, 1991) gene transcription. In the present study we have observed that GnRH primary transcript is also a short-lived nuclear RNA species (Figure 3). Indeed, we have observed that there are parallel changes of GnRH primary transcript levels and its rate of transcription as measured by nuclear run-on assay, in response to phorbol esters (Yeo et al, submitted) and serum (Yeo and Roberts, unpublished observations). These results strongly support the notion that the levels of primary transcript do reflect the rate of gene transcription. The measurement of primary transcript for addressing the changes in gene transcription in response to hormones is particularly advantageous because in many circumstances the more direct methods for measuring the rate of gene transcription, such as nuclear run-on assay, are not sensitive enough or not feasible. Even though transient transfection analysis using promoter driven reporter gene constructs has been very useful for addressing transcriptional regulation, however, it is not effective for addressing rapid and/or transient transcriptional changes. In addition, the presence of regulatory sequences within the coding regions (including introns and exons), or 3' end may produce misleading results if only the conventional promoters (5' flanking regions) are used. Furthermore, for *in vivo* analysis of transcriptional effects, the measurement of primary transcripts is technically much easier as compared to *in vivo* transfection or nuclear run-on assays.

Probes against the intervening sequences of the GnRH gene were first established in the rat (Jakubowski et al, submitted). Examination of the GnRH nuclear transcripts by reverse transcription coupled with polymerase chain reaction (RT-PCR) revealed that intron B is the first intron to be spliced out from

the precursor RNA in rat brain fractions including the preoptic area-anterior hypothalamus and basal olfactory area (Jakubowski et al, submitted). Since GnRH primary transcript is the only nuclear RNA species containing intron B in the rat brain, a riboprobe containing this sequence can be used to accurately measure the levels of GnRH primary transcript. We were interested in developing a similar assay system for the measurement of the mouse GnRH RNA transcripts. The availability of transgenic mice techniques and the presence of hypogonadal mice (caused by a 33.5 kb deletion of the distal half of the GnRH gene, Mason et al, 1986) have provided the opportunity for further genetic manipulation of the GnRH system as compared to the existing rat models. In addition, the recent production of the mouse hypothalamic neuronal GT1 cell line which provides an invaluable tool for elucidating the regulation of GnRH gene expression at the cellular and molecular levels (Mellon et al, 1990; Weiner et al, 1992).

Similar strategies to that used in the rat were taken to characterize the mouse GnRH transcripts in the present study. We found that, at least in GT1 cells, the processing patterns of the mouse GnRH precursor RNA and the relative abundance of different transcripts are different from the rat. Unlike in the rat brain where intron B is the first intron to be spliced out, there does not appear to be an absolute order of intron excision for the processing of GnRH primary transcript in GT1 cells, as independently determined by RT-PCR and RNase protection assay; all possible processing intermediates exist at some level. The decay patterns of nuclear transcripts in the presence of RNA synthesis inhibitors also indicate that there was not one exclusive nuclear RNA processing pathway (data not shown). This phenomenon has been reported in the analysis of the steady-state populations of the β -globin (Zeitlin et al, 1984) and interleukin-3

(Lang et al, 1987) transcripts. However, while the A2B or B3C probe can not exclusively measure the primary transcript, since processing intermediates contribute little to the total levels because their levels are very low (Table I). For example, the A2B probe will protect either primary transcript or a processing intermediate where only intron C has been removed. But from Table I it can be seen that the transcript without intron C and with intron B is present at less than 5% of the primary transcript. The same argument holds for the results obtained using the B3C probe. Therefore, the full-length fragments protected by these probes can closely reflect the levels of primary transcript. Furthermore, since they contain sequences spanning the intron/exon/intron junctions which protects not only the primary transcript but also the processing intermediates as described above, use of these probes in RNase protection assays will be able to monitor changes in transcription (primary transcript), nuclear processing (processing intermediates), and mRNA levels. In this study we have also found that in the mouse hypothalamic GT1 cells, the intron-containing GnRH RNA transcripts are present only in the nucleus. These data support previous findings that the cytoplasmic intron-containing RNA species (intron A) are present only in the human placenta (Radovick, 1990) or ovary, testes and mammary tissues (Dong, in press) but not in the hypothalamic tissues in the rat (Jakubowski, submitted) and in human (Radovick, 1990, Dong, in press), suggesting a tissue-specific intron retention mechanism.

In addition to the differences in nuclear RNA processing patterns, the relative abundance between several nuclear transcripts and between nuclear and cytoplasmic GnRH transcripts is also different between the rat and the mouse hypothalamic GT1 cells. In the rat, GnRH nuclear RNA transcripts comprise about 30% of the total cellular GnRH transcripts and GnRH primary transcript,

processing intermediates, and nuclear mRNA each contributes to about one-third of the total nuclear transcripts (Jakubowski et al, submitted). However, in the GT1 cells, only 5% of the total cellular GnRH RNA transcripts are nuclear (Table 1), with primary nuclear mRNA comprising the vast majority of GnRH nuclear transcripts (approximately 90% of total GnRH nuclear transcripts). One possible explanation for these differences is that the GT1 cells were derived from hypothalamic tumors which may not necessarily have identical features to mature GnRH neurons. The other possibility is that the contacts with other neurons and cells in the *in vivo* system may have different effects on the rate of GnRH RNA synthesis, nuclear RNA processing and turnover when compared to the GT1 cells which are grown in the dishes as a pure population. These issues will require further investigation in order to clarify the cause of the differences between the rat and the GT1 cells.

The cytoplasmic level of an mRNA, which provides the templates for protein synthesis, is determined by the synthetic rate of its precursor, the primary transcript, the rate of nuclear RNA splicing, and its rate of degradation (turnover). Even though the study of gene regulation has been primarily focused on transcription, in recent years it has been recognized that post-transcriptional control, particularly cytoplasmic mRNA turnover, also contributes significantly to the regulation of gene expression in many systems (for review, see Nielson et al, 1990). Numerous examples have been found in the neuroendocrine systems of the hormonal regulation of mRNA stability (Krane et al, 1991; Carroll et al, 1991; Ayoubi et al, 1992; Murphy et al, 1992; Attardi et al, 1993). In this study we have used several approaches to measure the turnover rate of GnRH mRNA which can be applied in future studies for elucidating whether similar effects on mRNA

stability as observed in other systems also play an role in mediating the levels of GnRH gene expression.

The half-life of cytoplasmic mRNA can be determined by several methods, including the administration of RNA synthesis inhibitors, pulse-labeling techniques, use of an inducible promoter system, and theoretical calculation of approach to steady-state kinetics (Rodgers et al, 1985; Shapiro et al, 1985; Pikielny et al, 1985; Helms et al, 1990; Harrold et al, 1991) Initially we used the RNA synthesis inhibitor actinomycin D to measure the half-life of GnRH mRNA. Since only a slight change in the level of GnRH mRNA was observed with 24 hours of actinomycin D treatment, we were concerned that it might be an artifact produced by the cytotoxic effect of actinomycin D. Thus another RNA synthesis inhibitor DRB, which has very a different mode of action on RNA synthesis inhibition and is less toxic to the cells (Attardi et al, 1993), was used. The results obtained from DRB treatment are in complete agreement with those from actinomycin D, that GnRH mRNA is a relatively stable molecule in GT1 cells. It is clear that this is not caused by a general inhibition of the turnover of RNA polymerase II (Pol II) transcripts by either of the inhibitors, since both rat GnRH/Tag and cyclophilin mRNA turnover much more rapidly in the cytoplasm (Figure 4).

It is possible that the RNA synthesis inhibitors may specifically block the decay of GnRH mRNA: as reported in the case of the cyclic nucleotide-induced increase in plasminogen activator inhibitor 1 mRNA turnover is blocked by actinomycin D (Heaton et al, 1992). To avoid this pitfall of inhibition based studies, a theoretical approach using steady-state kinetics (Pikielny et al, 1985) was subsequently used. Using initial rates of decay after actinomycin D or DRB

treatment we were able to calculate the turnover rate of the GnRH primary transcript, the initial precursor to GnRH mRNA, to be 170 molecules per hour in GT1 cells. Since at steady-state the rate of synthesis and degradation of GnRH mRNA must be equal, unless there are additional degradation pathways in the nucleus where some of the primary transcript, processing intermediates or mRNA is destroyed, the turnover rate of the primary transcript will be the same as the synthetic rate of cytoplasmic mRNA and the same as its rate of turnover. The theoretical half-life of GnRH mRNA suggests that it would take approximately 40 hours to replace 50% of the 13,400 GnRH mRNA molecules in a GT1 cell. If any additional nuclear GnRH RNA degradation pathways existed, this calculated half-life would be an underestimation of the true $t_{1/2}$ since there would be fewer new GnRH mRNAs making it to the cytoplasm. This estimate of the minimum $t_{1/2}$ of at least 40 hours is in agreement with the observed $t_{1/2}$ (70 hours) using the RNA synthesis inhibitors. In order to absolutely determine the $t_{1/2}$ of GnRH mRNA, pulse-labeling techniques will be required, although because of its long half-life, it will be difficult to get sufficient label into the GnRH mRNA. Nevertheless, the long cytoplasmic half-life of GnRH mRNA is not an unusual phenomenon since several other Pol II transcripts have been identified to have relatively long cytoplasmic half-lives, including globin (Volloch et al, 1981), growth hormone (Helms et al, 1990), LH β , and alpha-subunit (Attardi et al, 1993). The long half-life of the GnRH mRNA raises interesting questions regarding the regulation of GnRH mRNA levels in the cytoplasm. It has been reported in the rat hypothalamus that the administration of the excitatory amino acid NMDA results in rapid changes in GnRH mRNA levels (within 15 minutes) as determined by in situ hybridization (Peterson et al, 1991). Similar rapid changes in GnRH mRNA levels were observed in our laboratory using RNase protection assay with a surprising finding that there was no significant changes in the levels of nuclear

primary transcript or processing intermediates. This suggests that the rapid changes in GnRH mRNA levels could be due to an alteration in the stability of cytoplasmic mRNA (Gore et al, submitted). To test this hypothesis, the GT1 cells would provide an ideal model for elucidating the cellular and molecular mechanisms regulating cytoplasmic GnRH RNA turnover in response to hormones or secretagogues.

In this report, we have defined the basic parameters and established assay methods for the analysis of GnRH RNA transcripts in the mouse hypothalamic neuronal GT1 cells. Nevertheless, the finding that GnRH mRNA has an extremely long half-life strongly suggests that the mechanisms of rapid regulation of cytoplasmic GnRH mRNA levels have to act at the post-transcriptional level. Taken together, these initial studies on the characterization of GnRH RNA transcripts will allow us to begin to dissect the nuclear and cytoplasmic sites of hormonal action in regulating the mRNA levels of this important neuroendocrine peptide.

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Figures and Figure Legends

Figure 1. Schematic illustration for the analysis of nuclear GnRH transcripts and the nuclear precursors present in the GT1 cells as detected by RT-PCR.

(a) The presence of different GnRH RNA precursors can be detected by RT-PCR using oligodeoxynucleotide primers corresponding to the intron or exon regions of the mouse GnRH gene. Boxes and lines represent exons and introns, respectively, with numbers or letters representing the order of each individual intron/exon in the genomic sequence. Arrows represent the regions in GnRH gene corresponding to the primer sequences. The size of each putative RNA precursor derived RT-PCR DNA fragment to be detected by the primer combination is shown underneath the primed regions.

(b) Products obtained from the RT-PCR reactions using nuclear RNA from the GT1 cells. Numbers of the lanes correspond to the primer pairs as described in (a). M1 and M2 represents sizes of double strand DNA molecular weight markers electrophoresed with the samples in the gels (markers not shown). M1 represents the combination of BstEII, BstNI, and MspI and M2 represents HaeIII digestion of pBR 322 DNA. Lane 1-4 were run in composite gels containing 0.5% agarose and 2% acrylamide and Lane 5 was run in a 2% agarose gel.

a.

Sense primer $\longrightarrow$
Antisense primer $\longleftarrow$

Lane	1	2	3	4	5
Priming region and oligo pairs					
Sizes of PCR products representing putative nuclear RNA precursors	 1770 bp	 1330 bp	 1600 bp	 1630 bp	 1700 bp
	 450 bp	 330 bp	 280 bp	 300 bp	 360 bp

b.

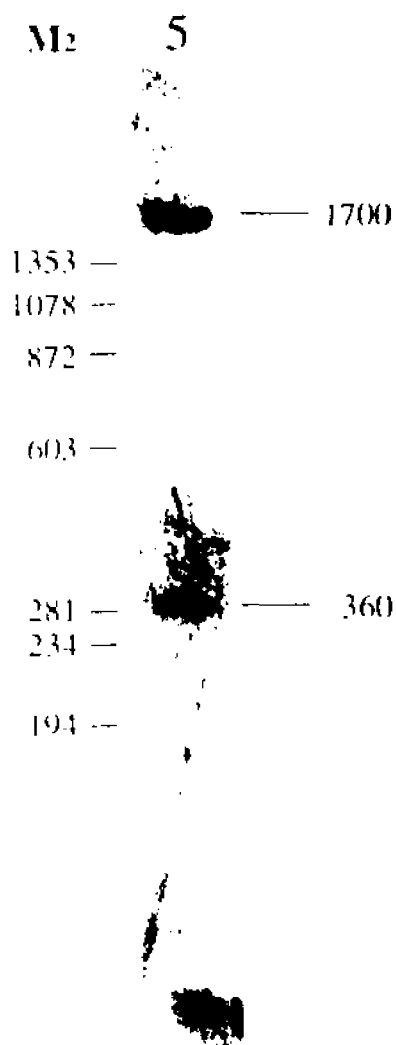
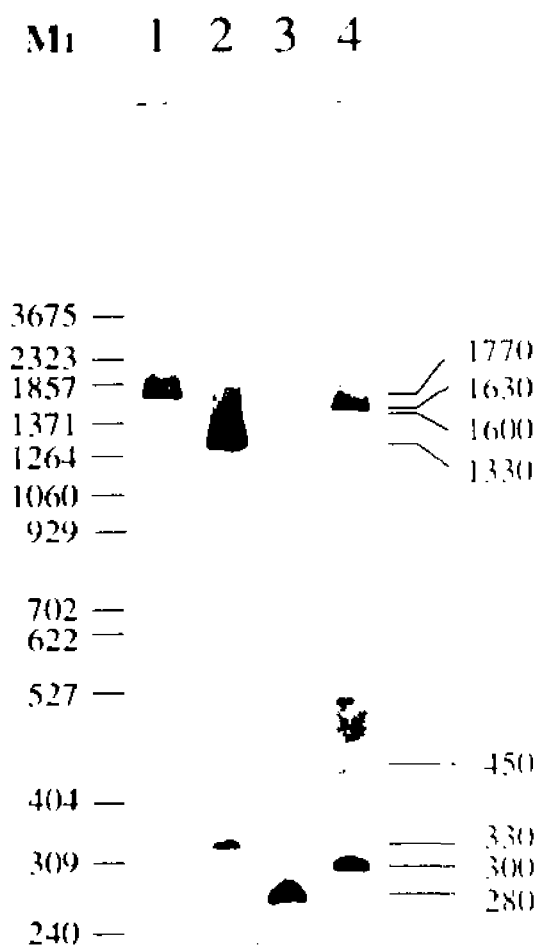


Figure 2. GnRH nuclear transcripts present in the GT1 cells as detected by RNase protection assay.

(a) Mouse GnRH gene structure and the probes designed for RNase protection assay for the analysis of GnRH nuclear precursors present in the GT1 cells. The numbers indicate the sizes of potential protected fragments by the A2B (intron A/exon 2/intron B) and the B3C (intron B/exon 3/intron C) probes. A2B probe will protect putative RNA transcripts containing intron A/exon 2/intron B (383 bp), exon 2/intron B (297 bp), intron A/exon 2 (230 bp), and exon 2 (144 bp). B3C probe will detect the presence of the following RNA species: intron B/exon 3/intron C (297 bp), exon 3/intron C (221 bp), intron B/exon 3 (172 bp), and exon 3 (96 bp).

(b) RNA isolated from GT1-7 cells (T: total RNA, C: cytoplasmic RNA, N: nuclear RNA isolated from one well or half a well) was hybridized to either A2B or B3C probes and digested with RNase A and T1. Samples were run in 5% acrylamide gels. The sizes were determined by the comigration of molecular weight marker MspI digest of pBR 322 DNA (M).

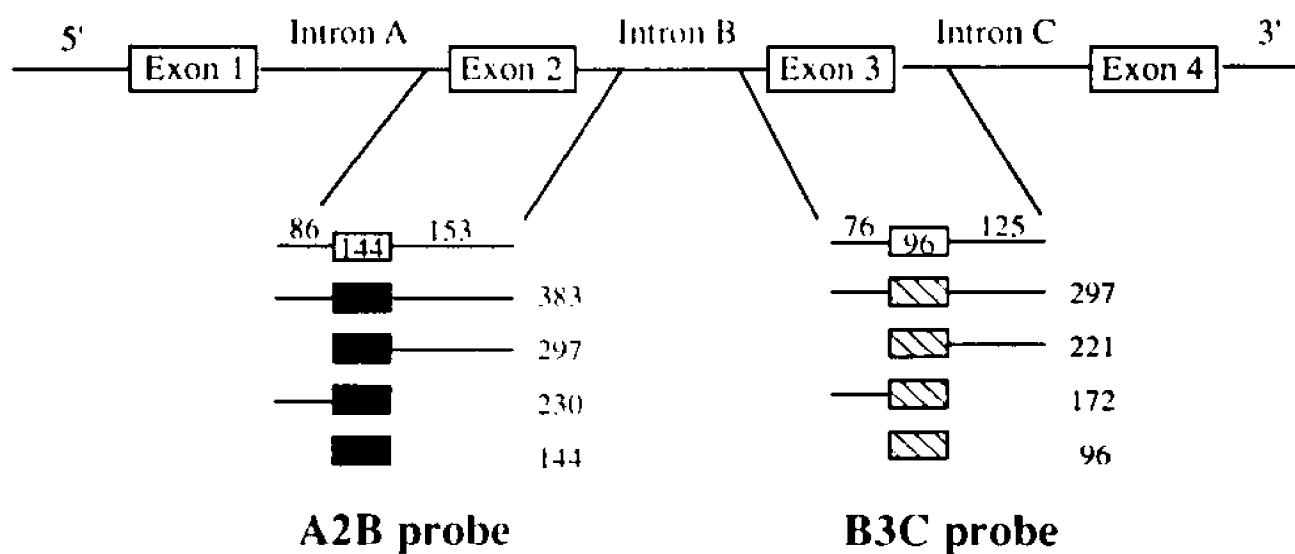
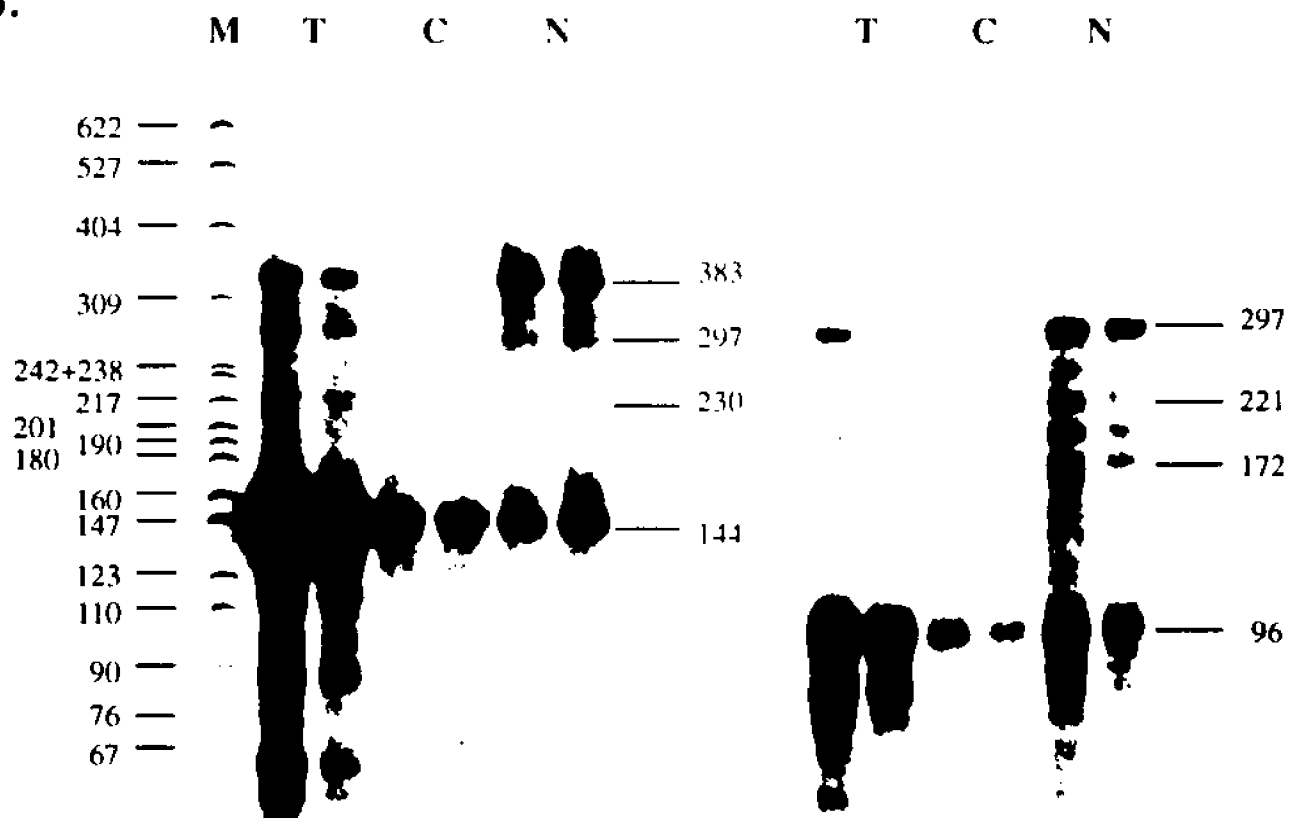
a.**b.**

Figure 3. Decay rates of GnRH primary transcript in GT1 cells in the presence of RNA synthesis inhibitors.

GT1-7 cells were treated with actinomycin D (5 $\mu\text{g/ml}$) or DRB (100 $\mu\text{g/ml}$) and the decay of GnRH primary transcript was measured by RNase protection assay using either A2B (a) or B3C (b) probe. The levels of GnRH primary transcript were expressed as % of control (pg/well) and plotted in semilog format against time.

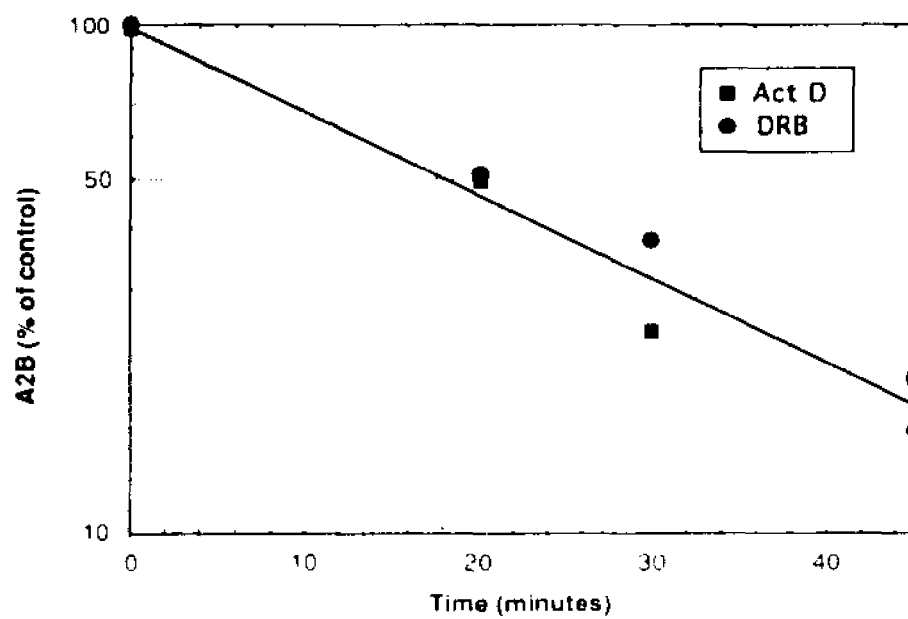
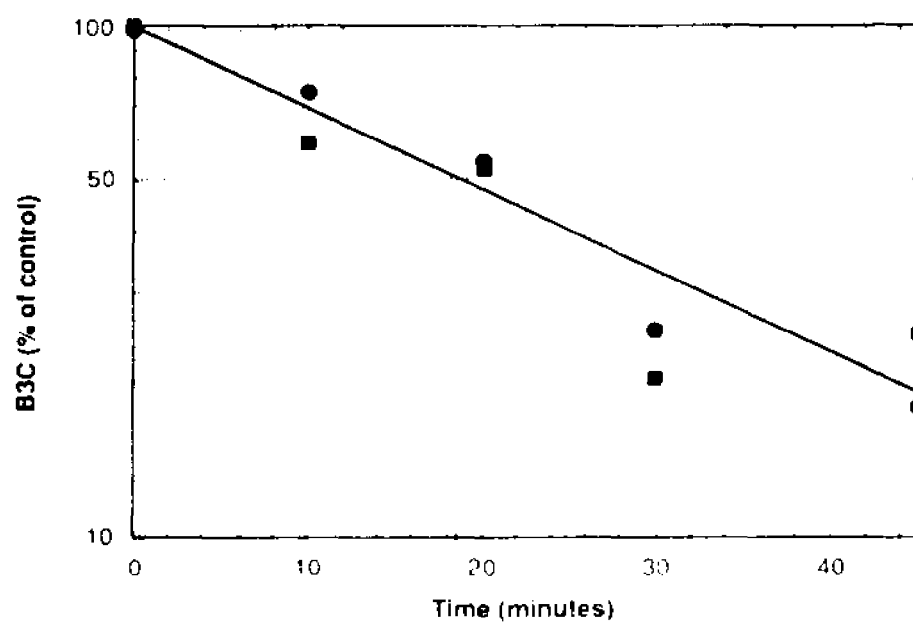
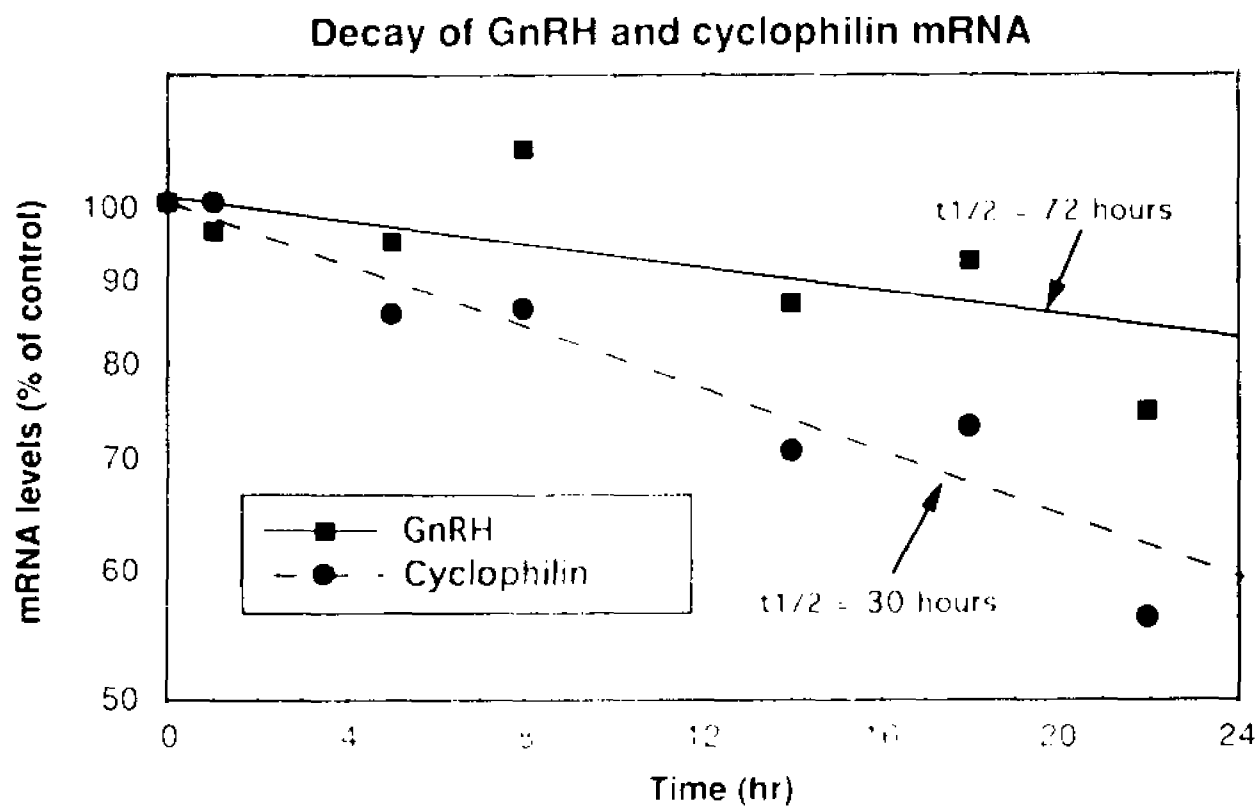
a. Decay of GnRH primary transcript**b.**

Figure 4. Decay rates of cytoplasmic GnRH, cyclophilin, and rat GnRH/TA_g mRNA in GT1 cells in the presence of the RNA synthesis inhibitor DRB.

(a) GT1-7 cells were treated with DRB (100 μ g/ml) and harvested at 0, 1, 5, 8, 14, 18, and 22 hours after treatment. Cytoplasmic RNA was hybridized to GnRH A2B and cyclophilin probes in one single RNase protection assay. The levels of GnRH and cyclophilin mRNA were measured (pg mRNA/ μ g cytoplasmic RNA) and expressed as % of control in a semilog scale. The half-lives of the two mRNAs were defined as the time when 50% of the mRNA is left through extrapolation of each line.

(b) DRB (100 μ g/ml) was added to GT1-7 cells, and the cells were harvested at 0, 8, and 16 hours after treatment and probed with the rat GnRH 5' probe. The levels of rat GnRH/TA_g mRNA were expressed as % of control (pg mRNA/ μ g cytoplasmic RNA).

a.



b.

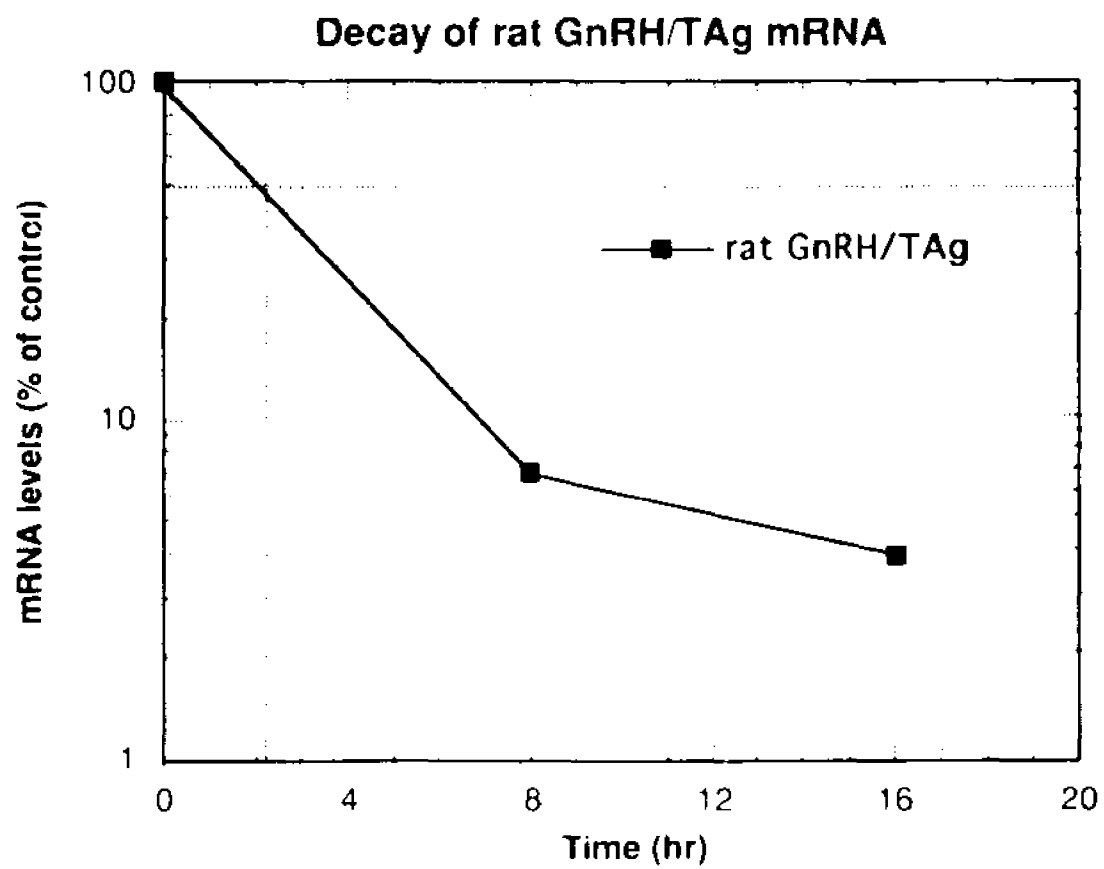


TABLE I

Levels of nuclear and cytoplasmic GnRH RNA transcripts in the GT1 cells determined by RNase protection assay

RNA species		pg/well	atmol/well	No. of molecules/cell	% of precursor	% of nuclear GnRH RNA	% of total GnRH RNA
Nuclear							
	A2B	5.8	46.8	94	91.3 %	11.2 %	0.7 %
	2B	0.3	3.2	6	5.8 %	0.7 %	< 0.05 %
	A2	0.1	1.4	3	2.9 %	0.4 %	< 0.05 %
	Exon 2	17.1	368	738		88 %	5.2 %
	B3C	5.5	57.7	116	89.9 %	12.5 %	0.8 %
	3C	0.2	2.8	6	4.7 %	0.6 %	< 0.05 %
	B3	0.2	3.6	7	5.4 %	0.8 %	< 0.05 %
	Exon 3	12.4	398	800		86 %	5.6 %
Cytoplasmic							
	Exon 2	310.3	6670	13400			94 %

The levels of nuclear GnRH RNA precursors and cytoplasmic mRNA were determined by RNase protection assay. For analysis of nuclear RNA species, A2B or B3C probe was hybridized to equal amounts of nuclear RNA. Calculation of numbers of molecules per cell is based on the estimation of 300,000 cells per well in 6-well plates. The molecular weight of a single nucleotide of 323 was utilized.

Chapter IV

Regulation of Gonadotropin Releasing Hormone Gene Expression by Protein Kinase A Pathway in Mouse Hypothalamic GT1 cells

Tracy T.S. Yeo, Mariann Blum and James L. Roberts

Abstract

We have used the GT1 cells as an *in vitro* model for studying the regulation of gonadotropin releasing hormone (GnRH) gene expression by the activation of protein kinase A pathway. We previously reported that prolonged treatment of the GT1 cells with the GnRH secretagogues forskolin (10 μ M) in a static incubation system caused a significant decrease of GnRH mRNA levels. The cellular and molecular mechanisms responsible for the forskolin-effect on GnRH mRNA levels were elucidated in the present study. Time course analysis showed that there was a gradual loss of GnRH mRNA levels in the presence of forskolin as compared to control, decreasing to about 40% of control levels by 24 hours. Similar results were observed when the cAMP-mimicking compound 8-bromo-cAMP was used. The level of the endogenous rat GnRH/Tag mRNA, which is driven by the homologous rat GnRH promoter, was also inhibited by the two drugs following similar time course with the mouse GnRH mRNA, suggesting that the inhibitory effects were transcriptional. No significant change in cyclophilin mRNA levels was observed throughout the time course, suggesting that this is a specific effect of forskolin on the two GnRH promoter-driven genes. To verify the nuclear site of action of cAMP, the levels of nuclear GnRH RNA precursors were measured. The levels of GnRH primary transcript did not change within the first 6 hours of forskolin treatment but decreased in a time-dependent manner afterwards. Long-term treatment with forskolin also decreased the levels of GnRH nuclear mRNA. These results suggest that the decrease of GnRH mRNA levels by the activation of protein kinase A is due, at least in part, to the inhibition of GnRH gene transcription.

Introduction

Gonadotropin releasing hormone (GnRH) is a hypothalamic neuropeptide which regulates the release of luteinizing hormone (LH) and follicle stimulating hormone (FSH) from the anterior pituitary to coordinate vertebrate reproductive functions. It has been suggested that the cyclic nucleotide cAMP, an intracellular second messenger which is known to activate the protein kinase A (PKA) pathway (for review, see McKnight, 1991; Borrelli et al, 1992), may play an important role in this hypothalamic control of anterior pituitary hormonal functions (Weissman et al, 1976; Naor et al, 1979). The correlation of hypothalamic cAMP levels with plasma estradiol levels and adrenergic activities (the two key factors involved in regulating the bulk release of GnRH in the afternoon of proestrus to trigger the LH surge) strongly suggests involvement of cAMP in regulating GnRH functions (Weissman et al, 1975; Kimura et al, 1980; Kant et al, 1981; Zubin et al, 1983). This hypothesis is confirmed by the observation that direct intraventricular injection of cAMP stimulated LH release *in vivo* (Ojeda et al, 1974) and stimulated GnRH release from median eminence *in vitro* (Ramirez et al, 1985; Kim et al, 1985; Ojeda et al, 1986). Furthermore, it has been reported in an *in vitro* superfusion study that activation of the PKA pathway by forskolin caused a significant increase in GnRH mRNA levels in rat hypothalamus (Lee et al, 1990). This suggests that in addition to the stimulation of GnRH release, activation of PKA pathway may also regulate GnRH functions by the alteration of its gene expression.

It has been found in many neuropeptide systems that the trans-synaptic activation of the cAMP/PKA pathway can regulate the biosynthesis of these peptides at the transcriptional (for review, see Comb et al, 1987) and post-

transcriptional levels (Fossom et al, 1992). The fact that forskolin causes a significant increase in GnRH mRNA levels indicates that activation of the cAMP/PKA pathway can also have similar effects on GnRH gene expression. However, due to the paucity of GnRH neurons *in vivo*, mechanisms involved in mediating the cAMP/PKA effects on GnRH gene expression have been difficult to study. In addition, the complex neuronal connections to the GnRH neuronal system has also made it difficult to determine whether the observed effects on GnRH gene expression are mediated by direct activation of the cAMP/PKA pathway in the GnRH neuron or are secondary to the activation of interneurons.

The availability of an immortalized hypothalamic neuronal GnRH-secreting cell line, the GT1 cells (Mellon et al, 1990), has provided the opportunity to examine and delineate the effect of the activation of the cAMP/PKA pathway in a pure population of GnRH neurons by which the cellular and molecular mechanisms can be assessed. We have used GT1 cells as an *in vitro* model to study the regulation of GnRH peptide secretion and mRNA levels by the activation of several second messenger pathways and found that activation of the cAMP/PKA pathway by forskolin caused a decrease in GnRH mRNA levels (Yu et al, submitted). We have further examined the mechanisms contributing to this forskolin-induced decrease of GnRH mRNA levels in the present study.

Results

Suppression of GnRH mRNA levels by forskolin and 8-bromo-cAMP

Two pharmacological compounds which can activate the cAMP/protein kinase A pathway were used in the GT1-3 cells to examine the effect of PKA activation on GnRH mRNA levels. GT1-3 cells were treated with forskolin (10 μ M) or 8-bromo-cAMP (1 mM) and harvested at various times after drug treatment. Cytoplasmic RNA was isolated and the levels of GnRH mRNA were measured by RNase protection assay. There was a gradual decrease in GnRH mRNA levels in GT1-3 cells in the presence of forskolin, reaching about 40% of control levels at 24 hours (Figure 1a). Similar effects on GnRH mRNA levels was also observed in GT1-3 cells treated with 8-bromo-cAMP (Figure 1b).

Forskolin and 8-bromo-cAMP suppress rat GnRH/TA_g mRNA levels

Because GT1 cells also carry a transgene (SV40 T-antigen) driven by a rat GnRH promoter (Mellon et al, 1990) which is highly homologous to the mouse promoter (Dong et al, 1993), comparing its mRNA levels to the endogenous mouse GnRH mRNA can help to distinguish whether the observed forskolin effects are acting at the promoter level or at the levels of cytoplasmic RNA turnover. Since this transgene product has a completely different sequence after the Hind III site of the first exon (+88 bases in the rat; corresponding to the Xho I site, +23 bases in mouse exon 1; the difference between the mouse and the rat is due to the usage of different transcription start sites) when compared to the endogenous mouse GnRH mRNA, its post-transcriptional regulation is probably different from the mouse GnRH mRNA. A probe against the rat GnRH mRNA 5' region which does not effectively cross hybridize with the endogenous mouse mRNA was used for the RNA measurement of this transgene. As shown in Figure 2, there was a time-dependent gradual decrease in the levels of the rat

GnRH/TA γ mRNA in the presence of forskolin or 8-bromo-cAMP as compared to control, similar to the trend of the mouse GnRH mRNA.

Effect of forskolin on cyclophilin mRNA levels

In order to determine whether the inhibitory effects of forskolin on both mouse GnRH and rat GnRH/TA γ mRNA levels were caused by cytotoxicity of the drug or were a general effect on RNA polymerase II transcripts, the levels of cyclophilin mRNA were measured as an internal control. As shown in Figure 3, there was no significant change in the levels of cyclophilin mRNA throughout the time course. Similar effects were observed in GT1-3 cells treated with 8-bromo-cAMP (data not shown).

Forskolin decreases GnRH primary transcript and nuclear mRNA levels

As shown in Figures 1 and 2, the similarity between the mouse GnRH and rat GnRH/TA γ mRNA in response to forskolin suggests that this may be due to a transcriptional effect, since only the promoter and partial exon 1 sequences (up to +88 in the rat and +23 in the mouse) are homologous. Since the levels of GnRH primary transcript can be used as an indirect indication of transcription (Yeo et al, 1993a; see chapter III), a time course study was performed to determine whether the inhibitory effect of forskolin on GnRH mRNA levels was due to the inhibition of transcription. In addition, the levels of nuclear GnRH mRNA were also measured for comparison of the nuclear site of action to the cytoplasmic effect. There were no significant changes in the levels of GnRH primary transcript in the first 6 hours of forskolin treatment; however, at 8 and 24 hours, forskolin caused a significant decrease in GnRH primary transcript levels (Figure 4a). A significant decrease of nuclear mRNA levels was found only after 24 hour incubation with forskolin (Figure 4b).

Discussion

We have previously reported that the administration of forskolin, a pharmacological compound which can induce the elevation of intracellular cAMP levels through the activation of adenylate cyclase, in a static incubation system resulted in a significant increase of GnRH peptide release from GT1 cells (Yu et al, submitted). This stimulation of GnRH secretion by forskolin is attenuated with prolonged treatment, presumably by a decrease in GnRH biosynthesis and thus the material available for release. The observation that long-term forskolin treatment significantly decreased the levels of GnRH mRNA and primary transcript (Yu et al, submitted) suggests that, indeed, the attenuation of GnRH release is due to the decrease of GnRH biosynthesis at the RNA level. The mechanisms contributing to the decrease of GnRH RNA transcripts in response to forskolin were further examined in the present study. We found that this down-regulation is specific to the GnRH transcripts, and is attributed, at least in part, to an action at the promoter level to inhibit GnRH gene transcription.

In order to further investigate the effect of the activation of cAMP/PKA pathway on GnRH mRNA levels, two pharmacological compounds forskolin and 8-bromo-cAMP which activate the cAMP/PKA pathway via different mechanisms, were used in parallel experiments. Extensive time course analysis using either drug reveals similar, if not identical, inhibitory effects on GnRH mRNA levels, suggesting a correlation between the activation of the intracellular cAMP/PKA pathway and the down-regulation of GnRH mRNA levels in GT1 cells. In order to identify whether this decrease of GnRH mRNA levels is attributed to the inhibition of GnRH promoter activity or the alteration of GnRH RNA processing or stability, we analyzed the levels of another transcript, the rat GnRH/SV40 T antigen

mRNA (rat GnRH/TA_g, Mellon et al, 1990), a transgene driven by a truncated 2.3 kb rat GnRH promoter which is highly homologous to the mouse GnRH promoter and thus may have similar transcriptional control mechanisms. Furthermore, the RNA transcript transcribed from this transgene contains only part of the mouse GnRH exon 1 sequence (23 bases 5' of the Xho I site in mouse GnRH exon 1; 88 bases 5' of the Hind III site in rat GnRH exon 1) with the rest of the coding region substituted by the SV40 T antigen sequence, suggesting that the post-transcriptional control of this RNA can be different from the mouse GnRH transcript. Thus, unless the first 88 bases in rat GnRH exon 1 are involved in regulation of mRNA turnover, we can assume that the changes in the rat GnRH/TA_g mRNA levels are solely a promoter-driven event. Nevertheless, this phenomenon is supported by the finding that several potential elements (two putative hairpin loops within the coding region and an AUUUA-related AUUUCA element in the 3' untranslated region) as revealed by sequence analysis of GnRH mRNA, could be responsible for altering mRNA stability (for review, see Brawerman, 1987; Sachs, 1993), are located downstream from the sequences present in the hybrid gene. Thus, by comparing the levels of the rat GnRH/TA_g mRNA to the mouse GnRH mRNA in response to hormonal manipulation, one should be able to distinguish the promoter event from a dual contribution by RNA transcription and processing/stability. The observation that the levels of these two RNAs are down-regulated by both forskolin and 8-bromo-cAMP following a similar time course strongly suggest that the effects of cAMP/PKA on GnRH mRNA are at the level of gene transcription.

It is possible that the down-regulation of GnRH mRNA levels after long-term forskolin treatment may not be a specific effect but a phenomenon of an overall decrease in RNA polymerase II mediated transcription. This was tested

with a parallel measurement of the levels of GnRH mRNA and cyclophilin mRNA, an abundant and apparently non-regulated mRNA species (Jakubowski et al, 1991), to distinguish the specificity of the forskolin effect. Since cyclophilin mRNA level remained unchanged when GnRH mRNA levels were decreasing significantly under the same treatment, it suggests that the forskolin inhibitory effect on the two transcripts driven by two similar GnRH promoters is a specific effect.

We measured levels of GnRH primary transcript and nuclear mRNA in response to forskolin in a time course analysis. The results indicate that activation of the cAMP/PKA pathway has a nuclear site of action on GnRH gene expression since both the GnRH primary transcript and nuclear mRNA levels were decreased in the presence of forskolin. This observation further supports the result obtained from the analysis of the rat GnRH/TA_g mRNA, that a decrease of GnRH gene transcription is the major factor contributing to lowered GnRH mRNA levels in long-term incubation. Recently, we have directly elucidated the effect of cAMP/PKA activation on GnRH promoter activity using transient transfection analysis and found that both forskolin and 8-bromo-cAMP significantly decreased mouse and rat GnRH promoter activity (Dong et al, manuscript in preparation). Thus activation of the cAMP/PKA pathway inhibits GnRH gene transcription in the GT1 cells.

The effects of PKA activation by forskolin on GnRH mRNA levels have been reported by other laboratories with controversial results. Lee and colleagues reported that a two-hour perfusion of rat hypothalami with forskolin resulted in a significant increase in GnRH mRNA levels as determined by RNA blot analysis (Lee et al, 1990). On the other hand, Wetsel and colleagues

observed no change in GnRH mRNA levels in GT1 cells treated with forskolin for 48 hours as determined by Northern blot analysis (Wetsel et al, 1993). One likely reason for the discrepancy between our study and that of Lee's group is that we used an immortalized hypothalamic neuronal cell line and conducted our studies using a static culture system while their results were obtained from perfused rat hypothalami. It is also possible that the immortalization of neurons may interfere with the normal regulatory functions. One cannot exclude the possibility that the stimulation of GnRH mRNA observed in their studies may be an indirect effect due to the activation of non-GnRH neurons. The difference between our results and those of Wetsel and colleagues (Wetsel et al, 1993) is more puzzling. One possible explanation is that there may be differences in the nature of the GT1 sublines used in the two studies: their studies were conducted in GT1-7 cells, while ours are in the GT1-3 cells. Another possibility is the fluctuation of the basal levels of GnRH mRNA in the control group as we have previously observed (data not shown). In several repeated experiments, we have control groups for each time point matched to the drug treatment groups for comparison. In at least two experiments we have observed a rise of basal levels of GnRH mRNA levels during the time course. In these two experiments forskolin still exerts about two- to three- fold inhibition on GnRH mRNA levels as compared to control (data not shown). This inhibitory effect will look much smaller if only the forskolin-treated groups are compared, as described by Wetsel and colleagues. However, in all our experiments forskolin and 8-bromo-cAMP clearly exert consistent significant two- to three-fold inhibitory effect on GnRH mRNA, rat GnRH/TA_g mRNA, GnRH nuclear mRNA and primary transcript as determined by quantitative RNase protection assay and on GnRH promoter as determined by transient transfection assay.

Activation of the cAMP/PKA pathway is known to have positive or negative effects on gene transcription through the regulation of transcription factors binding to discrete DNA elements (Taylor et al, 1990; Borrelli et al, 1992; Lamph et al, 1990; Foulkes et al, 1991). However, the time course by which GnRH gene transcription is inhibited by cAMP/PKA activation indicates a slow and gradual response, raising the possibility that it may be a secondary effect of cAMP/PKA activation. Crosstalk between different second messenger pathways has also been reported in several systems (For review, see Berridge, 1987; Schule et al, 1991; Lucas et al, 1992). It has also been found that the activation of cAMP/PKA pathway can increase cytosolic calcium levels (Luini et al, 1985); the interactions between the cAMP/PKA and diacylglycerol/PKC pathways have also been reported in many systems (Comb et al, 1986; Lee et al, 1990; McKnight, 1991). We have observed that the activation of calcium and diacylglycerol/PKC pathway by pharmacological compounds result in rapid (within 1 hour) inhibition of GnRH gene transcription in the GT1 cells (Yu et al, submitted; Yeo et al, submitted, see Chapter V). Comparison to the slow response to the activation of cAMP/PKA pathway which does not inhibit GnRH gene transcription until 8 hours after drug treatment, suggests that cAMP/PKA activation may activate/inhibit other second messenger systems which have inhibitory effects on GnRH gene transcription in the GT1 cells.

It is rather surprising to see that the stimulation of GnRH release did not lead to an increase of biosynthesis, at least at the RNA level, in GT1 cells. The bi-phasic regulation of initial rapid non-genomic activation and the late genomic inhibition have been suggested in the regulation of the GnRH system by progesterone (Ramirez et al, 1985). It is proposed that depending on the high/low doses, pulsatile/constant and short/long-term exposure to modulators,

there can be dramatic differences in regulating GnRH neuronal functions. This phenomenon has been reported in the regulation of gonadotropin secretion and biosynthesis from the anterior pituitary by GnRH (Weiss et al, 1992; Weiss et al, 1993). Further experiments need to be conducted for better understanding of the mechanisms regulating the separation of non-genomic from genomic events on the control of GnRH gene expression.

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Figures and Figure Legends

Figure 1. Effect of forskolin or 8-bromo-cAMP on GnRH mRNA levels: a time course analysis

GT1-3 cells were treated with either forskolin (10 μ M, 1a) or 8-bromo-cAMP (1 mM, 1b), and harvested at 0-24 hours after treatment. For the control group, DMSO was administered as vehicle and the cells were harvested together with the experimental groups at every time point. 3 μ g of cytoplasmic RNA was used in RNase protection assays and GnRH mRNA levels were measured using the mouse GnRH intron A/exon 2/ intron B probe. The time course of the levels of GnRH mRNA (pg protected fragments/ μ g RNA) in the presence of forskolin and 8-bromo-cAMP are expressed separately as the percentage of that measured in the control group. Two-way analysis of variance (two-way ANOVA) showed that there were significant changes in GnRH mRNA levels in the presence of forskolin (treatment: $p=0.0001$; time and treatment: $p=0.0003$) and 8-bromo-cAMP (time: $p=0.0444$; treatment: $p=0.0001$; time and treatment: $p=0.0001$). Post-hoc analysis showed that forskolin and 8-bromo-cAMP significantly suppressed mouse GnRH mRNA levels in the GT1 cells. *, $p<0.05$; ***, $p<0.0001$ vs control.

Mouse GnRH mRNA levels

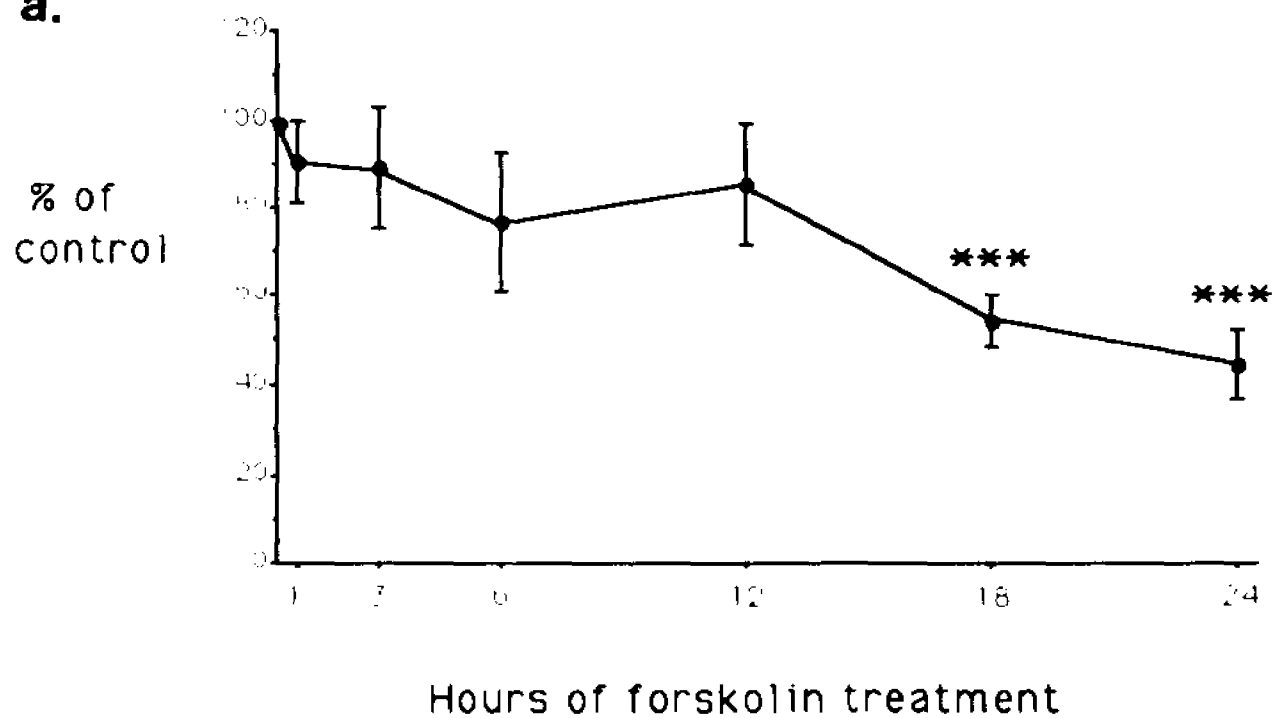
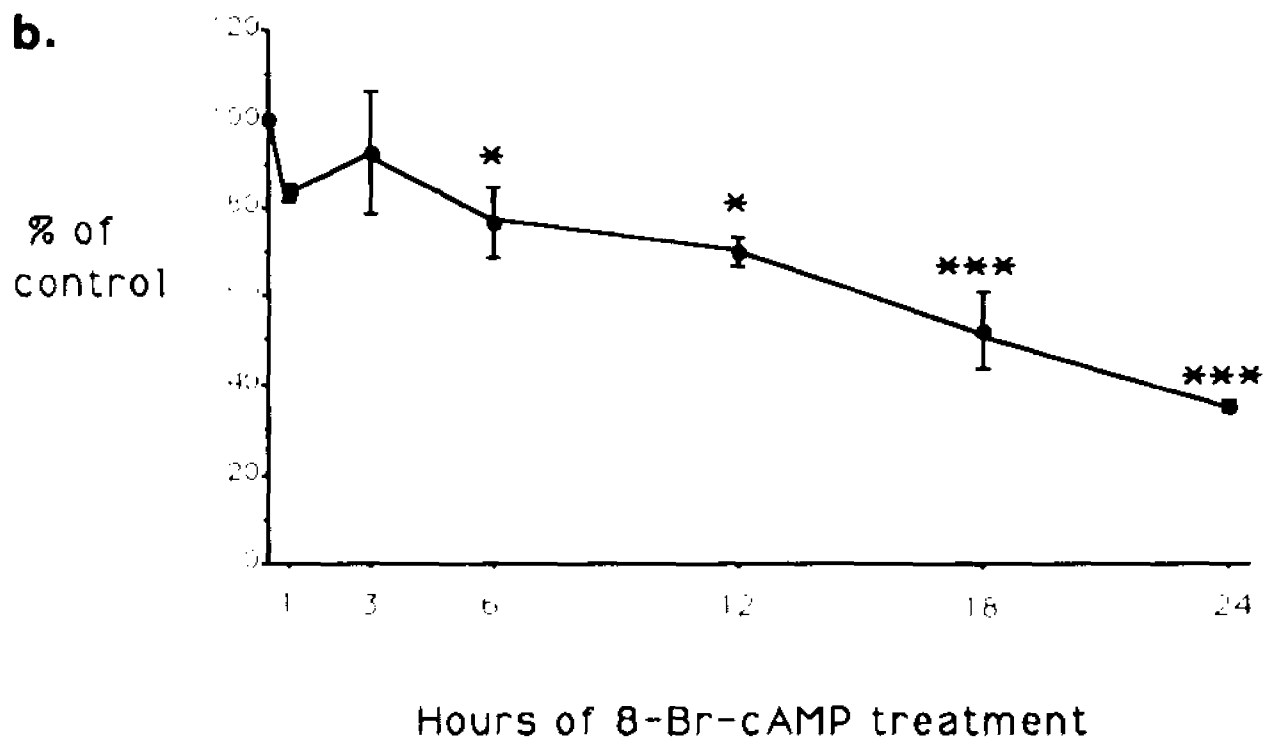
a.**b.**

Figure 2. Effect of forskolin and 8-bromo-cAMP on the rat GnRH/Tag mRNA levels

Equal amounts of cytoplasmic RNA isolated from GT1-3 cells in the same experiment shown in Figure 1 were probed with the rat GnRH 5' probe in RNase protection assay to measure the levels of the rat GnRH/Tag mRNA. The time course of mRNA levels in the presence of both drugs was expressed separately as the percent of control. Forskolin (2a) and 8-bromo-cAMP (2b) decreases the levels of the rat GnRH/Tag mRNA. Two-way ANOVA showed that there were significant changes in ratGnRH/Tag mRNA levels in the presence of forskolin (time: $p=0.0001$; treatment: $p=0.0004$; time and treatment: $p=0.0001$) and 8-bromo-cAMP (time: $p=0.0001$; treatment: $p=0.0001$; time and treatment: $p=0.0001$). Post-hoc analysis showed that forskolin and 8-bromo-cAMP significantly suppressed rat GnRH/Tag mRNA levels in GT1 cells. *, $p<0.05$; ***, $p<0.0001$ vs control.

Rat GnRH 5'/T-Ag mRNA levels

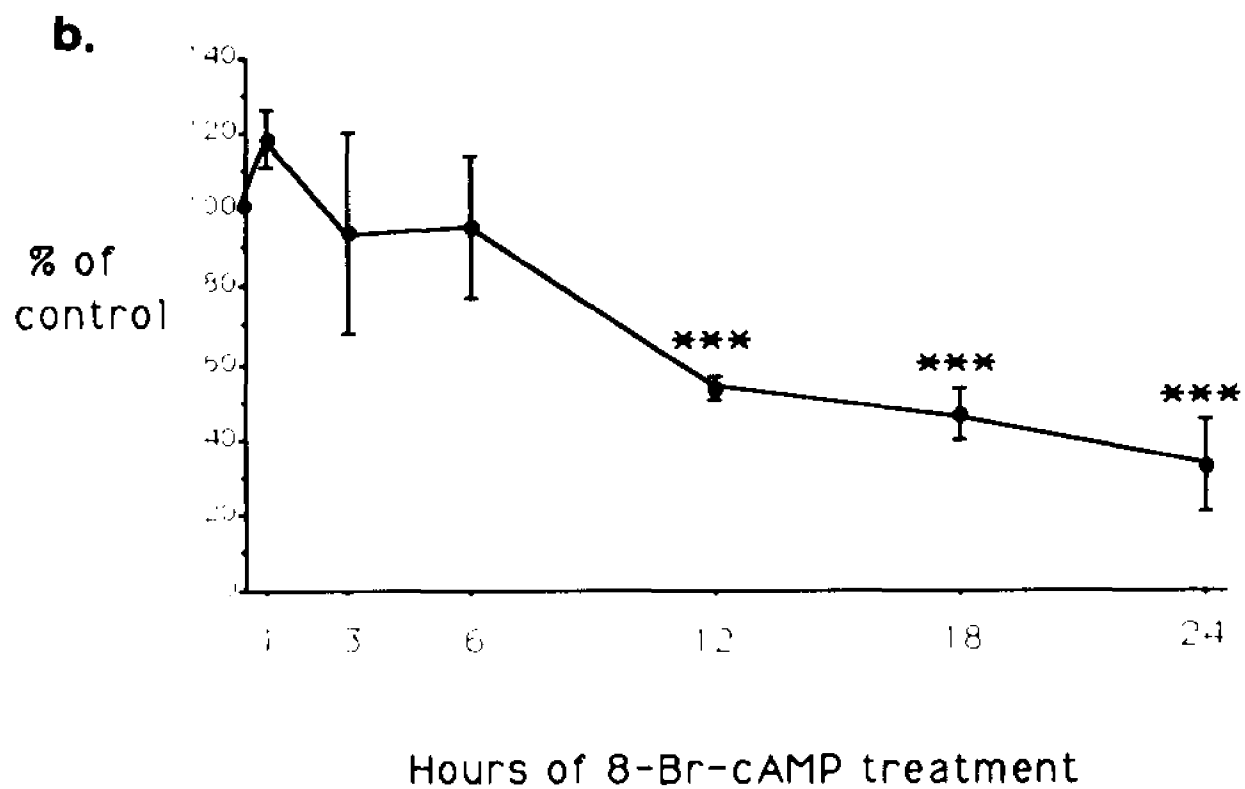
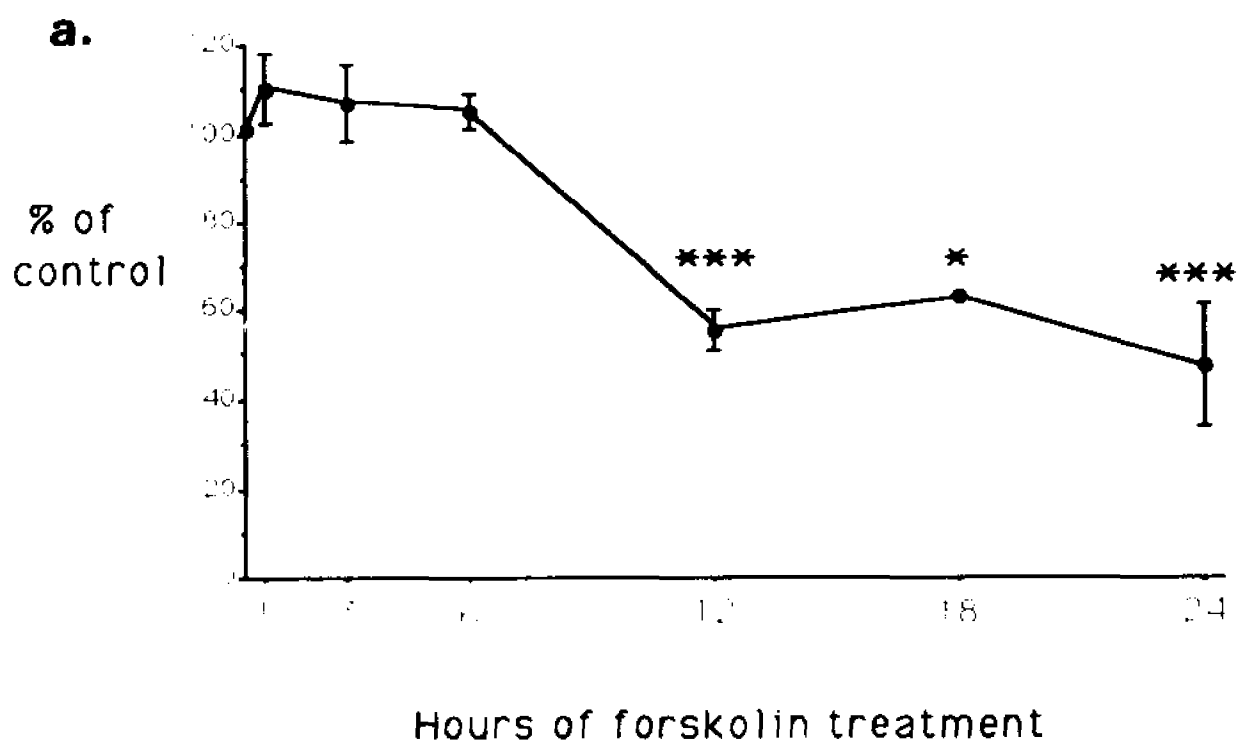


Figure 3. Effect of forskolin on cyclophilin mRNA levels

GT1-3 cells were treated with forskolin (10 μ M) and harvested at 1.5, 3, 6, 8, and 24 hours after drug treatment and cyclophilin mRNA levels were measured by RNase protection assay. There was no significant change in cyclophilin mRNA levels in the presence of forskolin throughout the time course as indicated by two-way ANOVA (time: $p=0.1756$; treatment: $p=0.7632$; time and treatment: $p=0.4564$) and post-hoc analysis. The significance value was set at 95%.

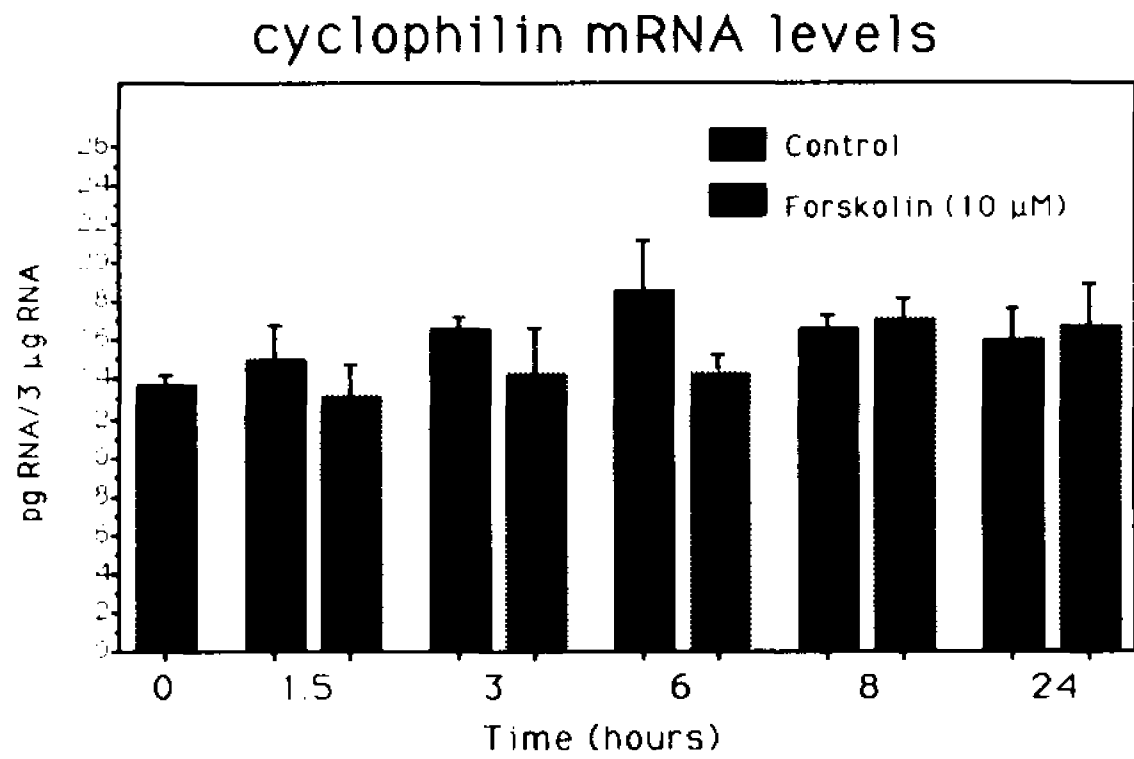
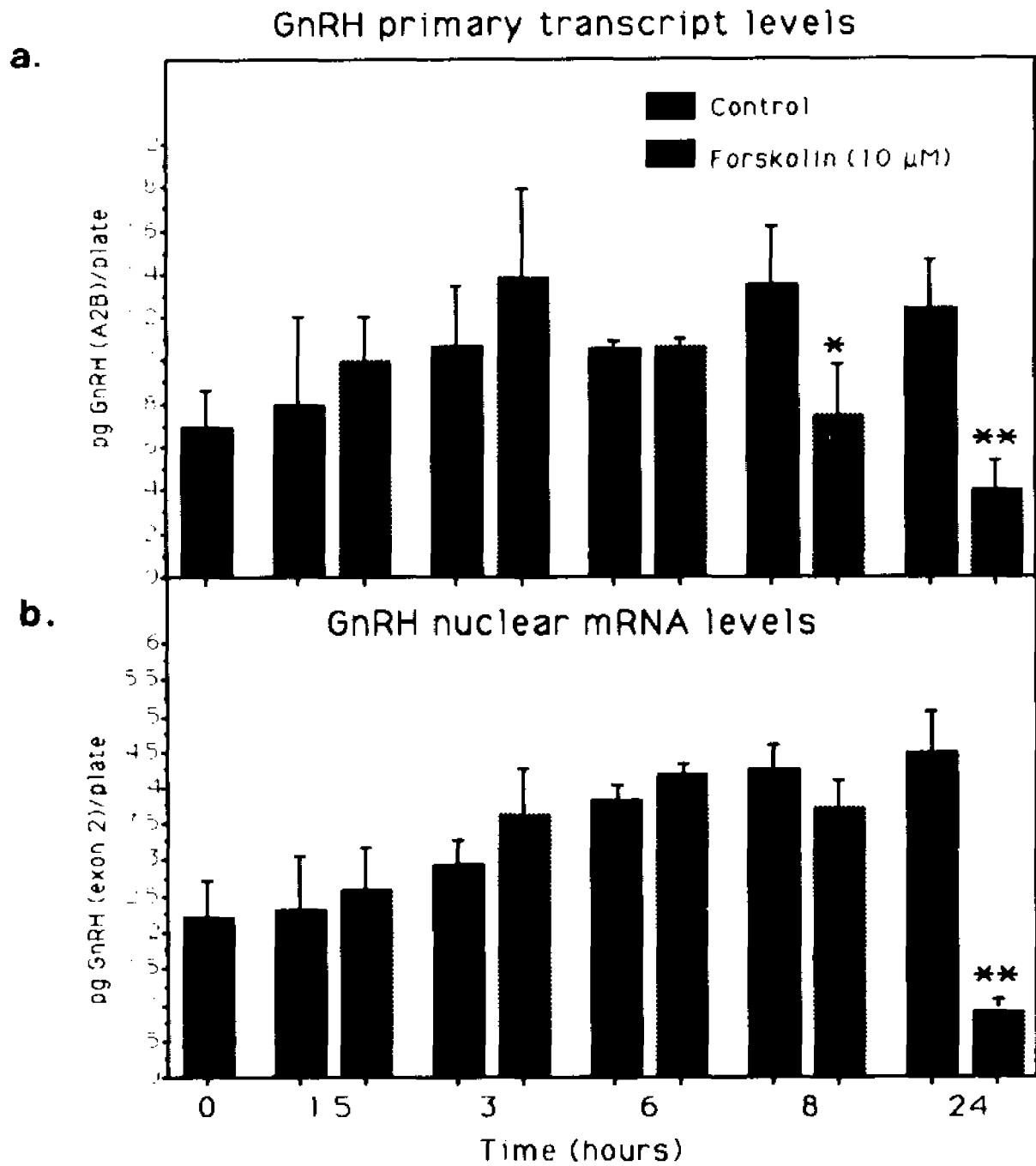


Figure 4. Effect of forskolin on GnRH primary transcript and nuclear mRNA levels

Nuclear RNA isolated from the same experiment as described in Figure 3 was used to measure the levels of GnRH primary transcript and nuclear mRNA levels using the mouse GnRH intron A/exon 2/intron B probe in an RNase protection assay. Two-way ANOVA showed that there were significant changes in primary transcript (treatment: $p=0.0135$; time and treatment: $p=0.0185$) and nuclear mRNA levels (time: $p=0.0008$; time and treatment: $p=0.0008$) in the presence of forskolin. Post-hoc analysis showed that forskolin significantly suppressed both GnRH primary transcript and nuclear mRNA levels. *, $p<0.05$; **, $p<0.001$; ***, $p<0.0001$ vs control.



Chapter V

Multiple Mechanisms of Regulation of Gonadotropin Releasing Hormone Gene Expression by Protein Kinase C Pathway in Mouse Hypothalamic GT1 cells

**Tracy T.S. Yeo, Ke Wen Dong, Zhouwen Zeng, Mariann Blum
and James L. Roberts**

Abstract

We and others have previously reported that long-term treatment of GT1 cells with the phorbol ester PMA resulted in decreased GnRH peptide secretion and a parallel decrease of GnRH mRNA levels, suggesting an inhibitory effect on GnRH gene expression. The cellular mechanisms contributing to this decrease were further investigated in the present study at both transcriptional and post-transcriptional levels. Time course analysis showed that PMA significantly suppressed both GnRH primary transcript and mRNA levels. Maximal inhibition of GnRH primary transcript by PMA was observed within 3 hours and the inhibition gradually attenuated in a time-dependent manner. GnRH mRNA levels were not changed within the first 3 hours of PMA treatment but decreased afterwards to 25% of control levels with a half-time of decline of about 5-6 hours and remained suppressed throughout the time course. A similar time course of suppression of GnRH primary transcript and mRNA was also observed after only a 1 hour-pulse of PMA. The inhibitory effects of PMA on GnRH gene transcription, as shown by the levels of primary transcript, were verified by nuclear run-on assay and transfection assay using a mouse GnRH promoter/luciferase reporter construct. Both confirmed that PMA inhibited GnRH gene expression at the transcriptional level. The inhibitory effect of PMA on GnRH gene transcription was not abolished by the protein synthesis inhibitor cycloheximide. We have observed that GnRH mRNA is a long-lived transcript; thus the rapid decline in GnRH mRNA elicited by PMA in the present study suggests an enhancement in the rate of cytoplasmic GnRH mRNA turnover. This post-transcriptional effect of PMA on GnRH gene expression was further examined using two different RNA synthesis inhibitors, actinomycin D and DRB (5,6-dichloro-1- β -ribofuranosylbenzimidazole). Interestingly, both inhibitors

abolished the PMA-mediated decrease in GnRH mRNA, indicating that it is an RNA synthesis dependent event. We conclude that both transcriptional and post-transcriptional mechanisms contribute to the PMA-induced down regulation of GnRH mRNA in GT1 cells.

Introduction

Gonadotropin releasing hormone (GnRH) is a hypothalamic decapeptide which regulates reproductive function. It is synthesized in a subpopulation of hypothalamic neurons and released into portal blood vessels which flow into the anterior pituitary, where GnRH stimulates the secretion of the gonadotropins into circulating blood with subsequent effects on the gonads. Numerous substances have been reported to regulate this axis at the hypothalamic level by regulating GnRH peptide secretion (for review, see 1-3) and gene expression (4-9). Several intracellular second messenger pathways involved in mediating the actions of those factors regulating GnRH secretion and gene expression have also been studied, including cAMP-protein kinase A (PKA), phosphoinositol turnover-protein kinase C (PI/PKC), and calcium (10-13).

The PI/PKC pathway has long been considered to play an important role in regulating GnRH release and gene expression since the action of several GnRH-regulating substances can be transduced by the PI/PKC pathway (for review, see 14). This includes endothelin (15), serotonin (16), neuropeptide Y (17), vasopressin (18), excitatory amino acids (19) and GnRH itself (20, 21). Direct stimulation of protein kinase C by pharmacological compounds results in significant effects on GnRH secretion (10-12, 22) and mRNA levels (13) in intact animals or animal tissues. However, the complex neuronal connections onto the GnRH neurons in both the hypothalamus and median eminence make it difficult to interpret results obtained from animal studies. The recent success of producing an immortalized GnRH-expressing neuronal GT1 cell line (23) has allowed us to study the regulation of GnRH gene expression directly in response to these factors in a pure population of GnRH cells.

The GT1 cells were produced by genetically targeted tumorigenesis using a 2.3 kb truncated rat GnRH promoter driven SV40 large T antigen oncogene (rat GnRH/Tag) which resulted in a hypothalamic GnRH-secreting tumor in a transgenic mouse. Three clonal cell lines were derived by serial dilution from a pure cell population of this tumor and extensively studied (GT1-1, GT1-3, GT1-7). Although quantitative variations exist, they all possess a neuronal phenotype, express the endogenous mouse GnRH gene as well as the rat GnRH/Tag transgene, and secrete GnRH (for review, see 24). Numerous studies have been published on the characterization of the three GT1 sublines, including their neuronal phenotype (25), episodic GnRH secretion (26, 27), membrane receptor characterization and the regulation of GnRH peptide secretion by neurotransmitters (15, 28-31). Effects of the activation of several second messenger pathways on GnRH secretion and gene expression have also been reported (32-34).

It has been observed in several laboratories that the activation of PKC by phorbol esters in the GT1 cells results in a rapid stimulation of GnRH secretion followed by a decline in peptide secretion and a progressive decrease of GnRH mRNA levels in long-term treatments (32-34). The effect of phorbol esters on GnRH mRNA levels was shown to be specific to the GnRH system since the levels of several other RNA polymerase II transcripts were not altered under the same treatment, including β -actin (32), histone (33), and the SH locus transcript (32, 35, 36). These data indicate that the activation of PKC pathway by phorbol esters may have inhibitory effects on GnRH biosynthesis in the GT1 cells. In the present study we report that administration of the phorbol ester PMA to GT1 cells

inhibited GnRH gene expression at both transcriptional and post-transcriptional levels which resulted in the subsequent decrease in GnRH mRNA levels.

Results

PMA suppressed both GnRH primary transcript and mRNA levels

The effects of PMA on GnRH primary transcript and mRNA levels were measured by a quantitative solution hybridization/ribonuclease protection assay (RNase protection assay) using an antisense riboprobe spanning the intronA-exon2-intronB region of the mouse GnRH gene (mouse GnRH A2B probe) which protects the primary transcript (37). The levels of cyclophilin mRNA were measured simultaneously as an internal control using a riboprobe synthesized from a cyclophilin cDNA sequence (38). GT1-7 cells were treated with 100 nM PMA and harvested at various time points after drug treatment. PMA significantly suppressed the levels of GnRH primary transcript. After 3 hours of PMA treatment the levels of GnRH primary transcript were about 20% of control levels; subsequently GnRH primary transcript levels started to increase in a time-dependent manner and reached about 60-80% of control levels by 21-24 hours (Figure 1a). A shorter time course analysis showed that the PMA-inhibitory effect on GnRH primary transcript levels can be readily observed within 1 or 1.5 hour of PMA (10 nM, see Figure 3; or 100 nM, data not shown) treatment in both GT1-3 and GT1-7 cells. PMA also suppressed GnRH mRNA levels with a slower time course than for primary transcript. A three-hour incubation of GT1-7 cells with PMA did not have significant effect on GnRH mRNA levels, while 10 hours or longer administration of PMA resulted in a decrease of GnRH mRNA levels in a time-dependent manner (Figure 1b), with a half-time of decrease of about 5-6 hours. Cyclophilin mRNA levels remained essentially unchanged throughout the time course (Figure 1c). Similar results were observed in the GT1-3 cells with either 10 or 100 nM PMA treatment (data not shown).

Effect of short-term exposure to PMA on GnRH primary transcript and mRNA levels

It has been reported that short-term (30 minute pulse) exposure of GT1 cells to PMA also resulted in the subsequent suppression of GnRH mRNA levels (32), suggesting that the inhibitory effect of PMA on GnRH mRNA levels was probably due to activation, not down-regulation, of the protein kinase C pathway. We have compared the effects of continuous and short-term PMA exposure on GnRH primary transcript and mRNA levels in GT1 cells. The levels of cyclophilin mRNA were also measured concomitantly to show the specificity of the PMA effect. GT1-7 cells were incubated either in the continuous presence of 100 nM PMA or for one-hour with PMA followed by change of medium. A one-hour pulse of the GT1-7 cells with PMA resulted in a decrease of GnRH primary transcript levels which was similar to that of continuous exposure at both 8 and 16 hours (Figure 2a). GnRH mRNA levels were also suppressed significantly by short-term exposure to PMA (Figure 2b). Again, there was no significant change in cyclophilin mRNA levels in any of the groups (Figure 2c).

PMA decreases the level of GnRH gene transcription

To verify that the decreases in GnRH primary transcript by PMA reflected transcriptional effects, a nuclear run-on assay was performed to determine the effect of PMA on GnRH gene transcription. GT1-3 cells were treated with vehicle (DMSO) as the control group or PMA (10 nM) for 1 hour and analyzed by *in vitro* transcription run-on assay using specific DNA filters containing mouse GnRH A2B fragment. pBR322 DNA was used to determine the background. GnRH gene transcription was significantly suppressed about two-fold with PMA treatment (Table 1).

PMA inhibits mouse GnRH promoter activity

To further verify that the PMA-inhibitory effect was directly on GnRH gene transcription, GnRH promoter/reporter constructs were used in transient transfection assays to confirm that it is a promoter-driven event. It has recently been reported that the activity of a construct containing a 3 kb Hind III fragment of the rat GnRH 5'/CAT (chloramphenicol acetyl-transferase) reporter gene was suppressed by phorbol ester TPA in the mouse hypothalamic GT1-7 cell line (33). The same construct has been generated in our laboratory and similar results were observed in both GT1-3 and GT1-7 cells (data not shown). Since there are discrete differences between the otherwise highly homologous rat and mouse GnRH promoter (39), it is important to examine whether a reporter gene driven by the mouse GnRH promoter will have the same response to phorbol esters in the GT1 cells. The effect of PMA on mouse GnRH promoter activity was examined by transient transfection analysis using luciferase as the reporter gene. A 3.4 kb mouse GnRH promoter/ luciferase reporter gene was transfected into GT1-7 cells by the calcium phosphate precipitation method, and cells were treated with vehicle (DMSO) or PMA (10 nM). Luciferase enzyme activity was analyzed after 24 hours as a measure of GnRH promoter activity. PMA significantly decreased the activity of the mouse GnRH promoter about two fold as compared to control (Table 1).

Effect of PMA and cycloheximide on c-fos mRNA and GnRH primary transcript levels

The mRNA levels of c-fos, a gene whose expression can be induced by the activation of protein kinase C pathway (40), were elevated 10 to 30 fold within 20-30 minutes of 10 nM PMA treatment in both the GT1-3 and GT1-7 cells (data not shown). The administration of a protein synthesis inhibitor cycloheximide

(100 µg/ml) to the GT1-3 cells for 15 minutes followed by the addition of PMA (10 nM) for another hour resulted in an elevation of basal and PMA-induced c-fos mRNA levels (Figure 3a), indicating that protein synthesis (including Fos protein) was indeed inhibited (41) by this treatment. Analysis of the nuclear RNA from the same experiment showed that basal levels of GnRH primary transcript were lower. However, the inhibitory effect of PMA on GnRH primary transcript levels was not abolished by cycloheximide (Figure 3b). Similar results were observed in the GT1-7 cells treated with cycloheximide for 15 minutes followed by 90 minutes of PMA treatment. The amplitude of PMA-induced c-fos mRNA levels was smaller in the GT1-7 cells (Figure 3c and 3d).

Inhibitory effect of PMA on GnRH mRNA levels was abolished by both actinomycin D and DRB

Since PMA induced a relatively rapid decrease in GnRH mRNA while our RNA turnover studies suggested it is a very stable mRNA (37), the effect of PMA on GnRH mRNA turnover was examined in the absence or presence of the RNA synthesis inhibitors actinomycin D or DRB (5,6-dichloro-1-β-ribofuranosylbenzimidazole). GT1-7 cells were treated with vehicle (DMSO), PMA (100 nM) or actinomycin D (5 µg/ml) alone, PMA together with actinomycin D, or 1 hour PMA followed by actinomycin D addition (Figure 5a, 5b). A parallel experiment was also performed with DRB (100 µg/ml) (Figure 5c, 5d). Cells were harvested at 8 and 16 hours after treatment and cytoplasmic RNA was assayed for GnRH mRNA. As expected, neither actinomycin D nor DRB treatment resulted in a significant alteration of GnRH mRNA levels and PMA significantly decreased GnRH mRNA levels. However, the inhibition of GnRH mRNA levels by PMA was abolished by the administration of either actinomycin D or DRB. Even though a one hour-pulse of PMA was sufficient to trigger the cellular factors responsible for

the suppression of GnRH primary transcript and mRNA levels, as shown in Figure 2, the PMA-inhibitory effect on GnRH mRNA was abolished in the GT1-7 cells treated by PMA for one hour followed with the addition of either RNA synthesis inhibitor.

Discussion

The administration of phorbol esters to activate the protein kinase C (PKC) system in the GT1 cells results in a bi-phasic response of GnRH secretion: initial stimulation and long-term suppression (32-34). Measurement of GnRH mRNA levels suggests that the later decrease of GnRH secretion may be due to the depletion of a releasable GnRH peptide pool (34) as a result of the decreased GnRH biosynthesis, due to the lower levels of GnRH mRNA (32-34). In the present studies we extend the previous findings (33) which suggested that this decrease in mRNA is due to the suppression of GnRH gene transcription, and show the time course of the transcriptional suppression of GnRH gene expression by the phorbol ester PMA as measured by primary transcript levels. Our data indicate that post-transcriptional effects on cytoplasmic mRNA turnover also play a significant role in GnRH mRNA regulation.

Using a rat GnRH gene promoter/reporter construct and transfection into GT1-7 cells, Wetsel and colleagues (33) showed that TPA caused a reduction of rat GnRH gene transcriptional activity, suggesting that the suppression of cytoplasmic GnRH mRNA observed in mouse GT1-7 cells was due at least in part to a transcriptional mechanism. We have further characterized the mouse GnRH promoter (39), sequencing an additional 3 kb upstream of the reported mouse GnRH promoter sequence (49). Despite extensive homologies between the rat and mouse genes, there are significant regions of difference. Moreover, the rat GnRH gene uses a different transcriptional start site relative to the mouse, presumably due to a mutation in the TATA box. Hence, we focused our studies on the mouse GnRH gene. Three different types of analysis showed that PMA had a 2-4 fold inhibitory effect on GnRH gene transcription in GT1 cells. The

most direct analysis, nuclear run-on assay, and an indirect assay quantitating primary transcript, both show that there is an inhibitory effect of PMA on GnRH transcription within one hour. This inhibition appears to attenuate over 24 hours of constant exposure to PMA, possibly due to down regulation of PKC activity at the later times of treatment. Using 3.4 kb of mouse GnRH promoter linked to a luciferase reporter, we also saw a two-fold suppression of promoter activity by PMA showing that the mouse GnRH 5' flanking region alone was sufficient to elicit the transcriptional effect seen in the run-on assay. Since identical effects with a similar rat GnRH gene promoter/CAT construct were reported by Wetsel and colleagues (33) and also observed by us in this study, this suggests that the PMA inhibitory effect is most likely mediated through the homologous regions between the rat and mouse GnRH gene promoters.

There is controversy about whether the phorbol ester-inhibitory effect on GnRH gene expression is due to the activation or the subsequent down-regulation of PKC activity. Staurosporine, a commonly used PKC inhibitor, had no significant effect on either basal GnRH secretion or mRNA levels and partially blocked the TPA-induced increase of GnRH peptide secretion and attenuated the decrease of GnRH mRNA levels, indicating that the PMA-inhibitory effect is mediated by the activation of PKC (32). However, another PKC inhibitor, NPC 15437, caused a significant suppression of both basal GnRH mRNA levels and the activity of a rat GnRH promoter/CAT reporter gene (33). This suppression was not augmented by TPA co-administration, suggesting that down-regulation of PKC activity may be inhibitory to GnRH gene expression (33). Direct measurement of PKC enzyme activity showed that high levels of PKC activity were present in GT1-3 cells after 1.5 and 3 hours of phorbol ester treatment (34). Even after 6 hours of TPA treatment, a significant dose-dependent elevation of

PKC activity can still be detected in GT1-7 cells (32). The fact that in the present studies the rate of GnRH gene transcription was inhibited two-fold within 1 hour of PMA treatment and maximal suppression of GnRH primary transcript was observed within 3 hours, when PKC enzyme activity was still elevated, suggests that at least the initial phase of the PMA-inhibitory effect on GnRH gene transcription was not due to the down-regulation of the PKC pathway. This hypothesis is strengthened by the observation that short-term (1 hour pulse) exposure of GT1-7 cells to PMA was sufficient to suppress GnRH primary transcript levels (Figure 2a) many hours after PMA had been removed from the culture.

Activation of the PKC pathway by phorbol esters can result in either activation or suppression of gene transcription. The induction of *c-fos* and *c-jun* gene expression by phorbol ester from which the resulting FOS and JUN proteins can form heterodimers to induce promoter activities in genes containing the AP-1 (FOS/JUN) enhancer element is well documented (40, 42). The induction and/or modification of cellular proteins by PKC can also have inhibitory effects on gene transcription by antagonizing the AP-1 activation or through binding to regions other than the AP-1 element (43). We observed in GT1 cells that *c-fos* mRNA levels were elevated many fold shortly after PMA administration, prior to the decrease of GnRH primary transcript levels (Figure 3). However, the inhibition of GnRH gene transcription by PMA clearly does not require new synthesis of FOS/JUN proteins because cycloheximide does not abolish the PMA-mediated inhibitory effect on GnRH primary transcript levels. It is evident that protein synthesis was inhibited with this dose of cycloheximide (100 µg/ml) in GT1 cells, as indicated by the super-induction of both basal and PMA-induced cytoplasmic *c-fos* mRNA levels. This phenomenon has been reported in other systems as a

result of the removal of the negative auto-feedback effect of Fos protein to its own promoter (41). Since PMA was able to suppress GnRH primary transcript levels in the absence of protein synthesis, this suggests that the inhibition of GnRH gene transcription by PMA is mediated by the modification of existing cellular factors. It is also interesting to note that basal GnRH primary transcript level is also significantly inhibited by cycloheximide treatment alone, implying that basal transcription levels are very sensitive to ongoing protein synthesis. It is possible that PMA can elicit its inhibitory transcriptional effect by enhancing the decay or inactivation of a labile basal transcription factor.

Even though most studies on hormonal regulation of gene expression have focused on the effects on transcription, it has been recognized in recent years that regulation of gene expression can also occur at other levels, including RNA processing, mRNA stability, rate of translation, and protein processing. In many cases, changes in cytoplasmic mRNA stability can significantly alter mRNA levels after hormonal manipulations (for review, see 44). For example, in the HPG (hypothalamic-pituitary-gonadal) axis it has recently been reported that the stability of follicle-stimulating hormone (FSH) β -subunit mRNA can be enhanced or reduced several-fold by activin or inhibin/follistatin, respectively, while the mRNA stability of two other related genes, the LH/FSH α -subunit and LH β -subunit, remained unchanged (45, 46). Indeed, the hormonal effect on gene expression is often mediated simultaneously at multiple levels, such as at the transcriptional and post-transcriptional levels (47, 48, 52-54). Such a mechanism can allow for rapid and sustained alterations in gene expression. In our initial studies (as shown in Figure 1) we found that while GnRH primary transcript levels slowly returned toward control values, the levels of cytoplasmic GnRH mRNA remained suppressed even at later times. This suggests that GnRH

mRNA might be turning over more rapidly in order to maintain the lower cytoplasmic mRNA levels in the presence of rebounding levels of gene transcription. Therefore, we hypothesize that PMA, in addition to its initial inhibitory effect on GnRH gene transcription, may also have effects at the post-transcriptional level.

Indeed, we have observed that GnRH mRNA is very stable in GT1 cells with a $t_{1/2}$ of greater than 24 hours (37). Thus the rapid effect of PMA in decreasing GnRH mRNA levels must for the most part be due to an enhanced degradation of the existing GnRH mRNA since the 2-4 fold transcriptional inhibition would take at least 24 hours to elicit a two-fold decrease in such a large cytoplasmic mRNA pool. When we attempted to prove this conclusion directly, we surprisingly found that the PMA-effect on GnRH mRNA turnover was abolished when RNA synthesis was inhibited, suggesting that PMA must induce the expression of another gene(s) whose product(s) could regulate the stability of GnRH mRNA. Furthermore, the observation that the addition of RNA synthesis inhibitors one hour after PMA treatment was still able to block the PMA effect on GnRH mRNA also suggests that the alteration in GnRH mRNA stability may be a delayed event requiring the accumulation of new gene products. A similar phenomenon has been reported by Lumelsky and Forget (54). Phorbol ester PMA negatively regulates γ -globin gene expression by both decreasing the rate of gene transcription and increasing the rate of mRNA degradation. The PMA-effect on increasing the degradation of the otherwise very stable γ -globin mRNA is abolished by the RNA synthesis inhibitor actinomycin D or the protein synthesis inhibitor cycloheximide. The similar finding of the negative post-transcriptional regulation of γ -globin and GnRH gene expression by PMA suggests that the PMA-effect on these two mRNAs may be mediated through

similar pathways. Further studies are necessary to delineate the mechanisms of the post-transcriptional effect of PMA on GnRH gene expression.

The large basal cytoplasmic pool of GnRH mRNA as measured in GT1 cells suggests that acute changes on GnRH mRNA levels by neurotransmitters or hormones may be mediated more efficiently and rapidly by changing the rate of cytoplasmic mRNA turnover rather than the rate of transcription. Indeed, the observation by Peterson and colleagues that the administration of NMDA in the rat resulted in a rapid increase in GnRH mRNA levels (7) together with our observation that NMDA and kainate induced rapid changes in GnRH mRNA levels without changes in nuclear GnRH primary transcript levels (55) support this possibility. However, chronic suppression of GnRH promoter activity by changes in physiological or environmental factors will eventually interfere with GnRH function as well. Thus, the parallel regulation of GnRH gene expression at different levels of its biosynthetic pathway is important for maintaining the function of this important neuroendocrine gene in response to various physiological demands.

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Figures and Figure Legends

Fig. 1. Effect of PMA on GnRH primary transcript, GnRH mRNA, and cyclophilin mRNA levels: a time course analysis.

GT1-7 cells were treated with either vehicle (DMSO) as the control group or PMA (100 nM), and harvested at 0-24 hours after drug treatment. About 15-25 µg of cytoplasmic RNA were recovered from each well and used for the RNase protection assay. The levels of GnRH primary transcript are expressed as the amount of protected fragments/well and mRNA levels are expressed as the amount of protected fragment/amount of RNA recovered in each sample. Two-way analysis of variance (two-way ANOVA) showed that there were significant changes in GnRH primary transcript (time: $p < 0.0168$; treatment and time: $p < 0.0023$) and mRNA levels (time: $p < 0.0001$; treatment and time: $p < 0.0002$). Post-hoc analysis showed that PMA significantly suppressed the levels of both GnRH primary transcript (1a) and mRNA (1b) without notable effect on cyclophilin mRNA (1c). *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$ vs control.

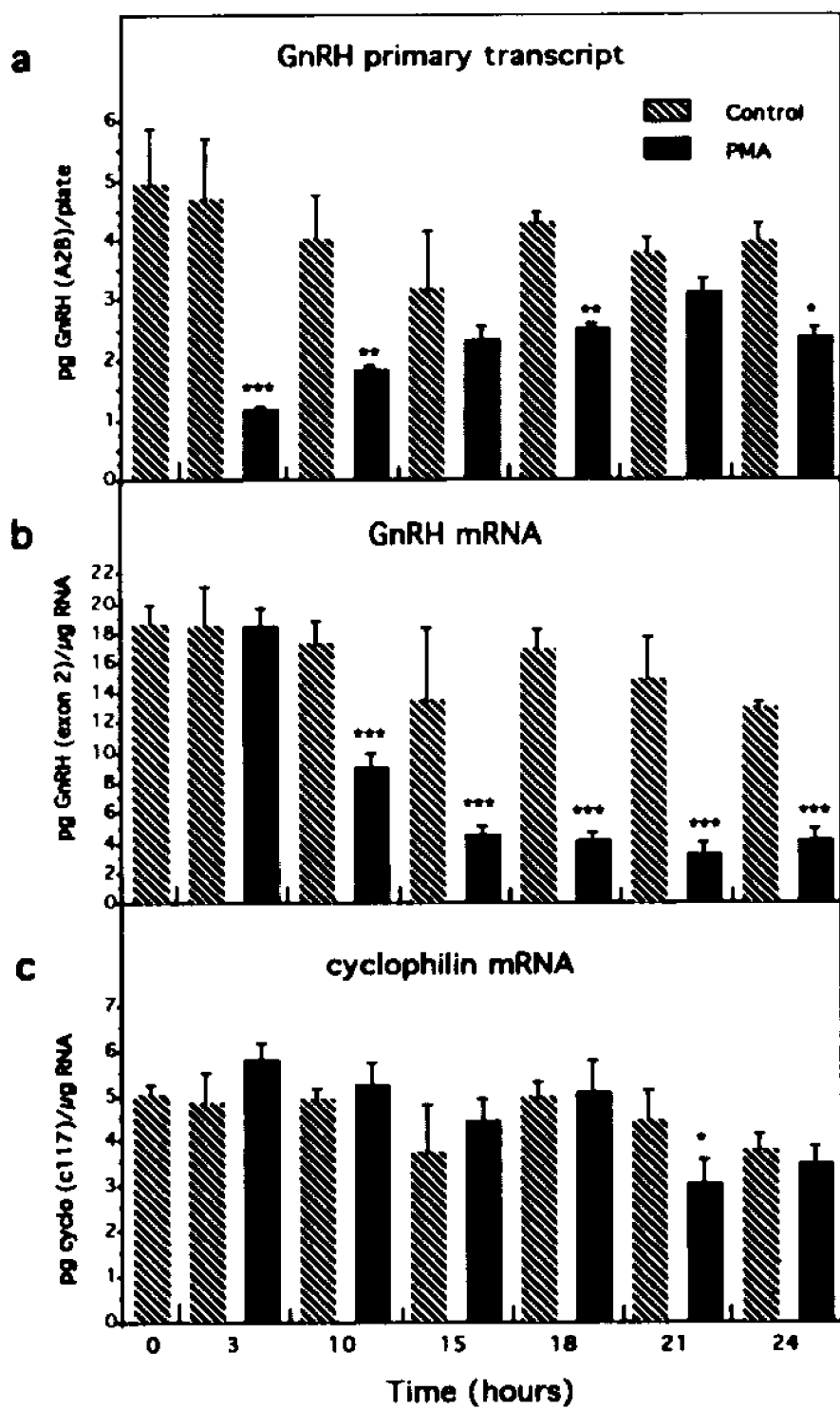


Figure 1
Yeo et al, GnRH regulation by PKC

Fig. 2. Effects of continuous and short-term (1 hour pulse) exposure to PMA on GnRH primary transcript, GnRH mRNA, and cyclophilin mRNA levels.

GT1-7 cells were treated with either vehicle (DMSO) as control group; PMA (100 nM) as the continuous treatment group; or pulse with PMA for 1 hour followed by change of medium as the short-term exposure group. Cells were harvested 8 or 16 hours after the initial drug treatment and about 4-8 μ g of cytoplasmic RNA was recovered and used in the RNase protection assay. Two-way ANOVA showed that there were significant changes in GnRH primary transcript (treatment: $p < 0.001$) and mRNA levels (treatment: $p < 0.0001$; time: $p < 0.0027$). Post-hoc analysis showed that both continuous and 1 hour PMA treatment resulted in a significant suppression of nuclear primary transcript (2a) and cytoplasmic GnRH mRNA (2b) levels at both 8 and 16 hours with no significant effect on cyclophilin mRNA levels (2c). *, $p < 0.05$; **, $p < 0.001$; ***, $p < 0.0001$ vs control.

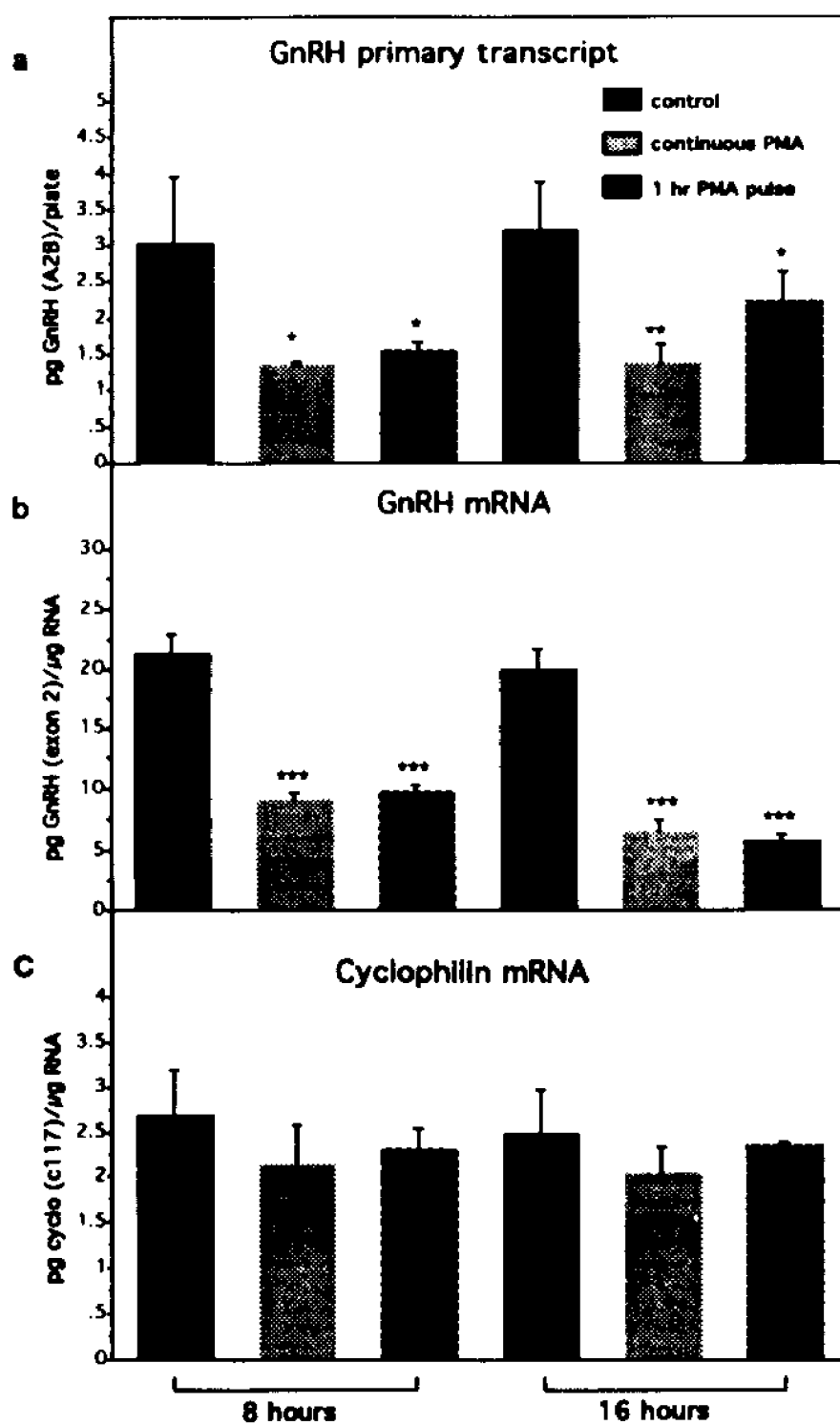


Figure 2

Yeo et al, GnRH regulation by PKC

Fig. 3. Effect of PMA and cycloheximide on c-fos mRNA and GnRH primary transcript levels.

GT1-3 and GT1-7 cells were treated with vehicle (DMSO) as control; PMA (10 nM); or pre-treated with cycloheximide (100 µg/ml) 15 minutes before the addition of vehicle, CHX; or PMA (10 nM), CHX+PMA. GT1-3 cells were harvested 1 hour and GT1-7 cells were harvested 90 minutes after vehicle or PMA treatment. About 40-80 µg of cytoplasmic RNA was recovered. 10 µg of cytoplasmic RNA was hybridized to c-fos probe to measure the levels of c-fos mRNA by RNase protection assay. Cycloheximide treatment increased basal and PMA-induced c-fos mRNA levels. The means of c-fos mRNA levels of each experimental group in the GT1-3 cells are: Control, 0.65; PMA, 9.01; CHX, 1.28; CHX+PMA, 52.8 pg/µg cytoplasmic RNA (3a); and in the GT1-7 cells are: Control, 0.65; PMA, 1.99; CHX, 1.15; CHX+PMA, 19.7 pg/µg cytoplasmic RNA (3c). Nuclear RNA isolated from the each plate was hybridized to GnRH A2B probe. One-way ANOVA showed a significant suppression of GnRH primary transcript by PMA as compared to control: GT1-3 cells, $p < 0.0001$ (3b); GT1-7 cells, $p < 0.05$ (3d), and by CHX+PMA as compared to CHX: GT1-3 cells, $p < 0.0001$ (3b); GT1-7 cells, $p < 0.05$ (3d). *, $p < 0.05$; ***, $p < 0.0001$.

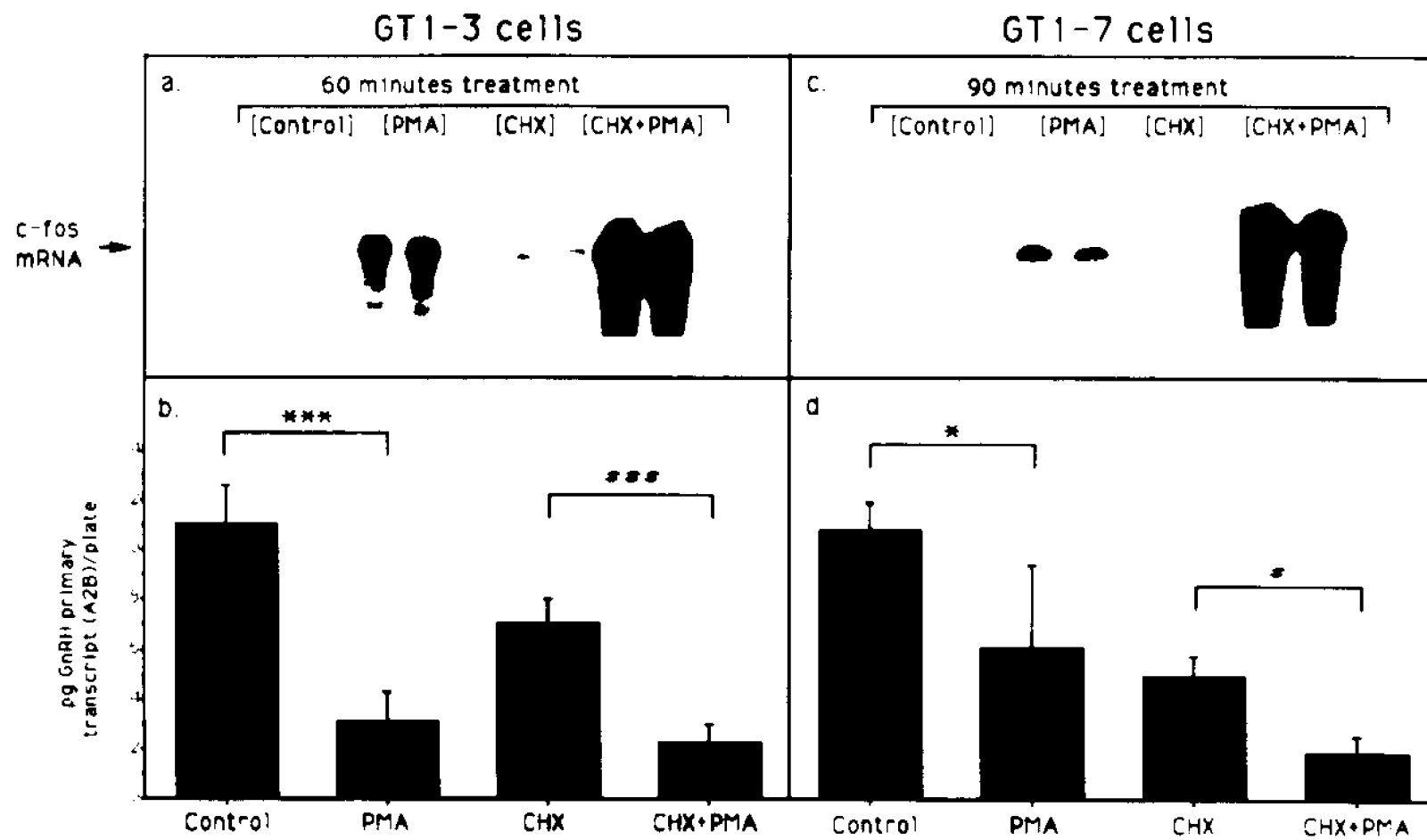


Figure 3
Yeo et al, GnRH regulation by PKC

Fig. 4. Effect of PMA and RNA synthesis inhibitors actinomycin D or DRB on GnRH mRNA levels in GT1-7 cells.

GT1-7 cells were treated with vehicle (DMSO) as control, Ctr; the RNA synthesis inhibitors actinomycin D (5 μ g/ml), Act-D, or DRB (100 μ g/ml); PMA (100 nM); PMA with actinomycin D, P+A, or with DRB, P+D; PMA for 1 hour followed by the addition of actinomycin D, P1+A, or DRB, P1+D. Cytoplasmic RNA was assayed by RNase protection assay. Two-way ANOVA showed that there were significant treatment differences among both actinomycin D and DRB treatment groups (actinomycin D, $p < 0.0001$; DRB, $p < 0.0001$). Post-hoc analysis showed that there was no significant difference between control and actinomycin D or DRB treated groups while PMA decreased GnRH mRNA levels significantly at both 8 (4a, 4c) and 16 hours (4b, 4d). ***, $p < 0.0001$ vs control. Co-treatment of PMA with either inhibitors, either simultaneously (P+A, P+D) or added one hour after PMA (P1+A, P1+D), significantly attenuated the PMA effect on GnRH mRNA levels. ##, $p < 0.001$; ###, $p < 0.0001$ vs PMA alone.

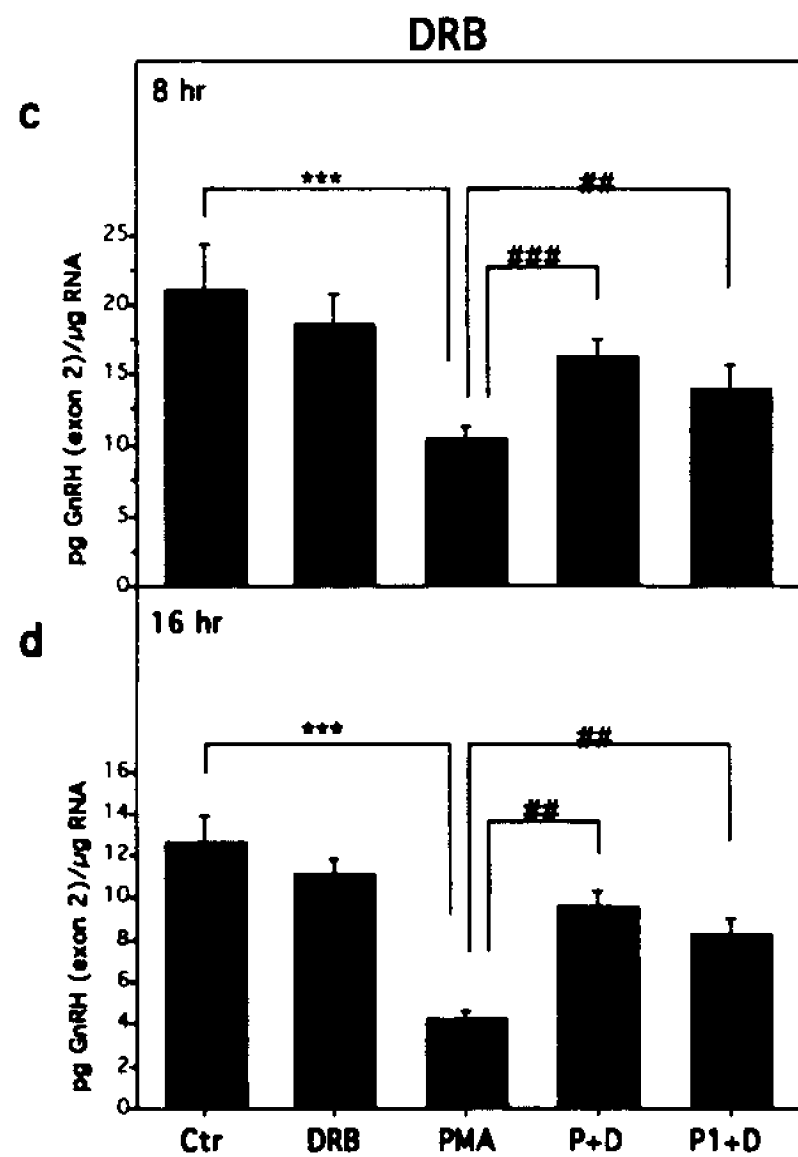
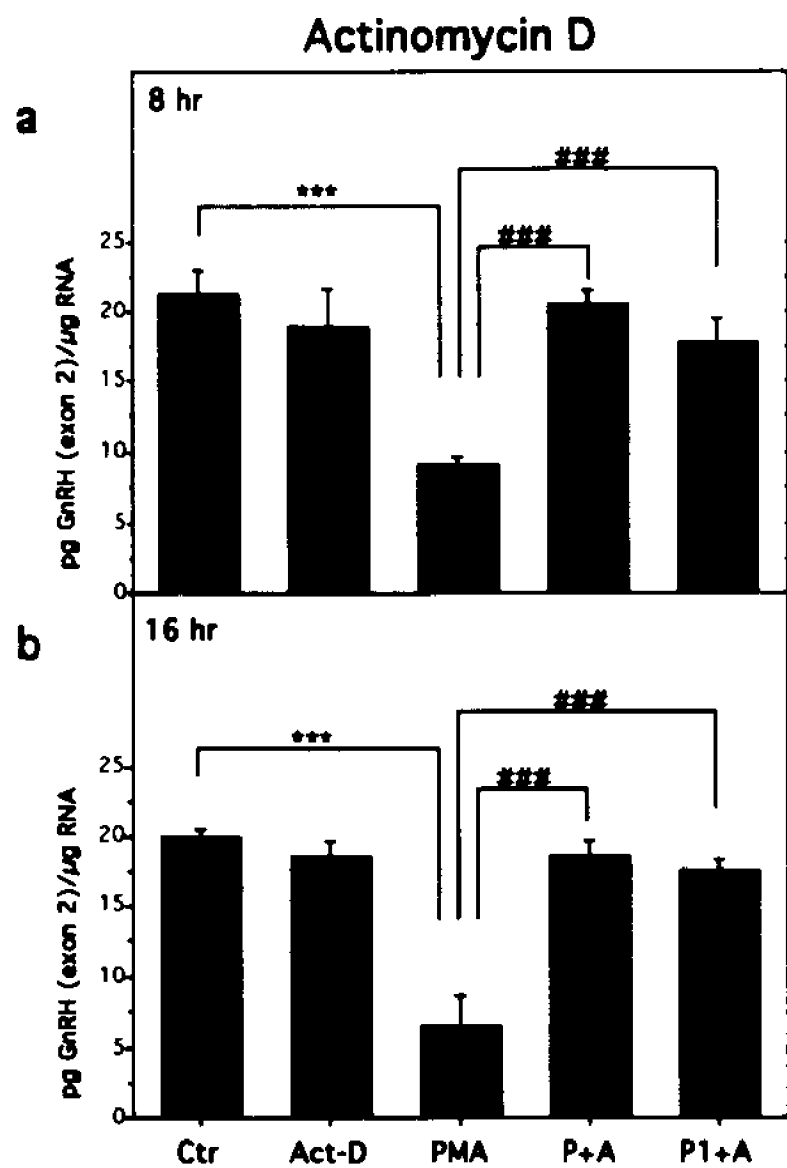


Figure 4
Yeo et al, GnRH regulation by PKC

Table 1. Effect of PMA on GnRH gene expression

Methods	Control	PMA
Nuclear run-on assay rate of GnRH gene transcription (relative intensity unit)	62711 ± 7912	31716 ± 1504 * (51% of control)
Transfection analysis GnRH promoter activity (luciferase enzyme activity)	3090 ± 529	1364 ± 168 * (44% of control)

a. Nuclear run-on assay: GT1-3 cells were treated with vehicle (DMSO) as the control group or PMA (10 nM) for 1 hour. Nuclei were isolated and nuclear run-on assay was performed to determine the effect of PMA on the rate of GnRH gene transcription. One-way ANOVA showed that 1 hour of PMA treatment resulted in significant decrease of GnRH gene transcription rate. *, p<0.05.

b. Transfection assay: GT1-7 cells were transfected with mouse GnRH promoter/ luciferase DNA construct and treated with either vehicle (DMSO) as control or PMA (10 nM). One-way ANOVA showed that GnRH promoter activity was significantly decreased under PMA treatment. *, p<0.05.

Chapter VI

General Discussion

The regulation of the GnRH neuronal system is a classic example of the complex, multi-factorial interactions of cells in the central nervous system to integrate the bulk of information generated from dynamic developmental, physiological, and environmental changes and to respond after it had "made the decision" by altering the release and/or the biosynthesis of neurohormones which subsequently affect the physiological functions. Thus the central nervous system can exert the control of its "messengers", the neurohormones, at multiple levels with different time courses. The most immediate yet transient effect is to alter the rate of hormone release which would directly affect the target cells. The change in hormone transport and storage, and in some cases prohormone production and processing, can also have fairly rapid physiological effects. The function of neurosecretory cells can also be regulated by slower but longer-term effects by changing the levels of cytoplasmic mRNA which provide the templates for the synthesis of prohormones.

The time course and the magnitude of the changes in cytoplasmic mRNA levels in response to hormonal manipulation can be controlled at multiple steps along its biosynthetic pathway: rate of transcription, rate of precursor processing, rate of nuclear to cytoplasmic transport, rate of cytoplasmic mRNA turnover, and the sizes of nuclear and cytoplasmic RNA pools relative to the rate of synthesis. For example, if a certain RNA species has a relatively small nuclear and cytoplasmic RNA pool, large increase in the rate of transcription will result in dramatic increase of cytoplasmic mRNA levels (assuming precursor processing and nuclear to cytoplasmic transport are not the rate-limiting steps). On the other

hand, as has been observed in many hormonal systems that the secretory cells tend to have fairly large basal cytoplasmic hormone encoding mRNA pools to ensure adequate supplies of hormones ready to be secreted. Then the most rapid and effective way to make sudden fluctuation of mRNA levels is to change its stability (rate of turnover). The processing rate of RNA precursors and the control of nuclear to cytoplasmic transport can also significantly alter cytoplasmic mRNA levels of a given gene if it has a large nuclear RNA pool. By this kind of multi-faceted control, the nervous system is able to regulate the biosynthetic rate of its RNAs to ensure appropriate physiological function.

In the past decade, with the development of recombinant DNA technology, there has been significant advancement in the understanding of neuropeptide hormone gene expression (for review, see Roberts et al, 1982; Blum, 1989; Mains et al, 1990). In particular, the availability of cell line models has provided invaluable tools for addressing the cellular and molecular aspects of hormonal gene regulation which is otherwise difficult to be performed in the animal. For example, AtT20 cells have been used for studying the regulation of the proopiomelanocortin (POMC) gene and GH₃/GC cells have been used for studying prolactin and growth hormone gene regulation.

As discussed in Chapter III, we have analyzed the basic characteristics of the GnRH gene transcripts in GT1 cells. We have established assay methods for the measurement of GnRH RNA transcripts in the GT1 cells, including primary transcript, processing intermediates, nuclear and cytoplasmic mRNA. These methods have enabled us to directly address the transcriptional and posttranscriptional (RNA processing, transport, and turnover) control of GnRH gene expression. We have proposed that the measurement of GnRH primary

transcript level should indirectly reflect the rate of GnRH gene transcription since the half-life of its precursor RNA should be short. We then determined the half-life of the GnRH primary transcript by measuring its decay in the presence of RNA synthesis inhibitors. The finding that indeed the half-life of GnRH primary transcript is very short (18 minutes) suggests that the assay would be valid. In a later chapter (Chapter V) we have performed two other assays (nuclear run-on assay and transient transfection promoter analysis) together with the measurement of primary transcript, to assess the transcriptional regulation of GnRH gene expression in response to PKC activation. The fact that three different assays all give the same result strengthens the ground for using the levels of GnRH primary transcript as an indicator of transcriptional activity. The establishment of this system enables us to analyze the hormonal control of GnRH gene expression in a single RNA assay system. Furthermore, as discussed earlier, the changes in cytoplasmic mRNA levels are the sum of changes at multiple steps along its biosynthetic pathway. Using this assay method to study the hormonal control of GnRH gene expression is particularly advantageous because the design of our intron/exon/intron probes enables us to monitor the levels of primary transcript, processing intermediates, nuclear and cytoplasmic mRNA in one assay.

It is interesting to note that our measurement of the basal levels and half-lives of GnRH cytoplasmic mRNA has raised an interesting question about the time course and mechanisms for regulating GnRH mRNA levels in GT1 cells. The large basal pool and long cytoplasmic half-life (>24 hours) of GnRH mRNA as compared to the short half-life and small nuclear pool of its precursor suggest that alteration of cytoplasmic stability is more effective for mediating rapid

changes in mRNA levels. However, persistent changes in the rate of gene transcription can also result in significant changes in GnRH mRNA levels.

In a study not included in this thesis, we found that the activation of three major intracellular second messenger pathways (forskolin and 8-bromo-cAMP for cAMP/protein kinase A, phorbol ester for diacylglycerol/protein kinase C, and ionomycin for calcium/CaM kinase) with pharmacological reagents result in stimulation of GnRH secretion with different time course and a slightly different degree in the GT1 cells (Yu et al, submitted). The administration of ionomycin in the GT1 cells causes a very rapid increase in GnRH secretion, while phorbol ester and forskolin have a slower stimulatory effect. In short-term (1-3 hour) treatment, phorbol ester triggers more GnRH release than forskolin. However, long-term treatment with all these three GnRH secretagogues results in the attenuation of GnRH secretion and by 24 hours, there is no significant difference between the accumulated release from control group and from the three treatment groups. Analysis of GnRH mRNA shows that GnRH mRNA levels decreased significantly in all three treatment groups as compared to control.

It is surprising to find that there is no subsequent increase of GnRH biosynthesis, at least at the RNA levels, to replenish the GnRH pool in response to stimulated secretion. Rather, these secretory stimulations all lead to a decrease in GnRH mRNA levels. We further examined the mechanisms which are responsible for the decrease of GnRH mRNA levels in response to these three GnRH secretagogues. We found that all these three secretagogues exert their effects on GnRH gene expression at different levels and with different time courses: the activation of the cAMP/PKA pathway (Chapter IV) results in a slow but gradual inhibitory effect on GnRH gene transcription; the activation of the

diacylglycerol/PKC pathway (Chapter V) results in a rapid (within one hour) but transient transcriptional repression and a slower effect on mRNA stability; the activation of the calcium/CaM kinase pathway (included in a co-author manuscript, Yu et al, submitted) results in a rapid and long-term inhibition on GnRH gene transcription.

This discrepancy of GnRH gene regulation in response to different second messenger activation has raised several interesting questions. The first question is whether the GnRH system can respond to the same signal differently depending on the length and continuity of exposure. It has been found that there are divergent responses in the levels of the three gonadotropin subunit mRNAs to GnRH administration, depending on whether GnRH is administered continuously or in a pulsatile manner (Weiss et al, 1990). The level of the common α -subunit mRNA is increased by both continuous or pulsatile GnRH; FSH- β mRNA decreases significantly under continuous GnRH but is stimulated 4-fold by pulsatile GnRH; LH- β mRNA does not respond to either treatment. It has also been proposed that the GnRH system can also respond to progesterone differently: short-term, pulsatile treatment results in a stimulatory effect while long-term, continuous treatment results in inhibition (Ramirez et al, 1985). Since our studies have been conducted in a static culture system, a perfusion system which allows the administration of pulses of drugs will be helpful in assessing whether the GT1 cells will respond to these three secretagogues differently.

The second question is whether there is crosstalk between second messenger systems which act in concert to give the final levels of GnRH gene expression, in particular with the PKA-inhibition of GnRH gene expression, since time course analysis indicates a slow yet specific effect on GnRH gene

transcription (Chapter IV). It has been reported that the elevation of intracellular cAMP levels can result in the increase of cytosolic calcium (Luini et al, 1985). It has also been observed that both PKC activity and its nuclear translocation can be affected by intracellular cAMP levels (Narindrasorasak et al, 1987; Cambier et al, 1987). Thus it is possible that the inhibitory effect of cAMP on GnRH primary transcript and mRNA levels in the GT1 cells is secondary, triggered by the activation of other second messenger pathways. Further experiments, including the use of specific enzyme inhibitors, need to be performed to address this question.

The third question is, what are the promoter elements in the GnRH gene which are responsible for mediating these inhibitory effects by the activation of these three pathways? In a study which is not included in this thesis, we have found that serum also has rapid inhibitory effects on GnRH gene transcription and mRNA levels (Yeo and Roberts, unpublished observations). It is possible that these signals all converge on the same regions of the GnRH promoter which have a dominant inhibitory effect on GnRH promoter activity. On the other hand, the transcriptional effects on the GnRH gene by these factors, like the c-fos gene where distinct regions in the promoter can respond to different signals and exert similar effects on gene transcription (for review, see Hershman, 1991), can be mediated by different DNA elements. Extensive promoter analyses, which are ongoing in our and several other laboratories, will help to answer these questions.

Finally, in the study elucidating the post-transcriptional effect of PKC activation on GnRH gene expression, we have found that the PMA-effect on GnRH mRNA is abolished by RNA synthesis inhibitors. This suggests there is a

requirement for the synthesis of new factors to exert the post-transcriptional effect. The observation that the addition of RNA synthesis inhibitors one-hour after PMA addition was still sufficient to inhibit the PMA-effect on mRNA turnover suggests that the induction of these new factors is not an immediate event. Detailed time course analysis defining the time of this induction and delineating the regions in GnRH mRNA which are responsible for this regulatory event will be helpful for understanding the mechanisms for post-transcriptional regulation of GnRH gene expression.

The GT1 cells have provided a wonderful tool by which, for the first time, many questions regarding the regulation of the GnRH system can be directly addressed. However, the discrepancy between some of the results obtained from *in vivo* studies (which is the sum of the behavior of a mixed neuronal population) and those from GT1 cells (a clonal population of immortalized neurons) has told us that caution has to be taken to interpret the results from either system. Like the study of many other hormonal systems, careful studies performed using both animal and cell line models will be able to compensate for the disadvantages and possible artifacts produced by using either system alone. Although there is still a long way to go before the cellular and molecular mechanisms underlying the regulation of expression of this important neuroendocrine peptide are fully revealed, many questions which have been difficult to address in the animal system, are being studied by the use of the GT1 cells. In order to better understand the complex, multi-faceted regulation of the GnRH system, it is important to first understand the cellular and molecular mechanisms mediating these events at the single cell level. Thus utilizing the GT1 cells, one can design more appropriate experiments and hopefully arrive at a better interpretation of the results than from an *in vivo* GnRH system.

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