AUTOCRINE REGULATION OF GONADOTROPIN-RELEASING HORMONE IN IMMORTALIZED HYPOTHALAMIC GT1-7 NEURONS

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DECLARATION

I, ***A.L.Pithey***, hereby declare that the work on which this thesis is based is my original work (except where acknowledgements indicate otherwise) and that neither the whole work nor any part of it has been, is being, or is to be submitted for another degree in this or any other University.

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"How should people know what is coming to pass within them,

when there are no words to grasp it?

How could the drops of water know themselves to be a river?

Yet the river flows on.

Antonie de Saint-Exupery, The Wisdom of the Sands.

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ABSTRACT

The existence of an ultrashort feedback mechanism regulating GnRH secretion has been supported from *in vivo* and *in vitro* studies. However, the complex synaptic connections of GnRH neurons with other neural elements made it difficult to determine whether the regulation was mediated by direct actions on the GnRH neurons or through actions on other interneurons. The recent development of the GnRH-secreting neuronal cell line, GT1, provided a model system for the study of neural regulation of a pure population of GnRH neurons. The present studies utilized GT1-7 cells to investigate whether GnRH (at the level of the nerve terminal) influences the control of its own release.

Preliminary studies determined the presence of GnRH mRNA in GT1-7 cells and established a cell culture system for the analysis of secretagogue-induced GnRH release. In this system GnRH release was shown to be spontaneous and was enhanced by the addition of K^+, L-GLU, forskolin and PMA. Furthermore, K^+- and forskolin-induced GnRH release was dependent on extracellular Ca^{2+}. For the analysis of an ultrashort feedback mechanism, GT1-7 cells were cultured in 6-well plates to near confluence and then incubated in serum-free medium in the presence (1nM-1μM) or absence of GnRH antagonist, Ant 27. Basal, K^+-and forskolin-induced secretion of GnRH was monitored with antiserum 1076 which does not cross-react with Ant 27 at > 1 μM. Ant 27 treatment increased basal, K^+- and forskolin-stimulated GnRH release in a dose-dependent manner. Total content was unaffected by 18 h treatment of GT1-7 cells with Ant 27. This suggests that the effects of Ant 27 are at the level of release and not biosynthesis. The presence of GnRH binding sites in the cells was demonstrated with ^{125}I-GnRH analog. These findings support the concept that GnRH, acting via autoreceptors, negatively controls its own release.

ABBREVIATIONS

AMPA	α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid
AMV	Avian myeloblastosis virus
AN-ME	Arcuate nucleus-median eminence
ANG II	Angiotensin II
BSA	Bovine serum albumin
$[Ca^{2+}]_i$	Intracellular Ca^{2+} concentration
CA	Catecholamines
cDNA	complementary DNA
CH_3CN	Acetonitrile
CNS	Central nervous system
CRH	Corticotropin-releasing hormone
DA	Dopamine
DBB	Diagonal band of Broca
DEPC	Diethyl pyrocarbonate
dH_2O	Distilled water
DMEM	Dulbecco's modified eagles medium
DNQX	6,7-dinitroquinoxaline-2,3-dione
DSIP	Delta sleep-inducing peptide
E_2	Estradiol
EDTA	Disodium ethylenediaminetetra acetic acid
EOP	Endogenous opioid peptides
FBS	Fetal bovine serum
FSH	Follicle-stimulating hormone
GABA	Gamma-aminobutyric acid
GAL	Galanin
GAP	GnRH-associated peptide
GLU	Glutamate
GnRH	Gonadotropin-releasing hormone
GRF	Growth hormone-releasing factor
GuSCN	Guanidinium thiocyanate
HA	Histamine
HPLC	High-performance liquid chromatography
IL	Infundibular lip
IP_3	Inositol 1,4,5-triphosphate
ir	immunoreactive
LH	Luteinizing hormone
MBH	Medial basal hypothalamus
ME	Median eminence
MK-801	(+)-5-methyl-10,11-dihydro-5H-dibenzo[A,D]cyclohepten-5,10-imine maleate
mRNA	messenger RNA
$Na_2S_2O_5$	Sodium metabisulphate
NAL	Naloxone
NE	Norepinephrine
NH_4Ac	Ammonium acetate
NMDA	N-methyl-D-aspartate

NPK	Neuropeptide K
NPY	Neuropeptide Y
NSB	Non-specific binding
NT	Neurotensin
OVX	Ovariectomized
PBS	Phosphate buffered saline
PCR	Polymerase chain reaction
PDGF	Platelet-derived growth factor
PIPES	Piperazine-N-N'-bis [2-ethane sulfonic acid]
PKA	Protein kinase-A
PKC	Protein kinase-C
PMA	Phorbyl-12-myristate-13-acetate
POA	Preoptic area
PPC	Push-pull cannulae
PRL	Prolactin
RIA	Radioimmunoassay
RNase	Ribonuclease
RT	Reverse transcriptase
S-ME	Stalk-median eminence
SDS	Sodium dodecyl sulphate
SP	Substance P
SS	Somatostatin
T	Testosterone
TBE	Tris-borate buffer
TGF-α,-B	Transforming growth factor-α,-B
TRH	Thyrotropin releasing hormone
VP	Vasopressin
VSCC	Voltage-sensitive Ca^{2+} channels

1: NEURAL REGULATION OF GnRH BIOSYNTHESIS AND RELEASE

1.1 INTRODUCTION

Gonadotropin-releasing hormone (GnRH) is a hypothalamic decapeptide that plays a pivotal role in controlling reproduction in a variety of animal species. GnRH acts on the anterior pituitary gland to stimulate the biosynthesis and release of the gonadotropins, follicle-stimulating hormone (FSH) and luteinizing hormone (LH). These hormones in turn stimulate gonadal steroidogenesis and gametogenesis. The steroids feed back at the pituitary and the hypothalamus to modulate further production of the gonadotropins and GnRH (Schally, 1978; Millar *et al.*, 1987).

GnRH perikarya have been localized in several brain regions, but are concentrated in the ventral forebrain. Here, cells are dispersed in several areas including the diagonal band of Broca (DBB), stria terminalis, medial preoptic area (POA), lateral POA, periventricular POA, and the ventral portions of the medial and lateral septal nuclei (Kalra and Kalra, 1983; Silverman, 1988). GnRH is synthesized as part of a larger prohormone in neuronal cell bodies, especially in the medial POA and the suprachiasmatic area. The human prohormone contains the sequence for GnRH and a sequence of 56 amino acids called GnRH-associated peptide (GAP) (Seeburg and Adelman, 1984). Following processing of the prohormone within the neurosecretory granules, GnRH is released into the hypophyseal portal circulation from nerve terminals within the median eminence (ME). This release is pulsatile and obligatory to pulsatile LH release. Control is exerted by neural pathways in the central nervous system (CNS) which converge onto the GnRH-secreting cells (Schally *et al.*, 1971; Knobil, 1980; Rangaraju *et al.*, 1991). The localization of GnRH and GnRH receptors in extrahypothalamic brain areas suggests that, in addition to being the regulator of LH and FSH release, GnRH might act as a central neuromodulator (Kalra and Kalra, 1983; Reubi and Maurer, 1984).

This literature review will discuss the neural systems which are responsible for the regulation of hypothalamic GnRH biosynthesis and release. The essence of the thesis describes an investigation into the role of GnRH in modulating its own secretion.

1.2 FACTORS REGULATING GnRH BIOSYNTHESIS AND RELEASE

Neuroendocrine regulation of GnRH secretion is the central control point in the complex modulation of gonadal function at all stages of life. These include the transitional states of puberty and seasonal or ovarian cyclicity (Wildt *et al.*, 1980; Lincoln and Short, 1980; Knobil, 1980). Various modulatory hormonal and neural inputs act to influence the amplitude/ frequency characteristics of GnRH discharge or to provide a " permissive milieu " that allows the GnRH neurons to produce and discharge optimal levels of neurohormone with inherent periodicity (Kalra and Kalra, 1983).

In several species the neuroendocrine mechanisms of pubertal development involve the integration of various processes at the levels of the hypothalamus, pituitary and gonads which occur at different times during the prepubertal period. These, in turn result in the culmination of a multitude of hormonal and anatomical developmental changes. A number of neuronal factors are implicated in the maturation of the GnRH surge centre. The final component elicits the increased frequency of pulsatile GnRH secretion responsible for triggering the prepubertal LH surge (Ojeda *et al.*, 1980; Bourguignon and Franchimont, 1984).

The GnRH neuron receives a sparse but chemically diverse synaptic input which includes the following:

1.2.1 NEUROTRANSMITTERS

CATECHOLAMINES (CA)

Subsequent to the initial observations that dibenamine, an α-adrenergic antagonist,

blocks ovulation in the rabbit, numerous investigators have suggested a role for CA in the reproductive process (Sawyer *et al.*, 1947; McCann *et al.*, 1975; Negro-Vilar *et al.*, 1979). CA terminals directly innervate GnRH neurons, providing the anatomical substrate for CA modulation of GnRH secretion, although neither norepinephrine nor dopamine is localized within GnRH neurons (Ajika, 1979; Hoffman *et al.*, 1982; Chen *et al.*, 1989a).

It is widely accepted that certain neuromodulators convey information regarding the steroidal milieu to the GnRH neurons, since few if any GnRH neurons concentrate steroids or contain steroid receptors (Shivers *et al.*, 1983; Bicknell, 1985; Herbison and Theodosis, 1992). Interestingly, CA neurons are capable of concentrating steroids raising the possibility that they may mediate GnRH release in response to changes in sex steroid levels (Sar and Stumpf, 1981; Sar, 1984). Further evidence to support this view are the findings by Moguilevsky *et al.* (1990) who showed that the effect of CA on GnRH- induced LH secretion changes from inhibition to stimulation during sexual maturation in the female rat. They postulate that these changes are related to modifications in the response of the CA neurons to ovarian hormones.

NOREPINEPHRINE (NE)

The GnRH cell bodies in the POA of the rat hypothalamus lie in close apposition to noradrenergic fibres which originate in the medulla and the locus ceruleus. This anatomical association supports the involvement of NE in modulating GnRH release (Palkovits, 1981; Hoffman *et al.*, 1982).

Ramirez *et al.* (1986) monitored pulsatile GnRH release by means of push-pull cannulae (PPC) implanted in the hypothalami of conscious, unrestrained female rabbits. Their data showed that infusion of NE (100 nM - 1 µM) induced a 2-fold increase in GnRH release. In a similar study, perfusion of NE into the medial basal hypothalamus (MBH) stimulated GnRH release, which is accompanied by an increase in plasma LH, in estradiol (E_2)-treated ovariectomized (OVX) rabbits, but not OVX rabbits (Pau and Spies, 1986). *In vivo* experiments by Terasawa *et al.* (1988)

demonstrated that NE acting through an α_1-adrenergic receptor stimulates pulsatile GnRH release in OVX rhesus monkeys, questioning the involvement of ovarian steroids in mediating this effect. In support of this observation Jarry *et al.* (1990) showed that NE via an α_1-adrenergic mechanism in the preoptic/anterior hypothalamus but not MBH is required to drive the GnRH pulse generator in OVX rats. Furthermore, studies by Levine *et al.* (1991) showed that treatment with an α_1-adrenergic antagonist reduced GnRH pulse generator activity in OVX rats, but was ineffective in gonadally intact female rats. Their explanation for this observation is that the ovarian hormones suppress the activity of the GnRH pulse generator by inhibiting NE synaptic transmission.

The literature is thus inconsistent regarding the effects of ovariectomy and ovarian steroid treatment on NE stimulated GnRH release.

Gore and Terasawa (1991) investigated whether changes in NE input to the GnRH neurosecretory system of female rhesus monkeys contributed to the known pubertal increase in GnRH release. Their results showed that the response of the prepubertal group to α_1-adrenoceptor stimulation was significantly greater than those of the older age groups. Secondly, they found that NE release increased significantly from the pre- and early pubertal to the midpubertal stage. These results lead them to propose that the increase in NE release during puberty contributes to the developmental increase in GnRH release.

Leposavic *et al.* (1990) using an *in vitro* superfusion system demonstrated that an α_1-agonist stimulated GnRH release from hypothalami from adult and peripubertal rats but inhibited the secretion from the limited stores available in hypothalami from immature rats. Hypothalami from castrated adult rats responded with a marked reduction in GnRH release to α_1-adrenoceptor stimulation *in vitro*. These results were similar to the *in vivo* stimulation of immature rats and support a role for testosterone (T) in this response. Binding studies revealed a striking increase in the density of α_1- and β-adrenoceptor binding sites in the adult but failed to demonstrate concomitant age-related changes in α_2-adrenoceptor binding. In addition to the above mentioned

proposal by Gore and Terasawa (1991), these observations raise the possibility that alterations in the nature of the GnRH response to α_1-adrenoceptor stimulation may be a contributory factor in the initiation of puberty.

Besides the effects on GnRH release, Weesner *et al.* (1991) showed that the administration of an α_1-adrenergic antagonist into the POA of OVX rats suppressed levels of GnRH messenger RNA (mRNA). Further studies by the same group have suggested that NE, acting through an α_1-adrenoceptor, is a mediator of the stimulatory effects of estrogen on GnRH biosynthesis and release and subsequent LH release (Weesner *et al.*, 1993).

DOPAMINE (DA)

Dopaminergic fibres end in close proximity to GnRH projections in the perivascular region of the ME and synaptic contacts between these systems have been reported in the ME of the ewe and also in the medial POA of the rat (Ajika, 1980; Watanabe and Nakai, 1986; Kuljis and Advis, 1989).

Evidence exists to support both an inhibitory and a stimulatory role for DA in GnRH release. This may be indirect since both Sarkar and Fink (1981) and Jarjour *et al.* (1986), using *in vivo* and *in vitro* systems in the rat, suggest that the DA-induced release of GnRH is most likely attributable to DA-induced release of NE. This is supported by the following observations: 1) an α-adrenergic antagonist prevented the DA effect (*in vitro*); and 2) normal NE synthesis is required for the occurrence of a full GnRH surge (*in vivo*). Recently Contijoch *et al.* (1992) reported that DA decreases GnRH release from denervated ME tissues in hens. However, at the level of biosynthesis, Li and Pelletier (1992) demonstrated that GnRH mRNA levels in male rats are positively regulated by DA.

The opposite effects of DA on GnRH biosynthesis and release are probably due to the existence of pharmacologically distinct DA receptors in the CNS.

SEROTONIN

Kiss *et al.* (1985) using a combination of immunocytochemistry and autoradiography demonstrated that serotoninergic axons terminate on GnRH neurons in the POA of the rat. However, recent studies were unable to detect mRNA encoding for serotonin 2, 1A or 1C receptor subtypes in GnRH neurons of both OVX and OVX-E_2 treated rats (Wright and Jennes, 1993).

Arias *et al.* (1990) using both *in vivo* and *in vitro* systems demonstrated that serotonin is involved in mediating GnRH release. Their results show age related opposite effects of serotonin on GnRH neurons in the female rat. Enhancement of GnRH release is observed at 14 days of age, while inhibition occurs in 30-day-old rats. They postulate that this switch in the changing effect of serotonin on GnRH release in juvenile rats may be involved in the onset of puberty.

Wright and Jennes (1993) have suggested that these effects are not mediated by direct activation of serotonin 2, 1A or 1C receptors on GnRH neurons. Instead serotonin may act via, yet unidentified serotonin receptor subtypes or via non-serotoninergic intermediary neurons.

GLUTAMATE (GLU)

GLU, aspartate and other excitatory amino acids are thought to act mainly as neurotransmitters at excitatory synapses in the vertebrate CNS. GLU has been found to activate ionotropic and/or metabotropic receptors in the CNS. GLU binding to ionotropic receptors results in Ca^{2+} entry through a channel within the receptor, as well as through voltage-sensitive Ca^{2+} channels (VSCC). N-methyl-D-aspartate (NMDA) is a selective agonist for one subtype of ionotropic receptor, the NMDA receptor, whereas α-amino-3-hydroxy-5-methyl-4-isoxazole propionic acid (AMPA) and/or kainate is a selecitve agonist for the other subtype, the AMPA/kainate receptor. GLU binding to metabotropic receptors may result in stimulation of inositol 1,4,5-triphosphate (IP_3) production and release of Ca^{2+} from intracellular stores (Mayer and

Miller, 1990). Furthermore, recent evidence has demonstrated that the GnRH neuronal network receives synaptic input from GLU nerve fibers, providing concrete anatomical evidence for direct excitatory GLU control of GnRH release (Van den Pol *et al.*, 1990; Jennes and Dupre, 1991; Goldsmith *et al.*, 1994).

Bourguignon *et al.* (1989a) provided the first direct physiological evidence that neuroexcitatory amino acids trigger the release of GnRH. *In vitro* incubation of hypothalami from 50-day-old male rats in the presence of increasing concentrations of kainate or NMDA resulted in a dose-related stimulation of GnRH release. At concentrations of 10, 50 and 100 mM, NMDA was about twice as potent as similar amounts of kainate in eliciting GnRH release. They concluded that the effects of kainate and NMDA are mediated through distinct receptor types, since the GnRH response to kainate was not affected by factors such as glycine or Mg^{2+} concentrations which did affect the response to NMDA. Studies using male rat arcuate nucleus-median eminence (AN-ME) fragments *in vitro* have demonstrated that, at this level, GnRH release is mediated via non-NMDA receptors (Donoso *et al.*, 1990; Lopez *et al.*, 1992). In addition, Bourguignon *et al.* (1989b) found that MK-801, a non-competitive NMDA antagonist, could suppress the pulsatile secretion of GnRH from hypothalamic explants. In contrast, pulsatile release of GnRH was not affected by the non-NMDA-receptor antagonist 6,7-dinitroquinoxaline-2,3-dione (DNQX).

Urbanski and Ojeda (1990) demonstrated that in prepubertal female rats the pulsatile administration of NMDA resulted in precocious activation of the reproductive system. Furthermore, a number of groups have shown that the administration of MK-801 delayed the subsequent onset of puberty in female rats (Urbanski and Ojeda, 1990; MacDonald and Wilkinson, 1990; Veneroni *et al.*, 1990; Meijs-Roelofs *et al.*, 1991). In addition, the administration of NMDA to 16- and 30-day-old female rats enhanced GnRH content in the anterior POA and MBH. Estrogen and progesterone pretreatment potentiated this increase of GnRH induced by NMDA in the 16-day-old-rats, but not in the 30-day-old rats (Carbone *et al.*, 1992). Similar studies by Bourguignon *et al.* (1990a) reported that the NMDA induced secretion of GnRH from hypothalamic explants of male rats required lower concentrations at 25 days than at 15 or 50 days

of age. Furthermore, this group have shown that NMDA inhibits the *in vitro* secretion of GnRH from hypothalami from immature male rats (Bourguignon *et al.*, 1992a). Their data indicate that the NMDA receptors involved in the stimulatory control of pulsatile GnRH secretion are increasingly activated between 15 and 25 days in the male rat, a period preceding the onset of puberty (Bourguignon *et al.*, 1990a). They conclude that a sequential reduction in activity of inhibitory and facilitatory NMDA receptors provides a developmental basis for the neuroendocrine mechanism of onset of puberty. Additional studies by Bourguignon *et al.* (1992b) have shown that the age-related changes in NMDA receptor activation occur in the absence of gonads, questioning the role of T in this response.

Furthermore, recent studies have reported that NMDA or kainate administration to rats rapidly increases GnRH mRNA levels in parallel with increases in GnRH release (Petersen *et al.*, 1991; Liaw and Barraclough, 1993; Roberts and Gore, 1993).

GAMMA-AMINOBUTYRIC ACID (GABA)

GABA is an inhibitory neurotransmitter and is thought to exert its effect directly on GnRH cells since GABA-ergic neurons make synaptic connections with GnRH cell bodies in the medial POA of the rat (Leranth *et al.*, 1985a). In addition, recent studies by Petersen *et al.* (1993) demonstrated the presence of $GABA_A$ receptor subunit mRNA in GnRH neurons in rat POA.

Evidence from *in vitro* and *in vivo* studies indicate that GABA both stimulates and inhibits GnRH release from ME. It appears that these opposite effects of GABA on GnRH release may be a result of differential activation of two classes of receptors. Studies with adult female rats demonstrate that the $GABA_A$ receptor mediates the stimulatory effect and the $GABA_B$ receptor the inhibitory effect (Masotto *et al.*, 1989). The effect of $GABA_A$ on *in vivo* GnRH release may be mediated by NE, since depletion of endogenous NE resulted in a 17-fold reduction of $GABA_A$ induced GnRH release from rat ME (Luo *et al.*, 1991). In addition, the inhibitory effect of $GABA_B$ receptors on GnRH cells may involve a presynaptic effect, whereby GABA tonically

inhibits those NE terminals which innervate GnRH neurons. This can be deduced from the fact that inhibition of GnRH release does not occur when presynaptic inhibition of NE release is absent (Nikolarakis *et al.*, 1988; Masotto *et al.*, 1989).

In addition, studies by Donoso *et al.* (1992) demonstrated that a $GABA_A$ agonist increased GnRH and enhanced L-GLU induced GnRH release from AN-ME fragments *in vitro*. A $GABA_B$ agonist, on the other hand, reduced L-GLU induced GnRH release. These results provide evidence for a functional link between GABA-ergic and GLU-ergic neurotransmission at the level of the AN-ME, an area known to be involved in the regulation of GnRH secretion.

Furthermore, since GABA neurons contain estrogen receptors, changes in plasma E_2 could affect GnRH release by modulating activity in GABAergic neurons (Flugge *et al.*, 1986).

Interestingly, studies by Moguilevsky *et al.* (1991) with female rats demonstrated that GABA exerts a stimulatory tone on gonadotropin secretion in prepubertal rats (16 days of age) and an inhibitory one in peripubertal animals (30 days old). This effect is most probably mediated by $GABA_A$ receptors. As suggested for the serotoninergic system, this change in effect which takes place during sexual maturation maybe related to the central mechanisms involved in the onset of puberty. This change could involve the development of a different subtype of this GABA receptor or, more likely, the maturation of interneuronal contacts of the GABAergic system with other neurotransmitter systems involved in the control of the GnRH-gonadotropin system (Olsen and Tobin, 1990; Moguilevsky *et al.*, 1991).

Bergen *et al.* (1991) demonstrated that activation of $GABA_B$ receptors in the POA of OVX rats resulted in inhibition of GnRH gene expression. It is not yet known whether this effect is due to a presynaptic effect (i.e. a decrease in excitatory tone to the GnRH cells) or whether $GABA_B$ receptors located on the GnRH cells mediate this effect directly.

HISTAMINE (HA)

Studies by Miyake *et al.* (1987) have showed that HA, via H1 receptors, induced the *in vitro* release of GnRH from rat MBH. They suggest that the H1 receptors are involved in both the basal and E_2-induced GnRH release.

1.2.2 NEUROPEPTIDES

ENDOGENOUS OPIOID PEPTIDES (EOP)

Three major groups of EOP are known: the endorphins, enkephalins and dynorphins. These peptides are produced in separate neuronal systems and each group is found within the hypothalamus. Much of the evidence suggests that EOP may be an integral component of the neural circuitry which exerts an inhibitory control on GnRH release from the ME. Previously a large body of evidence suggested that EOP may exert their effects on GnRH secretion indirectly via modulation of NE release, however, more recent work has focused on both the independent effects, as well as the interaction of these two systems (Kalra and Kalra, 1983; Bicknell, 1985).

There are at least two sites where opioids may affect GnRH neurons, i.e. in the vicinity of the cell bodies and in the region containing their nerve terminals. Chen *et al.* (1989b) demonstrated that β-endorphin-containing neurons constitute almost 10% of the synaptic input to GnRH neurons in the DBB/POA of the male rat. Similarly, Thind and Goldsmith (1988) reported the presence of symmetrical synapses between opioid and GnRH neurons at the infundibular lip (IL) in juvenile rhesus monkeys. The IL is situated just below the AN and it is suggested that these opioid neurons directly inhibit the nearby GnRH pulse generator. Although these data provide anatomical evidence in support of a direct modulation of GnRH release by opiates they do not, however, rule out a possible intermediary effect of opiates on other neuronal systems known to be involved in the control of GnRH release.

Morphine, an opiate agonist, inhibits GnRH release from rat and human MBH *in vitro*.

The suppression of pulsatile GnRH release induced by morphine in human MBH was reversed by the opiate μ-receptor antagonist, naloxone (NAL) (Rotsztejn *et al.*, 1978; Rasmussen *et al.*, 1990). NAL can also stimulate the *in vitro* release of GnRH from POA-MBH fragments of both intact and castrated rats (Kalra *et al.*, 1987). In addition, *in vivo* studies showed that NAL induced GnRH release (by increasing GnRH pulse amplitude) and was equally effective in both intact and OVX rats (Karahalios and Levine, 1988).

The interaction between opioid and noradrenergic systems on GnRH release by POA-MBH explants from intact postpubertal female rats was investigated by Clough *et al.* (1990). Their results demonstrate a potentiating interaction between opioid antagonism and α-adrenergic stimulation on the release of GnRH *in vitro*. This suggests that separate mechanisms of GnRH control by each class of neurotransmitter is present. Studies involving the electrical activity of the GnRH pulse generator by Nishihara *et al.* (1991), lead them to suggest that the noradrenergic and opioid peptidergic systems have tonic facilitatory and inhibitory influences, respectively, on GnRH pulse generator activity in OVX rats. Neither of these neuronal systems themselves appear to be the pulse generator, but both appear to be modulators of the pulse generator. In contrast, *in vitro* studies with hypothalami from adult and prepubertal male rats found that the ability of NAL to release GnRH was inhibited by an α_1-antagonist, suggesting that the μ-receptor-mediated opioid inhibition of GnRH secretion involves an intermediary CA mechanism (Leposavic *et al.*, 1991). To date it remains unclear where the opioid and noradrenergic systems interact in the regulation of GnRH release.

Furthermore, the opioid neurons, analogous to CA neurons, concentrate steroids and are thus implicated in mediating the effects of gonadal steroids on GnRH secretion (Bicknell, 1985).

Hahn and Fishman (1985) have suggested that changes in the number of brain opiate receptors may be involved in gonadal steroid modulation of the sensitivity of the GnRH pulse generator to EOP. In addition, whereas EOP tonically inhibits GnRH release in adult male rats it fails to do so in immature animals. However, hypothalamic

fragments obtained from immature male rats treated *in vivo* with T were capable of responding to NAL with GnRH release *in vitro*. This data suggests that in the rat the maturation of the EOP system is a component of male puberty that is induced by the peripubertal T rise (Nazian, 1992).

NEUROPEPTIDE Y (NPY)

Several studies have demonstrated that NPY, a 36 amino acid peptide, is involved in the control of GnRH release. NPY may act directly on GnRH neurons since synaptic contacts between NPY and GnRH neurons have been found in the sheep and rat POA (Norgren and Lehman, 1989; Tsuruo *et al.*, 1990). Alternatively, since NPY and NE co-exist in certain neurons, NPY may act either co-operatively or via the adrenergic pathways to stimulate GnRH release (Everitt *et al.*, 1984; Allen *et al.*, 1987).

Exposure to 1 μM NPY stimulated the *in vitro* release of GnRH from MBH fragments obtained from steroid-replaced OVX rats, but not from hormonally untreated OVX rats (Crowley and Kalra, 1987; Sabatino *et al.*, 1989). Similarly, *in vitro* perfusion experiments indicated that isolated hypothalami from intact, but not OVX rabbits, responded to NPY with an increase in GnRH release (Khorram *et al.*, 1988). Furthermore, the same group reported that NPY has a bimodal effect on MBH release of GnRH in PP perfusates, producing a stimulation of GnRH release in intact rabbits while inhibiting its release in OVX rabbits (Khorram *et al.*, 1987). These results suggest that NPY is a steroid-dependent modulator of GnRH release in rats and rabbits. In support of this, studies by Sar *et al.* (1990) have shown the co-localization of NPY and E_2 in the hypothalamus of the rat. In contrast, the presence of steroid hormones is not required for the stimulatory action of NPY on GnRH release in non-human primates. This is supported by experiments by Woller and Terasawa (1991) who found that the direct infusion of NPY into the stalk-median eminence (S-ME) increased GnRH release in both gonadectomized male and female rhesus monkeys. Further studies by Woller *et al.* (1992) using the same experimental system lead them to conclude that NPY is an important component of the GnRH pulse generating system since: 1) NPY release in the S-ME was pulsatile, 2) NPY pulses occur

synchronously with GnRH and LH pulses, and 3) immunoneutralization of endogenous NPY into the S-ME suppressed pulsatile GnRH release.

The infusion of prazosin, an α_1-antagonist, into the MBH of OVX E_2-treated rabbits blocked the expected NPY-induced GnRH release. These results by Berria *et al.* (1991) suggest that NPY-stimulated MBH-GnRH release is mediated by or dependent upon α_1-adrenergic pathways. In addition, Sahu *et al.* (1990) found that NAL evoked a concurrent release of both NPY and GnRH from MBH of E_2-primed rats. This finding lead them to propose that a decreased inhibitory opioid tone results in an increase in both NPY and NE release with the subsequent enhancement of GnRH release.

Sutton *et al.* (1988) observed that, after birth, the content of NPY increases in parallel with that of GnRH in the hypothalamus and POA until stabilizing around puberty. Furthermore, the preovulatory GnRH surge is accompanied by a surge of NPY in the pubertal female rat. The same group examined the effects of centrally administered anti-NPY in pubertal female rats. Anti-NPY reduced the magnitude of both the proestrous -GnRH and -LH surges. Together these findings suggest that NPY plays a facilitatory role in the initiation of the GnRH surge that leads to the onset of puberty (Minami *et al.*, 1990).

GALANIN (GAL)

Merchenthaler *et al.* (1990) reported that GAL neurons appear to directly innervate GnRH neurons in the rat. Furthermore, they noted GAL is co-localized in a subset of GnRH neurons in the POA. These studies support the idea that GAL may play a role in regulating GnRH secretion.

Merchenthaler (1991) also reported the following results concerning the differential co-expression of GAL and GnRH in the rat brain: 1) the number of neurons containing both peptides in females, regardless of the stage of the estrous cycle, is 3-4 fold higher than in males; 2) ovariectomy results in a decline in the number of neurons co-expressing GAL and GnRH to levels similar to those seen in the male; 3) E_2 or T

replacement of adult gonadectomized animals enhances co-expression in female but not male animals. The sexual dimorphism and the estrogen-dependent co-expression of GAL indicates the importance of E_2 in the regulation of the co-expression of GAL in a subpopulation of GnRH neurons.

In addition, Lopez and Negro-Vilar (1990) showed that GAL stimulated the *in vitro* release of GnRH from male rat AN-ME fragments. This effect appears to involve the activation of α_1-adrenergic receptors since prazosin, an α_1-antagonist, completely blocked the effect of GAL on GnRH release.

CORTICOTROPIN-RELEASING HORMONE (CRH)

A role for CRH, a 41 amino acid peptide, has been implicated in the mechanism by which stress inhibits the reproductive axis. Direct synaptic contacts between CRH and GnRH containing neurons have been demonstrated in the POA of the rat (MacLusky *et al.*, 1988; Csiffary *et al.*, 1990).

In vivo results have shown that CRH inhibits the release of GnRH into the hypophyseal portal circulation in female rats (Petraglia *et al.*, 1987). In support of this observation *in vitro* evidence has demonstrated that CRH inhibits GnRH release from rat hypothalami (Nikolarakis *et al.*, 1986; Gambacciani *et al.*, 1986). Further experiments by the same group demonstrated that *in vitro* treatment of hypothalamic slices from male rats with the CRH receptor antagonist, CRF9-41 (1 μM), resulted in a significant increase in GnRH release. Comparable results were obtained *in vivo* during push-pull perfusion of the AN-ME region with CRF9-41 (Nikolarakis *et al.*, 1988). In addition, CRH suppressed the pulsatile release of GnRH from macaque MBH *in vitro* and decreased the *in vivo* GnRH pulse generator activity in OVX rhesus monkeys (Williams *et al.*, 1990; Mershon *et al.*, 1991).

GONADOTROPIN-RELEASING HORMONE (GnRH)

Demonstration of GnRH nerve endings in synaptic contact with medial preoptic GnRH-

containing perikarya provides morphological evidence to support the hypothesis that an intrinsic GnRH network is present (Leranth *et al.*, 1985b; Pelletier, 1987).

In vitro experiments using GnRH agonists have shown that GnRH inhibits its own release from rat MBH. The agonists suppressed both basal and K^+-stimulated GnRH release (DePaolo *et al.*, 1987; Zanisi *et al.*, 1987). Studies with rat hypothalami by Bourguignon *et al.* (1987) demonstrated that the frequency of GnRH secretory pulses *in vitro* is reduced in the presence of GnRH agonists. In addition, Valenca *et al.* (1987) observed that a GnRH antagonist enhances GnRH secretion from rat ME fragments *in vitro* in a concentration-dependent manner. Moreover, *in vivo* studies by Sarkar (1987) showed that administration of a GnRH agonist into the third venticle of OVX rats decreased both the amplitude and the frequency of endogenous GnRH pulses in portal blood and this effect was prevented by the simultaneous administration of a GnRH antagonist.

Together the above data support the existence of a negative ultrashort feedback mechanism for GnRH at the level of the hypothalamus.

OTHER PEPTIDES

NEUROTENSIN (NT): As GnRH neurons within the preoptic periventricular area of the male mouse are contacted by NT axons and a sub-population of rat GnRH neurons express NT receptors, NT is believed to affect GnRH secretion directly (Hoffman, 1985; Beaudet *et al.*, 1993). Recent experiments demonstrated that infusion of NT into the S-ME of OVX rhesus monkeys stimulated GnRH release (Pu and Terasawa, 1993). Furthermore, Herbison and Theodosis (1992) have shown that in the rat POA NT neurons possess estrogen receptors, and may therefore be involved in mediating the effects of gonadal steroids on GnRH biosynthesis and release.

ANGIOTENSIN II (ANG II): Steele *et al.* (1992) demonstrated that, in E_2 and progesterone-treated OVX rats, intracerebroventricular infusion of ANG II stimulates GnRH release from the ME.

VASOPRESSIN (VP): Thind *et al.* (1991) provided evidence that VP-immunoreactive (ir) boutons formed symmetrical synapses with GnRH-ir cell bodies in the supraoptic nucleus of cynomolgus monkeys. Whether VP itself and/or co-existent neuroeffectors act directly on GnRH secretion remains to be determined.

SUBSTANCE P (SP): Tsuruo *et al.* (1991) found that in the septo-preoptic area SP neurons make synaptic contact with GnRH-containing perikarya and dendritic processes in the rat. SP has also been reported to stimulate GnRH release from rat MBH *in vitro* (Ohtsuka *et al.*, 1987).

NEUROPEPTIDE K (NPK): Sahu and Kalra (1992) observed that NPK acting via NK-2 receptors suppressed both the basal and KCl-induced GnRH release from female rat AN-ME *in vitro*.

PROLACTIN (PRL): Azad *et al.* (1990) showed that *in vitro* immunoneutralization of hypothalamic PRL significantly decreased the release of GnRH from male rat hypothalami, suggesting that hypothalamic PRL has a stimulatory effect on GnRH release.

DELTA-SLEEP-INDUCING PEPTIDE (DSIP): Vallet *et al.* (1990) demonstrated that DSIP and GnRH are co-localized in the same neurosecretory vesicles in the rat ME. This result implies that these peptides are co-secreted.

1.2.3 STEROIDS

It is clear that the interplay between the multiple neuronal systems modulating the activity of GnRH synthesizing and releasing neurons is complex and subject to regulation by gonadal steroids. Aside from modifying the rhythmic GnRH release by modulating other systems affecting GnRH neurons, gonadal steroids may directly influence the activity of the GnRH neurons through feedback mechanisms exerted at hypothalamic and/or pituitary level (Zanisi and Messi, 1991).

Recently, a number of *in vivo* and *in vitro* studies have demonstrated that steroid hormones can affect GnRH biosynthesis and release. The use of different model systems by various groups has lead to conflicting results, most of which are contrary to the assumption that gonadal steroids act in the CNS to restrain GnRH secretion (for review see Kalra and Kalra, 1989). The ensuing discussion is a brief overview of the more substantiated work conducted to date in male and female rats.

Numerous studies in the male rat have shown that castration leads to a decrease in the MBH GnRH content and a gradual decrease in the amounts of GnRH in nerve terminals in the ME, while in the POA the levels remain unchanged (Chen *et al.*, 1977; Gross, 1980; Bourguignon *et al.*, 1984). Ramirez *et al.* (1991) have suggested that castration does not lead to changes in frequency of the GnRH pulse generator, but that the main control of the testis is either by regulating the amount GnRH discharge in response to an action potential and/or in regulating the number of active GnRH neurons. In contrast, recent work by Phelps *et al.* (1992) found that at multiple days after castration that there was no consistent change detected in the overall amount, pulse amplitude or frequency of the GnRH signal reaching the pituitary. At the level of GnRH biosynthesis, neuronal GnRH mRNA levels are either increased (Toranzo *et al.*, 1989), unchanged (Wiemann *et al.*, 1990; Malik *et al.*, 1991) or even decreased (Rothfeld *et al.*, 1987; Park *et al.*, 1988) following castration. In addition, contrary to expectation, the suppression of hypothalamic GnRH release or levels *in vitro* or *in vivo* has not been observed after T treatment in castrated rats. More surprisingly, when T replacement is initiated immediately after castration, it readily prevents the castration-induced fall in the MBH GnRH levels and release both *in vivo* and *in vitro* and release in response to a number of secretagogues. However, this example of T replacement is no longer effective if a longer interval (>1-2 weeks) is allowed to elapse after castration (Kalra and Kalra, 1989).

As in male rats, gonadal ablation in female rats eventually leads to a decrease in hypothalamic GnRH output in association with the fall in hypothalamic GnRH content (Kalra, 1976). Furthermore, ovariectomy results either in unchanged (Kelly *et al.*, 1989) or reduced GnRH mRNA levels (Roberts *et al.*, 1989; Kim *et al.*, 1989; Park *et*

al., 1990). There is also considerable agreement that E_2 treatment in OVX rats increases GnRH output and that progesterone can release GnRH *in vitro* only from hypothalami primed with estrogen (Kim and Ramirez, 1982; Drouva *et al.*, 1985; Kalra and Kalra., 1989). As well as stimulating the release of GnRH, E_2 and progesterone also increase the levels of GnRH mRNA (Kim *et al.*, 1989; Rosie *et al.*, 1990). In addition, it is becoming more evident that *in vivo* output of the GnRH pulse generator can be differently controlled by estrogen and progesterone. For instance, estrogen appears to increase the frequency of the signal, whereas progesterone predominantly changes the amplitude of the GnRH signal (Ramirez *et al.*, 1985; Dluzen and Ramirez, 1986). Progesterone administered systemically or in short pulses directly into the hypothalamus can trigger remarkable changes in the amplitude of the GnRH signal without measurable changes in frequency (Ramirez *et al.*, 1985).

Recently morphological evidence has shown that GnRH-ir terminals provide synaptic input to estrogen receptor-ir cells in a restricted part of the medial POA in the female guinea pig. Since GnRH-ir cells are thought to be regulated by estrogen responsive neurons, these results potentially identify an anatomical substrate for a regulatory feedback circuit between these two systems (Langub *et al.*, 1991).

Although gonadal steroids exert a negative effect on gonadotropin release from the pituitary, it seems that in general, steroids have a positive effect on GnRH biosynthesis and release in most model systems investigated. In summary: 1) in the absence of gonads the neurosecretory activity of GnRH neurons is markedly diminished; 2) steroids increase GnRH secretion in gonadectomized rats. These results have lead to the proposal that T, E_2 and progesterone, either on their own or together with other steroidal or non-steroidal secretions of the gonads, may in fact augment the rate of GnRH neurosecretion (Kalra and Kalra, 1989).

Clearly, further studies are necessary to provide a unified view on the exchange of information between the gonads and hypothalamus for control of reproduction.

1.3 CONCLUSION

This review has considered the potential role(s) of various neuroendocrine factors in the physiological regulation of pulsatile GnRH/LH release. In summary, the GnRH neurosecretory system appears to be regulated at many levels, and by a variety of neural and endocrine factors with a complex interplay amongst stimulatory and inhibitory systems (see Table 1).

The various effects observed on GnRH release by the same neurotransmitters and neuropeptides are possibly due to different experimental conditions i.e. age, sex, species and the endocrine status of the animal. Interestingly, the latter may play an important role in the regulation of hypothalamic neuropeptide receptors. The up and/or down regulation of receptor densities may be part of the mechanisms mediating the effects of gonadal steroids.

Also, the different effects of various hormones on the hypothalamic activity of some neural systems i.e. DA, Serotonin and GABA during sexual maturation have been observed. It has been proposed that sexual maturation and the onset of puberty involve qualitative changes in the effect of sexual hormones on the neural systems regulating GnRH release (Carbone *et al.*, 1992). These effects may include the maturation of different receptor subtypes and/or the formation of new interneuronal contacts involved in the control of GnRH.

What are the reasons for such an intricate system? The GnRH neurosecretory system is required to perform a variety of biological tasks. First and foremost, the GnRH neuronal population is charged with the responsibility of maintaining basal activity within the hypothalamic-hypophyseal-gonadal axis. The GnRH neuron is also called on to execute major shifts in the activity of the axis- from dormancy to activity and back to dormancy, and perhaps in some cases from one of these states to some physiological shade of gray in between. Puberty and seasonality are, of course, two examples of situations in which such shifts occur.

Table 1: Summary of neural systems involved in GnRH biosynthesis and release.

	Anatomical evidence	**Stimulates (+) and/or Inhibits(-)**	**Inter-action with..**	**Onset of puberty**	**Co-local-ized**
NE: α_1	Apposition	+	See below	?	
DA	Synaptic contact	+/-	NE		
Serotonin	"	+/-		?	
GLU	Apposition	+	GABA	Yes	
GABA: A B	Synaptic contact	+/- -	NE/GLU NE/GLU	?	
HA: H1		+			
EOP	Synaptic contact	-	NE/NPY	?	
NPY	"	+	NE/EOP	Yes	
GAL	"	+	NE		Yes
CRH	"	-			
GnRH	"	-		Yes	
NT	Synaptic contact	+			
ANG II		+			
VP	Synaptic contact	?			
SP	"	+			
NPK: NK-2		-			
PRL		+			
DSIP		?			Yes

In primates, the neural mechanism that governs pulsatile release of GnRH, the so-called GnRH pulse generator, is held in a state of "suspended animation" during the greater part of prepubertal development. This mechanism requires a very efficient regulatory process. Furthermore, the GnRH neurosecretory system is required to produce an abrupt signal for the initiation of the LH surge and must also be able to respond to acute (stress) and chronic (prolonged reduction of circulating gonadal hormones) perturbations.

Since all endocrine and neural systems in vertebrates have evolved to a degree of great complexity, it is not surprising that neuroendocrine cells which are at the interface of these two systems should be regulated by a plethora of neural, endocrine and other factors. To identify the mechanisms underlying the GnRH pulse generator, the current study focuses on the *in vitro* autoregulation of GnRH using an immortalized GnRH secreting cell-line.

2: DEVELOPMENT OF PROTOCOLS FOR GnRH mRNA DETECTION AND ANALYSIS OF MODULATED GnRH RELEASE USING A GT1-7 CELL CULTURE SYSTEM

2.1 INTRODUCTION

The scarcity and the diffuse anatomical distribution of GnRH- containing neurons in the forebrain has precluded extensive characterization of their cell biology (Silverman, 1988). Thus, the activation of GnRH neurons, while implied by increased GnRH output, remains a matter of hypothesis and conjecture, largely due to the absence of an experimental technique for measuring the activity of individual GnRH neurons. To obtain significant numbers of GnRH neurons for experimental purposes, Mellon *et al.* (1990) recently developed an immortalized hypothalamic GnRH-secreting cell line.

Briefly, these investigators constructed a hybrid gene that contained 2 kb of the rat GnRH promoter coupled to the coding region for the large T-antigen of simian virus (SV)-40. This construct was introduced into transgenic mice, and some of the offspring developed anterior hypothalamic tumours. These tumours were then used for the development of clonal, differentiated, neurosecretory cell lines (GT1-1, GT1-3 and GT1-7). These cells are phenotypically and immunologically neuronal, secrete GnRH and express its mRNA (Mellon *et al.*, 1990). As depicted in Figure 1, cultured GT1-7 cells exhibit the classical neuronal appearance of GnRH neurons. In addition, Liposits *et al.* (1991) have shown that these cells establish numerous interconnections. Neighbouring neurons are coupled by tight junctions, while more distant cells are interconnected with neuronal axon-like processes and collaterals. This cellular organization could serve to coordinate GnRH secretion from individual neurons. In support of this concept of synchronized release, studies by Martinez de la Escalera *et al.* (1992a) and Wetsel *et al.* (1992) have shown that these cells maintain the pulsatile pattern of GnRH secretion that is critical for successful reproduction.

The concentration of GnRH which reaches the anterior pituitary via the hypothalamo-hypophyseal portal system appears to be controlled in part by the rate of GnRH degradation within the hypothalamus and/or pituitary (Griffiths *et al.*, 1975; Lasdun *et al.*, 1989). The metalloendopeptidase EC 3.4.24.15 (EP 24.15), which cleaves the Tyr^5-Gly^6 bond of GnRH is the primary degrading enzyme in pituitary and hypothalamic membrane preparations (Griffiths *et al.*, 1975; Lasdun *et al.*, 1989). In addition, GT1-7 cells were found to secrete EP 24.15 (M.J. Glucksman, personal communication). This section describes the development of experimental protocols for GnRH mRNA detection, followed by methodology for the establishment of the GT1-7 cell culture system for the analysis of secretagogue-induced GnRH release. EP 24.15 by modifying endogenous GnRH levels could interfere with investigations into the effects of exogenous factors on GnRH release. Thus, the absence of EP 24.15 (or any other GnRH-degrading enzymes) in the proposed experimental system was determined prior to the initiation of the aforementioned studies. Results using the developed cell culture system showed the presence of GnRH mRNA as well as spontaneous GnRH release. Furthermore, this release was augmented by the addition of K^+, L-GLU, forskolin and phorbyl-12-myristate-13-acetate (PMA). Lastly, K^+- and forskolin-induced GnRH release was shown to be dependent on extracellular Ca^{2+}.

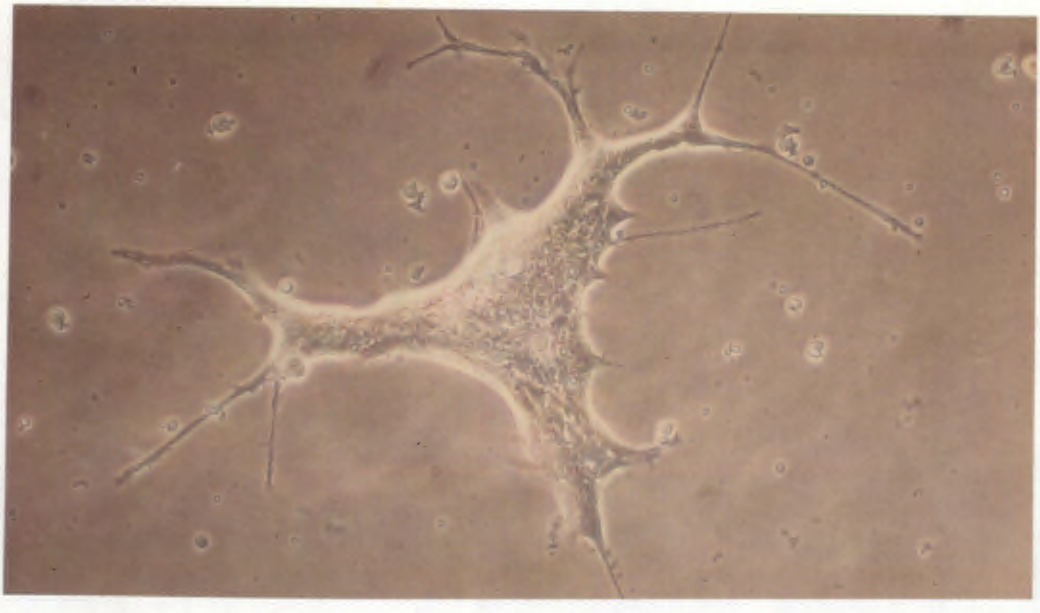


Figure 1: Phase-contrast micrograph of a representive GT1-7 cell in culture. Note the distinct neuronal appearance, including neurite formation; Magnification: 450x (a gift from Dr. N.Illing).

2.2 MATERIALS AND METHODS

2.2.1 *CHEMICALS AND BUFFERS*

Reagents

Activated charcoal, α-chymotrypsin, PMA and forskolin (dissolved in ethanol) (Sigma, St. Louis, MO); Dextran T70 (Pharmacia, Uppsala, Sweden). All other reagents were obtained from commercial sources and were of the highest purity available.

Composition of buffers

Phosphate buffered saline (PBS) (10x) for cell culture was 1.5 M NaCl, 0.03 M KCl, 0.1 M Na_2HPO_4, 0.02 M KH_2PO_4, pH 7.4.

Buffer I consisted of 140 mM NaCl, 4 mM KCl, 1 mM $MgCl_2$, 1 mM $CaCl_2$, 8.3 mM D-glucose, 20 mM Hepes (pH 7.4), 6 mg/l phenol red, and 0.1% (w/v) Bovine serum albumin (BSA) (fatty acid free, Pentax fraction V, Miles Laboratories, Elkhardt, IN). For 60 mM K^+ stimulations the NaCl concentration was adjusted to 80 mM to maintain isotonicity of the buffer.

PBS-gelatin buffer for radioimmunoassay (RIA) was 0.04 M $NaH_2PO_4.2H_2O$, 0.15 M NaCl, 0.01 M disodium ethylenediaminetetra acetic acid (EDTA), 0.1% (w/v) NaN_3, pH 7.0, with 0.1% gelatin (w/v), 0.1% (v/v) Triton X-100 (BDH Chemicals Ltd., Poole, UK).

Lysis buffer for total cellular RNA extraction consisted of 4 M guanidinium thiocyanate (GuSCN), 0.025 M sodium citrate (pH 7.0), 0.5% (w/v) N-Lauroylsarcosine, 0.1 M β-mercaptoethanol.

Diethyl pyrocarbonate (DEPC)-H_2O for use in RNA extraction was prepared as follows: 300 µl DEPC (BDH Chemicals Ltd., Poole, U.K) was added to 500 ml distilled water (dH_20), mixed and incubated at 37°C for at least 12 h and then autoclaved for 40 min.

Hybridization solution used for ribonuclease (RNase) protection assay consisted of 4 M GuSCN, 0.1 M EDTA, 30 mM NaCl, 3 mM sodium citrate (pH 7.0), 0.02% (v/v) DEPC.

RNase solution used in RNase protection assay was 10 mM Tris-HCl (pH 8.0), 300 mM NaCl, 5 mM EDTA, 40 µg/ml RNase A and 2 µg/ml RNase T1 (Sigma, St. Louis, MO)

Gel-loading buffer used for electrophoresis was 95% (v/v) formamide, 20 mM EDTA, 0.05% bromophenol blue, 0.05% xylene cyanol.

Tris-Borate buffer (TBE) for gel electrophoresis was 0.89 M Tris-HCl (pH 8.3), 0.89 M Boric acid, 0.02 M EDTA.

PIPES (piperazine-N-N'-bis [2-ethane sulfonic acid]) buffer used for Northern blot analysis consisted of 50 mM PIPES , 100 mM NaCl, 50 mM sodium phosphate, 1 mM EDTA and containing 5% sodium dodecyl sulphate (SDS).

1 x SSC used for Northern blot analysis was 0.15 M NaCl with 15 mM sodium citrate (pH 7.0)

2.2.2 *PEPTIDES AND ANTISERA*

GnRH pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-GlyNH_2
(R.C. deL. Milton, University of Cape Town, Cape Town, South Africa)

Antiserum 1076, was provided by Dr. R.P. Millar (MRC Regulatory Peptides Research Unit, University of Cape Town (UCT) Medical School, Cape Town, South Africa). This antiserum requires the region Trp^3 to Pro^9 for effective binding, and therefore recognizes both precursor and processed GnRH, as well as certain fragments of GnRH (King and Millar, 1982). Antiserum 678, was kindly provided by Dr. J.A. King (University of Cape Town, Cape Town, South Africa). This antiserum recognizes the N and C termini of GnRH and therefore detects only processed GnRH decapeptide (Dr.J.A.King, personal communication).

2.2.3 *CELL CULTURE*

Preparation of GT1-7 monolayered networks was a modification of the method as described by Mellon *et al.* (1990). Briefly, GT1-7 cells supplied by P.L. Mellon

(Regulatory Biology Laboratory, Salk Institute, La Jolla, CA) were maintained in 175 cm^2 tissue culture flasks (Nunc, Copenhagen, Denmark) at 37°C in a humidified atmosphere of 5% CO_2/95% air in Dulbecco's Modified Eagle's Medium (DMEM) with 1 mg/ml D-glucose, 100 U/ml penicillin, 100 µg/ml streptomycin (Gibco, Grand Island, NY) and 10% (v/v) fetal bovine serum (FBS) (Delta bioproducts, South Africa).

For experimental purposes cells were dislodged from confluent flasks using 0.02% EDTA in 1 x PBS and dispersed in medium using a Pasteur pipette. The suspension was centrifuged at room temperature for 10 min at 800 x g and the pellet was resuspended in medium. An aliquot was taken for cell counting (1.5 x 10^7 cells/flask) and assesment of viability using the trypan blue exclusion technique (90%). Dispersed cells were resuspended in culture medium to give 2 ml/well for plating at a density of 3 to 4 x 10^5 cells/well in 6-well plastic culture plates (Nunc, Copenhagen, Denmark). After 3 days in culture the cells formed a monolayered network in which each neuron made multiple interconnections with neighbouring cells (Figure 1). At this stage the medium was replaced with serum-free medium and incubated for a further 18 h.

2.2.4 *CULTURE STUDIES*

The following protocol was adhered to for experiments conducted in GT1-7 cells, any modifications within experiments are stated.

After 18 h in serum-free medium the cells were washed twice for 10 min in buffer I (1 ml/well) on a rotary shaker (60 rpm) at 37°C without CO_2. The cells were stimulated for 15 or 30 min in 1 ml buffer I with or without secretagogues at 37°C (as for washes). After stimulation, the medium was collected in glass tubes and centrifuged at 4°C for 10 min at 800 x g to remove detached cells. The supernatants were stored in vials at -20°C prior to RIA for secreted precursor and processed GnRH. For the determination of total protein content the cells were lysed with 1 ml/well 1% (v/v) Triton X-100. To determine the effect of extracellular Ca^{2+} on GnRH release, GT1-7 cells were stimulated in buffer I with or without 1 mM $CaCl_2$ for 15 min.

2.2.5 *PROTEIN DETERMINATION*

In order to determine whether cell numbers between wells varied, the total protein content of each well was measured using the Bio-Rad protein assay system (Bio-Rad, Munich, Germany). Briefly, a standard curve was prepared using BSA as the standard (0 to 40 µg/ml in 2 ml dH_20). The samples were diluted 1:40 in 2ml dH_20. 0.5 ml dye reagent concentrate was added to the standards and diluted samples and mixed by vortexing. After a period of 5 to 30 min the absorbance at 595 nm was measured using a spectrophotmeter (UV-160A, Shimadzu, Japan). The intra-assay well to well coefficient of variation was 4.3%.

2.2.6 *RIA OF GnRH*

The method of RIA used was a modification of that described by Hendricks *et al.*, 1975).

Iodination and purification of GnRH

Synthetic GnRH was labelled with ^{125}I (Amersham, England) and purified by high-performance liquid chromatography (HPLC). Reagents were added to the iodination vial in the following order: 5 µg GnRH in 40 µl 0.5 M (NaH_2PO_4) (pH 7.6); 10 µl ^{125}I (1 mCi); 10 µl chloramine T (BDH Chemicals Ltd., Poole, U.K)(12 mg/5 ml 0.5 M NaH_2PO_4 (pH 7.6)), mixed and the reaction was stopped after 10 sec by the addition of 20 µl sodium metabisulphate ($Na_2S_2O_5$)(Calbiochem, La Jolla, CA) (4 mg/5 ml 0.5 M NaH_2PO_4 (pH 7.6)). The iodination mixture was applied to a Vydac C-18 reverse phase column (0.46 x 25 cm; 5 µm particle size; The Separations Group, Hesperia, CA) equilibrated with 0.01 M ammonium acetate (NH_4Ac) (pH 4.6). Forty 1.5 ml fractions were eluted from the column with a flow rate of 1.5 ml/min using a gradient of 0 to 60% acetonitrile (CH_3CN) in 0.01 M NH_4Ac (pH 4.6) over 40 min.

The radioactivity of each fraction was determined in an autogamma spectrometer (Riastar, Packard Instruments). Two well-separated peaks of radioactivity were

yielded (Figure 2). The first peak represented unreacted iodide, and the second peak ^{125}I-GnRH. Fractions containing the ^{125}I-GnRH were pooled, aliquoted into vials (4 x 10^6 cpm/vial), and stored at -20°C.

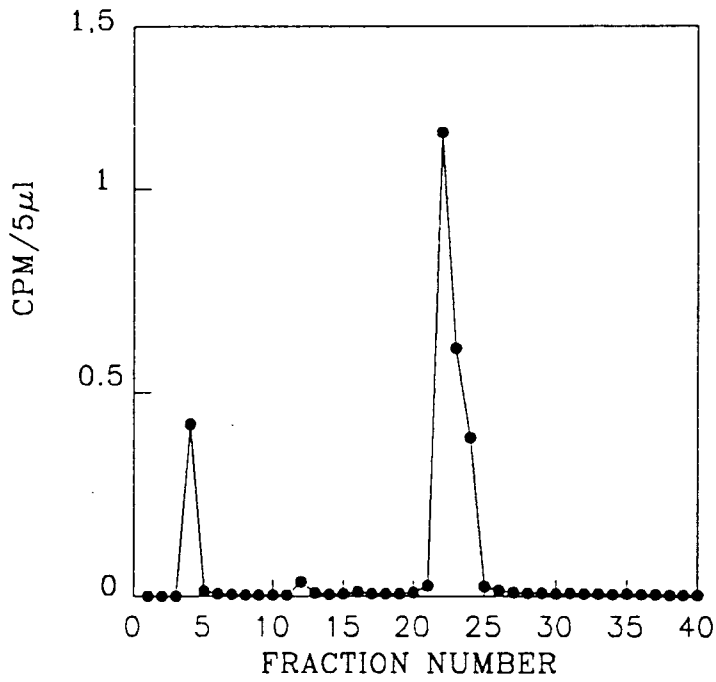


Figure 2: Iodination of mGnRH. The first peak shows unreacted iodide and the second ^{125}I-GnRH.

Assay protocol

100 µl standard (GnRH) serially diluted in PBS-gelatin buffer, or sample, 200 µl PBS-gelatin buffer and 100 µl of antiserum 1076 (1:10 000 initial dilution) and 100 µl ^{125}I-GnRH (10 000 cpm) were incubated in glass tubes overnight at 4°C. Non-specific binding (NSB) was assessed by incubation of 100 µl ^{125}I-GnRH with 400 µl PBS-gelatin buffer. Separation of antibody-bound and free ^{125}I-GnRH was achieved by the addition of a donkey anti-rabbit γ-globulin serum (Sac-Cel, IDS, Washington, England; 100 µl of a 1:2 solution in PBS-gelatin buffer). Tubes were incubated at room temperature for 30 min, 1 ml dH_20 added and centrifuged at 4°C for 15 min at 2000 x g. The supernatants were decanted and the pellets counted for 1 min in an autogamma spectrometer. The assay sensitivity (96% of total binding) was 1.2 pg/tube, with 50% inhibition of total binding occuring at 15.7 pg. The intra-assay coefficient of variation was 4.1%. Each sample from an experiment was measured in duplicate in a single assay. All test compounds used in the experimental system were examined for possible interference in the RIA. None of the compounds displayed any significant interference in the assay system.

2.2.7 *DEGRADATION STUDIES*

To investigate the presence of GnRH-degrading enzymes within the experimental system, the following approach was employed. Firstly, in order to achieve optimal binding of processed GnRH decapeptide, an assay system was developed using ^{125}I-GnRH and antiserum 678. This involved titration of antiserum 678 (1: 50 to 51200 final dilution) in the presence of a constant amount of ^{125}I-GnRH (iodination procedure as in **2.2.6**). As shown in Figure 3 maximal binding (85-89% of total counts (TC)) of labeled peptide was achieved by incubation at 4°C for 18 h, followed by separation of antibody-bound from free ^{125}I-GnRH. This required the addition of a 0.5 ml charcoal (0.5% w/v)-dextran (0.05% w/v) mixture, incubating at 4°C for 10 min, followed by centrifugation at 4°C for 15 min at 2000 x g. Thereafter, supernatants were poured over and counted in an autogamma spectrometer (*). Secondly, maximal binding of ^{125}I-GnRH in the presence of 2 ng/ml GnRH (estimated concentration within experimental system) was assessed. As shown in Table 2, a final dilution of 1: 50 (antiserum 678) was sufficient to achieve maximal binding of ^{125}I-GnRH in the presence of 2 ng/ml GnRH.

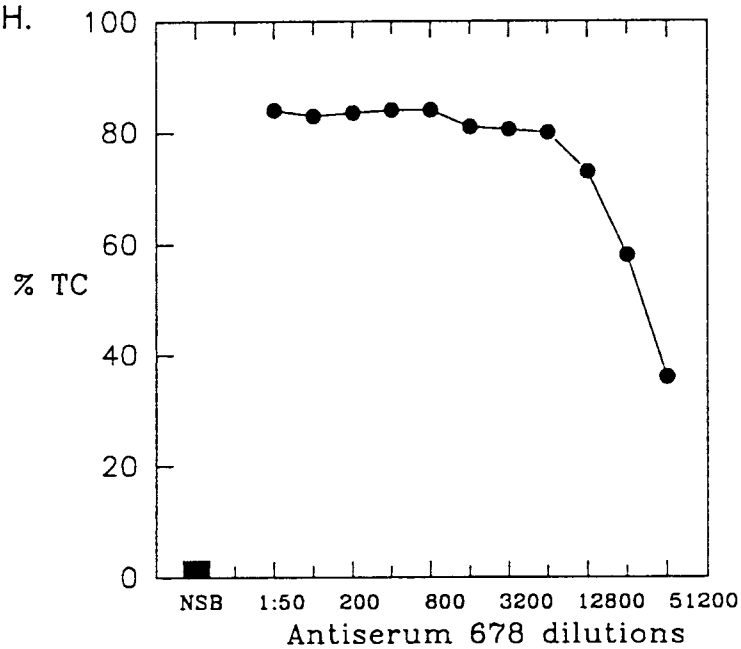


Figure 3: Titration of antiserum 678 in the presence of constant ^{125}I-GnRH. Increasing dilutions of antiserum 678 were incubated in the presence of constant ^{125}I-GnRH at 4°C for 18 h. NSB (3-4%). Separation of antibody-bound from free ^{125}I-GnRH was achieved as described in the text (**2.2.6**).

Table 2: % Binding of ^{125}I-GnRH to antiserum 678 in the presence of 2 ng/ml GnRH.

	% TC
NSB	3.2
1:50 678	86.5
1:50 678 + 2ng/ml GnRH	86.0

Following the completion of the initial studies, degradation of ^{125}I-GnRH by GT1-7 cells was investigated. Pancreatic α-chymotrypsin, known to cleave the Tyr^5-Gly^6 and Trp^3-Ser^4 bonds of GnRH, served as the +ve control (Schally *et al.*, 1971). The experiment was performed as follows: Buffer I was incubated in the presence or absence of GT1-7 cells for 30 min at 37°C. Buffer I from either set was then divided into four groups (in duplicate) for: 1) NSB; 2) +ve control (2 µg/ml chymotrypsin); 3) -ve control (boiled for 15 min to inactivate any peptidases which may be present) and 4) untreated medium. ^{125}I-GnRH (10 000 cpm/100 µl) was added to aliquots (100 µl) from each group and incubated at 37°C for 30 min. Thereafter, aliquots from group 2 were boiled to inactivate the chymotrypsin. The following was added to each aliquot (200 µl), 100µl (not NSB) antiserum 678 (1:10 initial dilution) and 200 µl (300 µl for NSB) PBS-gelatin buffer before incubation at 4°C for 18 h. Separation of antibody-bound from free ^{125}I-GnRH was achieved as described above (*).

2.2.8 *EXTRACTION OF TOTAL CELLULAR RNA*

The method employed for extraction of total cellular RNA from rat hypothalamus and GT1-7 cells was a modification of the method described by Chomczynski and Sacchi (1987). Dissected tissue was chilled on ice and homogenized in 0.5 ml lysis buffer. GT1-7 cells grown to 80% confluency in 80 cm^2 tissue culture flasks (Nunc, Copenhagen, Denmark) were washed twice with ice-cold 1 x PBS and lysed in 1 ml lysis buffer at room temperature. Both tissue and cell lysates were transferred to polypropylene tubes before the addition of 100 µl 2 M sodium acetate (pH 4.0). The lysates were mixed by inversion and extracted by the addition of 1 ml phenol (DEPC-

H_20 saturated), 200 µl $CHCl_3$:isoamylalcohol (49:1) and mixed vigorously for 10 sec. After 15 min on ice the mixture was centrifuged at 10 000 x g for 20 min at 4°C, to separate the RNA from DNA and proteins. The RNA was recovered from the aqueous phase by the addition of 1 ml isopropanol, mixed by inversion and incubated on dry ice for 10 min, the precipitate was collected by centrifugation at 10 000 x g for 20 min at 4°C. The pellet was resuspended in 500 µl lysis buffer, before the addition of 500 µl isopropanol, mixed, incubated on dry ice for 10 min and then centrifuged at 10 000 x g for 20 min at 4°C. The pellet was washed in 75% ethanol (made in DEPC-H_2O) by centrifugation at 10 000 x g for 5 min at room temperature. After removal of ethanol by aspiration the pellet was air dried and resuspended in 100 µl DEPC-H_2O. The concentration of RNA was determined by spectrophotometric (UV-160A, Shimadzu, Japan) absorbance at 260 nm, where one absorbance unit is equivalent to 40 µg RNA/ml (Sambrook *et al.*, 1989). RNA yields were between 100 and 200 µg per flask with the ratio of 260 nm/280 nm ranging between 1.8 and 2.0. RNA was stored at -70°C until needed.

2.2.9 *SYNTHESIS OF PROBE AND REFERENCE RNAs*

The method employed for synthesis of probe and reference RNA was similar to that described by Roberts *et al.* (1989). Template DNA for the *in vitro* synthesis of probe (antisense) and reference (sense) RNA was a complementary DNA (cDNA) clone (pGEM3-RAT), representing 338 bp of the rat POA GnRH mRNA inserted into plasmid GEM3 (Promega, Madison, WI) at restriction sites *Sph* I and *Bam*HI (Figure 4) (provided by Dr. C. Dutlow, MRC Regulatory Peptides Research Unit, UCT Medical School, Cape Town, South Africa).

The construct was linearized (*Nhe* I) and probe RNA (642 bp) labelled with [α-^{32}P]UTP (3000 Ci/mmole) (Amersham, England) to a high specific activity (2.0 x 10^8 cpm/µg) was transcribed using bacteriophage T7 RNA polymerase. For synthesis of reference RNA, the above construct was linearized with *Bam*HI and low-specific-activity (2.0 x 10^5 cpm/µg) reference RNA (364 bp) was transcribed using bacteriophage SP6 RNA polymerase and a trace amount of [α-^{32}P]UTP. Transcription was terminated by

digesting the template DNA with RNase-free DNase I (Boehringer Mannheim, Mannheim, Germany). Probe and reference RNAs were phenol-chloroform extracted and purified from the mononucleotides through two sequential ethanol precipitations in the presence of 5 M NH_4Ac and redissolved in 100-200 µl DEPC-H_2O. Reference RNA was quantitated using absorbance at 260 nm, aliquoted, and stored at -70°C in the presence of 10-20 U RNasin (Promega).

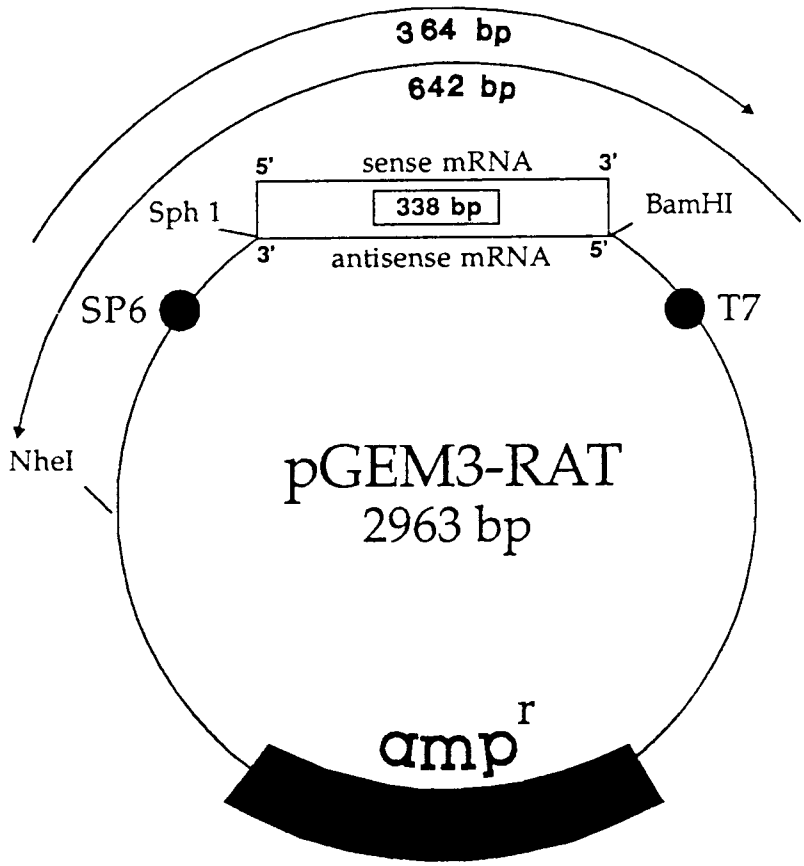


Figure 4: Diagram of pGEM-RAT showing sense (364 bp) and antisense (642 bp) transcripts used in the RNase protection assay.

The antisense oligonucleotide (5'-CC ATA GGA CCA GTG CTG GC-3', # 1, Figure 5) used for Northern analysis was directed against the sequence encoding mammalian GnRH -1 to 6. The oligonucleotide was 5'-end-labelled using T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (3000 Ci/mmole) (Amersham) (Maxam and Gilbert, 1980). Following purification through a Sephadex G-10 (Pharmacia, Uppsala, Sweden) spun column the ^{32}P 5'-end-labelled probe was ready for use.

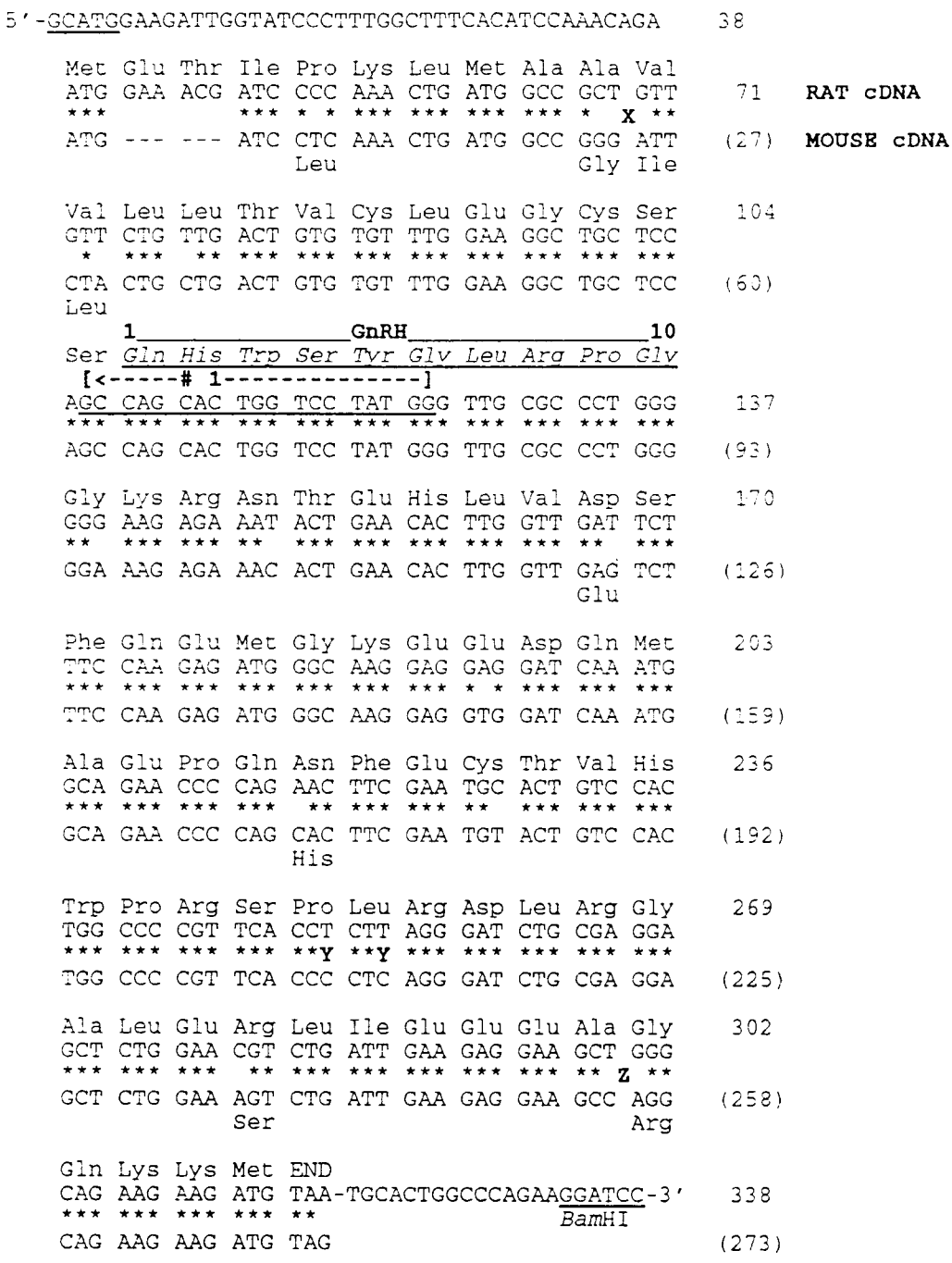


Figure 5: Nucleotide and amino acid sequence of rat and mouse cDNA encoding GnRH, indicating the binding site for the oligonucleotide used for Northern analysis. *Arrow* shows the direction and length of the oligonucleotide. *Underlined* are the *Sph* 1 (blunted) and *Bam*H1 restiction sites (for cloning in pGEM3) and the GnRH decapeptide.

2.2.10 *RNase PROTECTION ASSAY*

The probe (5 x 10^5 cpm/rxn) was mixed with the total RNA in a final volume of 25 µl hybridization solution. For standard curves, the same amount of probe was mixed with increasing known amounts of reference RNA (0-50pg) with 20 µg yeast *t*RNA added as carrier. After hybridization for 12 h at 34°C, the unprotected (single-stranded) RNA was removed by incubation at 34°C for 1 h with 300 µl RNase solution (diluting the DEPC concentration 13-fold to a level which does not inhibit the activity of the RNases). The reaction was terminated by the addition of 16 µl 10% SDS and 80 µg proteinase K (Sigma, St. Louis, MO), followed by incubation at 37°C for 15 min. Samples were phenol-chloroform extracted and precipitated with ethanol and 5 M NH_4Ac in the presence of 20 µg carrier yeast *t*RNA, washed with 75% ethanol, dried, and dissolved in 5 µl gel-loading buffer. The protected RNA hybrids were electrophoresed through a 6% polyacrylamide gel containing 50% (w/v) urea before exposure overnight at -70 °C to X-ray film with intensifying screens. Total RNA from hypothalamus and muscle (or E.coli) served as positive and negative controls, respectively. ^{32}P-labelled DNA markers ranging from 100 to 700 bases were used as size standards.

2.2.11 *NORTHERN BLOT ANALYSIS*

The method used was a modification of that described by Virca *et al.*, (1990). Thirty µg total RNA was denatured and electrophoresed through a 1.4% (w/v) agarose, 2.2 M formadlehyde gel and transferred to Hybond-N nylon membranes (Amersham, Arlington Heights, IL) (Sambrook *et al.*, 1989). Ethidium bromide staining confirmed that the ribosomal RNA's were intact. RNA blots were prehybridized in PIPES buffer at 42°C for 15 min before hybridization with the ^{32}P-labelled DNA probe for a further 18 h. Conditions ensured probe excess. After hybridization, blots were washed twice for 10 min in 5% SDS and 1 x SSC at the hybridization temperature. The blots were then exposed overnight to X-ray film with an intensifying screen at -70 °C.

2.2.12 *DATA ANALYSIS AND STATISTICS*

Results of the stimulation experiments are expressed as GnRH released (pg/ml), with each figure showing results from a single experiment which was representative of three experiments. Data are presented as the mean ± SEM (n = 3 to 6). Statistical significance of data was evaluated using one-way analysis of variance (ANOVA), followed by modified *t*-test.

2.3 RESULTS AND DISCUSSION

An important part in understanding the modulation of GnRH biosynthesis and release involves knowledge of those factors which influence the regulation of GnRH mRNA gene expression. Prior to the availability of the GT1-7 cell line methodologies for the detection of GnRH mRNA in hypothalami were limited by the sensitivity levels of the assays. GnRH-synthesizing neurons of which there are only about 1300 in the adult rat hypothalamus, show a scattered distribution in the POA and AN (Wray and Hoffman, 1986; Merchenthaler, 1991). Due to this small population of GnRH neurons, only pg quantities of GnRH mRNA can be isolated from each hypothalamus. Thus, a method to detect low levels of GnRH mRNA was developed. The approach involved optimizing the RNase protection assay, such that GnRH mRNA from individual rat hypothalami could be detected.

After several adjustments to increase the sensitivity of the RNase protection assay the methodology as described in sections **2.2.9** and **2.2.10** was selected. The rat cDNA probe together with known amounts of reference RNA (0-50 pg) was used to generate a standard curve. Figure 6A shows the gel after 1 (probe and standard curve) and 3 day exposure (hypothalamus and muscle). The sensitivity of the assay was in the 1-2 pg range, allowing the detection of GnRH mRNA from an individual rat hypothalamus. With the availability of the GT1-7 cells, the RNase protection assay was used to detect GnRH mRNA in these cells. As shown in Figure 6B, two bands of smaller size than expected were detected, indicating that the protected fragment had been digested.

As depicted in Figure 5, the sequences of rat and mouse cDNA's encoding GnRH are not 100% homologous (Adelman *et al.*, 1986; Mason *et al.*, 1986). As RNase A is an endoribonuclease that specifically attacks single-stranded RNA 3' to pyrimidine residues, the most likely area of cleavage would be regions of two or more base mismatches. As indicated in Figure 5, there are at least three sites where RNase A could cleave the RNA:RNA hybrid. Cleavage between X and Y or X and Z would result in fragments as shown in Figure 6B. As cleavage of the protected fragment may result in data that is quantitatively inaccurate, this method was deemed unsuitable for use in the GT1-7 cells. However, an alternative approach using Northern blot analysis allowed for the detection of a single transcript (± 600 bp) in GT1-7 cells (Figure 7), confirming the observations by Mellon *et al.* (1990).

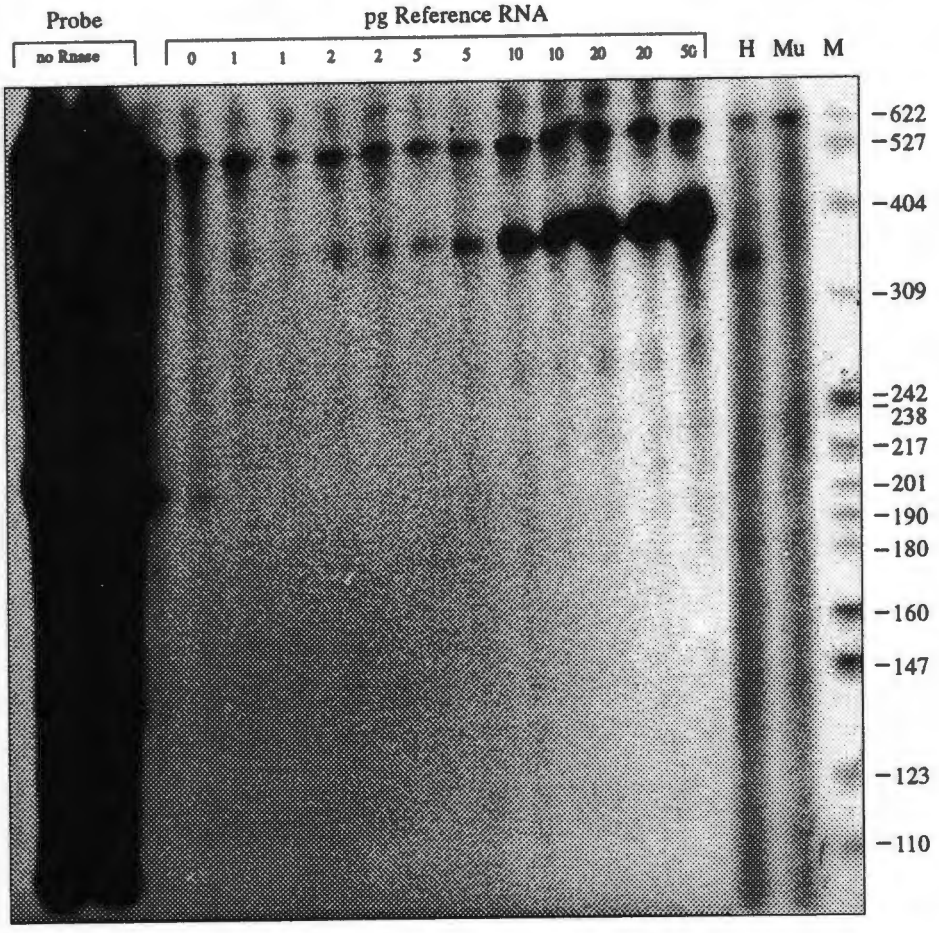


Figure 6A: Autoradiogram of gel electrophoresis of GnRH mRNA protected from RNase digestion. The gel shows probe and reference RNA (0-50 pg) after 1 day exposure, and total RNA from H (hypothalamus) and Mu (Muscle) after 3 day exposure. M, ^{32}P-labelled DNA marker (*Msp*I digest of pBR322).

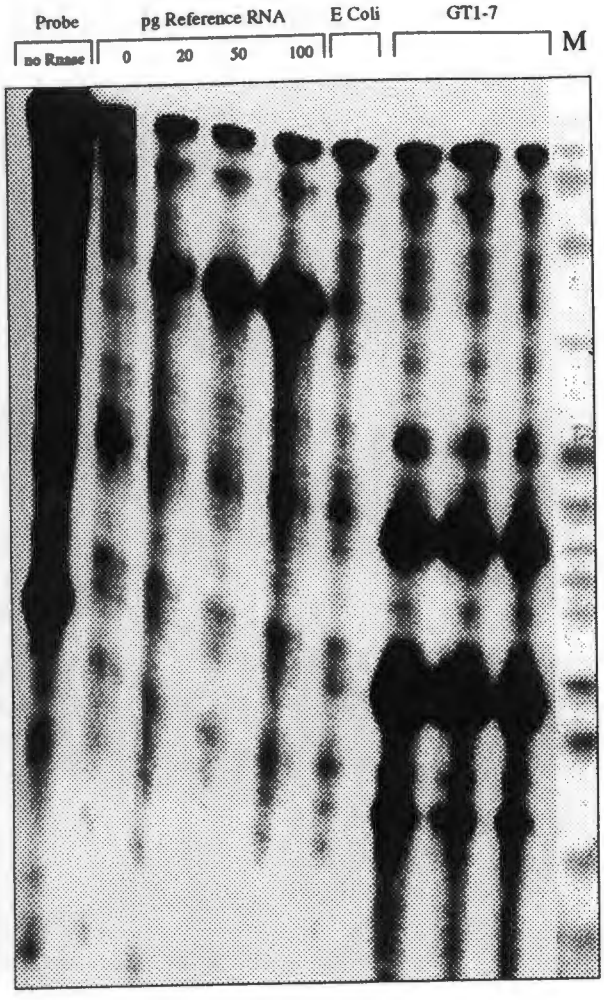
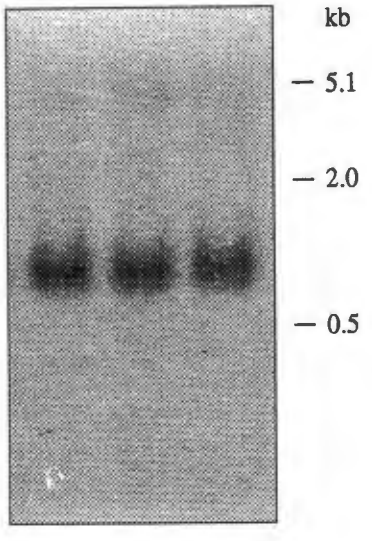


Figure 6B: Autoradiogram of gel electrophoresis of GnRH mRNA protected from RNA digestion in GT1-7 cells. The gel shows probe, reference RNA (0-100 pg), GT1-7 and E.coli total RNA. As shown GT1-7 GnRH mRNA was cleaved to give two smaller fragments. M, ^{32}P-labelled DNA marker (*Msp*I digest of pBR322).

Figure 7: Northern blot analysis of GT1-7 cells showing expression of GnRH mRNA. Thirty μg of total cellular RNA was analyzed by RNA blot analysis using antisense oligonucleotide as probe. Each lane represents RNA isolated from individual flasks of cells. Positions of the rRNA markers are indicated. The GnRH mRNA is approximately 600 bp.



GnRH functions as a central neuromodulator, as well as a hypothalamic hormone. The former action of GnRH is probably terminated by enzymatic degradation by EP 24.15, recently reported to be secreted by GT1-7 cells (M.J.Glucksman, personal communication). Since alterations in the rate of GnRH degradation by EP 24.15 may play a role in the regulation of its activity (Griffiths *et al.*, 1975; Lasdun *et al.*, 1989), it was necessary to resolve whether EP 24.15 was present in the experimental system.

Evidence against appreciable degradation was provided by incubation of ^{125}I-GnRH for 30 min at 37°C as described in **2.2.7.** As illustrated in Figure 8, no loss of immunoreactivity of labeled GnRH was observed upon subsequent testing of either group 3 or 4. Hence, it was concluded that EP 24.15 or any other GnRH-degrading enzymes have no observable influence in the experimental system, a prerequisite for the reliable detection of modulated GnRH levels by method of RIA (**2.2.6**).

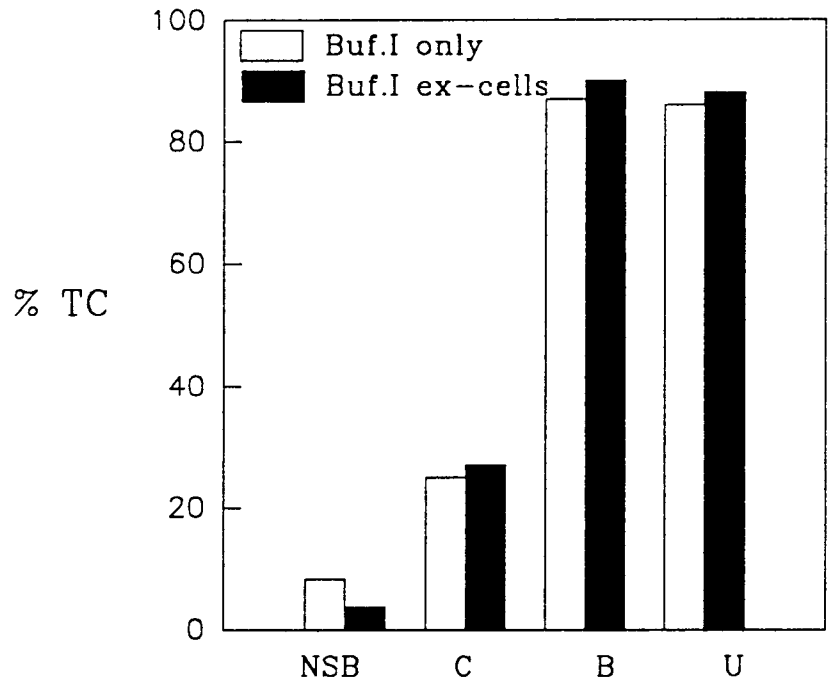


Figure 8: Stability of secreted GnRH from GT1-7 cells.
GnRH degrading activity was investigated by incubation of Buffer I in the presence or absence of GT1-7 cells for 30 min. This medium was divided into 4 groups: 1) NSB; 2) C (chymotrypsin 2µg/ml); 3) B (boiled); 4) U (untreated). After a 30 min incubation with ^{125}I-GnRH (all groups), antiserum 678 was added for 18 h at 4°C for detection of ^{125}I-GnRH decapeptide.

As discussed in the review there are a number of neuroendocrine factors involved in the regulation of GnRH secretion from the hypothalamus. These factors interact at the membrane with their receptors which are linked to the activation of various signal transduction pathways. Previous *in vitro* studies have shown that the protein kinase-A and -C (PKA and PKC) pathways are dually involved in the regulation of GnRH gene expression and release (Ojeda *et al.*, 1986; Lee *et al.*, 1990). Furthermore, studies using hypothalamic slices have shown that depolarization of GnRH neurosecretory neurons with high concentrations of K^+ causes rapid release of GnRH via VSCC (Drouva *et al.*, 1981). The present study examined basal GnRH release from GT1-7 cells and responses to the following secretagogues: L-GLU for activation of ionotropic and metabotropic GLU receptors, K^+ for depolarization, forskolin for activation of the cAMP-PKA pathway and PMA for the PKC-dependent pathway. The dependency of GnRH secretion on extracellular Ca^{2+} was also investigated.

GnRH release (as detected by RIA) from static GT1-7 cells incubated in Buffer I was monitored over 15 min (Figure 9A) or 30 min (Figure 9B) periods. The cells released GnRH spontaneously (basal release) and treatment with 10 mM L-GLU, 60 mM K^+, 10 μM forskolin and 50 nM PMA all significantly stimulated release above basal levels ($n=3$, $p<0.05$, $p<0.005$, $p<0.02$, $p<0.001$, respectively). These data corroborate the work by Mellon *et al.* (1990), who found that GT1-7 cells secrete GnRH spontaneously and that this release is linked to propagated action potentials involving fast Na^+ channels. The observation that L-GLU enhances GnRH secretion is consistent with recent studies by Spergel *et al.* (1994). This group observed that activation of NMDA and AMPA/kainate receptors lead to Ca^{2+} influx followed by an increase in GnRH secretion. In addition, these results show that both the PKC and PKA pathways are functional in the GT1-7 cells. Interactions between both transducing systems may provide a mechanism for enhanced GnRH release (Ojeda *et al.*, 1986; Lee *et al.*, 1990). Recent studies using static GT1-7 or GT1-3 cells have reported that although activation of the PKC pathway acutely increased GnRH release, prolonged treatment resulted in a progressive reduction in GnRH mRNA levels (Bruder *et al.*, 1992; Wetsel *et al.*, 1993; Yu *et al.*, 1994). Also, while PKA activation stimulated GnRH release, GnRH mRNA levels were either unchanged (Wetsel *et al.*, 1993) or decreased (Yu *et al.*, 1994).

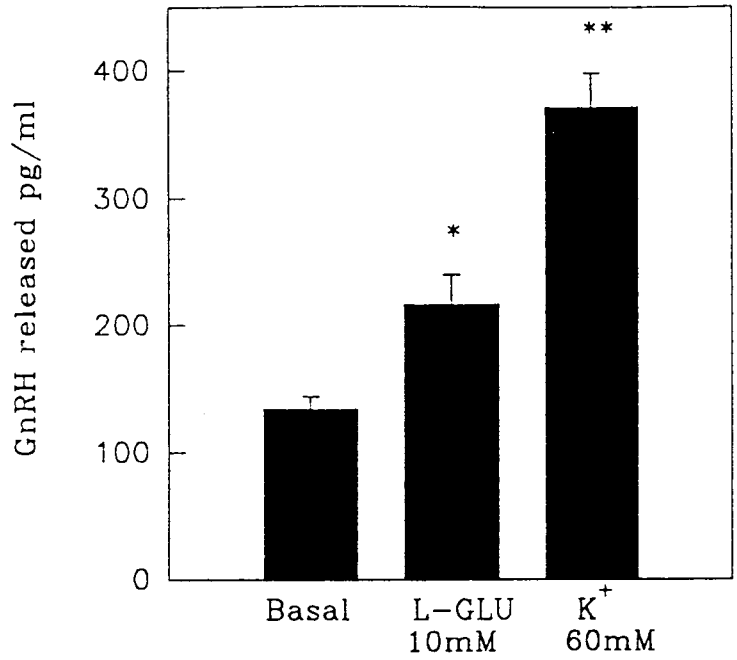


Figure 9A: Effect of L-GLU and K^+ on GnRH release from GT1-7 cells. Treatment of GT1-7 cells with 10 mM L-GLU and 60 mM K^+ for 15 min. *, P< 0.05; **, P< 0.005 vs. control (Basal) (n=3 for each group).

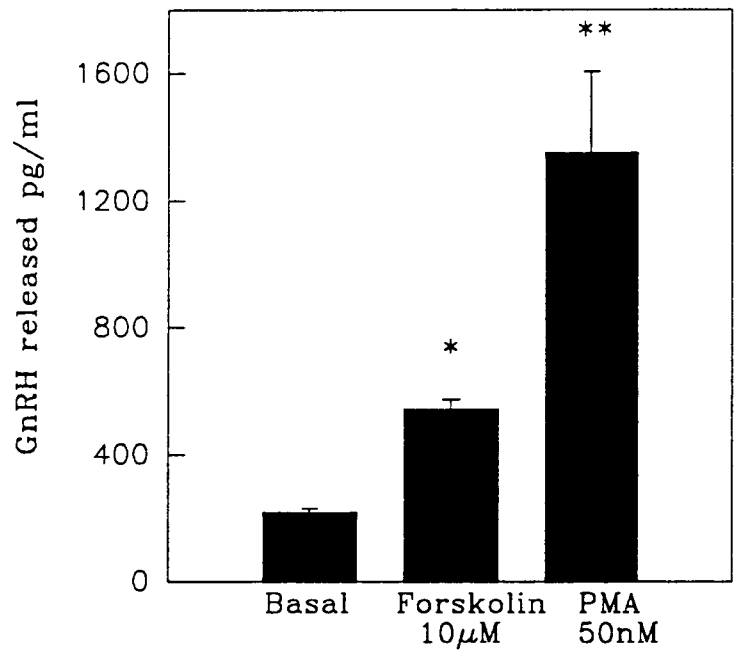


Figure 9B: Effect of forskolin and PMA on GnRH release from GT1-7 cells. Treatment of GT1-7 cells with 10 μM forskolin and 50 nM PMA for 30 min. *, P< 0.02; **, P< 0.001 vs. control (Basal) (n=3 for each group).

The role of extracellular Ca^{2+} in mediating basal, K^+- and forskolin-stimulated release of GnRH from GT1-7 cells was investigated over a 15 min period (Figure 10). The removal of Ca^{2+} from Buffer I had no effect on basal GnRH release, but resulted in a significant reduction to basal levels for the K^+- and forskolin- stimulated GnRH release ($n=3$, $p< 0.001$). The results demonstrate that influx of extracellular Ca^{2+} is required for the K^+- and forskolin-enhanced GnRH release. In support of these findings, Martinez de la Escalera *et al.* (1992a) and Wetsel *et al.* (1992) have shown that removal of extracellular Ca^{2+} for a prolonged period (4h) results in the progressive reduction of the amplitude and eventually in the absence of pulsatile GnRH release from GT1-7 cells. The unchanged basal levels to short-term (15 min) removal of Ca^{2+}, suggest that intracellular Ca^{2+} stores (or residual extracellular Ca^{2+}) can transiently maintain episodic GnRH release. Taken together, these observations suggest that activation of voltage-dependent high-threshold-Ca^{2+} conductances are rapidly triggered by membrane depolarization, resulting in an increased rate of Ca^{2+} influx and quantal release of GnRH.

In other studies pulsatile GnRH release from GT1-7 cells was markedly attenuated in the presence of nifedipine, an antagonist of dihydropyridine VSCC (Krsmanovic *et al.*, 1992). Nifedipine also reduced both intracellular Ca^{2+} concentration $[Ca^{2+}]_i$ and basal GnRH release in a dose-dependent manner. Also, depolarization with high K^+ increased $[Ca^{2+}]_i$ and GnRH release in an extracellular Ca^{2+} dependent and nifedipine-sensitive manner. Such a profile of Ca^{2+} response to depolarization by high K^+ is consistent with the presence of L-type VSCCs. Further studies with GT1-7s and rat AN-ME using Bay K 8644, a dihydropyridine Ca^{2+} channel agonist, increased basal and K^+-induced elevations of $[Ca^{2+}]_i$ and GnRH secretion. These effects are a result of Bay K 8644 increasing entry of extracellular Ca^{2+} via L-type VSCCs (Kalra *et al.*, 1991; Krsmanovic *et al.*, 1992). Kalra *et al.* (1991) also demonstrated that ω-conotoxin, an antagonist at N- and L-type VSCC, decreased the KCl-induced GnRH release by 40-60% from *in vitro* rat AN-ME. These results suggest that both N- and L- Ca^{2+} channels are present in GnRH neurons and that influx of extracellular Ca^{2+} via these channels is an important event in depolarization-induced release of GnRH from the AN-ME of male rats.

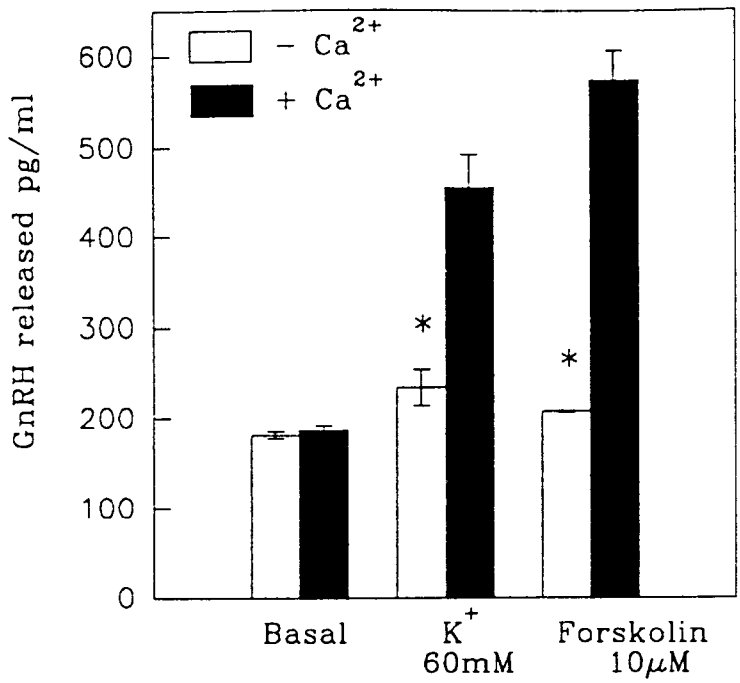


Figure 10: Effect of extracellular Ca^{2+} removal on GnRH release from GT1-7 cells. Basal, K^+ and forskolin stimulated release was determined in the presence of (+) or absence (-) of extracellular Ca^{2+} for 15 min. *, $P< 0.001$ vs. control ($+Ca^{2+}$) (n=3 for each group).

Ionomycin which stimulates Ca^{2+} influx independent of VSCC, significantly increased GnRH release from GT1-3 cells in a time-dependent fashion. The maximal increase in GnRH release by 1 μM ionomycin was observed after only 10 min of treatment. Interestingly, as mentioned for activation of the PKC and PKA pathways, ionomycin resulted in a dose-dependent reduction in GnRH mRNA expression after 24 h of treatment (Yu *et al.*, 1994). It is postulated that some of the secretagogues may transactivate other second messenger pathways, however, the exact mechanism of the divergent effects on the synthesis and secretion of GnRH remain to be elucidated.

Collectively, the above results characterized the intracellular responses to various secretagogues in GT1-7 cells. Knowledge of the molecular mechanisms by which second messenger pathways regulate the transcription, processing, and secretion of GnRH forms a foundation for understanding the physiological basis for the neuroendocrine regulation of reproduction. Clearly, these cells provide an excellent opportunity for the analysis of these mechanisms.

3: DEMONSTRATION OF GnRH BINDING SITES AND EFFECTS OF GnRH ANALOGS ON GnRH RELEASE FROM GT1-7 CELLS.

3.1 INTRODUCTION

The ability of cells to regulate their own metabolism and growth via cellular products has been termed autofeedback or autocrine regulation. Classically the term autocrine has referred to work within the fields of oncogene and growth factor research. Researchers within the field of neuroendocrinology however, introduced the concept of "ultrashort loop feedback", whereby hypothalamic hormones could regulate their own synthesis, storage, and/or release (Hyppa *et al.*, 1971; Sporn and Roberts, 1985).

The factors that are known to function via an autocrine mechanism include bombesin, transforming growth factor-α,-β (TGF-α, TGF-β) and peptides related to platelet-derived growth factor (PDGF) (Sporn and Roberts, 1985). In addition, the presence of ir-GnRH and GnRH mRNA in breast carcinoma cells known to have GnRH-binding sites has lead to the suggestion that GnRH may serve an autocrine role within these cells (Harris *et al.*, 1991). There are also several reports that document the possible existence of ultrashort feedback circuits for a number of neurotransmitters and neuropeptides. These include NE (via α_2-adrenoceptors), β-endorphin (via μ-receptors), somatostatin (SS), growth hormone-releasing factor (GRF), thyrotropin releasing hormone (TRH), oxytocin, GAL and CRH (Lumpkin *et al.*, 1981; Lumpkin *et al.*, 1983; Moos *et al.*, 1984; Ohta *et al.*, 1985; Lumpkin *et al.*, 1985; Ono *et al.*, 1985; Epelbaum *et al.*, 1986; Richardson and Twente, 1986; Kelly *et al.*, 1990; MacDonald *et al.*, 1992; de los Frailes *et al.*, 1992; Lopez *et al.*, 1992).

The review showed that the excitation of GnRH neurons is subject to modulation by hormones or neurotransmitters released by other cells. Moreover, as supported from *in vitro* and *in vivo* studies, GnRH has been proposed to exert an inhibitory action on its own secretion (**1.2.2**). Prior to an investigation into the existence of an ultrashort

feedback mechanism within GT1-7 cells it was essential to determine the presence of specific GnRH receptors (binding sites) and GnRH receptor mRNA in GT1-7 cells.

The availability of a GnRH-responsive pituitary cell line of the gonadotrope lineage (αT3-1) provided a model system for comparative GnRH receptor binding studies with the putative binding site on the GT1-7 cells (Windle *et al.*, 1990). The receptors in αT3-1 cells show specificity for different GnRH analogs, with dissociation constants very similar to those found in normal rat and mouse pituitary (Table 3).

Table 3. Comparison of the GnRH receptor on mouse, αT3-1, and rat anterior pituitary membrane homogenates (Horn *et al.*, 1991).

	Mouse	αT3-1	Rat
K_d (nM)	0.51	0.50	0.20

K_d, Dissociation constant of [DAla^6,Me-Leu^7,Pro^9-NEt]-GnRH

GnRH receptor binding studies were thus performed on both the αT3-1 and GT1-7 cell lines. In addition, cloning of the mouse GnRH receptor provided the necessary tools to determine by method of polymerase chain reaction (PCR) if the GT1-7 cells express mRNA for this receptor (Tsutsumi *et al.*, 1992). The results demonstrated that the GnRH analog, Ant 26, bound to specific receptors in GT1-7 cells and that these cells have the capacity to synthesize receptors for GnRH.

The above findings enabled an investigation to determine whether GnRH influences the control of its own release through autoreceptors located on GnRH-containing neurons. This was accomplished by examining basal and secretagogue-induced GnRH release from GT1-7 cells in the presence or absence of various GnRH analogs. Exposure of static GT1-7 cells to the GnRH antagonist (Ant 27) resulted in enhanced basal, K^+- and forskolin-stimulated GnRH release. However, GnRH content was shown to be unchanged by the analog. A preliminary account of some of these results has been reported (Pithey *et al.*, 1992).

3.2 MATERIALS AND METHODS

The methodology used was identical to that described in section **2.2**, except for the following:

3.2.1 *CHEMICALS AND BUFFERS*

Poly-L-ornithine (Sigma, St. Louis, MO); **2.5 x reverse transcriptase (RT) Taq buffer** for RT-PCR consisted of 168 mM Tris-HCl (pH 8.8), 41.5 mM $(NH_4)_2SO_4$, 16.8 mM $MgCl_2$, 25 mM β-mercaptoethanol, 0.42 mg/ml BSA.

3.2.2 *PEPTIDES AND ANTISERA*

Ant 26	Ac-D-p-ClPhe-Ac-D-p-ClPhe-D-Trp-Ser-Tyr-D-Lys-Leu-Arg-Pro-D-AlaNH_2 (D.H. Coy, Tulane University Medical Center, New Orleans, LA)
GnRH[His^{5}D-Arg^6-Trp^7-Tyr^8]	pGlu-His-Trp-Ser-His-D-Arg-Trp-Tyr-Pro-GlyNH_2 (R.C. deL. Milton)
GnRH[D-Ala^6-N-MeLeu^7, Pro^{9}NEt]	pGlu-His-Trp-Ser-Tyr-D-Ala-N-MeLeu-Arg-ProNEt (R.C. deL. Milton)
Ant 27	Ac-D-Nal-D-Me-4-ClPhe-D-Trp-Ser-N-IsopropylLys-D-Tyr-Leu-Arg-Pro-D-AlaNH_2 (R.C. deL. Milton)
Ant 140-36	Ac-D-2-Nal-D-Me-ClPhe-D-Trp-Ser-D-Pro-D-N-IsopropylLys-Leu-N-IsopropylLys-Pro-D-AlaNH_2 (R.W. Roeske, Indiana University, Indianapolis, IN)

Three of the above GnRH analogs had highly increased biopotency, while one was without biological activity. The binding potencies relative to GnRH were 6, 12, 11 and 32 times for GnRH[His^{5}D-Arg^6-Trp^7-Tyr^8], GnRH[D-Ala^6-N-MeLeu^7,Pro^{9}NEt], Ant 27 and Ant 26, respectively. These potencies were assessed by receptor binding to rat pituitary GnRH receptor (Perrin *et al.*, 1980; C. Flanagan, personal communication).

The inactive analog was Ant 140-36. The greater biological activity of GnRH analogs having amino acid substitutions in positions 5 and 6 indicate that their increased biological activity (compared to those having a Tyr^5-Gly^6 bond) might be caused by an increased resistance to enzymatic degradation. Stock solutions of peptides (1 mM) were made up in dH^2O or 5% (v/v) acetonitrile/5% (v/v) methanol (agonists), or 20 % (v/v) propylene glycol (antagonists), and stored at -20°C. For experimental purposes stock solutions were dissolved just before use in incubation medium.

Antiserum EL-14 (1: 50 000 initial dilution), was provided by W.E. Ellinwood (Department of Physiology, Oregon Health Sciences University, Portland, OR). This antiserum requires that both the N- and C-termini of GnRH be intact and thus recognizes only processed GnRH and not GnRH fragments (Ellinwood *et al.*, 1985). To assess whether any of the GnRH analogs might interfere with the measurement of endogenous GnRH by RIA, the displacement of ^{125}I-GnRH from the anti-GnRH antisera by each analog was determined. The results are summarized in Table 4.

Table 4 : Percentage binding of ^{125}I-GnRH to anti-GnRH antisera in the presence of GnRH analogs.

GnRH analog	GnRH[His^5DArg^6 Trp^7Tyr^8]	GnRH[DAla^{6}NMe Leu^7,Pro^9]	Ant 140-36	Ant 27	
Antisera	1076	1076	1076	1076	EL14
Conc.[M]	% BINDING				
10^{-9}	100	100	100	100	100
10^{-8}	100	100	100	100	100
10^{-7}	100	100	100	100	96*
10^{-6}	100	100	100	100	85*

* displacement of ^{125}I-GnRH by analog, mimicking presence of GnRH within RIA.

Since Ant 27 cross-reacts with EL-14 the antiserum of choice for all the analogs was 1076. No difference in levels of secreted GnRH was detected by antiserum EL-14 or 1076 (data not shown). Ab 678 was not available for these studies.

3.2.3 *BINDING STUDIES*

GT1-7 and αT3-1 cells (supplied by P.L. Mellon) were maintained and cultured for experimental purposes as described in **2.2.3**, except that the 6-well plates had previously been coated with poly-L-ornithine (15 µg/ml). In addition, the cells were cultured to confluence, after which serum-free medium was added for a further 24 h. After 24 h the cells were washed twice in buffer I (1 ml/well). Binding was initiated by the addition of 1 ml buffer I containing dilutions of unlabeled Ant 26 and ^{125}I-Ant 26 (1 x 10^5 cpm/well). After 1 h at 4°C the buffer was aspirated off and the cells were solubilized with a 1% Triton X-100 / 0.05% NaOH solution. The cell lysates (containing the bound (B) Ant 26) were counted in an autogamma spectrometer. All binding studies were performed in triplicate. Maximal binding (B_o) (20-21% of TC) was determined in the absence of unlabeled Ant 26 and non-specific binding (NSB) (8-10% of TC) by the addition of 10^{-6} M Ant 26.

Iodination of Ant 26 was carried out as described in **2.2.6** for GnRH, however 50 µl $Na_2S_2O_5$ (6 mg/ 5ml 0.5 M NaH_2PO_4 (pH 7.6)) was added to stop the reaction and a gradient of 0 to 80% CH_3CN was run for elution of the peptide from the column. Figure 11 shows two well-separated peaks of radioactivity, the first representative of unreacted iodide, and the second ^{125}I-Ant 26.

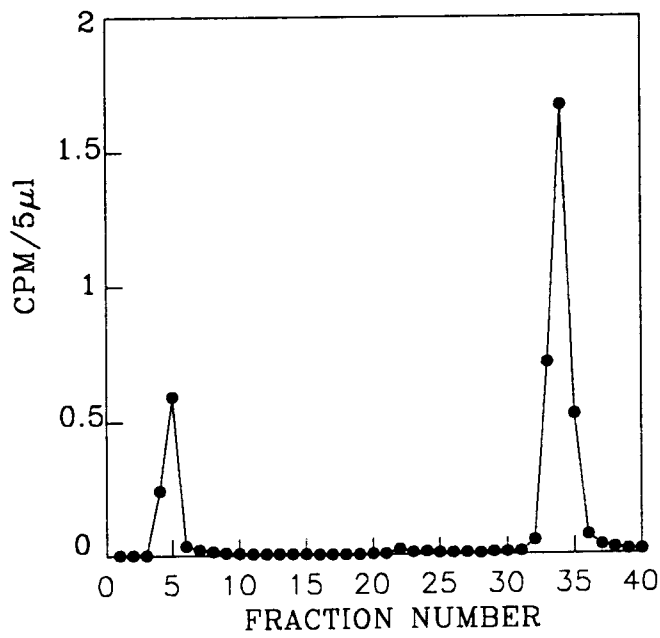


Figure 11: Iodination of Ant 26. The first peak shows unreacted iodide and the second ^{125}I-Ant26.

3.2.4 *RT-PCR OF GnRH RECEPTOR mRNA*

Total RNA from GT1-7 cells was prepared as described in section **2.2.8**. For RT-PCR reactions, two oligonucleotide GnRH primers were synthesized based on the reported cDNA sequence for the mouse GnRH receptor (Tsutsumi *et al.*, 1992). As depicted in Figure 12, the sense oligonucleotide (5'-CAGTGGTATGCTGGGG-3', *primer1) overlaps with the post transmembrane loop II, whereas the antisense oligonucleotide (5'-TTCTTGG ACTGATTCATCTG-3', primer 2) corresponds to post transmembrane loop V. These primers yield a 458 bp PCR product. Primer 1 was 5'-end-labelled using T4 polynucleotide kinase in the presence of [γ-^{32}P]ATP (3000 Ci/mmole) (Amersham) (Maxam and Gilbert, 1980). Following purification through a Sephadex G-10 (Pharmacia, Uppsala, Sweden) spun column the ^{32}P 5'-end-labelled primer (*primer1) was ready for use. cDNA synthesis was performed using sense (0.1 ng/ml) or total RNA (1 µg/ml) in a RT reaction. 10 µl RNA (10 ng for total, 1 pg for sense) was added to 10 µl 2.5 x RT Taq buffer containing 1 µl primer 2 (50 pmoles), heated to 94°C for 2 min and snap-cooled on dry ice. 10 µl of 2.5 x RT Taq buffer with 3 U avian myeloblastosis virus (AMV) RT (Life Sciences, St. Petersburg, FL), 1 U RNasin, and 1mM each of dATP, dCTP, dGTP, and dTTP was added to the cold annealing mix and incubated at 42°C for 15 min, 50°C for 15 min and 55°C for 30 min. The cDNA product was then amplified by the addition of 4 µl 2.5 x RT Taq buffer, 5 µl *primer1 and 2.5 U *Taq* DNA polymerase (Promega). A paraffin oil overlay was applied and 20 PCR cycles were performed. Each cycle of amplification consisted of a 40 sec denaturation at 94°C, followed by 2 min annealing (56°C) and 2 min extension (72°C) steps. After PCR, 8 µl gel-loading buffer was added to the samples before heating to 90°C for 2 min before electrophoresis through a 6% polyacrylamide, 50% (w/v) urea, 1 x TBE gel. The gel was dried under vacuum and exposed to X-ray film.

3.2.5 *ANALOG STUDIES*

The protocols described in sections **2.2.4**, **2.2.5**, and **2.2.6** were adhered to except where stated. Experiments involving the use of agonists and antagonists required preincubation for 18 h or 30 min (as indicated in the appropriate figure legends) in

```
                                                    ACGAGAGGGACTCCACTCTTGAAGCCTGTCCTTGGAGAAAT  -1
Met Ala Asn Asn Ala Ser Leu Glu Gln Asp Pro Asn His Cys Ser Ala Ile Asn Asn   19
ATG GCT AAC AAT GCA TCT CTT GAG CAG GAC CCA AAT CAC TGC TCG GCC ATC AAC AAC   57

Ser Ile Pro Leu Ile Gln Gly Lys Leu Pro Thr Leu Thr Val Ser Gly Lys Ile Arg  .38
AGC ATC CCC TTG ATA CAG GGC AAG CTC CCG ACT CTA ACC GTA TCT GGA AAG ATC CGA  114
                                                  I
Val Thr Val Thr Phe Phe Leu Phe Leu Leu Ser Thr Ala Phe Asn Ala Ser Phe Leu  .57
GTG ACC GTG ACT TTC TTC CTT TTC CTA CTC TCT ACT GCC TTC AAT GCT TCC TTC TTG  171

Leu Lys Leu Gln Lys Trp Thr Gln Lys Arg Lys Lys Gly Lys Lys Leu Ser Arg Met  .76
TTG AAG CTG CAG AAG TGG ACT CAG AAG AGG AAG AAA GGA AAA AAG CTC TCA AGG ATG  228
                                                                    II
Lys Val Leu Leu Lys His Leu Thr Leu Ala Asn Leu Leu Glu Thr Leu Ile Val Met  .95
AAG GTG CTT TTA AAG CAT TTG ACC TTA GCC AAC CTG CTG GAG ACT CTG ATC GTC ATG  285

Pro Leu Asp Gly Met Trp Asn Ile Thr Val Gln Trp Tyr Ala Gly Glu Phe Leu Cys  114
                                  [----*primer1------>]
CCA CTG GAT GGG ATG TGG AAT ATT ACT GTT CAG TGG TAT GCT GGG GAG TTC CTC TGC  342
                                 III
Lys Val Leu Ser Tyr Leu Lys Leu Phe Ser Met Tyr Ala Pro Ala Phe Met Met Val  133
AAA GTT CTC AGC TAT CTG AAG CTC TTC TCT ATG TAT GCC CCA GCT TTC ATG ATG GTG  399

Val Ile Ser Leu Asp Arg Ser Leu Ala Ile Thr Gln Pro Leu Ala Val Gln Ser Asn  152
GTG ATT AGC CTG GAC CGC TCC CTG GCC ATC ACT CAG CCC CTT GCT GTA CAA AGC AAC  456
                                                IV
Ser Lys Leu Glu Gln Ser Met Ile Ser Leu Ala Trp Ile Leu Ser Ile Val Phe Ala  171
AGC AAG CTT GAA CAG TCT ATG ATC AGC CTG GCC TGG ATT CTC AGC ATT GTC TTT GCA  513

Gly Pro Gln Leu Tyr Ile Phe Arg Met Ile Tyr Leu Ala Asp Gly Ser Gly Pro Thr  190
GGA CCA CAG TTA TAT ATC TTC AGG ATG ATC TAC CTA GCA GAC GGC TCT GGG CCC ACA  570

Val Phe Ser Gln Cys Val Thr His Cys Ser Phe Pro Gln Trp Trp His Gln Ala Phe  209
GTC TTC TCG CAA TGT GTG ACC CAC TGC AGC TTT CCA CAG TGG TGG CAT CAG GCC TTC  627
                                  V
Tyr Asn Phe Phe Thr Phe Gly Cys Leu Phe Ile Ile Pro Leu Leu Ile Met Leu Ile  228
TAC AAC TTT TTC ACC TTC GGC TGC CTC TTC ATC ATC CCC CTC CTC ATC ATG CTA ATC  684

Cys Asn Ala Lys Ile Ile Phe Ala Leu Thr Arg Val Leu His Gln Asp Pro Arg Lys  247
TGC AAT GCC AAA ATC ATC TTT GCT CTC ACG CGA GTC CTT CAT CAA GAC CCA CGC AAA  741

Leu Gln Met Asn Gln Ser Lys Asn Asn Ile Pro Arg Ala Arg Leu Arg Thr Leu Lys  266
    [<-------primer2---------]
CTA CAG ATG AAT CAG TCC AAG AAT AAT ATC CCA AGA GCT CGG CTG AGA ACG CTA AAG  798
                                                VI
Met Thr Val Ala Phe Ala Thr Ser Phe Val Val Cys Trp Thr Pro Tyr Tyr Val Leu  285
ATG ACA GTC GCA TTC GCT ACC TCC TTT GTC GTC TGC TGG ACT CCC TAC TAT GTC CTA  855

Gly Ile Trp Tyr Trp Phe Asp Pro Glu Met Leu Asn Arg Val Ser Glu Pro Val Asn  304
GGC ATT TGG TAC TGG TTT GAT CCA GAA ATG TTG AAC AGG GTG TCA GAG CCA GTG AAT  912
                    VII
His Phe Phe Phe Leu Phe Ala Phe Leu Asn Pro Cys Phe Asp Pro Leu Ile Tyr Gly  323
CAC TTT TTC TTT CTC TTT GCT TTC CTA AAC CCG TGC TTC GAC CCA CTC ATA TAT GGG  969

Tyr Phe Ser Leu                                                             327
TAT TTC TCT TTG TAGTTGGGAGACTACACAAGAACTCAGATAGAAATAAGGTAACTAATTGCACCAATTGA 1040

GAATAAACTCAAAGCTTTTGACACACTTATATACAAGGCAGGGTTTAAGGTTAGATTATCAACCTTGTTTTTGTA 1115
CAGAGTTTGTTGTTAGAGCTTCAGAAGACCTTCAAAAACAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAAA   1185
```

*primer1: 5'-CAGTGGTATGCTGGGG-3'
primer2 : 5'-TTCTTGGACTGATTCATCTG-3'

Figure 12: Nucleotide and deduced amino acid sequence of mouse cDNA encoding GnRH receptor, indicating the binding sites for primers used for RT-PCR. Putative transmembrane regions I-VII are *lined*.

serum-free medium with replacement of analogs for the stimulation period. For the determination of cellular GnRH content, cells were lysed with 1ml of 1% Triton X-100 per well before measurement by RIA (**2.2.6**).

3.2.6 *DATA ANALYSIS*

ED_{50} (effective dose required to displace 50% of ^{125}I-Ant 26) values were estimated by four-parameter non-linear curve fitting using Sigmaplot (Jandel Scientific, Corte Madera, CA). % Specific binding = (B-NSB/B_o-NSB) x 100. The SEMs for the binding studies are not included as they were extremely low and the error bars would not be visible on the graph. Data analysis for the analog studies were as described in **2.2.12.**

3.3 RESULTS AND DISCUSSION

Numerous neuroendocrine factors are implicated in the functional control of GnRH neurons. Many questions remain however, concerning the location of the receptors and whether the effects are exerted directly or indirectly on the GnRH neuron. While interconnections between GnRH neurons in the rat POA and in GT1-7 cells provide the neuroanatomical basis for GnRH autoregulation, the presence of GnRH receptors on these cells remain an integral component of this mechanism (Leranth *et al.*, 1985b; Liposits *et al.*, 1991). Thus, receptors for GnRH in GT1-7 cells were determined by binding studies and detection of GnRH receptor mRNA by method of PCR.

The GnRH antagonist (Ant 26) was used to characterize GnRH receptors in monolayered GT1-7 and αT3 cells. The displacement of ^{125}I-Ant 26 by unlabeled Ant 26 is illustrated in Figure 13. The estimated ED_{50} values for Ant 26 in αT3-1 and GT1-7 cells were 1.5 and 19 nM, respectively. The difference in apparent binding affinity suggest the presence of two classes of receptor. In addition, the cells were capable of binding GnRH in a similar manner on Day 3 or 4 of culture, indicating GnRH receptor integrity in this system. All these observations suggest that such an *in vitro* cell system would be appropriate for studying GnRH receptor characteristics under different physiological conditions.

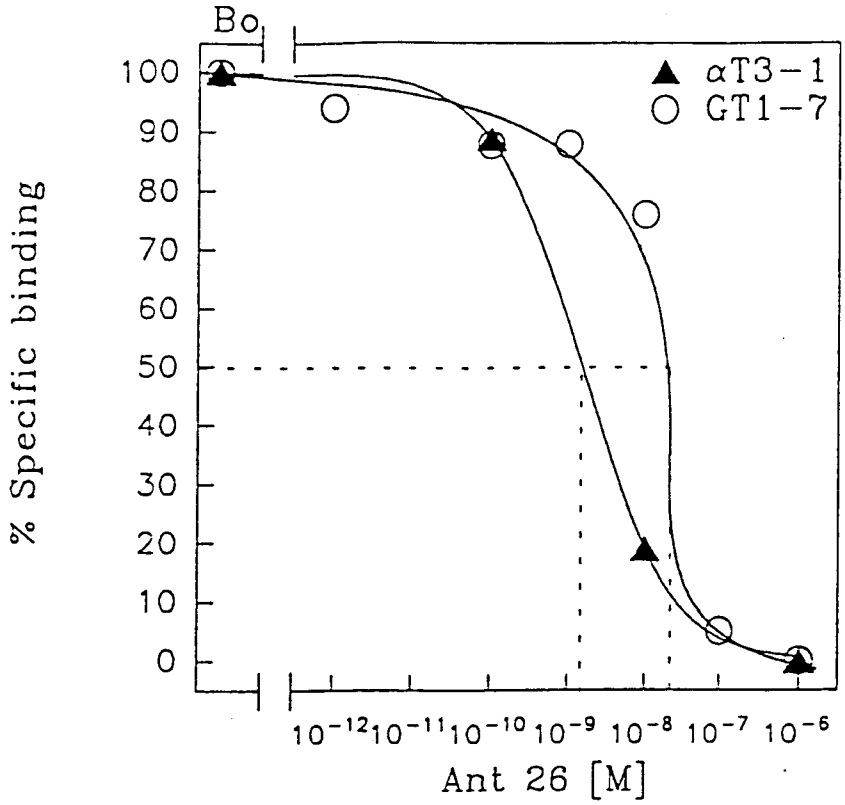


Figure 13: Demonstration of binding sites in GT1-7 and αT3-1 cells. GT1-7 and αT3-1 cells were incubated in the presence of different concentrations of Ant 26 together with ^{125}I-Ant 26 for 1h at 4°C. The above figure shows the displacement of ^{125}I-Ant 26 binding to GT1-7 and αT3-1 cells by unlabeled Ant 26. Dotted lines indicate ED_{50} values. B_o refers to binding of ^{125}I-Ant 26 in the absence of competing ligand.

Furthermore, the above studies are supported by recent work by Krsmanovic *et al.* (1993) who showed that GnRH binds with high affinity to specific receptors in GT1-7 cells in a dose-, time-, and temperature-dependent manner. Their results estimated IC_{50} values of 12 nM for GnRH, 2nM for the GnRH antagonist [D-pGly^1,D-Phe^2,D-Trp^{3,6}]GnRH, and 2.4 nM for the GnRH agonist des-Gly^{10}[DAla^6]GnRH *N*-ethylamide. No comparative studies with αT3-1 cells were presented.

As depicted in Figure 12, the isolated GnRH receptor cDNA encodes a 327 amino acid protein that has seven putative transmembrane regions. Intriguingly, it is unique among G protein-coupled receptors in that the predicted sequence lacks a carboxyl-terminal cytoplasmic domain (Tsutsumi *et al.*, 1992). For the detection of receptor

mRNA in GT1-7 cells, different methodologies were employed. Firstly, two other groups documented the expression of D_1-dopaminergic and β_1-adrenergic receptor mRNAs in GT1-7 cells by Northern analysis. Interestingly, β_1-adrenergic receptor mRNA was present in lower amounts in GT1-7 cells than in brain (Martinez de la Escalera *et al.*, 1992b; Findell *et al.*, 1993). In other studies, analysis of GT1-7 cells by PCR revealed the presence of NPY_1 and $GABA_A$ receptor subunit mRNA (Hales *et al.*, 1992; Besecke *et al.*, 1993). Thus, detection of mRNA encoding GnRH receptor in GT1-7 cells involved both Northern (**2.2.10**) and PCR (**3.2.4**) methodology. However, both approaches proved to be unsuccessful. It was concluded that the Northern was not sensitive enough to detect the low levels of GnRH receptor mRNA assumed to be present in GT1-7 cells. The PCR was done only once, the result of which was inconclusive. More than one band was present, suggesting non-specific binding to be the cause. Further optimization of the assay would include for example, changing the concentration of the oligonucleotide, its design, the Mg^{2+} concentration, or possibly the temperature of the reactions. In view of the other work being undertaken there was insufficient time to pursue this further. While the aforementioned methodologies were ineffective, analysis of GT1-7 RNA by Krsmanovic *et al.* (1993), revealed two mRNA species (1.6 and 3.5 kb) similar to those present in αT3-1 cells and in the mouse pituitary gland. The proportions of the receptor transcripts in αT3-1 and GT1-7 cell lines were also similar, with a predominance of the 3.5 kb species. In earlier studies, both transcripts were shown to encode functional receptors when expressed in *Xenopus laevis* oocytes (Reinhart *et al.*, 1992). It is proposed that the two mRNAs may have different stabilities or be differently regulated in response to various physiological situations *in vivo*.

The above findings have established that GT1-7 cells contain a specific binding site for GnRH as well as express mRNA for the GnRH receptor cloned from αT3-1 cells. Since GT1-7 and αT3-1 cells express similar GnRH receptor mRNA, but have distinct binding affinities for Ant 26, it is proposed that in the two cell lines the post-translational processing of the receptor differs. We cannot, however, discount the possibility that GT1-7 cells represent a transformed cell line that may express receptors not normally seen on GnRH neurons.

Although an ultrashort loop feedback inhibition of GnRH secretion has been demonstrated both *in vivo* and *in vitro*, the mechanism and circuitry of such an effect remain to be defined (**1.2.2**). Since the GT1-7 cells express the mRNA for GnRH and its receptor, the synapse-like connections observed among the GT1-7 cells may represent areas of the neuron where secretory vesicles congregate in close proximity to receptors (Mellon *et al.*, 1990; Liposits *et al.*, 1991). These findings provided the basis for the existence of a functional ultrashort feedback mechanism in the GT1-7 cell line. The current studies utilized GnRH analogs as tools to evaluate whether GnRH may regulate its own release via an ultrashort loop feedback mechanism.

Initially GT1-7 cells were exposed to either to GnRH[His^{5}D-Arg^6-Trp^7-Tyr^8] or the superactive GnRH agonist, GnRH[D-Ala^6-N-MeLeu^7,Pro^{9}NEt], for 18 h or 30 min, respectively. Thereafter, basal and secretagogue-stimulated GnRH release was determined over a 15 min or 30 min period. As shown in Figures 14A and B, neither agonist altered GnRH secretion under the conditions mentioned in the figure legends. It was concluded that the agonists were ineffective due to the high levels of endogenous GnRH. In other words, GnRH levels were already at maximal for the static culture system.

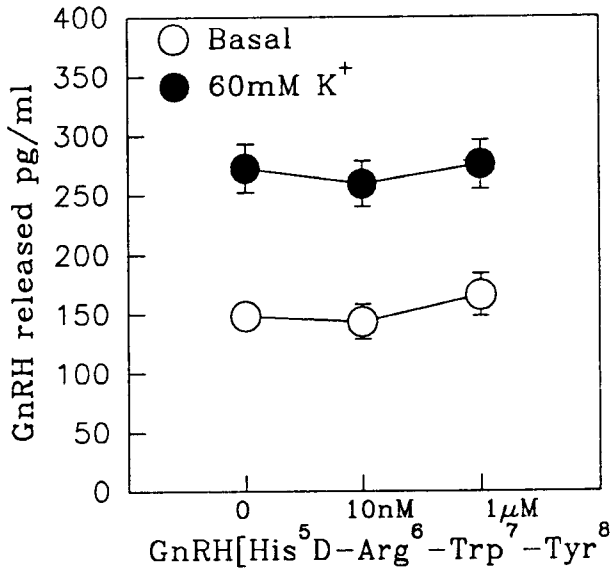


Figure 14A: Effect of different concentrations of GnRH[His^{5}D-Arg^6-Trp^7-Tyr^8] on GnRH release from GT1-7 cells.

After GT1-7 cells were preincubated with GnRH [His^{5}D-Arg^6-Trp^7-Tyr^8] for 18h, basal and K^+-stimulated GnRH release was determined over 15 min. No concentration-dependent effect was observed (n=3 for each group).

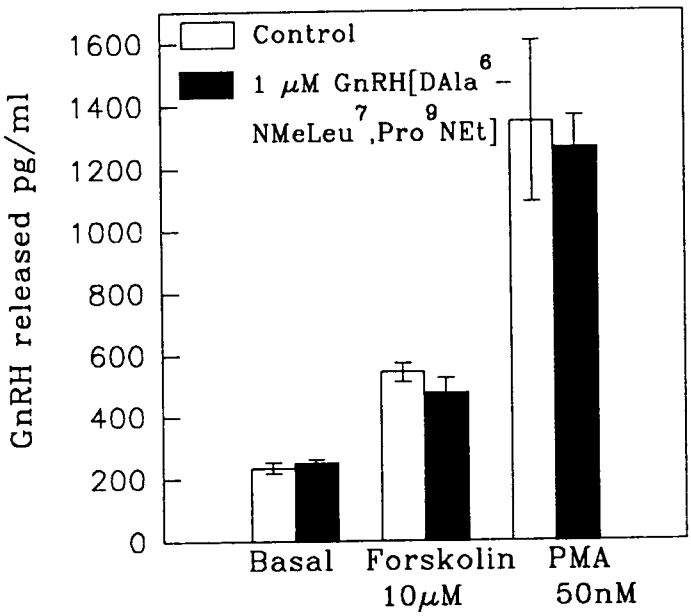


Figure 14B: Effect of 1 μM GnRH[DAla^6-NMeLeu^7,Pro^{9}NEt] on GnRH release from GT1-7 cells.

After GT1-7 cells were preincubated with GnRH[DAla^6-NMeLeu^7,Pro^{9}NEt] for 30 min, basal, forskolin and PMA-stimulated GnRH release was determined over 30 min. No significant difference to control groups (no agonist) was observed (n=3 for each group).

An alternative approach involved the use of a highly potent, but non-cross-reacting antagonist (Ant 27). After GT1-7 cells were co-incubated with Ant 27 for either 30 min or 18 h, basal and K^+-induced GnRH release was determined over 15 min. As shown in Figure 15A, Ant 27 significantly increased basal GnRH release over control levels at doses of 10 nM ($p< 0.05$), 100 nM and 1 μM ($p< 0.001$). Similarly, as shown in Figure 15B, preincubation for 18 h with Ant 27, resulted in a dose-dependent increase in basal GnRH release ($p< 0.001$). These results are consistent with the *in vitro* studies by Valenca *et al.* (1987), who showed that a different antagonist enhanced basal GnRH secretion in a concentration-dependent manner. In addition, Ant 27 further augmented K^+-induced GnRH release (Figures 15A and B). Preincubation for 30 min with 100 nM ($p< 0.01$) and 1 μM Ant 27 ($p< 0.001$) or 18 h with 1 μM Ant 27 ($p< 0.001$) enhanced K^+-stimulated GnRH release. These findings are in accord with the observations of Krsmanovic *et al.* (1993) who demonstrated that exposure of perifused GT1-7 neurons to a GnRH agonist analog (10 nM) caused a transient elevation of GnRH release and subsequent suppression of the basal pulsatile secretion. Their results are presented in Figure 16.

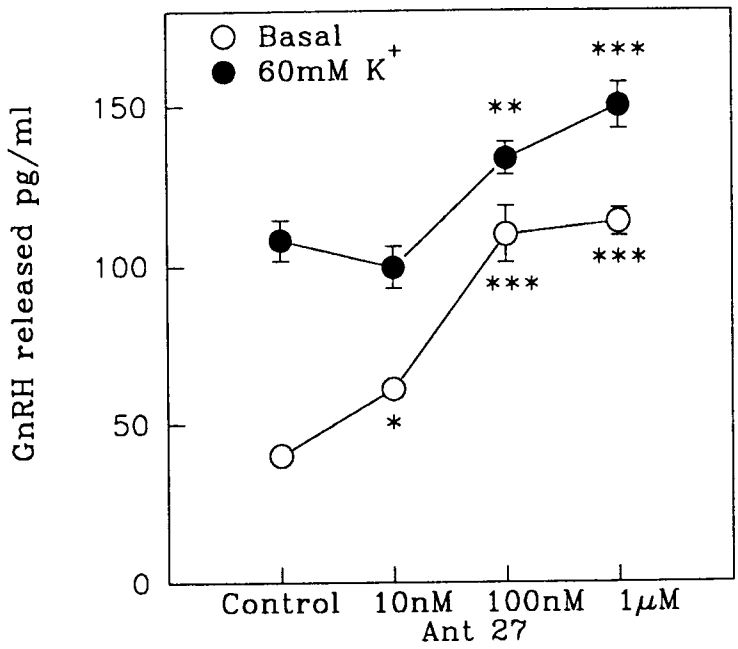


Figure 15A: Effect of different concentrations of Ant 27 on GnRH release from GT1-7 cells.

After GT1-7 cells were preincubated with Ant 27 for 30 min, basal and K^+-stimulated GnRH release was determined over 15 min. *, $P< 0.05$; **, $P<0.01$; ***, $P< 0.001$ vs. control group (no antagonist) (n=5 for each group).

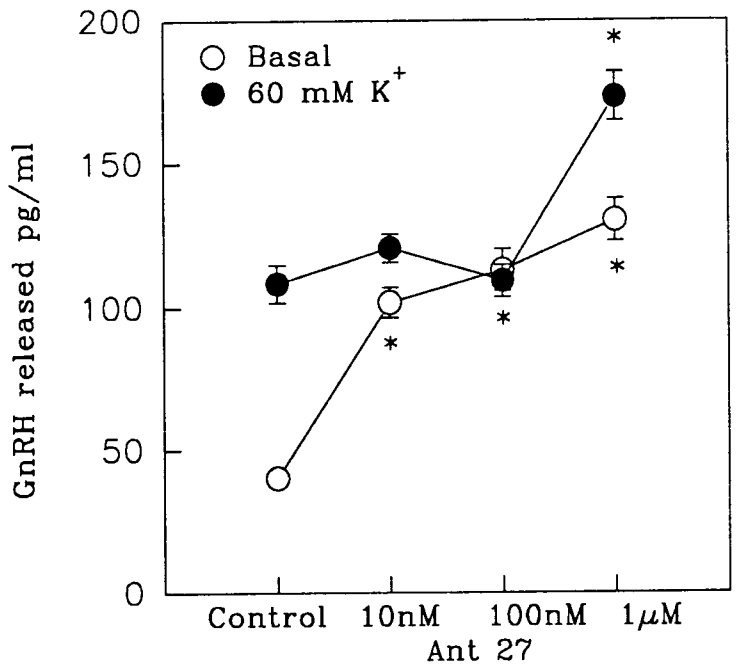


Figure 15B: Effect of different concentrations of Ant 27 on GnRH release from GT1-7 cells.

After GT1-7 cells were preincubated with Ant 27 for 18h, basal and K^+-stimulated GnRH release was determined over 15 min.*,$P< 0.001$ vs. control group (no antagonist) (n=5 for each group).

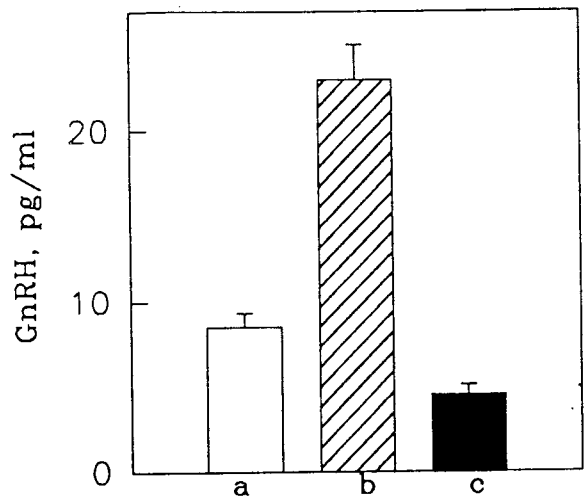


Figure 16: Averaged data (n=4) of GnRH release under basal conditions (bar a), and 10 min (bar b) and 60 min (bar c) after the beginning of agonist stimulation (Krsmanovic *et al.*, 1993).

Furthermore, as shown in Figure 17, co-incubation with 1 μM Ant 27 for 30 min, followed by a 30 min stimulation period, significantly increased GnRH release both basally and in response to 10 μM forskolin ($p < 0.001$). In contrast, 1 μM Ant 140-36 (inactive analog) was without effect. These results are supported by recent studies whereby preincubation of static GT1-3 cells for 1 h with 100 nM Ant 27 was shown to enhance basal and forskolin-stimulated GnRH release (after 1 h) (Yu *et al.*, 1994).

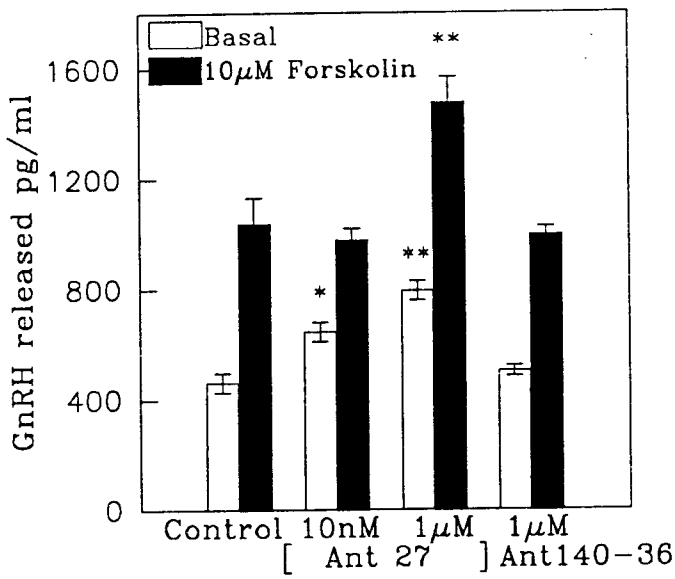


Figure 17: Effect of Ant 27 and Ant 140-36 on GnRH release from GT1-7 cells. After GT1-7 cells were preincubated with either Ant 27 or Ant 140-36 for 30 min, basal and forskolin-stimulated GnRH release was determined over 30 min. *, $P < 0.05$; **, < 0.001 vs. control group (no antagonists) (n=3 for each group).

Conversly, there was no significant effect on cellular GnRH content after 30 min or 18 h treatment with 10 nM, 100 nM or 1 μM Ant 27 (Figure 18). The above data support previous *in vitro* studies with rat MBH where although a GnRH agonist inhibited both basal and K^+-induced GnRH release, GnRH content remain unchanged (Zanisi *et al.*, 1987). However, Yu *et al.* (1994) recently demonstrated that prolonged treatment (24 h) with Ant 27 increased GnRH content, but not GnRH mRNA levels, suggesting that either the translational efficiency of GnRH mRNA or the processing of GnRH precursor protein may be under autofeedback regulation.

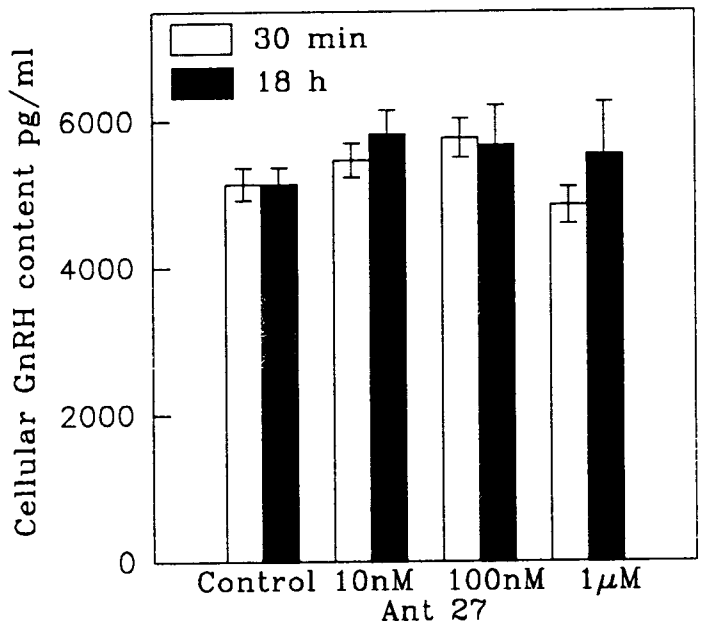


Figure 18: Effect of different concentrations of Ant 27 on cellular GnRH content of GT1-7 cells.
GnRH cellular content was determined after GT1-7 cells were co-incubated with Ant 27 for 30 min or 18 h. No significant difference to control groups (no antagonist) was observed (n=5 for each group).

Coupled with the anatomical evidence for direct contacts between GnRH neurons, the present findings clearly support a physiological role for a negative ultrashort loop feedback within the GnRH neuronal system. This mechanism may be a consequence of the ability of the GnRH autoreceptor to modulate the frequency and amplitude of the spontaneous GnRH release from these cells (Krsmanovic *et al.*, 1993).

4: CONCLUDING DISCUSSION

The neuroendocrine hypothalamus has been intensively studied using whole animals and tissue slices. However, direct analysis of the secretory activity of GnRH neurons has proved to be profoundly difficult due to their scattered topography and the physical inaccessibility of the hypothalamo-hypophyseal portal plexus. The recent development by Mellon *et al.* (1990) of GnRH-secreting neuronal cell lines (GT1-1, GT1-3 and GT1-7), derived by directed tumorigenesis in transgenic mice, provides the first *in vitro* model for the study of neural regulation of a pure population of GnRH neurons.

The extensive network of GnRH neurons and other neural elements act together to process a myriad of signals which influence the pulsatile release of GnRH into the pituitary portal circulation. Until recently, the anatomical, cellular, and molecular basis of the GnRH pulse generator remained largely unknown. Likewise, little is known of the inter- and intracellular routes through which the activity of GnRH pulse generator is regulated *in vivo*. These GnRH neuronal cells have provided a system in which to unravel the complex molecular and cellular mechanisms underlying GnRH gene expression, processing, and secretion. In addition, the cellular mechanisms responsible for the intrinsic rhythmic activity generating episodic GnRH release remain intriguing and important questions that should be addressable in GT1 cells (Martinez de la Escalera *et al.*, 1992a).

Recent studies have found pulsatile GnRH secretion to be an intrinsic property of GnRH neuronal networks and to depend on Ca^{2+} influx for its maintenance (Mellon *et al.*, 1990; Martinez de la Escalera *et al.*, 1992a; Wetsel *et al.*, 1992; Krsmanovic *et al.*, 1992). In addition to implying the existence of a single-cell oscillator, the observation of discrete pulses suggests the existence of a mechanism for synchronization of GnRH release from many neurons. This may be mediated either by synaptic mechanisms or electronic coupling via gap junctions (Wetsel *et al.*, 1992). Conceivably, a diffusible mediator such as GnRH itself, GAP or GAL may be involved. In accord with this, GnRH neurons *in vitro* are able to reorganize into interconnected clusters, forming contacts between their cell bodies and between developing and

mature processes (Liposits *et al.*, 1991). GnRH in axons of local circuit neurons could serve to co-ordinate GnRH secretion from individual cells and, thereby, orchestrate functions *in vivo* as diverse as the onset of puberty, the timing of ovulation, and the duration of lactational infertility. In addition, the synaptic connections could serve as a neuronal ultrashort loop feedback channel for the self-regulation of GnRH-synthesizing neurons. In turn, the autofeedback control may be differently regulated to achieve the variety of responses necessitated by the GnRH neurosecretoy system.

The complexity of GnRH action in perifused GT1-7 cells was recently highlighted by Krsmanovic *et al.* (1993). Their results suggest that the feedback effects of GnRH include both positive and negative components that exert an integrated action on its neurons and contribute to the operation of the GnRH pulse generator. However, in the present studies co-incubation of Ant 27 with static GT1-7 cells resulted in enhanced basal GnRH release. This result implies that GnRH by binding to autoreceptors in GT1-7 cells, negatively controls its own secretion.

Presynaptic inhibition of neurotransmitter release may involve either an enhancement of a K^+ conductance, reducing the ability of action potentials to invade the terminal, or inhibition of Ca^{2+} currents, directly reducing the influx of Ca^{2+} which is a prerequisite for transmitter release (Nicoll, 1988; Tsien *et al.*, 1988). Thus, the apparent paradox can be resolved by a model in which early activation of the GnRH receptor by the agonist causes an elevation of $[Ca^{2+}]_i$ (by releasing intracellular Ca^{2+} stores), resulting in enhanced GnRH release. The subsequent suppression of release is due to agonist-induced inhibition of Ca^{2+} entry (Krsmanovic *et al.*, 1993). Further the $[Ca^{2+}]_i$ response to GnRH was rapidly inhibited by prior addition of a potent GnRH antagonist. It is proposed that the enhanced GnRH release observed in the presence of Ant 27 is probably due to the removal of the inhibitory effect of GnRH on its own release. This may be accomplished by preventing the GnRH-induced inhibition of Ca^{2+} influx. As a result, Ca^{2+} influx may increase. In addition, since K^+- and forskolin-induced GnRH release is dependent on influx of extracellular Ca^{2+}, this hypothesis may explain the enhanced GnRH release by these secretagogues observed in the presence of Ant 27.

It would be reasonable to assume that activation of the negative ultrashort loop feedback is triggered once a certain threshold for GnRH concentrations is reached. This threshold is most likely rapidly attained in the static culture system. In this scenario the negative autofeedback would then be responsible for maintaining the steady-state levels required for basal activity. Furthermore, the juxtaposition of axon-like processess to cell bodies (mimicking the pattern of axo-somatic communication) provides the anatomical support for negative feedback effects on pulsatile GnRH secretion during periods of basal activity (Liposits *et al.*, 1991). Given the high degree of similarity between GT1-7 cells and GnRH neurons, the present data argues in favour of an inhibitory role of GnRH on its own secretion. However, further studies are necessary to identify precisely which cellular effector system(s) mediate the physiological actions of GnRH in GT1-7 cells.

An additional hypothesis involves the co-localization of GAL with GnRH in the GnRH neurons. Marks *et al.* (1993) propose a model which predicts that GAL expression in GnRH neurons is a prerequisite for producing the distinctly pulsatile pattern of GnRH release. GAL could most effectively alter GnRH secretion by acting directly on the GnRH neurons from which it is released. The degree of influence GAL has on GnRH release would be related to its concentration at the nerve terminal, which in turn, may depend on its relative rate of biosynthesis. Factors controlling this biosynthetic rate include ovarian steroids, which may act either directly on the GnRH neuron or indirectly on other neurons that provide afferent input to GnRH neurons. Although this model focuses on the effect of GAL expression in GnRH neurons on GnRH release, the precise functional role of GnRH autoregulation and the conditions under which it becomes operational remain to be defined.

Due to the intrinsic pulsatile nature of the GnRH secretory process it is obvious that, in addition to autoregulation, inhibitory as well as facilitatory pathways are required to coordinate such a delicate and precise secretory rhythm. The neuronal circuitry formed by the multiple neurotransmitter and neuromodulatory systems known to innervate GnRH neurons may form additional neural networks that are able to modify the frequency and amplitude of an inherent pulsatile rhythm. For example, changes

in inhibitory neural inputs from EOP and GABA neurons have been proposed as inhibitors of GnRH neuronal activity (Masotto *et al.*, 1989; Rasmussen *et al.*, 1990). Furthermore, hypophysiotropic peptidergic neurons have been shown to influence the activity of other intrahypothalamic peptidergic cells and to be themselves under peptidergic control. For instance, synaptic contacts have been demonstrated between SS and GRF, POMC and GnRH, and NPY and VIP-containing neurons. Interestingly, all of these cell types have been shown to synapse upon themselves indicating that they too are capable of synchronization and/or autoregulation. Taken together, these morphological data provide evidence for multiple levels of cross-talk between hypothalamic peptidergic neurons and indicate that such interactions may play a role in the regulation of the hypothalamo-hypophyseal axis (Beaudet, 1990). To date, the factors that have been shown to directly release GnRH from GT1-7 cells include the following. NE, Endothelin-3, NPY and a $GABA_A$ agonist all significantly stimulated GnRH release from static GT1 cultures (Martinez de la Escalera *et al.*, 1992b; Favit *et al*, 1992; Findell *et al.*, 1993; Moretto *et al*, 1993; Besecke *et al*, 1993).

Studies by Bourguignon *et al.* (1990b) lead them to suggest that the increased frequency of pulsatile GnRH secretion at the onset of puberty may be related to a reduced sensitivity of the hypothalamic pulse generator to an inhibitory autofeedback. This hypothesis is based on the observation that the inhibitory autofeedback of GnRH on pulsatility appears to be reduced in potency by NMDA. Interestingly, recent studies have indicated that GLU (via NMDA and AMPA/kainate receptors) can stimulate GnRH secretion by acting directly on GT1-7 cells (Spergel *et al.*, 1994). Results, in part, supported by work presented in section **2.3** demonstrating L-GLU enhanced GnRH release. As stated by Bourguignon *et al.* (1990b), "It is likely that the target of the autofeedback and the primary event of hypothalamic maturation at onset of puberty are closely related. This is why it is critical to further study autoregulation which can provide a clue of the central mechanism of puberty." Hence, an analysis combining the activation of the GLU receptor subtypes with GnRH analog studies in GT1-7 cells would provide essential information in this regard.

Further studies to elucidate the mechanism of autofeedback would include determining the effector systems that are coupled to the receptor and also, the location of the GnRH receptor (soma, dendrites, or axon?). The synapse-like contacts could perform a negative-feedback role to regulate not only the amount of GnRH released but also the molecular forms secreted. Since the cells also contain GAP, they will be valuable in studying the biosynthesis and processing of the GnRH molecule and its products. Also differences in the GAP/GnRH ratio in vesicles may influence the autofeedback mechanism. However, pro-GnRH processing may be more complete under perifusion than under static culture conditions. Indeed, perifusion serves to remove products (e.g., GnRH and GAP) from the cell that could feedback to inhibit some of the processing enzymes. For this reason, the perifusion system may provide a better physiological model to study GnRH secretion and processing than the conditions imposed under the static culture conditions (Wetsel *et al*, 1992).

In addition to numerous observations that steroids influence the activity of the neuroendocrine systems involved in the regulation of GnRH release (**1.2.3**), steroids seem to be critical for organizing and reorganizing the neuronal circuitary in the CNS (Matsumoto, 1991). These cells were, however, maintained in an environment relatively free of steroid hormone influences. Differences in receptor densities and/or receptor subtypes may exist when these cells are maintained under a different steroidal hormone milieu. GT1-7 cells cultured in isolation, almost certainly, represent only one potential state of a highly regulated cell. However, to date data obtained from experiments with GT1 cells are in good agreement with findings in animals, and they appear to be an important tool in furthering our knowledge concerning the neuroendocrine regulation of reproduction and the characteristics of an isolated CNS neuron.

To conclude:
" When you think about brain activity its correct to think about emergent properties at higher levels that depend on lower level phenomena in the system"

Patricia Churchland, Neurophilosophy.

Thus, the evidence presented for the existence of an ultrashort feedback at the level of the GnRH neuron is a significant step towards gaining insight into the mechanics of the system which has the GnRH pulse generator as its emergent property.

REFERENCES

Adelman J.P., Mason A.J., Hayflick J.S., Seeburg P.H. (1986). Isolation of the gene and hypothalamic cDNA for the common precursor of gonadotropin-releasing hormone and prolactin release-inhibiting factor in human and rat. Proc. Natl. Acad. Sci. (USA). 83: 179-183.

Ajika K. (1979). Simultaneous localization of LHRH and catecholamines in rat hypothalamus. J. Anat. 128: 331-347.

Ajika K. (1980). Relationship between catecholaminergic neurons and hypothalamic hormone-containing neurons in the hypothalamus. In Martini L., Ganong W.F. (eds). Frontiers in Neuroendocrinology. Raven Press, NY. p1

Allen L.G., Crowley W.R., Kalra S.P. (1987). Interactions between neuropeptide Y and adrenergic systems in the stimulation of luteinizing hormone release in steroid-primed ovariectomized rats. Endocrinology 121: 1953-1959.

Arias P., Szwarchfarb B., Rondina D.C., Carbone S., Sverdlik R., Moguilevsky J.A. (1990). *In vivo* and *in vitro* studies on the effect of the serotoninergic system on luteinizing hormone and luteinizing hormone-releasing hormone secretion in prepubertal and peripubertal female rats. Brain Res. 523: 57-61.

Azad N., Duffner L., Paloyan E.B., Reda D., Kirsteins L., Emanuele N.V., Lawrence A.M. (1990). Hypothalamic prolactin stimulates the release of luteinizing hormone-releasing hormone from male rat hypothalamus. Endocrinology 127: 1928-1933.

Beaudet A. (1990). Cross-talking peptidergic hypothalamic neurons. Abs. S11.1, 2nd International Congress of Neuroendocrinology, Bordeux, France.

Beaudet A., Leonard K., Faure M.P., Weiner R.I., Desjardins G.C. (1993). Binding and internalization of FITC-labeled neurotensin in a sub-population of GnRH-containing neurons: a confocal microscopic analysis. Abs. 571.17, 23rd Annual Meeting, Society for Neuroscience, Washington, D.C.

Bergen H.T., Hejtmancik J.F., Pfaff D.W. (1991). Effects of gamma-aminobutyric acid receptor agonists and antagonist on LHRH-synthesizing neurons as detected by immunocytochemistry and in situ hybridization. Exp. Brain Res. 87: 46-56.

Berria M., Pau K.-Y.F., Spies H.G. (1991). Evidence for α_1-adrenergic involvement in neuropeptide Y-stimulated GnRH release in female rabbits. Neuroendocrinolgy 53: 480-486.

Besecke L.M., Urben J.H., Levine J.E. (1993). Neuropeptide Y_1 receptors mediate NPY-stimulated LHRH release from superfused GT1-7 cells. Abs. 258.5, 23rd Annual Meeting, Society for Neuroscience, Washington, D.C.

Bicknell R.J. (1985). Endogenous opioid peptides and hypothalamic neuroendocrine neurones. J. Endocr. 107: 437-446.

Bourguignon J.P., Franchimont P. (1984). Puberty-related increase in episodic LHRH release from rat hypothalamus *in vitro*. Endocrinology 114: 1943-1945.

Bourguignon J., Gerard A., Franchimont P. (1984). Age-related difference in the effect of castration upon hypothalamic LHRH content in male rats. Neuroendocrinology 38: 376-381.

Bourguignon J.P., Gerard A., Debougnoux G., Rose J., Franchimont P. (1987). Pulsatile release of gonadotropin-releasing hormone (GnRH) from the rat hypothalamus *in vitro*: calcium and glucose dependency and inhibition by superactive GnRH analogs. Endocrinology 121: 993-999.

Bourguignon J.P., Gerard A., Franchimont P. (1989a). Direct activation of gonadotropin-releasing hormone secretion through different receptors to neuroexcitatory amino acids. Neuroendocrinology 49: 402-408.

Bourguignon J.P., Gerard A., Mathieu J., Simons J., Franchimont P. (1989b). Pulsatile release of gonadotropin-releasing hormone from hypothalamic explants is restrained by blockade of N-methyl-D,L-aspartate receptors. Endocrinology 125: 1090-1096.

Bourguignon J.P., Gerard A., Mathieu J., Mathieu A., Franchimont P. (1990a). Maturation of the hypothalamic control of pulsatile gonadotropin-releasing hormone secretion at onset of puberty. I. Increased activation of N-methyl-D-aspartate receptors. Endocrinology 127: 873-881.

Bourguignon J.P., Gerard A., Franchimont P. (1990b). Maturation of the hypothalamic control of pulsatile gonadotropin-releasing hormone secretion at onset of puberty. II. Reduced potency of an inhibitory autofeedback. Endocrinology 127: 2884-2890.

Bourguignon J.P., Gerard A., Alvarez-Gonzalez M.L., Franchimont P. (1992a). Neuroendocrine mechanism of onset of puberty. Sequential reduction in activity of inhibitory and facilitatory N-methyl-D-aspartate receptors. J. Clin. Invest. 90: 1736-1744.

Bourguignon J.P., Gerard A., Alvarez-Gonzalez M.L., Fawe L., Franchimont P. (1992b). Gonadal-independent developmental changes in activation of N-methyl-D-aspartate receptors involved in gonadotropin-releasing hormone secretion. Neuroendocrinology 55: 634-641.

Bruder J.M., Krebs W.D., Nett T.M., Wierman M.E. (1992). Phorbyl ester activation of the protein kinase C pathway inhibits gonadotropin-releasing hormone gene expression. Endocrinolgy 131: 2552-2558.

Carbone S., Szwarcfarb B., Losada M.E., Moguilevsky J.A. (1992). Effects of ovarian steroids on the gonadotropin response to N-methyl-D-aspartate and on hypothalamic excitatory amino acids levels during sexual maturation in female rats. Endocrinology 130: 1365-1370.

Chen H.T., Geneau J., Meites J. (1977). Effect of castration, steroid replacement and hypophysectomy on hypothalamic LHRH and serum LHRH. Proc. Soc. Exp. Biol. Med. 156: 127-131.

Chen W., Witkin J.W., Silverman A.J. (1989a). Gonadotropin releasing hormone (GnRH) neurons are directly innervated by catecholamine nerve terminals. Synapse 3: 288-290.

Chen W., Witkin J.W., Silverman A.J. (1989b). Beta-endorphin and gonadotropin-releasing hormone synaptic input to gonadotropin-releasing hormone neurosecretory cells in the male rat. J. Comp. Neurol. 286: 85-95.

Chomczynski P., Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium thiocyanate-phenol-chloroform extraction. Anal. Biochem. 162: 156-159.

Clough R.W., Hoffman G.E., Sladek C.D. (1990). Synergistic interaction between opioid receptor blockade and alpha-adrenergic stimulation on luteinizing hormone-releasing hormone (LHRH) secretion *in vitro*. Neuroendocrinology 57: 131-138.

Contijoch A.M., Gonzalez C., Singh H.N., Malamed S., Troncoso S., Advis J.P. (1992). Dopaminergic regulation of luteinizing hormone-releasing hormone release at the median eminence level: Immonocytochemical and physiological evidence in hens. Neuroendocrinology 55: 290-300.

Crowley W.R., Kalra S.P. (1987). Neuropeptide Y stimulates the release of luteinizing hormone-releasing hormone from medial basal hypothalamus *in vitro*: modulation by ovarian hormones. Neuroendocrinology 46: 97-103.

Csiffary A., Mezey E., Palkovits M. (1990). Corticotropin-releasing factor (CRF) immunoreactive nerve fibres innervate gonadotropin releasing hormone (GnRH)-containing neurons in the medial preoptic nucleus of the rat. Abs. P2.81, 2nd International Congress of Neuroendocrinology, Bordeux, France.

de los Frailes M.T., Cacicedo L., Fernandez G., Tolon R.M., Lorenzo M.J., Aguado F., Sanchez Franco F. (1992). Role of locally produced growth hormone-releasing factor in somatostatin regulation by fetal rat brain cells in culture. Neuroendocrinology 55; 221-229.

DePaolo L.V., King R.A., Carrillo A.J. (1987). *In vivo* and *in vitro* examination of an autoregulatory mechanism for luteinizing hormone-releasing hormone. Endocrinology 120: 272-279.

Dluzen D.E., Ramirez V.D. (1986). *In vivo* output of ovariectomized rats following estrogen treatment. Neuroendocrinology 43: 459-465.

Donoso A.O., Lopez F.J., Negro-Vilar A. (1990). Glutamate receptors of the non-N-methyl-D-aspartic acid type mediate the increase in luteinizing hormone-releasing hormone release by excitatory amino acids *in vitro*. Endocrinology 126: 414-420.

Donoso A.O., Lopez F.J., Negro-Vilar A. (1992). Cross-talk between excitatory and inhibitory amino acids in the regulation of luteinizing hormone-releasing hormone secretion. Endocrinology 131: 1559-1561.

Drouva S.V., Epelbaum J. Hery M., Tapia-Arancibia I., Laplante E., Kordon C. (1981). Ionic channels involved in the LHRH and SRIF release from rat mediobasal hypothalamus. Neuroendocrinology 32: 155-162.

Drouva S.V., Laplante E., Kordon C. (1985). Progesterone-induced LHRH release *in vitro* is an estrogen as well as Ca^{2+} and calmodulin-dependent secretory process. Neuroendocrinology 40: 325-331.

Ellinwood W.E., Ronnekleiv O.K., Kelly M.J., Resko J.A. (1985). A new antiserum with conformational specificity for LHRH: usefulness for radioimmunoassay and immunocytochemistry. Peptides 6: 45-52.

Epelbaum J., Tapia-Arancibia L., Alonso G., Astier H., Kordon C. (1986). The anterior periventricular hypothalamus is the site of somatostatin inhibition of its own release: an *in vitro* and immunocytochemical study. Neuroendocrinology 44: 255-259.

Everitt B.J., Hokfelt T., Terenius L., Tatemoto K., Mutt V., Goldstein M. (1984). Differential co-existence of neuropeptide Y (NPY)-like immunoreactivity with catecholamines in the central nervous system of the rat. Neuroscience 11: 443-462.

Favit A., Wetsel W.C., Negro-Vilar A. (1992). Stimulation of LHRH secretion by GABA receptor activation from immortalized hypothalamic neurons. Abs. 827, 74th Annual Meeting, The Endocrine Society, San Antonio, TX.

Findell P.R., Wong K.H., Jackman J.K., Daniels D.V. (1993). β_1-adrenergic and dopamine (D_1)-receptors coupled to adenyl cyclase activation on GT1 gonadotropin-releasing hormone neurosecretory cells. Endocrinology 132: 682-688.

Flugge G., Oertel W.H., Wuttke W. (1986). Evidence for estrogen-receptive GABAergic neurons in the preoptic/anterior hypothalamic area of the rat. Neuroendocrinology 43: 1-5.

Gambacciani M., Yen S.S.C., Rasmussen D. (1986). GnRH release from medial basal hypothalamus: *In vitro* inhibition by corticotropin-releasing factor. Neuroendocrinology 43: 533-536.

Goldsmith P.C., Thind K.K., Perera A.D., Plant T.M. (1994). Glutamate-immunoreactive neurons and their gonadotropin-releasing hormone-neuronal interactions in the monkey hypothalamus. Endocrinology 134: 858-868.

Gore A.C., Terasawa E. (1991). A role for norepinephrine in the control of puberty in the female rhesus monkey, *Macaca mulatta*. Endocrinology 129: 3009-3017.

Griffiths E.C., Hooper K.C., Jeffcoate S.L., Holland D.T. (1975). The effects of gonadectomy and gonadal steroids on the activity of hypothalamic peptidases inactivating luteinizing hormone-releasing hormone (LH-RH). Brain Res. 88: 384-385.

Gross D.S. (1980). Effect of castration and steroid-replacement on immunoreactive gonadotropin-releasing hormone in the hypothalamic and preoptic area. Endocrinology 106: 1442-1450.

Hahn E.F., Fishman J. (1985). Castration affects male rat brain opiate receptor content. Neuroendocrinology 41: 60-63.

Hales T.G., Kim H., Longoni B., Olsen R.W., Tobin A.J. (1992). Immortalized hypothalamic GT1-7 neurons express functional gamma-aminobutyric acid type A receptors. Mol. Pharmacol. 42: 197-202.

Harris N., Dutlow C., Eidne K., Dong K-W., Roberts J., Millar R. (1991). Gonadotropin-releasing hormone gene expression in MDA-MB-231 and ZR-75-1 breast carcinoma cell lines. Cancer Res. 51: 2577-2581.

Hendricks S., Millar R., Pimstone B. (1975). A specific radio-immunoassay for gonadotropin-releasing hormone. S. Afr. Med. J. 49: 1559-1562.

Herbison A.E., Theodosis D.T. (1992). Localization of oestrogen receptors in preoptic neurons containing neurotensin but not tyrosine hydroxylase, cholestokinin or luteinizing hormone-releasing hormone in the male and female rat. Neuroscience 50: 283-298.

Hoffman G.E., Wray S., Goldstein M. (1982). Relationship of catecholamines and LHRH: light microscope study. Brain Res. Bull. 9: 417-430.

Hoffman G.E. (1985). Organization of LHRH cells: Differential apposition of neurotensin, substance P and catecholamine axons. Peptides 6: 439-461.

Horn F., Bilezikjian L.M., Perrin M.H., Bosma M.M., Windle J.J., Huber K.S., Blount A.L., Hille B., Vale W., Mellon P.L. (1991). Intracellular responses to gonadotropin-releasing hormone in a clonal cell line of the gonadotrope lineage. Mol. Endocr. 5:347-355.

Hyppa M., Motta M., Martini L. (1971). 'Ultrashort' feedback control of follicle-stimulating hormone-releasing factor secretion. Neuroendocrinology 7: 227.

Jarjour L.T., Handelsman D.J., Raum W.J., Swerdloff R.S. (1986). Mechanism of action of dopamine on the *in vitro* release of gonadotropin-releasing hormone. Endocrinology 119: 1726-1732.

Jarry H., Leonardt S., Wuttke W. (1990). A norepinephrine-dependent mechanism in the preoptic/anterior hypothalamic area but not in the mediobasal hypothalamus is involved in the regulation of the gonadotropin-releasing hormone pulse generator in ovariectomized rats. Neuroendocrinology 51: 337-344.

Jennes L., Dupre E. (1991). Regulation of the GnRH-LH axis by glutamate. Abs. 1703, 73rd Annual Meeting, The Endocrine Society, Washington, D.C.

Kalra P.S., Crowley W.R., Kalra S.P. (1987). Differential *in vitro* stimulation by naloxone and K^+ of luteinizing hormone-releasing hormone and catecholamine release from hypothalami of intact and castrated rats. Endocrinology 120: 178-185.

Kalra S.P. (1976). Tissue levels of luteinizing hormone-releasing hormone in the preoptic area and hypothalamus, and serum concentrations of gonadotropins following anterior hypothalamic deafferentation and estrogen treatment of the female rat. Endocrinology 99: 101-107.

Kalra S.P., Kalra P.S. (1983). Neural regulation of luteinizing hormone secretion on the rat. Endocr. Rev. 4: 311-351.

Kalra S.P., Kalra P.S. (1989). Do testosterone and estradiol-17β enforce inhibition or stimulation of luteinizung hormone-releasing hormone secretion? Biol. Reprod. 41: 559-570.

Kalra S.P., Sahu A., Crowley W.R., Kalra P.S. (1991). Effect of calcium channel agonists and antagonists on hypothalamic NPY and LHRH release *in vitro*. Abs. 1024, 73rd Annual Meeting, The Endocrine Society, Washington, D.C.

Karahalios D.G., Levine J.E. (1988). Naloxone stimulation of *in vivo* LHRH release is not diminished following ovariectomy. Neuroendocrinology 47: 504-510.

Kelly M.J., Garrett J., Bosch M.A., Roselli C.E., Douglass J., Adelman J.P., Ronnekleiv O.K. (1989). Effects of ovariectomy on GnRH mRNA, proGnRH and GnRH levels in the preoptic hypothalamus of the female rat. Neuroendocrinology 49: 88-97.

Kelly M.J., Loose M.D., Ronnekleiv O.K. (1990). Opioids hyperpolarize β-endorphin neurons via μ-receptor activation of a potassium conductance. Neuroendocrinolgy 52: 268-275.

Khorram O., Pau K.-Y.F., Spies H.G. (1987). Bimodal effects of neuropeptide Y on hypothalamic release of gonadotropin-releasing hormone in conscious rabbits. Neuroendocrinology 45: 290-297.

Khorram O., Pau K.-Y.F., Spies H.G. (1988). Release of hypothalamic neuropeptide Y and effects of exogenous NPY on the release of hypothalamic GnRH and pituitary gonadotropins in intact and ovariectomized does *in vitro*. Peptides 9: 411-417.

Kim K., Ramirez V.D. (1982). *In vitro* progesterone stimulates the release of luteinizing hormone-releasing hormone from superfused hypothalamic tissue from ovariectomized estradiol-primed prepubertal rats. Endocrinology 111: 750-757.

Kim K., Lee B.J., Park Y., Cho W.K. (1989). Progesterone increases messenger ribonucleic acid (mRNA) encoding luteinizing hormone releasing hormone (LHRH) level in the hypothalamus of ovariectomized estradiol-primed prepubertal rats. Mol. Brain Res. 6: 151-158.

King J.A., Millar R.P. (1982). Structure of chicken hypothalamic luteinizing hormone-releasing hormone. J. Biol. Chem. 257: 10722-10728.

Kiss J., Halasz B. (1985). Demonstration of serotoninergic axons terminating on luteinizing hormone-releasing hormone neurons in the preoptic area of the rat using a combination of immunocytochemistry and high resolution autoradiography. Neuroscience 14: 69-78.

Knobil E. (1980). The neuroendocrine control of the menstrual cycle. Recent Prog. Horm. Res. 36: 53-88.

Krsmanovic L.Z., Stojilkovic S.S., Merelli F., Dufour S.M., Virmani M.A., Catt K.J. (1992). Calcium signaling and episodic secretion of gonadotropin-releasing hormone in hypothalamic neurons. Proc. Natl. Acad. (USA). 89: 8462-8466.

Krsmanovic L.Z., Stojilkovic S.S, Mertz L.M., Tomic M., Catt K.J. (1993). Expression of gonadotropin-releasing hormone receptors and autocrine regulation of neuropeptide release in immortalized hypothalamic neurons. Proc. Natl. Acad. Sci. (USA). 90: 3908-3912.

Kuljis R.O., Advis J.P. (1989). Immunocytochemical and physiological evidence of a synapse between dopamine- and LHRH-containing neurons in the ewe median eminence. Endocrinology 124: 1579-1581.

Langub M.C., Maley B.E., Watson R.E. (1991). Ultrastructural evidence for luteinizing hormone-releasing hormone neuronal control of estrogen responsive neurons in the preoptic area. Endocrinology 128: 27-36.

Lasdun A., Reznik S., Molineaux C.J., Orlowski M. (1989). Inhibition of endopeptidase 24.15 slows the *in vivo* degradation of luteinizing hormone-releasing hormone. J. Pharm. Exp. Therp. 251: 439-447.

Lee B.J., Kim K., Choo W.K. (1990). Activation of intracellular pathways with forskolin and phorbol ester increases LHRH mRNA level in the rat hypothalamus superfused *in vitro*. Mol. Brain Res. 8: 185-191.

Leposavic G., Dashwood M.R., Ginsburg J., Buckingham J.C. (1990). Peripubertal changes in the nature of the GnRH response to alpha-adrenoceptor stimulation *in vitro* and their modulation by testosterone. Neuroendocrinology 52: 82-89.

Leposavic G., Cover P.O., Buckingham J.C. (1991). *In vivo* and *in vitro* studies on the opioidergic control of the secretion of gonadotropin-releasing hormone and luteinizing hormone in sexually immature and adult male rats. Neuroendocrinology 53: 579-588.

Leranth C., MacLusky N.J., Sakamoto H., Shanabrough M., Naftolin F. (1985a). Glutamic acid decarboxylase-containing axons synapse on LHRH neurons in the rat medial preoptic area. Neuroendocrinology 40: 536-539.

Leranth C., Segura L.M.G., Palkovits M., MacLusky N.J., Shanabrough M., Naftolin F. (1985b). The LH-RH-containing neuronal network in the preoptic area of the rat: demonstration of LH-RH-containing nerve terminals in synaptic contact with LH-RH neurons. Brain Res. 345: 332-336.

Levine J.E., Bauer-Dantoin a.C., Besecke L.M., Conaghan L.A., Legan S.J., Meredith J.M., Strobl F.J., Urban J.H., Vogelsong K.M., Wolfe A.M. (1991). Neuroendocrine regulation of the luteinizing hormone-releasing hormone pulse generator in the rat. Recent Prog. Horm. Res. 47: 97-151.

Li S., Pelletier G. (1992). Role of dopamine in the regulation of gonadotropin-releasing hormone in the male rat brain as studied by *in situ* hybridization. Endocrinology 131: 395-399.

Liaw J-J., Barraclough C.A. (1993). N-methyl-D,L-aspartic acid differentially affects LH release and LHRH mRNA levels in estrogen-treated ovariectomized control and androgen-sterilized rats. Molec. Brain. Res. 17:112-118.

Lincoln G.A., Short R.V. (1980). Seasonal breeding: natures contraceptive. Recent Prog. Horm. Res. 36: 1

Liposits Z., Merchenthaler I., Wetsel W.C., Reid J.J., Mellon P.L., Weiner R.I., Negro-Vilar A. (1991). Morphological characterization of immortalized hypothalamic neurons synthesizing luteinizing hormone-releasing hormone. Endocrinology 129: 1575-1583.

Lopez F.J., Negro-Vilar A. (1990). Galanin stimulates luteinizing hormone-releasing hormone secretion from arcuate nucleus-median eminence fragments *in vitro*: Involvement of an α-adrenergic mechanism. Endocrinology 127: 2431-2436.

Lopez F.J., Donoso A.O., Negro-Vilar A. (1992). Endogenous excitatory amino acids and glutamate receptor subtypes involved in the control of hypothalamic luteinizing hormone-releasing hormone secretion. Endocrinology 130: 1986-1992.

Lopez F.J., Liposits Z., Merchenthaler I. (1992). Evidence for a negative ultrashort loop feedback regulating galanin release from arcuate nucleus-median eminence functional unit. Endocrinology 130: 1499-1507.

Lumpkin M.D., Negro-Vilar A., McCann S.M. (1981). Paradoxical elevation of growth hormone by intraventricular somatostatin: possible ultrashort-loop feedback. Science 211: 1072-1074.

Lumpkin M.D., Samson W.K., McCann S.M. (1983). Hypothalamic and pituitary sites of action of oxytocin to alter prolactin secretion in the rat. Endocrinology 112: 1711-1717.

Lumpkin M.D., Samson W.K., McCann S.M. (1985). Effects of intravenntricular growth hormone-releasing factor on growth hormone release: further evidence for ultrashort loop feedback. Endocrinology 116: 2070-2074.

Luo L.G., Zhao B.G., Zhao W., Zhu X.Z. (1991). A study of the regulatory role of GABA on the LHRH release in the median eminence of rats. Sheng Li Hsueh Pao 43: 205-212.

MacDonald M.C., Wilkinson M. (1990). Peripubertal treatment with N-methyl-D-aspartic acid or neonatally with monosodium glutamate accelerates sexual maturation in female rats, an effect reversed by MK-801. Neuroendocrinology 52: 143-149.

MacDonald A., Daly C.J., Bulloch J.M., McGrath J.C. (1992). Contributions of α_1-adrenoceptors, α_2-adrenoceptors and P_{2x}-purinoceptors to neurotransmission in several rabbit isolated blood vessels: role of neuronal uptake and autofeedback. Br. J. Pharmacol. 105: 347-354.

MacLusky N.J., Naftolin F., Leranth C. (1988). Immunocytochemical evidence for direct synaptic connections between corticotropin-releasing factor (CRF) and gonadotropin-releasing hormone (GnRH) containing neurons in the preoptic area of the rat. Brain Res. 439: 391-395.

Malik K.F., Silverman A.J., Morrell J.I. (1991). Gonadotropin-releasing hormone mRNA in the rat: distribution and neuronal content over the estrous cycle and after castration of males. Anat. Rec. 231: 457-466.

Marks D.L., Smith M.S., Clifton D.K., Steiner R.A. (1993). Regulation of gonadotropin-releasing hormone (GnRH) and galanin gene expression in GnRH neurons during lactation in the rat. Endocrinology 133: 1450-1458.

Martinez de la Escalera G., Choi A.L., Weiner R.I. (1992a). Generation and synchronization of gonadotropin-releasing hormone (GnRH) pulses: intrinsic properties of the GT1-1 GnRH neuronal cell line. Proc. Natl. Acad. Sci. (USA). 89: 1852-1855.

Martinez de la Escalera G., Choi A.L.H., Weiner R.I. (1992b). β_1-adrenergic regulation of the GT1 gonadotropin-releasing hormone (GnRH) neuronal cell lines: stimulation of GnRH release via receptors positively coupled to adenylate cyclase. Endocrinology 131: 1397-1402.

Mason A.J., Hayflick J.S., Zoeller R.T., Young W.S., Phillips H.S., Nikolics K., Seeburg P.H. (1986). A deletion truncating the gonadotropin-releasing hormone gene is responsible for hypogonadism in the *hpg* mouse. Science 234: 1366-1371.

Masotto C., Wisniewski G., Negro-Vilar A. (1989). Different gamma-aminobutyric acid receptor subtypes are involved in the regulation of opiate-dependent and independent luteinizing hormone-releasing hormone secretion. Endocrinology 125: 548-553.

Matsumoto A. (1991). Synaptogenic action of sex steroids in developing and adult neuroendocrine brain. Psychoneuroendocrinology 16: 25-40.

Maxam A.M., Gilbert W. (1980). Sequencing end-labelled DNA with base-specific chemical cleavages. Methods Enzymol. 101: 20-78.

Mayer M.L., Miller R.J. (1990). Excitatory amino acid receptors, seceon messengers and regulation of intracellular Ca^{2+} in mammalian neurons. Trends Pharmacol. Sci. 11: 254-260.

McCann S.M., Moss R.L. (1975). Putative neurotransmitters involved in discharging gonadotropin-releasing neurohormones and the action of LH-releasing hormone on the CNS. Life Sci. 16: 833-852.

Meijs-Roelofs H.M.A., Kramer P., van Leeuwen E.C.M. (1991). The N-methyl-D-aspartate receptor antagonist MK-801 delays the onset of puberty and may acutely block the first spontaneous LH surge and ovulation in the rat. J. Endocr. 131: 435-441.

Mellon P.L., Windle J.J., Goldsmith P.C., Padula C.A., Roberts J.L., Weiner R.I. (1990). Immortalization of hypothalamic GnRH neurons by genetically targeted tumorigenesis. Neuron 5: 1-10.

Merchenthaler I., Lopez F.J., Negro-Vilar A. (1990). Co-localization of galanin and luteinizing hormone-releasing hormone (LHRH) in a subset of preoptic hypothalamic neurons: anatomical and functional correlates. Proc. Natl. Acad. Sci. (USA). 87: 6326-6330.

Merchenthaler I. (1991). Sex-linked differences in galanin and LHRH co-expression in preoptic-septal neurons are enhanced by estrogen. Abs. 1715, 73rd Annual Meeting, The Endocrine Society, Washington, D.C.

Merchenthaler I. (1991). Current status of brain hypophysiotropic factors: Morphological aspects. Trends Endocrinol. Metab. 2: 219-226.

Mershon J.L., Sehlhorst C.S., Rebar R.W., Liu J.H. (1991). CRH inhibits pulsatile release of GnRH from the macaque mediobasal hypothalamus. Abs. 1213, 73rd Annual Meeting, The Endocrine Society, Washington, D.C.

Millar R.P., King J.A., Davidson J.S., Milton R.C. de L. (1987). Gonadotropin-releasing hormone - diversity of functions and clinical applications. S. Afr. Med. J. 72: 748-756.

Minami S., Frautschy S.A., Plotsky P.M., Sutton S.W., Sarkar D.K. (1990). Facilitatory role of neuropeptide Y on the onset of puberty: Effect of immunoneutralization of neuropeptide Y on the release of luteinizing hormone and luteinizing-hormone-releasing hormone. Neuroendocrinology 52: 112-115.

Miyake A., Ohtsuka S., Nishizaki T., Tasaka K., Aono T., Tanizawa O, Yamatodani A., Watanabe T., Wada H. (1987). Involvement of H1 histamine receptor in basal and estrogen-stimulated luteinizing hormone-releasing hormone secretion in rats *in vitro*. Neuroendocrinology 45: 191-196.

Moguilevsky J.A., Arias P., Szwarcfarb B., Carbone S., Rondina D. (1990). Sexual maturation modifies the catecholaminergic control of gonadotropin secretion and the effect of ovarian hormones on hypothalamic neurotransmitters in female rats. Neuroendocrinology 52: 393-398.

Moguilevsky J.A., Carbone S., Szwarcfarb B., Rondina D. (1991). Sexual maturation modifies the GABAergic control of gonadotrophin secretion in female rats. Brain Res. 563: 12-16.

Moos F., Freund-Mercier M.J., Guerne Y., Guerne J.M., Stoeckel M.E., Richard Ph. (1984). Release of oxytocin and vasopressin by magnocellular nuclei *in vitro*: specific facilitatory effect of oxytocin on its own release. J. Endocr. 102: 63-72.

Moretto M., Lopez F.J., Negro-Vilar A. (1993). Endothelin-3 stimulates luteinizing hormone-releasing hormone (LHRH) secretion from LHRH neurons by a prostaglandin-dependent mechanism. Endocrionology 132: 789-794.

Nazian S.J. (1992). Development and control of the opioid inhibition of gonadotropin secretion during sexual maturation of the male rat. Neuroendocrinology 55: 613-620.

Negro-Vilar A., Ojeda S.R., McCann S.M. (1979). Catecholaminergic modulation of luteinizing hormone-releasing hormone release by median eminence terminals *in vitro*. Endocrinology 104: 1749-1757.

Nicoll R.A. (1988). The coupling of neurotransmitter receptors to ion channels in the brain. Science 241: 545-551.

Nikolarakis K.E., Almeida O.F.X., Herz A. (1986). Corticotropin-releasing factor (CRF) inhibits gonadotropin-releasing hormone (GnRH) release from superfused rat hypothalami *in vitro*. Brain Res. 377: 388-390.

Nikolarakis K.E., Loeffler J-Ph., Almeida O.F.X., Herz A. (1988). Pre- and post-synaptic actions of GABA on the release of hypothalamic gonadotropin-releasing hormone (GnRH). Brain Res. Bull. 21: 677-683.

Nikolarakis K.E., Almeida O.F., Sirinathsinghji D.J., Herz A. (1988). Concomitant changes in the *in vitro* and *in vivo* release of opioid peptides and luteinizing hormone-releasing hormone from the hypothalamus following blockade of receptors for corticotropin-releasing factor. Neuroendocrinology 47: 545-550.

Nishihara M., Hiruma H., Kimura F. (1991). Interactions between the noradrenergic and opioid peptidergic systems in controlling the electrical activity of luteinizing hormone-releasing hormone pulse generator in ovariectomized rats. Neuroendocrinology 54: 321-326.

Norgren R.B., Lehman M.N. (1989). A double-label pre-embedding immunoperoxidase tecnique for electron microscopy using diaminobenzidine and tetramethylbenzidine as markers. J. Histochem. Cytochem. 37: 1283-1289.

Ohta H., Kata Y., Matsushita N., Shimatsu A., Kabayama Y., Imura H. (1985). Central inhibitory action of TRH on prolactin secretion in the rat. Proc. Soc. Exp. Biol. Med. 179: 9.

Ohtsuka S., Miyake A., Nishizaki T., Tasaka K., Aono T., Tanizawa O. (1987). Substance P stimulates gonadotropin-releasing hormone release from rat hypothalamus *in vitro* with involvement of oestrogen. Acta. Endocrinol. 115: 247-252.

Ojeda S.R., Aguado L.I., Smith White S. (1980). Recent advances in the endocrinology of puberty. Endocr. Rev. 1: 228-257.

Ojeda S.R., Urbanski H.F., Katz K.H., Costa M.E., Conn P.M. (1986). Activation of two different but complementary biochemical pathways stimulates the release of hypothalamic luteinizing hormone-releasing hormone. Proc. Natl. Acad. Sci. (USA). 83: 4932-4936.

Olsen R.W., Tobin A.J. (1990). Molecular biology of GABA-A receptors. FASEB J. 4: 1469-1480.

Ono N., Bedran de Castro J.C., McCann S.M. (1985). Ultrshort-loop positive feedback of corticotropin (ACTH)-releasing factor to enhance ACTH release in stress. Proc. Natl. Acad. Sci. (USA). 82: 3528-3531.

Palkovits M. (1981). Catecholamines in the hypothalamus: an anatomical review. Neuroendocrinology 33: 123-128.

Park Y., Park S.D, Cho W.K., Kim K. (1988). Testosterone stimulates LH-RH-like mRNA level in the rat hypothalamus. Brain Res. 451: 255-260.

Park O.K., Gugneja S., Mayo K.E. (1990). Gonadotropin-releasing hormone gene expression during the rat estrus cycle: effects of pentobarbital and ovarian steroids. Endocrinology 127: 365-372.

Pau K.-Y.F., Spies H.G. (1986). Estrogen-dependent effects of norepinephrine on hypothalamic gonadotropin-releasing hormone release in the rabbit. Brain Res. 399: 15-23.

Pelletier G. (1987). Demonstration of contacts between neurons staining for LHRH in the preoptic area of the rat brain. Neuroendocrinology 46: 457-459.

Perrin M.H., Rivier J.E., Vale W.W. (1980). Radioligand assay for gonadotropin-releasing hormone: Relative potencies of agonists and antagonists. Endocrinology 106: 1289-1296.

Petersen S.L., McCrone S., Keller M., Gardner E. (1991). Rapid increase in LHRH mRNA levels following NMDA. Endocrinology 129: 1679-1681.

Petersen S.L., McCrone S., Coy D., Adelman J.P., Mahan L.C. (1993). $GABA_A$ receptor subunit mRNAs in cells of the preoptic area: colocalization with LHRH mRNA using dual-label *in situ* hybridization histochemistry. Endocrine J. 1: 29-34.

Petraglia F., Sutton S., Vale W., Plotsky P. (1987). Corticotropin-releasing factor decreases plasma luteinizing hormone levels in female rats by inhibiting gonadotropin-releasing hormone release into hypophyseal portal circulation. Endocrinology 120: 1083-1088.

Phelps C.P., Kalra S.P., Kalra P.S. (1992). *In vivo* pulsatile LHRH release into the anterior pituitary of the male rat: effects of castration. Brain Res. 569: 159 -163.

Pithey A.L., Becker I., Roberts J.L., Millar R.P. (1992). Direct evidence for an ultrashort negative feedback regulation of gonadotropin-releasing hormone. Abs. P12.03.035, 9th Internatioinal Congress of Endocrinology, Nice, France.

Pu S., Terasawa E. (1993). Effects of neurotensin and atrial natriuretic peptide on the *in vivo* release of luteinizing hormone-releasing hormone in the stalk-median eminence in ovariectomized female rhesus monkeys. Abs. 258.11, 23rd Annual Meeting, Society for Neuroscience, Washington, D.C.

Ramirez V.D., Kim K., Dluzen D.E. (1985). Progesterone action on the LHRH and the nigrostriatal dopamine neuronal systems: *in vitro* and *in vivo* studies. Recent. Prog. Horm. Res. 41: 421-461.

Ramirez V.D., Ramirez A.D., Slamet W., Nduka E. (1986). Functional characteristics of the luteinizing hormone-releasing hormone pulse generator in conscious unrestrained female rabbits: Activation by norepinephrine. Endocrinology 118: 2331-2339.

Ramirez V.D., Pickle R.L., Lin W.W. (1991). *In vivo* models for the study of gonadotropin and LHRH secretion. J. Steroid. Biochem. Mol. Bio. 40: 143-154.

Rangaraju N.S., Xu J., Harris R.B. (1991). Pro-gonadotropin-releasing hormone protein is processed within hypothalamic neurosecretory granules. Neuroendocrinology 53: 20-28.

Rasmussen D.D., Gambacciani M., Schwartz W., Tueros V.S., Yen S.S.C. (1989). Pulsatile gonadotropin-releasing hormone release from the human mediobasal hypothalamus *in vitro*: opiate receptor-mediated suppression. Neuroendocrinology 49: 150-156.

Reinhart J., Mertz L.M., Catt K.J. (1992). Molecular cloning and expression of cDNA encoding the murine gonadotropin-releasing hormone receptor. J.Biol.Chem. 267: 21281-21284.

Reubi J.C., Maurer R. (1984). Visualization of LHRH receptors in the rat brain. Eur. J. Pharmacol. 106: 453-454.

Richardson S.B., Twente S. (1986). Inhibition of rat hypothalamic somatostatin release by somatostatin: evidence for somatostatin ultrashort loop feedback. Endocrinolgy 118: 2076-2082.

Roberts J.L., Dutlow C.M., Jakubowski M., Blum M., Millar R.P. (1989). Estradiol stimulates preoptic area-anterior hypothalamic proGnRH-GAP gene expression in ovariectomized rats. Mol. Brain Res. 6: 127-134.

Roberts J.L., Gore A.C. (1993). Regulation of gonadotropin-releasing hormone gene expression by the excitatory amino acids kainic acid (KA) and N-methyl-D,L-aspartate (NMA) in the rat. Abs. 571.3, 23rd Annual Meeting, Society for Neuroscience, Washington, D.C.

Rosie R., Thomson E., Fink G. (1990). Oestrogen positive feedback stimulates the synthesis of LHRH mRNA in neurones of the rostral diencephalon of the rat. J. Endocr. 124: 285-289.

Rothfeld J.M., Hejtmancik J.F., Conn P.M., Pfaff D.W. (1987). LHRH messenger RNA in neurons in the intact and castrate male rat forebrain, studied by in situ hybridization. Exp. Brain Res. 67: 113-118.

Rotsztejn W.H., Drouva S.V., Pattou E., Kordon C. (1978). Effect of morphine on the basal and dopamine-induced release of LHRH from the mediobasal hypothalamic fragments in vitro. Eur. J. Pharmacol. 50: 285-286.

Sabatino F.D., Collins P., McDonald J.K. (1989). Neuropeptide-Y stimulation of luteinizing hormone-releasing hormone secretion from the medial eminence *in vitro* by estrogen-dependent and extracellular Ca^{2+}-independent mechanisms. Endocrinology 124: 2089-2098.

Sahu A., Crowley W.R., Kalra S.P. (1990). An opioid-neuropeptide-Y transmission line to luteinizing hormone (LH)-releasing hormone neurons: A role in the induction of LH surge. Endocrinology 126: 876-883.

Sahu A., Kalra S.P. (1992). Effects of tachykinins on luteinizing hormone release in female rats: Potent inhibitory action of neuropeptide K. Endocrinology 130: 1571-1577.

Sambrook J., Fritsch E.F., Maniatis T. (1989). Molecular Cloning. a Laboratory Manual, 2. Cold Spring Harbor Laboratory Press, Cold Spring Harbor.

Sar M., Stumpf W.E. (1981). Central noradrenergic neurones concentrate ^{3}H-oestradiol. Nature 289: 500-502.

Sar M. (1984). Estradiol is concentrated in tyrosine hydroxylase-containing neurons of the hypothalamus. Science 223: 938-940.

Sar M., Sahu A., Crowley W.R., Kalra S.P. (1990). Localization of neuropeptide Y immunoreactivity in estradiol-concentrating cells in the hypothalamus. Endocrinology 127: 2752-2756.

Sarkar D.K., Fink G. (1981). Gonadotropin-releasing hormone surge: Possible modulation through postsynaptic α-adrenoceptors and two pharmacologically distinct dopamine receptors. Endocrinology 108: 862-867.

Sarkar D.K. (1987). *In vivo* secretion of LH-RH in ovariectomized rats is regulated by a possible autofeedback mechanism. Neuroendocrinology 45: 510-513.

Sawyer C.H., Markee J.E., Hollinshead W.H. (1947). Inhibition of ovulation in the rabbit by the adrenergic-blocking agent dibenamine. Endocrinology 41: 395-402.

Schally A.V., Arimura A., Baba Y., Nair R.M.G., Matsuo H., Redding T.W., Debeljuk L., White W.F. (1971). Isolation and properties of FSH- and LH-releasing hormone. Biochem. Biophys. Res. Commun. 43: 393-399.

Schally A.V. (1978). Aspects of hypothalamic regulation of the pituitary gland. Science 202: 18-28.

Seeburg P.H., Adelman J.P. (1984). Characterization of the cDNA for the precursor of human luteinizing hormone releasing hormone. Nature 311: 666-668.

Shivers B.D., Harlan R.E., Morrell J.E., Pfaff D.W. (1983). Absence of oestradiol concentration in cell nuclei of LHRH-immunoreactive neurones. Nature 304: 345-347.

Silverman A. (1988). The gonadotropin-releasing hormone (GnRH) neuronal systems: Immunocytochemistry. In: The Physiology of Reproduction, edited by Nobil E. and Neill J.D.S., pp 1283-1304. Raven Press, New York.

Spergel D.J , Krsmanovic L.Z., Stojikovic S.S., Catt K.J. (1994). Glutamate modulates $[Ca^{2+}]_i$ and gonadotropin-releasing hormone secretion in immortalized hypothalamic GT1-7 neurons. Neuroendocrinology 59: 309-317.

Sporn M.B, Roberts A.B. (1985). Autocrine growth factors and cancer. Nature 313: 745-747.

Steele M.K., Stephenson K.N., Meredith J.M., Levine J.E. (1992). Effects of angiotensin II on LHRH release, as measured by in vivo microdialysis of the anterior pituitary gland of conscious female rats. Neuroendocrinology 55: 276-281.

Sutton S.W., Mitsugi N., Plotsky P.M., Sarkar D.K. (1988). Neuropeptide Y (NPY): A possible role in the initiation of puberty. Endocrinology 123: 2152-2154.

Terasawa E., Krook C., Hei D.L., Gearing M., Schultz N.J., Davis G.A. (1988). Norepinephrine is a possible neurotransmitter stimulating pulsatile release of luteinizing hormone-releasing hormone in the rhesus monkey. Endocrinology 123: 1808-1816.

Thind K.K., Goldsmith P.C. (1988). Infundibular gonadotropin-releasing hormone neurons are inhibited by direct opioid and autoregulatory synapses in juvenile monkeys. Neuroendocrinology 47: 203-216.

Thind K.K., Boggan J.E., Goldsmith P.C. (1991). Interactions between vasopressin- and gonadotropin-releasing-hormone-containing neuroendocrine neurons in the monkey supraoptic nucleus. Neuroendocrinology 53: 287-297.

Toranzo D., Dupont E., Simard J., Labrie C., Couet J., Labrie F., Pelletier G. (1989). Regulation of pro-gonadotropin-releasing hormone gene expression by sex steroids in the brain of male and female rats. Mol. Endocr. 3: 1748-1756.

Tsien R.W., Lipscombe D., Madison D.V., Bley K.R., Fox A.P. (1988). Multiple types of calcium channels and thier selective modulation. Trends Neurosci. 11:431-437.

Tsuruo Y., Kawano H., Kagotani Y., Hisano S., Daikoku S., Chihara K., Zhang T., Yanaihara N. (1990). Morphological evidence for neuronal regulation of luteinizing hormone-releasing hormone-containing neurons by neuropeptide Y. Neurosci. Lett. 110: 261-266.

Tsuruo Y., Kawano H., Hisano S., Kagotani Y., Daikoku S., Zhang T., Yanaihara N. (1991). Substance P-containing neurons innervating LHRH-containing neurons in the septo-preoptic area of rats. Neuroendocrinology 53: 236-245.

Tsutsumi M., Zhou W., Millar R.P., Mellon P.L., Roberts J.L., Flanagan C.A., Dong K., Gillo B., Sealfon S.C. (1992). Cloning and functional expression of a mouse gonadotropin-releasing hormone receptor. Endocrinology 6: 1163-1169.

Urbanski H.F., Ojeda S.R. (1990). A role for N-methyl-D-aspartate (NMDA) receptors in the control of LH secretion and initiation of female puberty. Endocrinology 126: 1774-1776.

Valenca M.M., Johnston C.A., Ching M., Negro-Vilar A. (1987). Evidence for a negative ultrashort loop feedback mechanism operating on the luteinizing hormone-releasing hormone neuronal system. Endocrinology 121: 2256-2259.

Vallet P.G., Charnay Y., Bouras C., Kiss J.Z. (1990). Co-localization of delta sleep-inducing peptide and luteinizing hormone-releasing hormone in neurosecretory vesicles in the rat median eminence. Neuroendocrinology 53: 103-106.

van den Pol A.N., Wuarin J-P, Dudek F.E. (1990). Glutamate, the dominant excitatory transmitter in neuroendocrine regulation. Science 250: 1276-1278.

Veneroni O., Cocilovo L., Muller E.E., Cocchi D. (1990). Delay of puberty and impairment of growth in female rats given a non-competitive antagonist of NMDA receptors. Life Sci. 47: 1253-1260.

Virca G.D., Northemann W., Shiels B.R., Widera G., Broome S. (1990). Simplified northern blot hybridization using 5% sodium dodecyl sulfate. Biotechniques 8: 370 - 371.

Watanabe T., Nakai Y. (1986). Electron microscopic cytochemistry of catecholaminergic innervation of LHRH neurons in the medial preoptic area of the rat. Arch. Histol. Jpn. 50: 103-112.

Weesner G.D., Bergen H.T., Pfaff D.W. (1991). Alpha-1 adrenergic regulation of luteinizing hormone-releasing hormone (LHRH) gene expression in the rat. Abs. 1019, 73rd Annual Meeting, The Endocrine Society, Washington, D.C.

Weesner G.D., Krey L.C., Pfaff D.W. (1993). α_1 adrenergic stimulation of estrogen-induced increases in luteinizing hormone-releasing hormone mRNA levels and release. Mol. Brain Res. 17: 77-82.

Wetsel W.C., Valenca M.M., Merchenthaler I., Liposits Z., Lopez F.J., Weiner R.I., Mellon P.L., Negro-Vilar A. (1992). Intrinsic pulsatile secretory activity of immortalized luteinizing hormone-releasing hormone-secreting neurons. Proc. Natl. Acad. Sci. (USA). 89: 4149-4153.

Wetsel W.C., Eraly S.A., Whyte D.B., Mellon P.L. (1993). Regulation of gonadotropin-releasing hormone by protein kinase A and -C in immortalized hypothalamic neurons. Endocrinology 132: 2360-2370.

Wiemann J.N., Clifton D.K., Steiner R.A. (1990). Gonadotropin-releasing hormone messenger ribonucleic acid levels are unaltered with changes in the gonadal hormone milieu of the adult male rat. Endocrinology 127: 523-532.

Wildt L., Marshall G., Knobil E. (1980). Experimental induction of puberty in the infantile rhesus monkey. Science 207: 1373

Williams C.L., Nishihara M., Thalabard J-C., Grosser P.M., Hotchkiss J., Knobil E. (1990). Corticotropin-releasing factor and gonadotropin-releasing hormone pulse generator activity in the rhesus monkey. Neuroendocrinology 52: 133-137.

Windle J.J., Weiner R.I., Mellon P.L. (1990). Cell lines of the pituitary gonadotrope lineage derived by targeted oncogenesis in transgenic mice. Mol. Endocr. 4: 597-603.

Woller M.J., Terasawa E. (1991). Infusion of neuropeptide Y into the stalk median eminence stimulates *in vivo* release of luteinizing hormone-releasing hormone in gonadectomized rhesus monkeys. Endocrinology 128: 1144-1450.

Woller M.J., McDonald J.K., Reboussin D.M., Terasewa E. (1992). Neuropeptide Y is a neuromodulator of pulsatile luteinizing hormone releasing hormone-releasing hormone release in the gonadectomized rhesus monkey. Endocrinology 130: 2333-2342.

Wray S., Hoffman G. (1986). A developmental study of the quantitative distribution of LHRH neurons within the central nervous system of postnatal male and female rats. J. Comp. Neurol. 252: 522-531.

Wray S., Kusano K., Gainer H. (1991). Maintenance of LHRH and oxytocin neurons in slice explants cultured in serum-free media: Effects of tetrodotoxin on gene expression. Neuroendocrinology 54: 327-339.

Wright D.E., Jennes L. (1993). Lack of expression of serotonin receptor subtype mRNAs in gonadotropin-releasing hormone producing neurons of the rat. Abs. 571.18, 23rd Annual Meeting, Society for Neuroscience, Washington, D.C.

Yu K.L., Yeo T.T.S., Dong K.-W., Jakubowski M., Lackner-Arkin C., Blum M., J.L. (1994). Second messenger regulation of gonadotropin-releasing hormone gene expression in immortalized mouse hypothalamic GT1-3 cells. Mol. Endocr. in press.

Zanisi M., Messi E., Motta M., Martini L. (1987). Ultrashort feedback control of luteinizing hormone-releasing hormone secretion *in vitro*. Endocrinology 121: 2199-2204.

Zanisi M., Messi E. (1991). Sex steroids and the control of LHRH secretion. J. Steroid. Biochem. Mol. Biol. 40: 155-163.