OPTIMIZATION OF AN ENZYME IMMUNOASSAY FOR THE DETECTION OF GONADOTROPIN-RELEASING HORMONE

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Optimization of an Enzyme Immunoassay for the Detection of Gonadotropin-releasing Hormone

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Optimization of an Enzyme Immunoassay for the Detection of Gonadotropin-releasing Hormone

A Thesis
Submitted to the Department of Biology
AUSTIN PEAY STATE UNIVERSITY
In Partial Fulfillment of the Requirements for the
Degree of Master of Science
in Biology
by

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November, 2008

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Optimization of an Enzyme Immunoassay for the Detection of Gonadotropin-releasing Hormone

By
Maryam Farsian

Submitted to the Department of Biology, Austin Peay State University
On November 2008 in Partial Fulfillment of the Requirements for the Degree of
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ABSTRACT

Gonadotropin-releasing hormone (GnRH) secretion by hypothalamic cells is responsible for the synthesis and release of gonadotropins from the anterior pituitary. In order to study the secretion of GnRH, an enzyme immunoassay (EIA) that is capable of detecting low levels of GnRH secretion is required. Recently, a GnRH EIA was developed. The objective of this project was to optimize this assay in order to increase sensitivity and decrease variability. Standard curves were constructed using three different concentrations of rabbit anti-GnRH primary antibody, donkey anti-rabbit secondary antibody, and biotinylated-GnRH. Optimal assay conditions were chosen based upon slope of the standard curve and the minimum detectable GnRH concentration. Based upon these criteria, the optimum concentrations of EIA reagents were 5 µg/ml donkey anti-rabbit antibody, rabbit anti-GnRH antibody at a 1:10,000 dilution, and a 1:5000 dilution of biotinyated-GnRH. The intra- and inter-assay coefficients of variation for this assay are 7.91 ± 4.4% and 24.91 ± 6.38%, respectively.

Thesis Advisor: Gilbert R. Pitts, Ph.D.
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Chapter 1. Introduction

Gonadotrpin-releasing hormone (GnRH) is a decapeptide that was initially isolated from porcine hypothalamic tissue (Baba *et al.,* 1971; Matsuo *et al.,* 1971). Its release into the hypophysial portal system stimulates the pulsatile release of follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the anterior pituitary (Schally *et al.,* 1971; Burgus *et al.,* 1972; Belchetz *et al.,* 1978; Clarke and Cummins,1982; Levine *et al.,* 1982; Pohl *et al.,* 1983). These two hormones target the gonads, directing gametogenesis and the production of estrogen, progesterone and testosterone. The frequency of GnRH pulses determines the relative levels of FSH and LH produced throughout the reproductive cycle, which can have significant effects on the gonads. In males, the pulse pattern is fairly regular and constant, producing periodic pulses of LH and FSH (Veldhuis and Johnson, 1988; Wu *et al.,* 1996). In females, however, there are substantial variations in the release of GnRH, provoking dramatic responses from the ovary throughout the follicular, ovulatory and luteal phases of the reproductive cycle (Reame *et al.,* 1984; Clarke *et al.,* 1987)

The physiological mechanisms that underlie GnRH pulse generation are still not completely understood. GnRH neurons exhibit spontaneous action potentials that depend upon Na^+, K^+, and Ca^{2+} concentrations and their movement through specific ion channels (Drouva *et al.,* 1981; Bosma 1993; Suter *et al.,* 2000). Because of the fundamental role that it plays in reproduction, GnRH pulse generation and the secretory profiles of GnRH neurons are areas of intense study. The pulse pattern of GnRH neurons can be examined for prolonged periods of time using the method of perfusion, in which cells are loaded into air-tight chambers and washed with a fluid medium. This perfusate solution is collected using a fraction collector and GnRH levels can then be measured in the samples. A benefit of perfusion is that the media can be supplemented with chemicals or additives to study possible effects on the GnRH secretory pattern.

A future goal of our lab is to perform a series of perfusion experiments examining the activities of a number of potential modifiers of GnRH secretion, and these experiments require a good method for quantifying GnRH. A GnRH enzyme immunoassay was recently developed by Tsai *et al.*, (2003) that provides a precise, sensitive, accurate and specific method for measuring GnRH. The purpose of this study is to optimize this enzyme immunoassay for use in our lab. The variables examined will be donkey anti-rabbit secondary antibody, R1245 primary antibody and biotinylated-GnRH concentration. Standard curves will be created with different concentrations of each variable. The optimized concentrations of each reagent will be chosen based upon slope of the standard curve and minimum detectable GnRH concentration of the standard curve. In addition, a number of methods to increase inter- and intra-assay variability will be assessed. This paper also presents preliminary perfusion data collected from an immortalized hypothalmic cell line, the GT1 cells.

Chapter 2. Literature Review

2.1 Introduction

Reproduction in both sexes is dependent upon a cascade of reactions initiated by the pulsatile release of gonadotropin-releasing hormone from the hypothalamus. The GnRH amino acid sequence is pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly-NH_2 (Baba *et al.,* 1971; Matsuo *et al.,* 1971). It has been demonstrated that the pulsatile release of GnRH into the hypophysial portal blood system controls both LH and FSH secretion from the anterior pituitary in a number of species (Schally *et al.,* 1971; Burgus *et al.,* 1972; Belchetz *et al.,* 1978; Clarke and Cummins, 1982; Levine *et al.,* 1982; Pohl *et al.,* 1983). These gonadotropins, in turn, target the gonads, where they stimulate gametogenesis and steroid hormone production.

The pulse pattern of GnRH differentially regulates both the synthesis and secretion of the gonadotropin hormones, as well as the responsiveness of the pituitary by altering the levels of GnRH receptor (GnRH-R) expressed by the gonadotrophs. This review begins by reporting the mechanism of GnRH synthesis and the model systems that can be used for the study of GnRH *in vitro*. Next, the pattern of GnRH release and how it is modified by signals that arise within both the GnRH neural network and individual GnRH neurons will be discussed. Several other modifiers of GnRH pulse pattern will be examined, including hormonal feedback mechanisms and GnRH autoregulation. The GnRH receptor and the effect of GnRH pulse pattern on gonadotropin secretion will be explored, and finally, methods for measuring GnRH will be discussed.

2.2 GnRH Synthesis

GnRH neurons originate outside of the central nervous system, in the medial olfactory placode of the developing nose (Schwanzel-Fukuda *et al.* 1989). During development, GnRH neurons migrate across the nasal septum to enter the forebrain and arch into the septal and preoptic areas in the hypothalamus (Schwanzel-Fukuda *et al.* 1989, Suter *et al.* 2000, Wray *et al.* 1989). This process is mediated by a variety of cell signaling pathways and chemotactic factors.

The human GnRH gene is located on the short arm of chromosome 8 at location p11.2 → p21 (Yang-Feng *et al.,* 1986). The GnRH precursor gene has been isolated from human and rat hypothalamic cDNA libraries (Adelman *et al.,* 1986). The precursor gene has an open reading frame of 276 nucleotides encoding a 92 amino acid protein. The precursor protein contains a cleavage site, separating the GnRH decapeptide from the 56 amino acid GnRH associated peptide (GAP) that has prolactin secretion inhibiting activity (Nikolics *et al.,* 1985). While the GnRH amino acid sequence is identical in rats and humans, the GAP protein contains 17 amino acid substitutions (Adelman *et al.,* 1986).

The GnRH gene has two promoter regions, an upstream segment that is active in reproductive tissues, such as the placenta, and a downstream region that is active in hypothalmic neurons (Dong *et al.,* 1997). The rat GnRH gene promoter contains a 173 base pair proximal promoter region that is evolutionarily conserved (Eraly and Mellon, 1995). Also, a 300 base pair region upstream from the promoter enhances GnRH gene transcription in an immortalized hypothalmic cell line (Whyte *et al.,* 1995). Both regions are important for expression of the GnRH gene specifically in neurons (Nelson *et al.,* 2000). Additionally, the GnRH gene is expressed in an intermittent manner within individual neurons (Nunez *et al.* 1998).

2.3 Model Systems for the Study of GnRH Secretion

GnRH has a very short half-life, and only a relatively small amount is released into the hypophysial portal system during any given pulse; therefore, *in vivo* analysis of GnRH release is difficult. To overcome this obstacle, a hypothalamic cell line has been established with properties similar to native GnRH neurons (Mellon *et al.,* 1990). This was accomplished by coupling the GnRH promoter to the SV40 T-antigen oncogene and using this construct to create transgenic mice. Some of these transfected mice developed hypothalamic tumors, from which the immortalized cell culture system was established. A pure neural cell population, known as GT1 cells were isolated and subcloned to three different cell lines (GT1-1, GT1-3 and GT1-7). GT1 cells express GnRH mRNA and release GnRH in a pulsatile manner and in response to depolarization (Mellon *et al.,* 1990). In culture, the GT1 cells form a monolayer, with the majority of the cells distributed homogenously and a few cells forming clusters of 5-10 neurons, growing very close together or on top of each other.

Several lines of evidence suggest that GT1 cells are closely related to GnRH neurons. GT1 cell bodies contain both forming and mature neurosecretory granules, Golgi apparatus and rough endoplasmic reticulum (Mellon *et al.,* 1990; Liposits *et al.,* 1991). Thick processes contain neurosecretory granules and are immunoreactive for GnRH and GAP, while thinner processes extending further away from the cell bodies often terminate in growth cones or bead-like dilations (Mellon *et al.,* 1990; Liposits *et al.,* 1991). GT1 cells contain significantly high levels of chromogranin B mRNA, a secretory granule matrix protein. They also express VAMP-2 mRNA, which is associated with synaptic membrane vesicles, and SNAP-25, a protein specific to neurons that is associated with the presynaptic membrane (Mellon *et al.,* 1990). Transmission electron microscope visualization of GT1 cells confirms that they are rich in ribosomes and contain several secretory vesicles and granules (Wetsel *et al.,* 1992). Further, GT1 cells are capable of differentiating and supporting gonadal development upon injection into mutant hypogonadal mice lacking the GnRH gene (Silverman *et al.,* 1992).

Taken together, these findings indicate that GT1 cells are neuronal cells with neurosecretory properties that may provide an excellent *in vitro* model for the study of GnRH release. It is important to note, however, that these are transformed cells, and this should be taken into account when interpreting data pertaining to these cells.

2.4 Pattern of GnRH Release

GnRH is released into the hypophysial portal blood circulation in a pulsatile manner (Carmel *et al.,* 1976; Urbanski *et al.,* 1988; Moenter *et al.,* 1992a; Moenter *et al.,* 1992b). GnRH is too dilute to measure in the systemic circulation, so it can only be measured in blood taken from the hypophysial portal vessels. Push-pull cannula (PPC) can be used to measure the release of neurohormones deep within the intact brain. A PPC consists of two parallel, side-by-side tubes that can be inserted into the hypophysial portal system, one tube is used to push a perfusion fluid into the capillary bed, while the other tube is used to pull fluid out for collection. The collection interval can be adjusted for the purposes of the study. In order to examine GnRH pulses *in vitro,* Levine *et al.* (1982) collected hypophysial portal blood from ovariectomized ewes at 10 minute intervals. These experiments revealed that GnRH was clearly released in an episodic manner, characterized by an interpulse interval between 36 and 44 min and amplitude fluctuating between 2 and 17 pg/min (Levine *et al.,* 1982). In similar experiments, Moenter *et al.,* (1992b) collected hypophysial portal blood from ovariectomized ewes at 30 sec intervals to better examine the minute-to-minute pulse pattern. Ovariectomized (OVX) and orchidectomized animals were used to eliminate feedback effects from the sex hormones, which will be discussed in future sections. Each observed GnRH pulse had an extremely steep rising edge, resulting in as much as a 50-fold increase above baseline levels within one minute of onset, a plateau period averaging 5.5 min (range 1.5-8.5 min), and then a rapid decline to baseline GnRH levels (Moenter *et al.,* 1992b).

Perfusion studies have also been used to examine secretion from GnRH neurons and GT1 cells. The cells are perfused with Krebs-ringer bicarbonate glucose buffer (Locke's solution), supplemented with bacitracin (2×10^{-5} M) to prevent enzymatic digestion of the GnRH peptide, enabling longer periods of perfusion (Rotsztejn *et al.*, 1976). Wetsel *et al.*, (1992) perfused GT1-7 cells at a rate of 100 µl/min and collected fractions at 5-minute intervals. The parameters describing GnRH pulses from GT1-7 cells are presented in Table 1. Perfusion of GT1-1 cells by de la Escalera *et al.*, (1992) revealed a mean interpulse interval of 25.8 ± 1.5 min, a mean duration of 18.8 ± 1.4 min, and a mean amplitude of 150.5 ± 6.0% above baseline.

Table 1. Pulse parameters for perfused GT1-7 cells

Parameter	Mean ± SEM
Frequency, pulses per hr	1.28 ± 0.18
Duration, min	20.00 ± 1.96
Interval, min	36.03 ± 3.00
Peak values, pg/min	13.90 ± 2.00
Trough values, pg/min	4.28 ± 0.59
Amplitude, pg/min	9.61 ± 1.528

* adapted from Wetsel *et al.,* 1992

2.5 GnRH Pulse Generator

Pulsatile GnRH release is inherent to native GnRH neurons as well as immortalized GnRH neurons, meaning that they do not require input from exogenous sources for patterned GnRH release (Charles and Hales, 1995; de la Escalera *et al.,* 1992; Kusano *et al.,* 1995; Vazquez-Martinez *et al.,* 2001). This activity is due to a proposed "pulse generator" that is intrinsic to GnRH neurons. Initially, many people thought that the GnRH pulse generator was controlled by cycles of transcription and translation, similar to the mechanism of circadian clocks. However, Pitts *et al.* (1992) demonstrated that neither inhibitors of transcription nor translation were able to prevent the episodic secretion of GnRH. Clearly, another mechanism must be involved (Pitts *et al.,* 1992).

Increased electrical activity in the arcuate nucleus of female OVX rhesus monkeys has been associated with subsequent increases of LH in the systemic circulation (Wilson *et al.,* 1984). Recordings of GT1-7 electrical activity revealed increases in firing rate of individual neurons or groups of neurons, with a mean interval of 46.9 ± 3.6 min (Nunemaker *et al.,* 2001). These findings correspond with the pattern of pulsatile GnRH release as well as gonadotrope secretion, suggesting that hypothalamic electrical activity is directly related to pituitary gonadotrope function (Wilson *et al.,* 1984; Moenter *et al.,* 1992; Wetsel *et al.,* 1992; Nunemaker *et al.,* 2001). GnRH neurons exhibit a resting membrane potential of approximately -65 mV, and Na^+, K^+, and Ca^{2+} ion channels are necessary for depolarization (Drouva *et al.,* 1981; Bosma 1993; Suter *et al.,* 2000). The activities of each ion are described below:

a. Sodium (Na^+)

Altering the activity of ion channels in the membrane of cells while simultaneously examining their electrical and/or pulsatile activity is a good way to identify if the movement of any of these ions through the

membrane channels is required for activities in the cell. The importance of Na^+ channels during release of GnRH has been confirmed by the application of tetrodotoxin (TTX), a sodium ion channel blocker, to GnRH neurons and GT1-7 cells. TTX inhibited GnRH neuron electrical activity and GnRH release (Drouva *et al.,* 1981; Mellon *et al.,* 1990; Bosma 1993; Suter *et al.,* 2000; Nunemaker *et al.,* 2001). Veratridine causes the opening of fast, TTX-sensitive Na^+ channels, which resulted in a significant increase in GnRH release from mediobasal hypothalamus slices during a 16 min period (Drouva *et al.,* 1981). Additionally, pretreatment with 50 µM TTX blocked both veratridine-stimulated and basal GnRH release (Drouva *et al.,* 1981; Mellon *et al.,* 1990). Taken together, this data suggests that, Na^+ ion channels have an important role in controlling GnRH release.

b. Potassium (K^+)

The concentration of K^+ and its ion channels are also involved in depolarization and hormone release. Depolarization, induced by incubating GT1-7 cells in Locke's medium containing 5.6 mM K^+ resulted in the measurable release of GnRH (Mellon *et al.,* 1990). In addition, increasing extracellular [K^+] caused a 4-fold increase in GnRH release within 15 min, with maximal secretion obtained at 56 mM K^+ (Rotsztejn *et al.,* 1976; Drouva *et al.,* 1981; Mellon *et al.,* 1990). GT1 cells express outward K^+ channels and both outward (repolarization) and inward (hyperpolarization) rectified K^+ currents (Bosma, 1993). The inward current has a time course of 15-20 msec at positive potentials and is suppressed by 200 nM and 2 µM GnRH and similar concentrations of somatostatin, an inhibitory hypothalmic peptide (Brazeau *et al.,* 1973; Bosma 1993). The action of K^+ is not inhibited by tetrodotoxin, suggesting that K^+-stimulated GnRH release is not dependent upon Na^+ ion channels (Mellon *et al.,* 1990). In addition, treatment of GT1-7 cells with a K^+

channel blocker increases both firing rate and GnRH secretion (Nunemaker *et al.,* 2001).

c. Calcium (Ca^{2+})

Ca^{2+} plays a key role in GnRH neuron electrical activity, hormone secretion in neurosecretory nerve terminals, transcription of the GnRH gene and GnRH mRNA stability in hypothalmic tissue and GT1 cells (Drouva *et al.,* 1981; de la Escalera *et al.,* 1992; Wetsel *et al.,* 1992; Charles and Hales, 1995; Sun *et al.,* 1998; Leclerc and Boockfor, 2007). It was first demonstrated in the squid giant axon that action potentials involve two phases of Ca^{2+} entry into the cell (Baker *et al.,* 1971). The early phase of Ca^{2+} entry into GT1-7 cells is blocked by TTX suggesting that it is associated with Na^{+} ion channels, while the late phase of Ca^{2+} entry occurs through voltage-sensitive Ca^{2+} channels (Charles and Hales, 1995; Watanabe *et al.,* 2004). Various studies have demonstrated that L-, T-, N-, P/Q- and R-type Ca^{2+} channels are present in GnRH neurons and are involved in pulse generation (Krsmanovic *et al.,* 1992; Charles and Hales, 1995; Van Goor *et al.,* 1999; Kato *et al.,* 2003; Watanabe *et al.,* 2004). Removal of extracellular Ca^{2+} gradually abolishes pulsatile secretion, including K^{+}- and veratridine-stimulated release, but pulsing resumes when Ca^{2+} is returned. Further, antagonist blockage of Ca^{2+} channels results in GnRH pulses with decreased amplitude (Rotsztejn *et al.,* 1976; Drouva *et al.,* 1981; de la Escalera *et al.,* 1992; Krsmanović *et al.,* 1992; Charles and Hales, 1995; Costantin *et al.,* 1999).

Charles and Hales (1995) demonstrated that GT1-7 cells exhibit oscillations in intracellular Ca^{2+} concentration. The Ca^{2+} oscillations were identified in individual cells and did not depend upon the activity of others, indicating that this activity is spontaneous and intrinsic to each cell. These fluctuations appeared every 3 to 120 sec and lasted for approximately 0.3 to 1 sec. Increasing extracellular $[K^{+}]$ resulted in an increase in Ca^{2+}

oscillations, and K^+ channel blockers caused either an increase or a sustained plateau of intracellular [Ca^{2+}] (Charles and Hales, 1995). GT1-1 cells also exhibit Ca^{2+} waves, and they occurred simultaneously with action currents. These waves of intracellular [Ca^{2+}] were localized within an individual neurons or group of neurons, and some were propagated to adjoining groups (Constantin and Charles, 1999). The waves are blocked by TTX, dependent upon extracellular [Ca^{2+}] and L-type Ca^{2+} channels, and can be modified by K^+ channels (Charles and Hales, 1995; Constantin and Charles, 1999; Constantin and Charles, 2001). These fluctuations in [Ca^{2+}] may serve two important purposes in GnRH secretion. First, they may facilitate secretion because high intracellular calcium is necessary for vesicular release. Second, while this high-frequency bursting pattern is much faster than the GnRH pulse pattern, it may be involved in generating the lower frequency action potentials that are associated with the release of GnRH. It appears that cultured embryonic GnRH neurons expressed high frequency [Ca^{2+}] oscillations that synchronized at a frequency of approximately 52.8 ± 3.0 min (Terasawa *et al.,* 1999). The synchronization started in a few cells and then spread wave-like to adjacent areas, usually with a larger amplitude than the normal [Ca^{2+}] oscillations. This pattern is similar to the GnRH pulse rate and may represent a mechanism whereby intrinsic activity within the GnRH network may lead to hormone release, but no study has yet linked the [Ca^{2+}] oscillations to GnRH secretion.

GT1-7 cells require network coordination for effective pulsing, and the secretory activity of GT1 neurons becomes progressively more synchronized as the time in culture increases (Vazquez-Martinez *et al.,* 2001). GT1 cells are known to be electrically coupled, with neighboring cells expressing coinciding fluctuations in spontaneous activity. The spread of the electrical signals between cells depends upon Na^+ channels and L-type Ca^{2+} channels but not K^+ channels (Hiruma *et al.,* 1997; Vazquez-Martinez *et al.,* 2001). Gap junctions are connections between

cells that allow small molecules such as ions or second messengers to move from one cell into another. Phase contrast and fluorescence microscope analyses of GT1-7 cells revealed synapse-like connections between adjacent cells and dye coupling, an indicator of gap junctions (Wetsel *et al.,* 1992). Gap junctions consist of smaller proteins called connexins (Cx). Approximately 75% of GT1-7 cells in culture are connected via gap junctions when grown to 70% confluency, and the most abundant connexin expressed in these cells is Cx26 (Hu *et al.,* 1999). Further support for gap junctional communication in GnRH neurons is provided by studies in which gap junction activity was blocked in GT1 neurons, resulting in a loss of synchronization among neurons within the neuronal network (Vazquez-Martinez *et al.,* 2001). It is through the gap junctions that action potentials may spread throughout the neural network, initiating GnRH release.

2.6 Modulators of GnRH pulse rate

1. Effects of steroidal feedback on GnRH

    a. Testosterone

    Testosterone (T) is the predominant gonadal hormone produced in males. It is converted via aromatization to estradiol or to dihydrotestosterone (DHT) in many cell types, including hypothalmic neurons (Selmanoff *et al.,* 1977). GT1-7 cells express an androgen receptor (AR) and an AR-specific coactivator known as ARA70 (Belsham *et al.* 1998, Shakil *et al.,* 2002). Binding of DHT to plasma membrane receptors decreases GnRH mRNA levels (Belsham *et al.,* 1998; Shakil *et al.,* 2002). GnRH mRNA levels decrease by approximately 45% at 24-36 h after administration of DHT, an effect that is completely abolished after 48 h (Belsham *et al.,* 1998). Given these results it is surprising to see that there is an immediate increase in GnRH secretion from GT1-7 cells upon administration of DHT or T, an effect that is probably mediated by an increase in intracellular [Ca^{2+}] that has been observed following exposure

to androgen (Shakil *et al.*, 2002). These differing effects of androgen on GnRH secretion and transcription are likely mediated via nuclear and plasma membrane receptors that initiate different intracellular responses (Shakil *et al.*, 2002). The net effect of T is negative and is likely mediated by estrogen, resulting in a decrease in GnRH pulse frequency from the hypothalamus and a decrease in pituitary responsiveness (Hayes *et al.*, 2000; Pielecka and Moenter, 2006).

b. Estrogen

GnRH neurons are known to possess the estrogen receptor-α (ER-α) and estrogen receptor-β (ER-β) (Roy *et al.*, 1999; Hrabovszky *et al.*, 2007). Estrogen (E_2) exerts both negative and positive feedback on GnRH release during different times of the reproductive cycle. E_2 is known to inhibit pulse generator electrical activity in rhesus monkeys as well as LH pulse frequency, two indicators of GnRH neuron activity (Kesner *et al.*, 1987; Kato *et al.*, 1994; Nunemaker *et al.*, 2002). The decrease in pulse generator activity is characterized by an increase in the interpulse interval (Nunemaker *et al.*, 2002). The result is a suppression of GnRH secretion, occurring in a dose-dependent manner, characterized by a decrease in GnRH pulse amplitude (Evans *et al.*, 1994). Further, exposing GT1-7 cells and rat hypothalmic sections to E_2 represses levels of GnRH mRNA (Zoeller *et al.*, 1988; Roy *et al.*, 1999).

Interestingly, as E_2 levels increase during the late follicular phase, E_2 exerts a positive feedback on GnRH secretion in ewes, resulting in the preovulatory GnRH surge (Moenter *et al.*, 1990). The mechanism whereby E_2 can exert both positive and negative feedback on GnRH secretion is still unclear. In the classical model of estrogen signaling, binding to an estrogen responsive element (ERE) in the genome alters the transcription of certain genes. In the non-classical model, ER binds with proteins or other transcription factors to exert specific effects (Stein and

Yang, 1995; Ray *et al.,* 1997). The GnRH promoter contains a stimulatory estrogen-responsive element (ERE) located between -547 and -516 bp (Radovick *et al.,* 1991). Recent evidence indicates that positive feedback and ovulation require binding of an estrogen responsive element (ERE) in the GnRH promoter and that this activity is mediated by binding of the ER-α. On the other hand, negative feedback is mediated via ER-α in both ERE-dependent and ERE-independent mechanisms (Glidewell-Kenney *et al.,* 2007; Christian *et al.,* 2008).

c. Progesterone

Progesterone (P_4) alone does not appear to have an effect on the GnRH secretory rate, as determined by measuring LH levels. However, when administered in combination with E_2, P_4 produces an inhibitory effect on pulsatile GnRH secretion (Nippoldt *et al.,* 1989). This is likely due to modulation of P_4 receptor mRNA expression by both E_2 and P_4 (Romano *et al.,* 1989; Simerly *et al.,* 1996). In knockout mice lacking ER-α, P_4 receptor mRNA is expressed in low levels. Similarly, OVX wild-type rats expressed low P_4 receptor mRNA levels. Upon administration of exogenous E_2, however, P_4 receptor mRNA increased in the wild-type but not the knockout mice (Shughrue *et al.,* 1997). Thus, both E_2 and P_4 exert a negative feedback on hypothalmic pulsatile activity as their levels rise with progressing follicular development and luteal activity during the reproductive cycle.

2. Effects of non-steroidal feedback on GnRH

a. Leptin

Leptin is a protein that is produced by adipocytes and is involved in food intake and metabolic regulation (Pelleymounter *et al.*, 1995). Leptin binds to a high affinity receptor (OB-R) that is present in the hypothalamus and GT1-7 cells (Tartaglia *et al.*, 2004; Yang *et al.,* 2005). In small doses, leptin results in significant increases in GnRH release but not gene

expression (Yu *et al.*, 1997; Magni *et al.*, 1999; Yang *et al.*, 2005). This is followed by LH and FSH release; however, increasing the dose resulted in a decrease in GnRH, with the highest dose of 10^{-6} M resulting in a smaller amount of GnRH levels than that of the controls (Yu *et al.*, 1997). Through its effects of GnRH secretion, leptin may represent a mechanism whereby nutritional status can influence on reproductive capabilities.

b. Stress

It is well known that stress exerts a negative influence on ovarian cyclicity in a number of animal models. Glucocorticoids are hormones that are released from the adrenal cortex in times of physiological stress. The most well-known of these glucocorticoids is cortisol. In the OVX ewe and gonadally-intact ewes, acute administration of cortisol at levels present during immune stress appears to inhibit pituitary responsiveness to GnRH (Breen and Karsch, 2004, Oakley *et al.*, 2008). Chronic administration of cortisol to gonadally-intact ewes and OVX ewes reduced GnRH pulse frequency, an effect that was greatly enhanced in the OVX animals when supplemented with E_2 and P_4 to mimic follicular phase levels of ovarian steroids (Oakely *et al.*, 2008). Bacterial endotoxin [lipopolysaccharide (LPS)] is a highly immunogenic molecule that can be given to simulate the immune system challenge and stress that takes place during an infection. OVX goats challenged with LPS, exhibited elevated cortisol and showed decreased hypothalamic electrical activity (Takeuchi *et al.*, 1997). This represents a mechanism whereby stress/illness could decrease GnRH pulse frequency, resulting in gonadal suppression.

c. Melatonin

Melatonin is a hormonal product of the pineal gland, produced during times of decreased light stimulation of the retina. Melatonin receptors are present in the suprachiasmatic nuclei and median eminence of rats as well as GT1-7 cells (Weaver *et al.*, 1989; Roy *et al.*, 2001). Melatonin directly

affects GnRH secretion at the neuronal level and in a cyclical manner, resulting in a substantial decrease in GnRH mRNA transcription at 12h after melatonin exposure (Roy *et al.,* 2001). The result is a decrease in GnRH levels by approximately 45% in GT1-7 neurons (Roy and Belsham, 2002). This mechanism of GnRH regulation is particularly important to seasonal breading animals, whose reproductive status depends upon photoperiod, and its role in human reproduction is not fully understood.

d. GnRH

GnRH acts through an ultra-short feedback loop to modulate GnRH secretion from the hypothalamus (DePaolo *et al.,* 1987). GnRH neurons targeted with green fluorescent protein and extracted from mice hypothalmic slices were shown to express GnRH receptors and to respond to GnRH, with low doses acting to suppress electrical activity and high doses increasing firing rate (Xu *et al.,* 2004). GT1-7 cells also express GnRH receptors, and perfusion with a GnRH agonist resulted in an initial small increase in GnRH release, followed by a decrease in basal GnRH release and ultimately completely absent pulses (Krsmanovic *et al.,* 1993). Infusion of GnRH into the third ventricle resulted in a drastic increase in GnRH mRNA levels in the ventromedial nucleus, but not the preoptic area, and an increase in GnRH-R mRNA throughout the entire hypothalamus (Lopot *et al.,* 2008).

2.7 GnRH Receptor

The human GnRH receptor (GnRH-R) is expressed in highest levels in the anterior pituitary, but is also present in ovary, testis, breast, and prostate tissue, indicating that GnRH serves a variety of reproductive functions (Kakar 1992). GnRH-R cDNA was first cloned from RNA extracted from the murine gonadotrope cell line, αT3-1 (Windle *et al.,* 1990; Reinhart *et al.,* 1992; Tsutsumi *et al.,* 1992). The human cDNA is approximately 1550 nucleotides in length and has a poly(A^+)-tail at the 3´ end (Kakar *et al.,* 1992). The human GnRH-R cDNA contains an open reading frame that encodes a 328 amino acid protein that

contains seven regions of highly hydrophobic amino acids and has 89-90% amino acid homology with the mouse GnRH receptor (Kakar *et al.,* 1992). Comparison of the amino acid sequences of the mouse and human GnRH-R reveal that the transmembrane domains are more highly conserved than the extra- and intracellular domains, suggesting that the transmembrane domains are likely involved in the normal functioning of the receptor (Kakar *et al.,* 1992; Chi *et al.,* 1993). GnRH-R belongs to a family of rhodopsin-like G protein-coupled receptors (GPCR), with 20-30% sequence similarity to other GPCRs, all of which contain seven putative transmembrane domains. GnRH-R is among the smallest of the GPCRs and lacks a C-terminal cytoplasmic domain, which prevents the receptor from undergoing agonist-dependent phosphorylation, resulting in a resistance to rapid desensitization (Kakar *et al.,* 1992; Tsutsumi *et al.,* 1992; Willars *et al.,* 1999). In addition, the GnRH-R has two other unique differences in highly conserved regions, the replacement of Tyr by Ser in transmembrane helix 3, and the reciprocal substitution of Asp in transmembrane helix 2 and Asn in transmembrane helix 7, which appear to be involved with receptor structural integrity (for review see Karges *et al.,* 2003; Millar *et al.,* 2004).

It is believed that GnRH binding involves several amino acids in both the extracellular and transmembrane domains. In addition, disulfide bonding between conserved Cys residues in the extracellular domains is necessary for binding and activation (Cook and Eidne, 1997). The GnRH-R is also glycosylated at several sites, increasing molecular stabilization and receptor expression, but not GnRH binding (Davidson *et al.,* 1995). Ligand binding to the GnRH-R results in the activation of many signal transduction pathways, ultimately leading to gonadotropin release.

The number of GnRH-Rs expressed varies with GnRH pulse frequency (Pieper *et al.,* 1982; Kaiser *et al.,* 1993; Yasin *et al.,* 1995). *In vitro* studies using superfused primary monolayer cultures of rat pituitary cells demonstrated that

expression of GnRH-R mRNA increased with pulsatile, and not continuous, GnRH administration (Kaiser *et al.,* 1993). Maximum GnRH-R levels were expressed when the GnRH pulse period was 30 min, while faster or slower frequencies decreased GnRH-R levels in the anterior pituitary of castrated male rats (Katt *et al.,* 1985; Kaiser *et al.,* 1997). In female rat pituitaries, the production of GnRH-Rs appears to be more sensitive than males to varying GnRH pulse frequency or amplitude. In addition, E_2 acts in a synergistic manner with GnRH to increase GnRH-R mRNA (Yasin *et al.,* 1995). These effects may influence GnRH-R levels during the rat estrous cycle. Further, the ability of the pituitary to bind GnRH changes throughout the estrous cycle, with binding ability highest during diestrus and proestrus, and lowest during estrus and metestrus (Savoy-Moore *et al.,* 1980).

2.8 GnRH stimulation of gonadotropin secretion

GnRH pulses are strictly episodic in males and in females, and they regulate the transcription, posttranscriptional modification, and pulse frequency of LH and FSH release from the anterior pituitary.

1. Effects of GnRH on FSH and LH synthesis

LH and FSH are dimers composed of an alpha glycoprotein subunit that is common to both gonadotropins, plus a β-subunit that is specific to each, LHβ and FSHβ (Godine *et al.*, 1982; Jameson *et al.*, 1984; Maurer and Beck, 1986). It has been demonstrated both *in vivo* and *in vitro* that pulsatile GnRH release stimulates LH and FSH synthesis by increasing the transcription of the α and β subunits (Shupnik 1990; Weiss *et al.*, 1990; Haisenleder *et al.*, 1991; Burger *et al.,* 2001). Disconnection of the blood supply from the hypothalamus to the pituitary results in decreased circulating levels of mRNA for both α- and β-subunits (Hamernik *et al.,* 1986). The common α-subunit mRNA levels increase with either pulsatile or continuous GnRH release, but they are highest at faster pulse frequencies (Lalloz *et al.*, 1988; Dalkin *et al.*, 1989; Weiss *et al.*, 1990). LH-β mRNA and LH secretion are maximally stimulated with a GnRH pulse

occurring every 30 min, while FSHβ mRNA levels and secretion are maximally stimulated by lower frequencies of GnRH secretion. In addition, both β- subunits are suppressed by continuous GnRH release (Lalloz *et al.*, 1988; Dalkin *et al.*, 1989; Weiss *et al.*, 1990; Kaiser *et al.*, 1997; Burger *et al.*, 2002). Thus, transcription of gonadotropin subunit mRNAs is regulated differentially by GnRH pulse frequency, with LH-β subunit transcription favored at a higher GnRH pulse frequency.

2. Effects of GnRH on FSH and LH secretion

In GnRH deficient primates, administration of exogenous GnRH pulses once per hour led to LH and FSH release, while continuous GnRH infusion suppressed gonadotropin secretion; therefore, pulsatile GnRH secretion must be required for gonadotropin release (Belchetz *et al.*, 1978). In OVX adult rhesus monkeys that underwent hypothalamic radioablation to cease GnRH and gonadotropin release, the intermittent administration of GnRH once per hour stimulated the release of both LH and FSH. Increasing pulse frequency to 2, 3 or 5 pulses/hour resulted in a gradual decrease in circulating levels of both gonadotropins, with this effect most pronounced at the higher frequency rates. On the other hand, decreasing GnRH pulse frequency to one pulse every 3 hours resulted in a decrease in LH levels but an increase in FSH (Wildt *et al.*, 1981). Pulsatile GnRH results in discrete LH pulses, with each observed LH pulse preceded by a GnRH pulse. In fact, comparison of blood collected from ovariectomized ewes reveals a one-to-one relationship between GnRH pulses in the hypophysial portal system and LH pulses in the peripheral circulation (Clarke and Cummins,1982; Levine *et al.*, 1982). The secretion of FSH is not as obviously linked to GnRH and has both episodic and basal elements (Levine *et al.*, 1982; Padmanabhan *et al.*, 1997). These results indicate that GnRH pulse frequency differentially regulates gonadotropin release, with LH release favored at a higher GnRH pulse frequency and FSH favored at a lower pulse frequency.

In order to examine the effect that GnRH pulse frequency has on gonadotropin release as well as follicular development, ovulation and E_2 levels, Pohl *et al.,* (1983) administered GnRH pulses of different frequency to adult female rhesus monkeys that had hypothalmic lesions. One GnRH pulse per hour stimulated normal follicular development resulting in ovulation and normal E_2 levels peaking at 232 ± 15 pg/ml. Decreasing pulse rate to once every 90 min resulted in an initial cycle of ovulatory development followed by mostly anovulatory cycles of follicular development. The successful initial cycle suggests that it was influenced by the GnRH pulse pattern of the cycle(s) preceding it. During the ovulatory cycles, peak E_2 concentrations reached 284 ± 29 pg/ml, and during the anovulatory cycles, E_2 levels were lower, at 173 ± 19 pg/ml. Further reducing the frequency to one pulse every 2 h resulted in limited or no follicular development, the absence of ovulation, and peak E_2 concentrations of 102 ± 11 pg/ml. One pulse every 3 h resulted in completely absent follicular development in four animals, but one monkey did have anovulatory follicular development with a peak estadiol level of 116 pg/ml. In these same animals, LH release pattern was dependent upon hypothalmic signaling, with a decrease in GnRH pulse frequency corresponding to a decrease in circulating LH. The pattern of FSH release was not affected by decreasing GnRH pulse frequency, staying relatively high at all pulse rates (Pohl *et al.,* 1983). Table 2 contains the mean gonadotropin concentrations during times of lowest estradiol as measured by Pohl *et al.,* (1983). In order to examine the effect of GnRH pulse amplitude, the animals were administered GnRH at 0.1 µg/min, resulting in declines in levels of both gonadotropins, while a 10-fold increase in GnRH pulse amplitude caused a decrease in circulating FSH levels with no change in LH levels (Wildt *et al.,* 1981).

Table 2. Mean plasma gonadotropin concentrations during periods of lowest plasma estradiol levels in adult rhesus monkeys bearing hypothalmic lesions that abolish GnRH secretion

Frequency of GnRH administration	Follicular development	n	Estradiol (pg/ml)	LH (ng/ml)	FSH (ng/ml)
1 pulse/hr (control)	Ovulatory	13	50 ± 5	8.8 ± 1.8	18.1 ± 2.1
1 pulse/2 h	Anovulatory	4	40 ± 5	3.6 ± 0.7	21.6 ± 0.9
1 pulse /3 h	None	4	18 ± 3	2.9 ± 0.7	17.7 ± 2.6

* adapted from Pohl *et al.*, 1983

3. GnRH pulse frequency throughout the female reproductive cycle

GnRH pulse frequency and multi-unit electrical activity is high and regular during the follicular phase of the ovarian cycle relative to the luteal phase (Clarke *et al.,* 1987; O'Byrne *et al.,* 1991). The result is approximately one GnRH pulse per hour. This slow pulse rate stimulates higher levels of FSH than LH from the hypophysis, leading to follicular development and low levels of E_2 in the systemic circulation (Pohl *et al.,* 1983; Reame *et al.,* 1984; O'Byrne *et al.,* 1991). As the follicular phase continues, GnRH pulse frequency increases while the amplitude decreases, causing the pituitary to secrete higher levels of LH relative to FSH (Reame *et al.,* 1984; Clarke *et al.,* 1987; Moenter *et al.,* 1991). It is believed that this gradual increase in GnRH pulse frequency is due to a decrease in P_4 from the previous luteal cycle, lowering progesterone's inhibitory effect on GnRH secretion (McCartney *et al.,* 2002). As ovarian follicles continue to develop, they secrete progressively higher levels of E_2, initiating a large surge of GnRH concomitant with the preovulatory LH surge (Sarkar *et al.,* 1976; Levine *et al.,* 1985; Moenter *et al.,* 1990; Moenter *et al.,* 1992). The link between the GnRH and LH surges was confirmed by simultaneously measuring portal blood levels of GnRH and jugular blood levels of LH during the preovulatory period in ewes. In most instances, the GnRH surge consisted of sustained 100- to 500-fold greater levels than baseline that extended well beyond the end of the preovulatory LH surge (Moenter *et al.,* 1991). During this GnRH surge period, pulsatile secretion is replaced by continuous release, and supporting evidence suggests that increasing levels of E_2 occurring in the late follicular phase inhibit all hypothalmic multi-unit electrical activity that is associated with pulsatile release (Kesner *et al.,* 1987; Moenter *et al.,* 1990; O'Byrne *et al.,* 1991; Moenter *et al.,* 1991; Moenter *et al.,* 1992). The sustained GnRH surge continues beyond the end of the LH surge, and the decrease in LH during this period is not clearly understood but may be due to pituitary desensitization, receptor down-regulation, depletion of cellular stores of LH, biochemical changes in the LH isoform, and/or exogenous forces in the portal blood that inhibit LH secretion (Belchetz *et al.,* 1978; Moenter *et al.,* 1991). GnRH release frequency is more irregular and the amplitude is

higher during the luteal period, possibly due to increasing levels of circulating estrogen and progesterone (Hauger *et al.,* 1977; Clarke *et al.,* 1987). Both LH and FSH levels decline during this phase, but the corpus luteum continues to secrete large amounts of E_2 and P_4 until luteolysis (Reame *et al.,* 1984). GnRH secretion throughout the sheep estrous cycle is summarized in Table 3, and Table 4 presents the gonadotropin and steroid hormone levels throughout the cycles of adult women.

Table 3. Analysis of GnRH secretion during the sheep estrous cycle. Day 0 is day of estrus.

Stage of cycle	Sheep (n)	Avg. GnRH (pg/ml)	Avg. GnRH (pg/min)	GnRH pulse amplitude (pg/ml)
Follicular	6	3.0 ± 0.44	0.68 ± 0.10	2.8 ± 0.34
LH Surge	6	9.5 ± 3.00	2.90 ± 1.03	6.1 ± 0.57
Day 1	1	5.2	0.73	8.2
Luteal	8	3.6 ± 0.44	0.58 ± 0.08	6.1 ± 1.37

* adapted from Clarke *et al.*, 1987.

Table 4. Plasma hormone concentrations during the same menstrual cycle in eight normal women

Hormone	LH (mIU/ml)	FSH (mIU/ml)	E_2 (pg/ml)	P (ng/ml)
Days 3-5	6.7 ± 1.0	6.1 ± 1.4	79 ± 6.5	0.6 ± 0.6
Days 10-12	9.0 ± 1.4	3.1 ± 0.4	154 ± 23	0.5 ± 0.1
Days 18-20	7.7 ± 2.0	2.1 ± 0.5	179 ± 16	11.8 ± 1.5
Days 24-26	5.3 ± 0.9	2.0 ± 0.6	159 ± 21	11.5 ± 2.8

* adapted from Reame *et al.*, 1984.

2.9 Measuring GnRH Release

There are two main techniques for measuring GnRH in samples collected either *in vivo* or *in vitro,* the radioimmunoassy (RIA) and the enzyme immunoassay (EIA) (Nett *et al.,* 1973; Tsai *et al.,* 2003). Both methods are similar in that they employ specific antibodies to bind GnRH and a tracer that competes with GnRH for binding sites on the antibodies. The tracer, or indicator, is actually a GnRH protein that has been labeled with some other molecule that can be easily detected. The RIA uses a radioactively labeled GnRH (^{125}I) that can be detected with a gamma counter, and the EIA uses a biotinylated-GnRH that can be measured by creating a luminescence reaction through an avidin-horseradish peroxidase intermediate. The levels of these tracer GnRHs are measured and compared using standard curves, giving an indirect measure of a sample's GnRH concentration.

The RIA has a sensitivity range of 0.5 to 1.9 pg/100 µl. The intra-assay coefficient of variation is 5.9% and an inter-assay coefficient of 14.7% (Nett *et al.,* 1977). RIA is the most frequently used method for measuring GnRH in research, but Tsai *et al.,* (2003) recently developed an EIA for measuring GnRH from hypothalmic explants. It uses donkey anti-rabbit secondary antibody (5 µg/ml), rabbit anti-GnRH primary antibody (1:200,000), and bt-GnRH (1:40,000) that is created in the lab and separated by high-performance liquid chromatography (HPLC). The EIA has a minimum detectable GnRH concentration of 0.1 pg/100 µl, and the intra- and inter-assay coefficients of variation were 13.82 ± 9.9% and 7.4 ± 3.0%, respectively. Figure 1 provides a representation of the series of reactions that occur in an individual well on the multiwell plate used for the EIA.

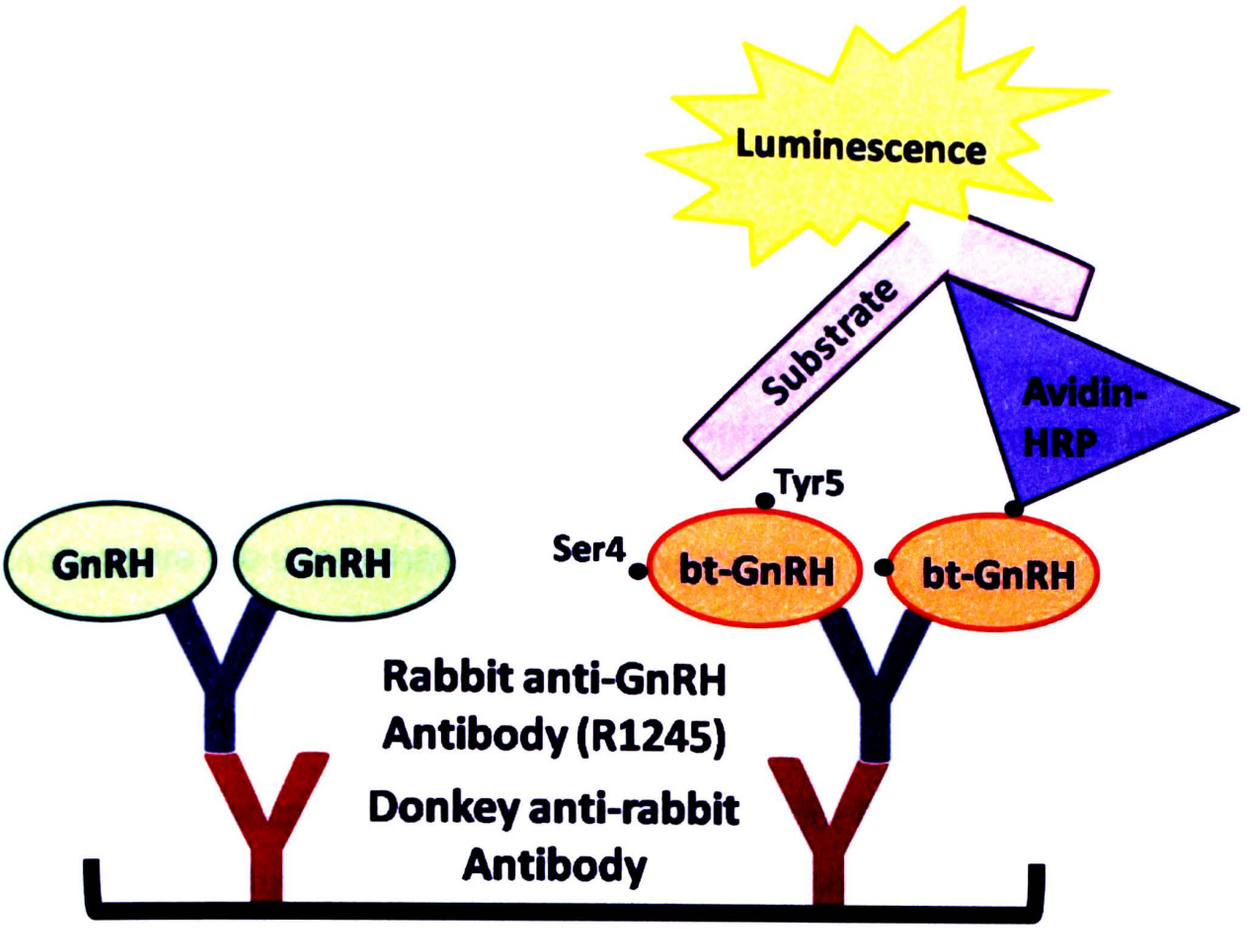


Figure 1. Representation of an enzyme immunoassay. The wells are first coated with donkey anti-rabbit secondary antibody, followed by incubation with R1245 rabbit anti-GnRH primary antibody. Then the unknown GnRH samples are added, and the tracer (bt-GnRH) is added to compete with naïve GnRH for primary antibody binding sites. The biotin will complex with avidin-horseradish peroxidase, that is added next, and the peroxide cleaves an ELISA Femto maximum sensitivity substrate, which emits a chemiluminescent signal that can be detected using a luminometer.

RIA is costly because working with radioactivity requires a Specific Radioactive Material License from the Division of Radiological Health, as mandated by the Tennessee Department of Environment and Conservation. The license can cost anywhere from $300 to $375,000, depending on the use of the radioactive material. In addition, laboratory technicians must be properly trained for working with radioactive material, and gamma counters are costly and must be calibrated frequently. On the other hand, EIA is a safe, inexpensive way to measure GnRH that requires minimal technician training and no special licensing because it does not require the use of hazardous materials.

2.10 Summary

GnRH is a decapeptide hormone responsible for the release of LH and FSH from the anterior pituitary. LH release is highly dependent upon the frequency of GnRH pulses, with slight deviations in pulse frequency and amplitude altering LH levels. FSH secretion is less tightly regulated by GnRH pulse frequency and is favored at a slower pulse rate. GnRH-R levels vary under a variety of circumstances. When few GnRH-Rs are present, all three gonadotropin mRNA subunits(α, LH-β and FSH-β) are expressed; however, when GnRH-Rs are more numerous, α- and LH-β levels are further stimulated. The differential release of LH and FSH is due to changes in both GnRH-R numbers and gonadotropin subunit gene expression.

The electrophysiological mechanisms which underlie the pulsatile release of GnRH are still not completely understood but do include an internal pulse generator that is intrinsic to the GnRH neuronal network. GnRH neuronal action potentials involve the movement of Na^+, K^+, and Ca^{2+} through specific ion channels, and gap junctional communication between individual neurons is necessary for effective pulse generation. Further, there are a variety of external factors that can modulate GnRH pulse generation, including both steroidal and non-steroidal compounds.

Perfusion is a useful method for collecting GnRH *in vitro*, and it can be used for examining the effects of various substances on GnRH secretion. In order to measure GnRH from samples collected during future perfusion experiments, we have optimized an immunoassay that is specific to GnRH. This study examines the effects of using donkey anti-rabbit secondary antibody, rabbit anti-GnRH antibody, and bt-GnRH at three different concentrations, each. Additionally, we have examined the effect of various washing methods in an effort to decrease intra- and inter-assay variation. Finally, preliminary perfusion data from the GT1-7 cells has been included.

Chapter 3. Materials and Methods

3.1 GnRH Immunoassay

Reacti-bind white opaque 96 well plates (Pierce Biotechnology, Rockford, IL) were coated with donkey anti-rabbit antibody (100 µl/well, Jackson Laboratories, West Grove, PA) dissolved in coating buffer (100 mM $NaHCO_3$ and 100 mM Na_2CO_3) for 24h at room temperature. The plates were rinsed twice and washed three times for 60 sec in 200 µl of assay buffer (100 mM sodium phosphate buffer, 150 mM NaCl, 0.1% tween 20, and nanopure water). Each plate was then incubated for 24h at 4˚C with 100 µl of rabbit anti-GnRH antibody (R1245) (Colorado State University) dissolved in assay buffer. After washing each plate three times for 60 sec with assay buffer, GnRH standards (BaChem, Torrance, CA) were mixed in 100 µl of assay buffer, added to the plates and incubated for 24h at 4˚C. Biotinylated GnRH (bt-GnRH, 50 µl) was added to each well and the plates were incubated at room temperature for one hour. The plates were then washed three times for 60 sec in assay buffer, and 100 µl of avidin D-HRP (1:5000, Vector Laboratories Inc, Burlingame, CA) was added to each well. The plates were incubated at room temperature for one hour and washed eight times for 60 sec with assay buffer. Super Signal ELISA Femto Maximum Sensitivity Substrate (100 µl/well, Pierce Biotechnology) was added, and the plates were incubated for four minutes at room temperature before luminescence values were obtained using a Bio-Tek Synergy HT plate reader (Winooski, VT).

3.2 Culture of GT1 Cells

GT1 cells were cultured in Dulbecco's modified Eagle medium nutrient mixture (DMEM/F12; Invitrogen, Grand Island, NY) with 10% heat-inactivated fetal bovine serum, penicillin (100 U/ml) and streptomycin (100 µg/ml). Cells were incubated at 37°C in a humidified 5% CO_2 environment and passaged when they reached approximately 70-90% confluence. To prepare for perfusion, 12-well plates were coated with Matrigel (1:10) (BD Biosciences, Bedford, MA) and allowed to dry for

3-5 h. Cells were added to Matrigel-coated wells and allowed to incubate until they reached 80-90% confluence. The medium was then replaced with OptiMEM I (Invitrogen, Grand Island, NY) supplemented with penicillin (100 IU/ml) and streptomycin (100 µg/ml) for serum deprivation for 24 hours.

3.3 Perfusion of GT1 cells

GT1-7 (passage 22) cells were grown on a Matrigel-coated plate, and perfused when they reached a confluence of 80-100% following 24 hour period of serum deprivation. The cells were perfused with Locke's buffer (containing (in mM): 154 NaCl, 5.6 KCl, 2.2 $CaCl_2$, 1 $MgCl_2$, 2 HEPES, 0.6 $NaHCO_3$, 10 D-glucose, and 0.02 bacitracin) in a Brandel Suprafusion 1000 perfusion system (Brandel, Inc., Gaithersburg, MD) with a flow rate of 100 µl/min. Cells were perfused for 30 min prior to sample collection to allow for stabilization. Control samples were collected at 4 minute intervals into ice-chilled tubes. Samples were stored at 2O°C and were assayed within 10 days of freezing. Following perfusion, cells were stained with 0.4% trypan blue for viability and checked for confluence.

3.4 Statistical Analysis

All data is presented as mean ± standard error. Statistical analysis was performed using the General Linear Models procedure of the Statistical Analysis System (SAS). Post-hoc comparisons were performed using Tukey tests. A P-value of 0.05 was considered to be statistically significant.

In order to assess variability, standard curves were created in triplicate, and the outlier was removed from each data set. The standard deviation of these two remaining data points was divided by the mean of the standard curve to calculate variability.

Chapter 4. Results

4.1 Effect of secondary antibody

The first objective in assay optimization was to determine the optimal concentrations of primary antibody (R1245, rabbit anti- GnRH) and secondary antibody (donkey anti-rabbit). Assays were performed using 10, 5, or 0.5 µg/ml secondary antibody and three different dilutions of R1245 (1:10,000, 1:50,000, or 1:100,000). Each standard curve was performed in triplicate, and each assay was performed 5 to 13 times. The secondary antibody concentration did not have a significant effect on the slope of the standard curve ($P>0.1$). However, the primary antibody concentration had a significant effect ($P<0.01$) as did the interaction between the primary and secondary antibody concentrations ($P<0.01$). The steepest slope (-6349.7) was observed when the primary antibody was diluted 1:10,000 ($P<0.05$). Slopes of -5190 and -4874.5 were observed when the primary antibody was diluted 1:50,000 and 1:100,000, respectively.

The minimum detectable GnRH concentrations were 0.449, 0.437, and 2.197 pg/100 µl when secondary antibody was used at concentrations of 0.5, 5, and 10 µg/ml, respectively. The minimum detectable GnRH concentration was significantly affected by the secondary antibody concentration ($P<0.01$), but not the primary antibody concentration or the interaction of the two ($P>0.1$).

Figure 3A depicts the effects of different dilutions of primary and secondary antibodies on the slopes of the standards. When either 5 or 10 µg/ml of secondary antibody was used, the 1:10,000 dilution of primary antibody yielded the greatest slope ($P<0.05$). The slope was not affected by primary antibody concentration when the secondary antibody concentration was 0.5 µg/ml ($P>0.05$). The effects of the primary and secondary antibody concentrations on the minimum detectable concentration of GnRH are shown in Fig. 3B. The 10 µg/ml donkey anti-rabbit secondary antibody concentration produced a curve with slightly flatter slope (-6807 versus -7193), but the 5 µg/ml concentration had a

significantly lower minimum detectable GnRH concentration (0.49 pg/100 µl versus 1.43 pg/100 µl). A graph of the data for these curves with plateau values removed is presented in Figure 4. The data clearly shows that the 10 µg/ml concentration had a steeper slope than the 5 µg/ml, but the latter had a greater range of detectability. The minimum detectable GnRH level was not affected by primary antibody concentration or secondary antibody at any concentration ($P>0.05$). For these reasons, we determined that the optimum combination of donkey anti-rabbit secondary was 5 µg/ml, and the optimum dilution of R1245 rabbit anti-GnRH primary antibody was 1:10,000.

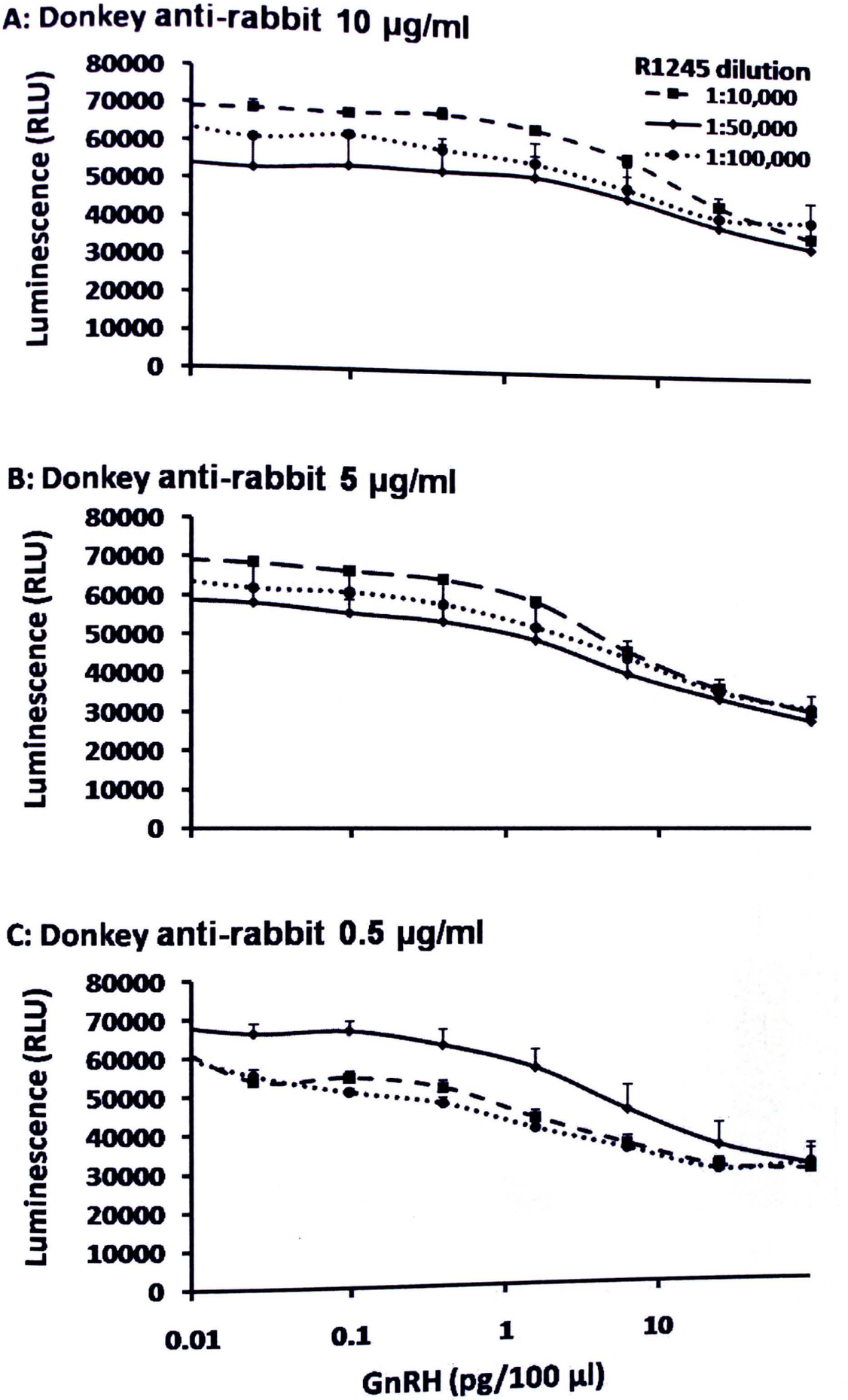


Figure 2. Luminescence values obtained from assays of discrete amounts of GnRH. These lines were used to calculate the standard curves for each EIA. Each line represents 5 to 13 standard curves, each performed in triplicate. Data are presented as mean ± standard error. Standard curves were created using murine GnRH. All curves were created using bt-GnRH diluted 1:2,500.

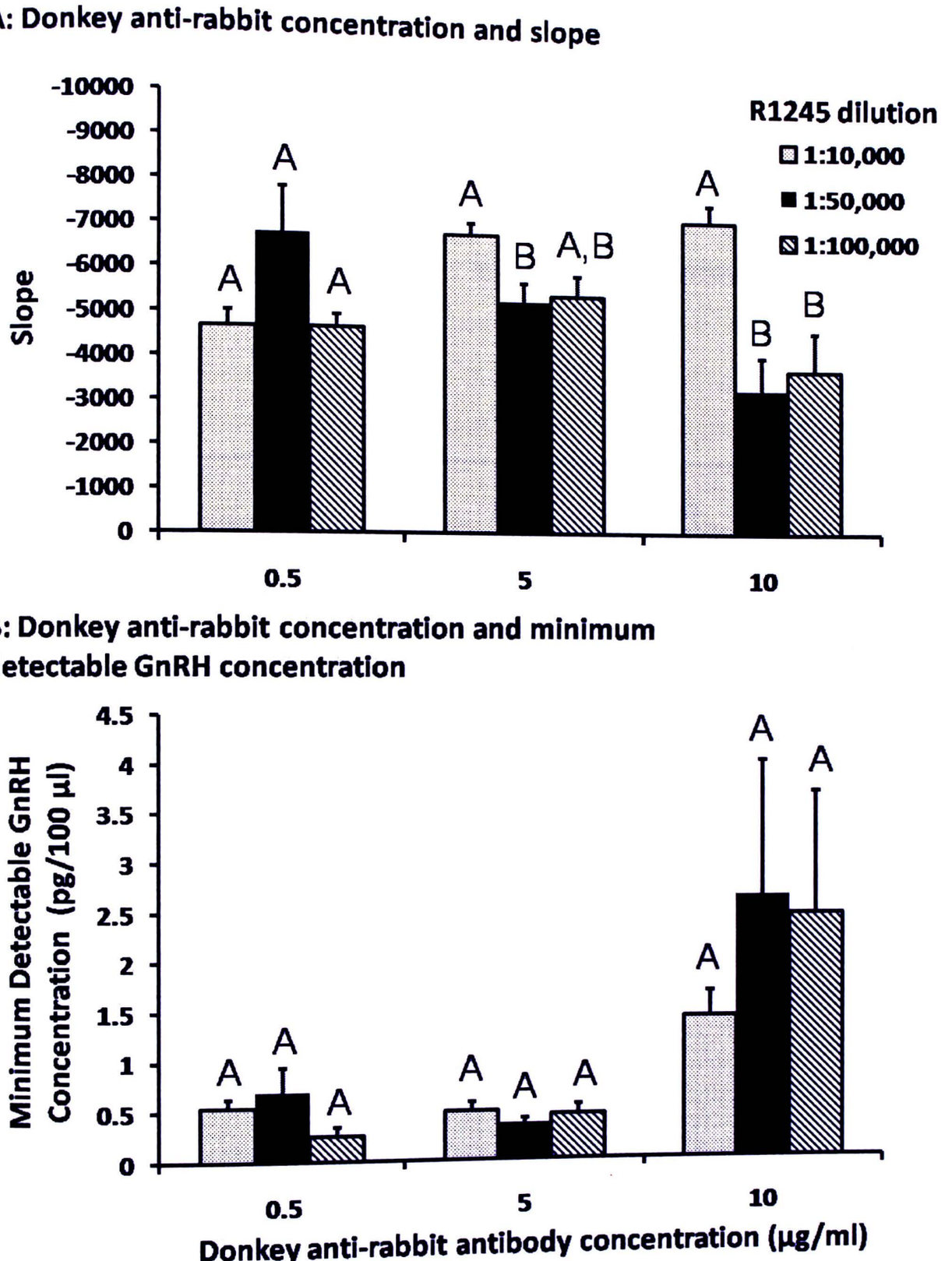


Figure 3. . Effect of donkey anti-rabbit secondary antibody concentration and R1245 rabbit anti-GnRH antibody dilution on the slope of the standard curve. B. Effect of donkey anti-rabbit secondary antibody concentration and R1245 rabbit anti-GnRH antibody dilution on the minimum detectable GnRH concentration of the standard curve. Each bar represents 5 to 13 standard curves, each performed in triplicate. Standard curves were created using murine GnRH and were edited to remove plateau values. Data are presented as mean ± standard error. Significant differences at a particular concentration of secondary antibody are denoted by different letters of the alphabet ($P>0.05$). All curves were created using bt-GnRH diluted 1:2,500.

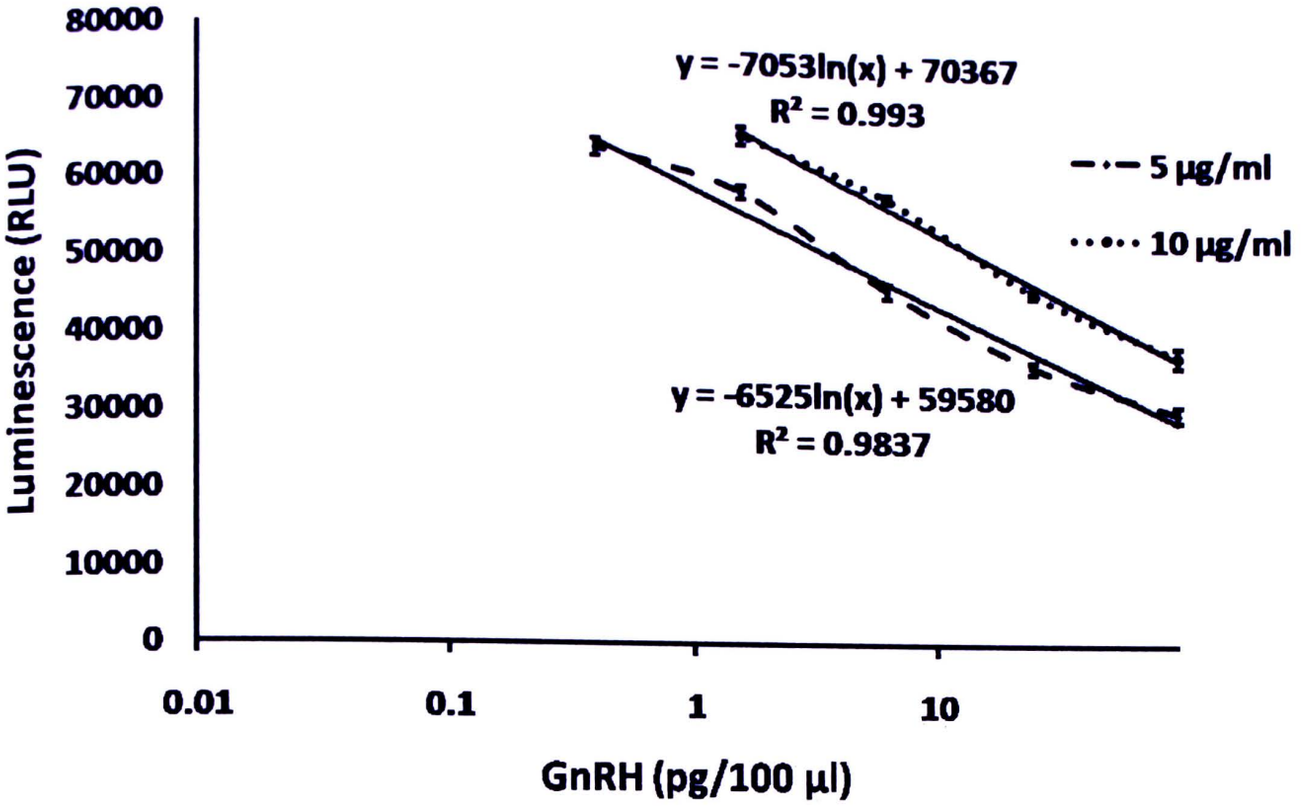


Figure 4. The lines represent donkey anti-rabbit secondary antibody at 5 µg/ml and 10 µg/ml and R1245 primary antibody diluted to1:10,000. Each bar represents 5 standard curves performed in triplicate. Standard curves were created using murine GnRH and were edited to remove plateau values. Data are presented as mean ± standard error. All curves were created using bt-GnRH diluted 1:2,500.

4.2 Effect of bt-GnRH

Another objective for this assay optimization was to determine the optimal interaction of primary antibody (R1245, rabbit anti-murine GnRH) and biotinylated GnRH. Assays were performed using biotinylated-GnRH diluted to 1:1000, 1:2500 or 1:5000 and R1245 diluted to 1:10,000, 1:50,000, or 1:100,000. Each standard curve was performed in triplicate, and each assay was performed 5 to 13 times. The standard curves of these assays are presented in Figure 5. The primary antibody and b-GnRH dilutions had a significant effect on the slope of the curve ($P<0.05$), but the interaction of the two did not have a significant effect ($P>0.1$). The steepest slope (-7333.8) was observed when the primary antibody dilution was 1:10,000 ($P<0.05$). Slopes of -5377.6 and -4908.4 were observed at primary antibody dilutions of 1:50,000 and 1:100,000, respectively.

Primary antibody dilution also significantly affected the minimum detectable GnRH concentration ($P<0.01$), with the lowest detectable concentration of 0.3850 pg GnRH/100 µl observed at a primary antibody dilution of 1:50,000 ($P<0.05$). The primary antibody dilution of 1:100,000 had a minimum detectable GnRH concentration of 0.4704 pg/100 µl, while the 1:10,000 dilution had a minimum detectable GnRH concentration of 0.6845 pg/100 µl GnRH. Neither bt-GnRH dilution alone ($P>0.1$) nor the combination of bt-GnRH with primary antibody concentration ($P>1$) had a significant effect on minimum detectable GnRH concentration.

Figure 6A depicts the effects of different dilutions of primary antibody and bt-GnRH on the slopes of the standards. The steepest slope was observed when every dilution of bt-GnRH was used in conjunction with a 1:10,000 dilution of primary antibody ($P>0.05$). The effects of the primary antibody and bt-GnRH dilutions on the minimum detectable concentration of GnRH are shown in Figure 6B. The minimum detectable GnRH concentration was significantly lower at a primary antibody dilution of 1:50,000 ($P<0.05$), but only at a bt-GnRH dilution of

1:5,000. There was no statistically significant effect at any other dilution of bt-GnRH.

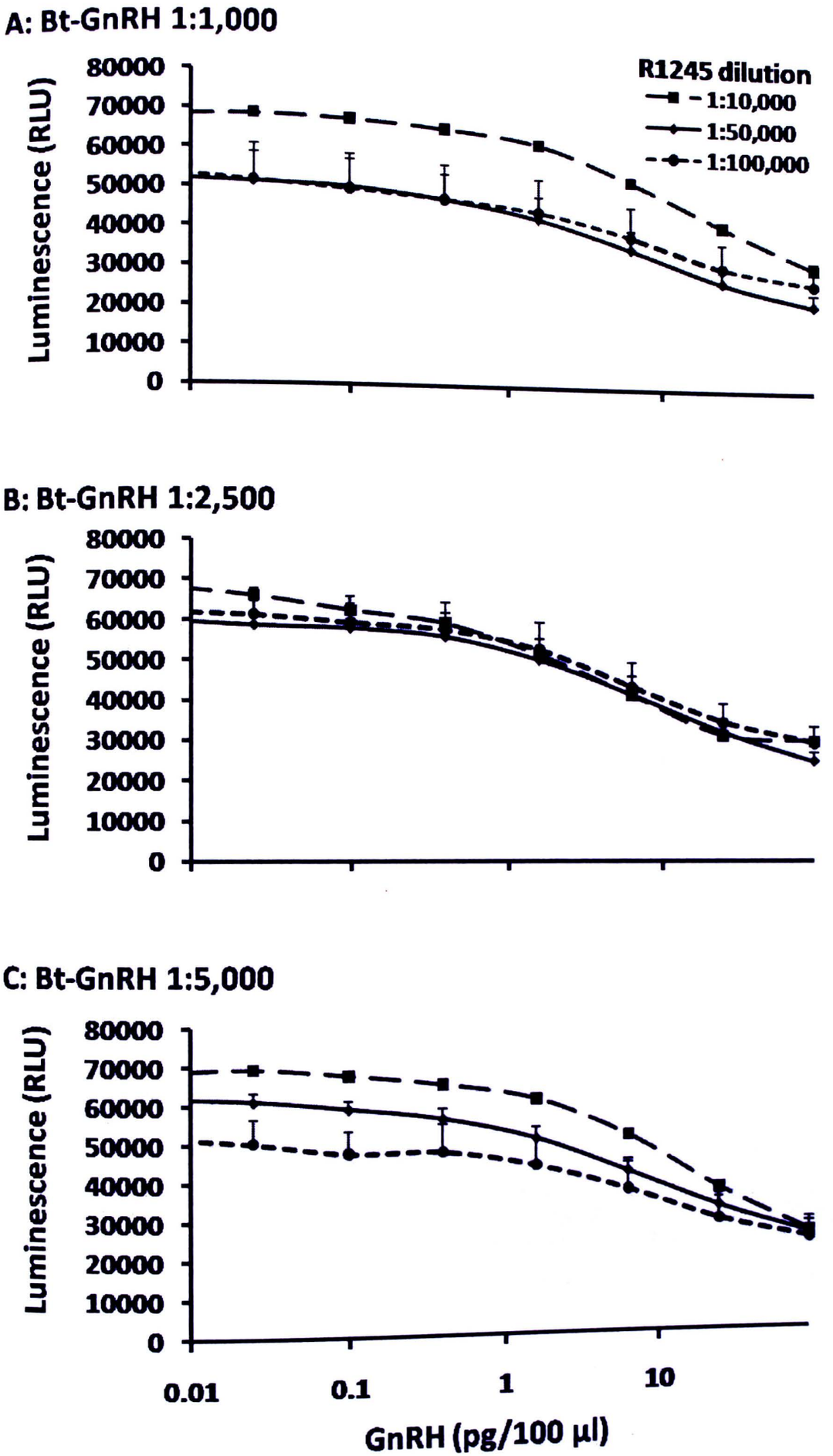


Figure 5. Each line represents 5 to 13 standard curves, each performed in triplicate. Data are presented as mean ± standard error. Standard curves were created using murine GnRH. All curves were created using donkey anti-rabbit secondary antibody at a concentration of 5 µg/ml.

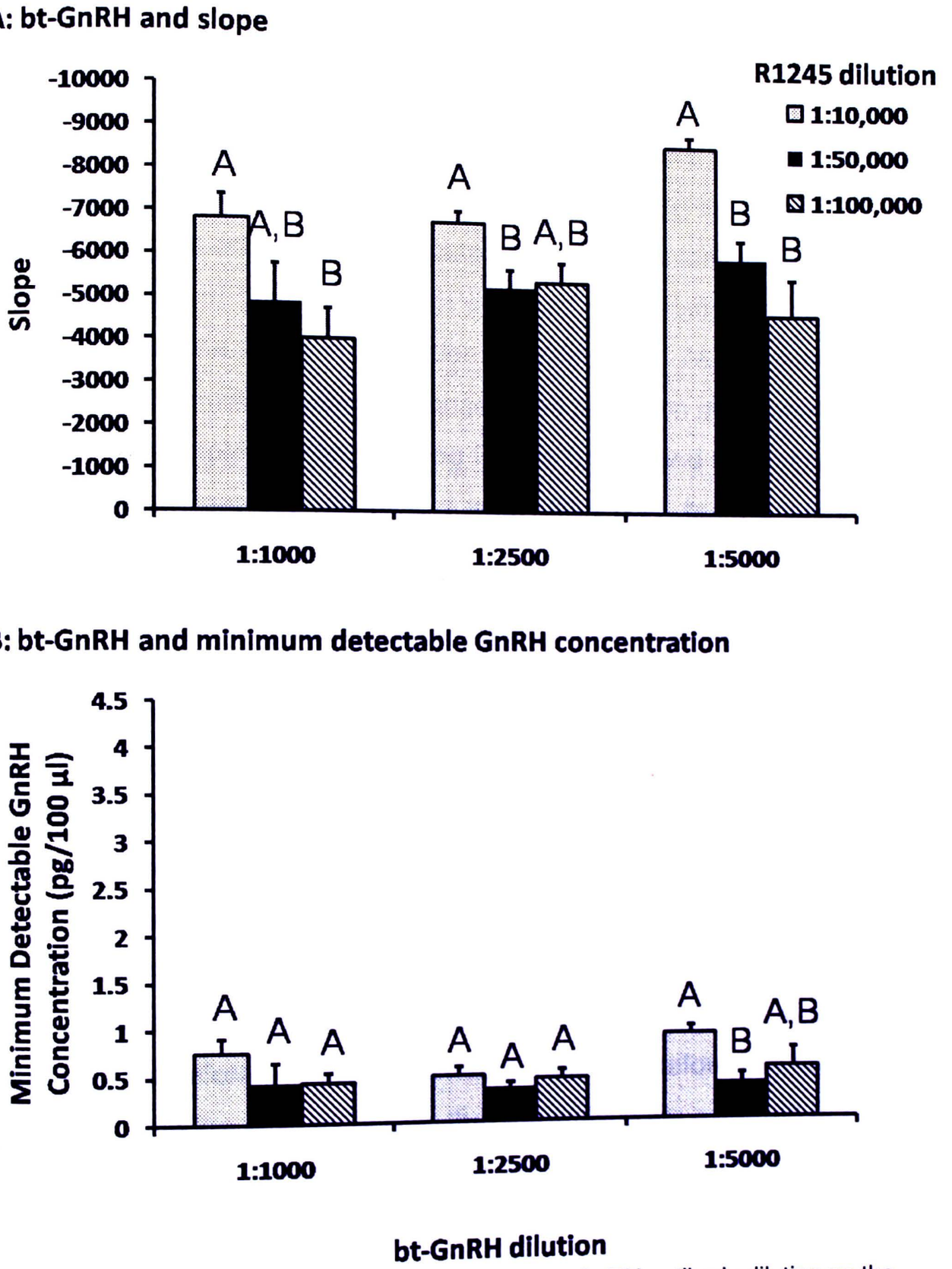


Figure 6. A. Effect of bt-GnRH dilution and R1245 rabbit anti-GnRH antibody dilution on the slope of the standard curve. B. Effect of bt-GnRH dilution and R1245 rabbit anti-GnRH antibody dilution on the minimum detectable GnRH concentration of the standard curve. Each bar represents 5 to 13 standard curves, each performed in triplicate. Standard curves were created using murine GnRH and were edited to remove plateau values. Data are presented as mean ± standard error. Significant differences at a particular concentration of secondary antibody are denoted by different letters of the alphabet ($P>0.05$). All curves were created using donkey anti-rabbit secondary antibody at a concentration of 5 µg/ml.

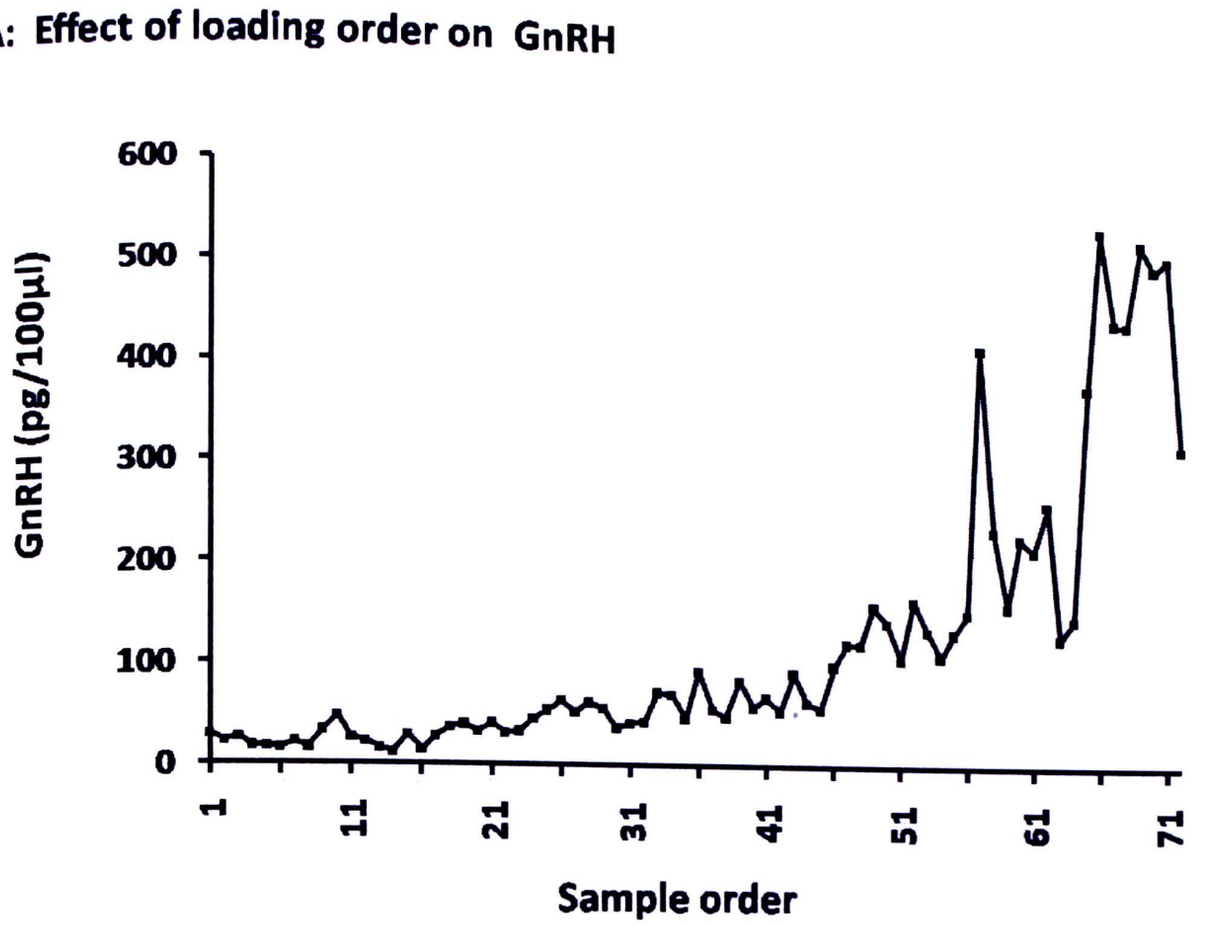
4.3 Decreasing intra- and inter-assay variation

From our first attempts to measure unknown GnRH samples, we observed that there was substantial variability within each plate as well as among serial plates. We tried a variety of methods to decrease this variation. We observed that the range increased dramatically depending upon location on the multiwell plate, with samples located toward the right edge of the plate exhibiting much greater variation than samples on the left. We tested the effects of loading sequence by placing a known amount of GnRH in each well, and then plotting the amount of GnRH obtained from the assay in the order of loading.(Fig. 7A). Figure 7B shows the effect of transition between consecutive plates on measured GnRH concentration. All samples contained 2 pg GnRH/100 µl, and the average calculated GnRH concentration for plate 1 was 1.95 ± 0.97, but the average for plate 2 was 5.31 ± 1.95.

Long term perfusion requires collecting samples for extended periods of time, so we need for our assay to be consistent for each multiwell plate and across several plates. Our original washing method involved upending the plate, pouring the liquid out of the wells, and whacking the plate on dry paper towels to remove excess fluid. Then new buffer or reagent was added using a multichannel pipette, starting on the left side of the plate and moving towards the right side, and, as mentioned previously, this is also the direction that individual samples were added to the plate. We suspected that the variation was due to the wells drying out before each reagent was added. Therefore, we focused our efforts on decreasing the amount of time that the wells were exposed to air. We tried a variety of methods, including washing the plates without whacking on paper towels, washing the plates by immersing them in a trough containing assay buffer, and using one multichannel pipette to remove the reagent from a column of wells and another to immediately add new reagent to the wells. The only method that was able to decrease variability sufficiently was the latter, using one multichannel pipette in the left hand for removing fluid from the wells, and the other in the right hand for adding fluids. Further, to decrease any antigen-

antibody reactions that could potentially take place while the samples were being added, we chilled the plates on ice when adding samples. Prior to optimization, the intra- and inter-assay variation were substantial. In order to determine the intra-assay variation, the mean GnRH concentration and standard deviation was determined for each dilution in the edited standard curve. The curves were edited to remove outliers at each dilution. The GnRH concentrations and standard deviation values were then averaged, and the average standard deviation for the entire curve was divided by the mean GnRH concentration for the entire curve. Intra-assay variation was determined by dividing the mean standard errors from each plate by the mean GnRH concentration from each plate. For 13 perfusions performed using the optimized assay procedure, the coefficients of intra- and inter-assay variation were 7.91 ± 4.4% and 24.91 ± 6.38%, respectively.

In order to compensate for such high inter-plate variability during analysis of a single perfusion run across several assay plates, we calculated the ratios of several known GnRH concentrations located at multiple locations across each plate. These ratios were then used to normalize the GnRH concentrations obtained on the second, and subsequent plates.



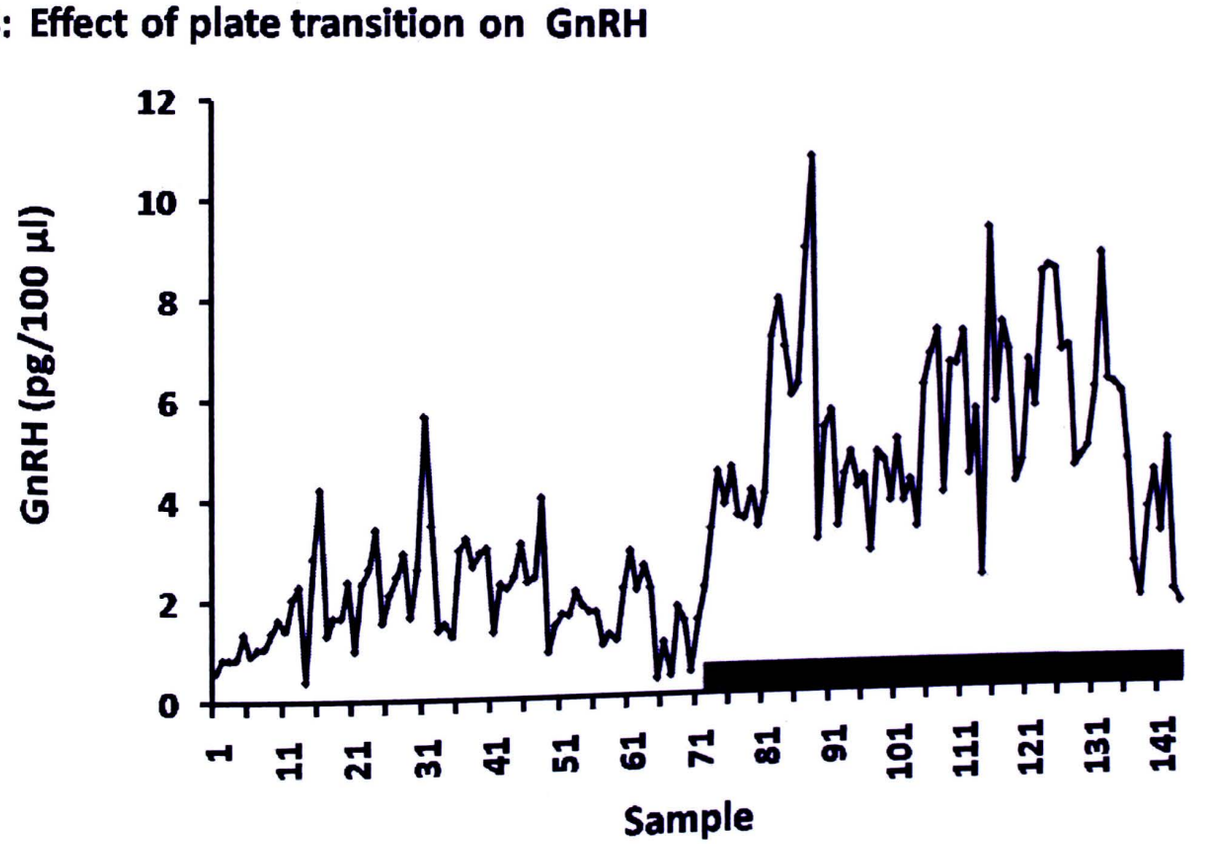


Figure 7. A. Effect of loading order and plate transition on measured GnRH concentration. 6A. All samples contained 33 pg/100 µl GnRH. The calculated GnRH values range from 11.87 to 550.09 pg/100 µl. B. All samples contained 2 pg/100 µl GnRH. The shaded bar identifies the samples from plate 2. The average GnRH concentration for plate 1 was 1.95 ± 0.97, and the average for plate 2 was 5.31 ± 1.95. Data are presented as mean GnRH concentration in pg/100 µl.

4.4 Perfusion of GT1 cells

Figure 8 shows the results of a representative preliminary perfusion experiment. GT1-7 cells were perfused with Locke's medium for a stabilization period of 30 minutes, followed by sample collection for 236 minutes. Pulses may be observed at times 32, 60, and 96 min. Potassium chloride (56 mM) was added after 192 min, evoking a substantial release of GnRH. Any values that were outside the range of detectability were replaced with the minimum detectable GnRH concentration for their corresponding plate.

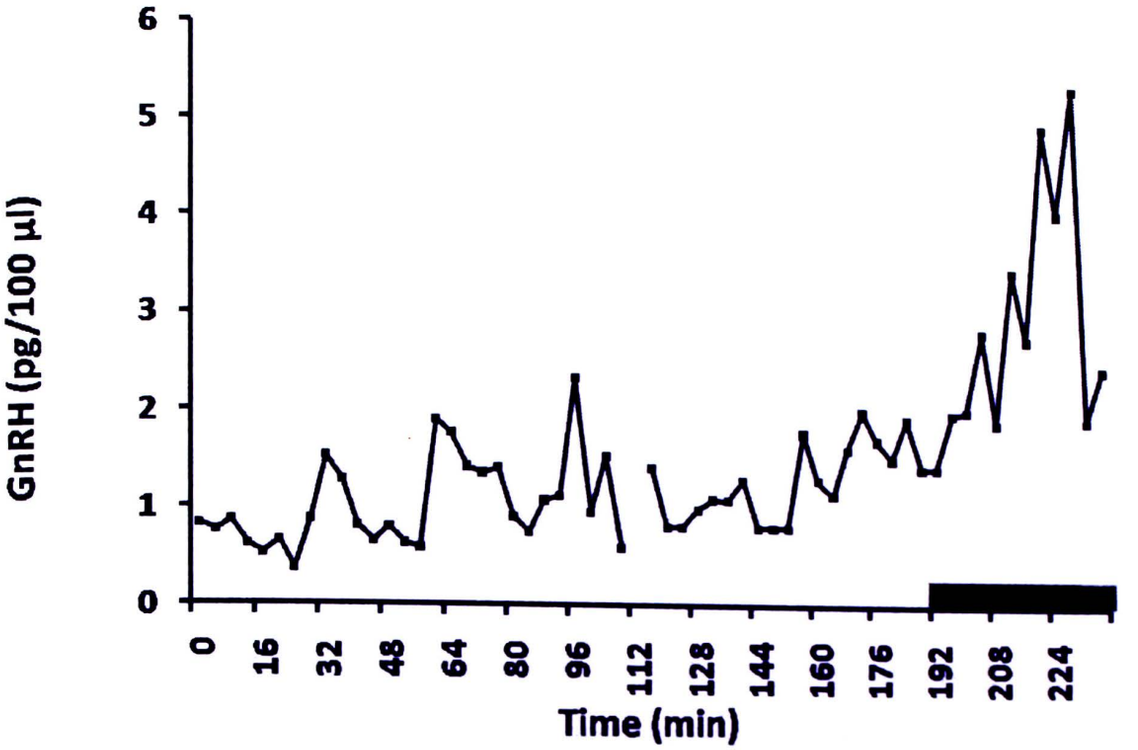


Figure 8. The GnRH pulse pattern collected from GT1-7 cells passage 22. Cells were allowed a stabilization period of 30 minutes, followed by sample collection every 4 minutes for a period of 236 minutes. To examine viability, the cells were challenged with KCl (56 mM). The gray shaded line represents the duration of the KCl challenge. Pulses may be observed at times 32, 60, and 96 min, and there was a substantial increase in GnRH secretion with the application of KCl.

Chapter 5. Discussion

The goal of this research was to optimize an enzyme immunoassay for measuring GnRH. We have successfully determined that the optimum concentrations of reagents is 5 µg/ml donkey anti-rabbit secondary antibody, 1:10,000 dilution of R1245 rabbit anti-GnRH primary antibody, and 1:5,000 dilution of bt-GnRH.

The two variables of importance in assessing standard curves are the slope and the range of concentration within which the target molecule can be detected. Steeper slopes have increased assay accuracy, and minimum detectable GnRH concentration is important because GnRH is released in very low levels. We need an assay that is sensitive enough to accurately measure very small amounts of the GnRH hormone. The concentration of secondary antibody did not affect either the slope or minimum detectable GnRH concentration of the standard curves, but the interaction of secondary and R1245 rabbit anti-GnRH primary antibody had an effect. The 5 and 10 µg/ml secondary antibody concentrations produced curves that were significantly steeper when used with the highest concentration of R1245 primary antibody, the 1:10,000 dilution. It is significantly more concentrated than the 1:200,000 dilution used by Tsai *et al.* (2003), but our optimum concentration of donkey anti-rabbit secondary antibody is the same as the one that they used.

At all dilutions of bt-GnRH, the 1:10,000 dilution of R1245 rabbit anti-GnRH primary antibody produced significantly steeper curves than either the 1:50,000 or 1:100,000 dilutions. This is in agreement with the data obtained from the experiments examining the effects of donkey anti-rabbit and R1245 rabbit anti-GnRH primary antibody, except at a secondary antibody concentration of 0.5 µg/ml where the primary antibody concentration of 1:50,000 was the steepest. The R1245 rabbit anti-GnRH primary antibody dilution of 1:50,000 produced a curve with the significantly lowest minimum detectable GnRH concentration. Our

optimized dilution of R1245 rabbit anti-GnRH antibody is the most concentrated dilution that we tested, and it is substantially more concentrated than the 1:200,000 dilution used by Tsai *et al.,* (2003).

Bt-GnRH dilution did not have an effect on slope or minimum detectable GnRH concentration at any dilution of rabbit anit-GnRH, so we determined that the most dilute concentration of 1:5,000 bt-GnRH would be the optimum concentration for our assay. This is significantly more concentrated than the 1:40,000 concentration that is employed by Tsai *et al.,* (2003).

The bt-GnRH used in this experiment is an *O*-biotinylated GnRH that was produced using a procedure described by Miller *et al.,* in 1992. Using this procedure, GnRH is biotinylated on Ser4 and to some extent Tyr5. Tsai *et al.,* (2003) used high performance liquid chromatography (HPLC) to purify their serine-biotinylated GnRH, the variant that provided them the highest sensitivity. The bt-GnRH that we used was column filtered, so it contains a mixture of both the serine- and tyrosine-biotinylated GnRH. This heterogenicity is a likely cause of the decrease in sensitivity between the two assays. Other, commonly used GnRH EIAs use a *N*-biotinylated D-Lys^6-GnRH (Li *et al.,* 1994; Maurer and Wray, 1999). These, too, are far less sensitive than the purified *O*-biotinylated GnRH.

Using this combination of reagents, the sensitivity of our assay is 0.6845 pg/100 µl GnRH. Nearly every curve that we have produced has had the maximum GnRH concentration of 100 pg/100 µl within the range of detectability, and maximum detectable dose is not really a limiting factor because any samples with GnRH to high to be detected could be diluted. Our minimum detectable concentration is higher than the range of 0.1 pg/100 µl reported for the EIA created by Tsai *et al.,* (2003), and it is comparable to the range of 0.5 to 1.9 pg/100 µl reported for the RIA developed by Nett *et al.,* (1973). Our assay is also more sensitive than a reported commercial assay (Peninsula Laboratories, Inc.; cat. no. S-1217) that has a minimum detectable range of 0.5-1 pg/100 µl.

The intra- and inter-assay coefficients of variation are 7.91 ± 4.4% and 24.91 ± 6.38%, respectively. The intra-assay variation is less than the 13.82 ± 9.9% reported by Tsai *et al.,* (2003), but the inter-assay coefficient of variation is substantially higher than their assay's variation of 7.4 ± 3.0%. Additionally, our variation was higher than the RIA intra-assay coefficient of variation of 5.9% and inter-assay coefficient of 14.7% reported by Nett *et al* (1977). It is important for a good GnRH EIA to have low variation on an individual plate as well as across several plates, as GnRH release is pulsatile, and long-term studies of pulse patterns require the collection and quantification of a great number of samples.

Perfusion is a useful method for collecting GnRH from hypothalmic explants or GT1 cells. From our preliminary perfusion data, shown in Figure 8, we can observe the pulsatile release of GnRH from the immortalized GT1-7 cells. KCl (56 mM) was added at 192 minutes (shaded bar), resulting in an increase in GnRH secretion. It is important to test for evoked GnRH secretion at the end of perfusion experiments to evaluate the viability of the cells. It is known that exposure to KCl increases neuronal release of GnRH, with a maximum response obtained at 56 mM KCl (Drouva *et al.,* 1981). While, veratridine opens Na^+ channels, causing an increase in GnRH secretion, and it is preferred over KCl because it results in a greater release of GnRH (Drouva *et al.,* 1981) we have not routinely used it as the use of K^+ is much more cost-effective. Another method that we used to check viability involved using trypan blue, a vital dye that can be used to differentiate living and dead cells. Trypan blue is negatively charged, and it will not interact with a cell unless the membrane is damaged. Thus, all cells that exclude the dye must be intact. Wells with a high percentage of dead cells should be excluded from data analysis because a change in pulsatile activity could be the result of decreasing numbers of living GnRH neurons.

This optimized assay can be used to measure GnRH from samples collected during perfusion experiments performed in our lab. This assay has a number of

additional potential applications, including measuring GnRH collected directly from cell culture media or during the course of push-pull cannula experiments. Optimization of this assay will be useful to us because we will be able to measure GnRH from samples collected via a number of experimental procedures, and since the EIA is specific for GnRH, there should be no reaction with agents used during the course of the experiments or introduced during the collection process. Our perfusion experiments will be initially focused on examining the electrophysiological mechanisms which underlie pulsatile GnRH release. Perfusion experiments will be performed using a specific hyperpolarization-activated ion channel blocker, and GnRH will be measured using this optimized EIA protocol. Any changes in the pulse rate can be detected and quantified.

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