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On the neuroendocrine regulation of reproduction: Functional characterization and kinetic studies of the lamprey gonadotropin -releasing hormone receptor and cloning and analysis of the cDNA encoding lamprey gonadotropin-releasing hormone-III

Matthew Ren Silver
University of New Hampshire, Durham

ON THE NEUROENDOCRINE REGULATION OF REPRODUCTION:

FUNCTIONAL CHARACTERIZATION AND KINETIC STUDIES OF THE LAMRPEY GONADOTROPIN-RELEASING HORMONE RECEPTOR AND CLONING AND ANALYSIS OF THE cDNA ENCODING LAMPREY GONADOTROPIN-RELEASING HORMONE-III

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MATTHEW REN SILVER

B.S., University of Connecticut, 2000

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This dissertation has been examined and approved.

Dissertation Director, Dr. Stacia A. Sower
Interim Associate Dean of Research of the College of Life Sciences and Agriculture, Professor of Biochemistry and Molecular Biology

Dr. William R. Trumble
Dean, College of Life Sciences and Agriculture, Professor of Biochemistry and Molecular Biology

Dr. William A. Condon
Professor of Animal and Nutritional Sciences

Dr. William K. Thomas
Associate Professor of Biochemistry and Molecular Biology

Dr. Karen L. Carleton
Research Associate Professor of Zoology

Nov 18, 2005
Date

DEDICATION

To Molly

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The vertebrate hypothalamic-pituitary-gonadal (HPG) axis is regulated by gonadotropin-releasing hormone (GnRH), a decapeptide that is produced and released from the hypothalamus. At the anterior pituitary, GnRH action is mediated through high affinity binding with the GnRH receptor, a rhodopsin-like seven transmembrane G-protein coupled receptor (GPCR). Interest in the evolution of reproductive physiology has led scientists to study the lamprey, a member of the oldest extant class of vertebrates, the agnathans. The studies presented herein contribute to the field of reproductive neuroendocrinology through developing our understanding of ancestral, or ancestral-like characteristics and mechanisms of the HPG axis. This dissertation is divided into two

major components: (1) functional characterization and kinetic studies of the lamprey GnRH receptor (chapters II and III), and (2) an analysis of the lamprey GnRH-III cDNA (chapter IV).

A type II lamprey GnRH receptor was recently identified via cDNA cloning, BLAST analysis and *in situ* hybridization, however the classification by these homology and expression studies was insufficient. Demonstration of function, through binding capacity or efficacy is a vital and required component of receptor characterization. To this end, a heterologous expression system was developed using COS7 cells transiently transfected with the lamprey GnRH receptor. The lamprey GnRH receptor was shown to be functional as well as lamprey GnRH-III selective based on a series of efficacy and kinetic studies. Ligand dependant internalization was characterized, which was dependant on a motif within the first forty amino acids of the C-terminal tail. Further function and kinetics studies were performed using C-terminal tail truncation mutants.

The objective of the second component of this dissertation was to clone and characterize the cDNA encoding lamprey GnRH-III from eight species of lamprey, which were analyzed by phylogenetics methodology to address the molecular evolution of the GnRH family and the lamprey lineage. The lamprey GnRH-III sequences formed three groups, supporting the current view of the lamprey lineage at the family level. Phylogenetic analysis of these sequences together with 64 previously described GnRH sequences suggested that the lamprey GnRHs are unique, as they group together separately from the three previously described paralogous lineages of the GnRH family.

CHAPTER I

BACKGROUND AND SIGNIFICANCE

Reproductive physiology is a fundamental component of life, evolution and medicine. Efficient and robust regulation of the mechanisms that control the reproductive system, from neural function to steroid synthesis, is essential for a species to survive and flourish, and therefore is subject to the unwavering scrutiny of selective pressure. In this light, the field of reproductive biology spills across disciplines and has been the focus of intense, multidisciplinary research which has been fed by the desire for basic scientific progress as well as the high pressure pursuit of pharmaceutical development. Like all other physiological systems, proper reproductive function relies on a highly organized mechanism of communication; hormones and their receptors provide this link between distantly located tissues that are coordinated in operation. This biochemical communication is governed by the hypothalamus, a region within the diencephalon (which sits at the base of the cerebral cortex), where integration and coordination of internal and environmental information occurs. In the control of the reproductive system, the hypothalamus produces the gonadotropin-releasing hormone (GnRH), which acts at the pituitary causing the synthesis and secretion of the gonadotropins, which in turn regulate gonadal function, which in turn feedback information, via gonadal hormones (steroid and protein) to the hypothalamus and pituitary. Consummate maintenance and regulation of this cycle is necessary and essential for proper reproductive function, and

therefore lies at the heart of fitness, selection and evolution as well as medicinal therapy development for the treatment of fertility disorders, reproductive abnormalities and sex steroid driven cancers.

In no field is the importance of comparative analysis as great as it is in reproductive biology, where the analysis of species across the vertebrate lineage has led to a more developed understanding through the description of systematic change through evolution. It is in this spirit that this dissertation intends to address and contribute to the field of molecular neuroendocrinology of reproduction through analysis of an ancestral model, the sea lamprey, which is one of the oldest living vertebrates, in order to better our understanding of more derived species.

Gonadotropin-Releasing Hormone

The gonadotropin-releasing hormone (GnRH-previously referred to as LRF or LHRH) is a ten amino acid peptide hormone that is produced in the hypothalamus and is the central regulator of the reproductive axis in all vertebrates. Since its discovery in the early 1970's (Burgus et al., 1972; Matsuo et al., 1971) GnRH has been the focus of intense research which has lead to a rapid advancement of this field. Released from the hypothalamus, GnRH stimulates the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH), which travel via the circulation system to the gonads where they stimulate gonadal steroidogenesis (i.e. testosterone or estradiol production) and gametogenesis (i.e. spermatogenesis or oogenesis) (See Figure 1). To date, twenty-four forms of GnRH have been identified,

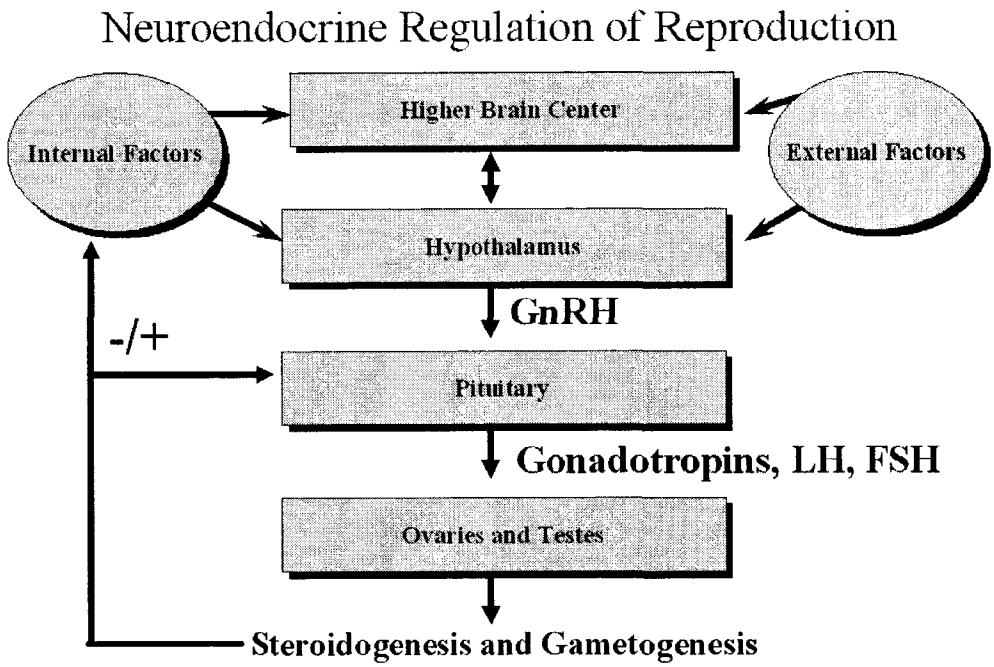

Figure 1. Schematic Representation of the Neuroendocrine Regulation of Reproduction. In all vertebrates, GnRH is the primary regulatory of the hypothalamic-pituitary-gonadal (HPG) axis, and therefore reproduction. GnRH is released from the hypothalamus and stimulates the synthesis and secretion of the gonadotropins (LH and FSH) from pituitary gonadotrope cells. The gonadotropins in turn control steoidogenesis and gametogenesis at the level of the gonads.

fourteen forms from vertebrates and ten from invertebrates, which have been named after the species they were identified (see Figure 2) (Gorbman and Sower, 2003).

After the isolation of mammalian GnRH in the early 1970's, two forms of GnRH were identified in the chicken in the early 1980's (Miyamoto et al., 1982; Miyamoto et al., 1984), at which time it was believed that only one form of GnRH was expressed within any given species, with a few exceptions that where accounted for as they arose, such as the lamprey (Sherwood et al., 1986; Sower et al., 1993) and certain teleosts (Bogerd et al., 1994; White and Fernald, 1998; White et al., 1995); which generally were thought of as unique non-mammalian variants. In time and in light of developing research powered by molecular biology, numerous sequences were cloned, most notably the cDNA encoding

Vertebrate	1			2	3	4	5	6	7	8	9	10
Mammal	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	GlyNH2
Guinea Pig	pGlu			Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	GlyNH2
Chicken - I	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	GlyNH2
Rana	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	GlyNH2
Seabream	pGlu			His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	GlyNH2
Salmon	pGlu			His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	GlyNH2
Medaka	pGlu			His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	GlyNH2
Catfish	pGlu			His	Trp	Ser	His	Gly	Leu	Asn	Pro	GlyNH2
Herring	pGlu			His	Trp	Ser	His	Gly	Leu	Ser	Pro	GlyNH2
Chicken -II	pGlu			His	Trp	Ser	His	Gly	Trp	Tyr	Pro	GlyNH2
Dogfish	pGlu			His	Trp	Ser	His	Gly	Trp	Leu	Pro	GlyNH2
Lamprey - III	pGlu			His	Trp	Ser	His	Asp	Trp	Lys	Pro	GlyNH2
Lamprey - I	pGlu			His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	GlyNH2
Whitefish	pGlu			His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	GlyNH2
Invertebrate	1			2	3	4	5	6	7	8	9	10
Octopus	PGlu	Asn	Tyr	His	Phe	Ser	Asn	Gly	Trp	His	Pro	GlyNH2
Tunicate - I	pGlu			His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	GlyNH2
Tunicate - II	pGlu			His	Trp	Ser	Leu	Cys	His	Ala	Pro	GlyNH2
Tunicate-III	pGlu			His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	GlyNH2
Tunicate-IV	pGlu			His	Trp	Ser	Asn	Glu	Leu	Thr	Pro	GlyNH2
Tunicate-V	pGlu			His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	GlyNH2
Tunicate-VI	pGlu			His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	GlyNH2
Tunicate-VII	pGlu			His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	GlyNH2
Tunicate-VIII	pGlu			His	Trp	Ser	Leu	Ala	Leu	Ser	Pro	GlyNH2
Tunicate-IX	pGlu			His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	GlyNH2

Figure 2. GnRH Family of Peptides. To date, 24 different forms of GnRH have been identified, which are named after the species from which they were first discovered. 14 Forms have been identified from vertebrates, and 10 from invertebrates. Highlighted residues indicated variation relative to mammalian GnRH. All forms, except from the octopus, are decapeptides with highly conserved N- and C-termini and Ser^4. Image design by Scott I. Kavanaugh.

chicken GnRH-II from shrew, human and monkey (Kasten et al., 1996; Urbanski et al., 1999; White et al., 1998), which led to the general conclusion that species expressing multiple forms of brain GnRH is common, with few exceptions (i.e. rat and mouse only express mammalian GnRH) (Silver et al., 2004). Within these multiple-GnRH expressing species, typically one form is hypothalamic and regulates the pituitary-gonadal axis, while the others are non-hypothalamic in expression and operate in some neuromodulatory function.

The hypothalamic form of GnRH is expressed in the preoptic-anterior hypothalamic region of the diencephalon (mammalian GnRH in all tetrapods except guinea pig (guinea pig GnRH) and bullfrog (rana GnRH); variable forms in fish including mammalian GnRH, sea bream GnRH, herring GnRH, whitefish GnRH, medaka GnRH, salmon GnRH and dogfish GnRH; and lamprey GnRH-I and -III in lamprey) (Fernald and White, 1999; Parhar, 2002). In tetrapods, these GnRH expressing neurons have axons that extend caudally and impinge and synapse on the median eminence, a neural-hemal organ, which is a portal blood vessel that is fed by the superior hypophysial artery and runs from the hypothalamus to the adenohypophysis (anterior pituitary). At the level of the adenohypophysis, GnRH diffuses through fenestrations in the capillary bed and interacts with gonadotropes via a specific 7-transmembrane G-protein coupled receptor (which is described in detail bellow) (Millar et al., 2004; Sealfon et al., 1997). This delivery system and hypothalamic GnRH distribution pattern [at a superficial level] has remained conserved within the tetrapod lineage (Nozaki et al., 1994). The Agnathan (lamprey and hagfish) and teleost (a radiation of the osteichthyes) hypothalamic GnRH system is highly conserved, however their mechanisms of delivery to the adenohypophysis are unique since neither group has a median eminence. Teleost have adapted to the lack of the portal system via neurohypophysial axon extension and penetration of the pituitary, creating a direct intervention (Kah et al., 1986; Peter et al., 1990). In lamprey, GnRH is released from the hypothalamus and diffuses through a thin layer of epithelial glandular cells, to the pituitary (Nozaki et al., 1994).

The second form of GnRH, chicken GnRH-II, is the most ubiquitously expressed form, which has been identified in all classes of vertebrates except Agnatha (Fernald and

White, 1999; Parhar, 2002). The sequence of chicken GnRH-II has remained unchanged throughout vertebrate evolution from cartilaginous fish through mammals, which would suggest a strong selective pressure, however its function is, to this date, rather ambiguous (Millar et al., 2004). The expression pattern of chicken GnRH-II varies drastically from species to species, but generally is expressed in the midbrain and numerous peripheral tissues (Parhar, 2002). For example, chicken GnRH-II is only expressed in the midbrain and testis of *Haplochromis burtoni* (White and Fernald, 1998), while it is expressed in the brain, testi, gonad, kidney, liver, pancreas, stomach, intestine, heart, lung, parathyroid, thymus and spleen of the leopard gecko (Ikemoto and Park, 2003). The function of chicken GnRH-II in peripheral tissues is largely unknown, but is thought to function in a paracrine/autocrine fashion. As a neuromodulator, chicken GnRH-II has been shown to be involved in behavior and possibly as a link between energy state and reproduction. For example, in poecilid (live bearing) fish, neurons that regulate sperm duct and oviduct contraction are impinged on by chicken GnRH-II immunoreactive cells located in the midbrain (Miller and Kriebel, 1986), and in newts, during courtship activity, chicken GnRH-II immunoreactivity relocates from midbrain soma to their axons (Kasten et al., 1996; Muske and Lancaster, 1993). In a few cases chicken GnRH-II has been detected in the median eminence, which suggests that, in these species, it may have some pituitary-gonadal axis regulatory function. In the goldfish, chicken GnRH-II immunoreactivity was detected in the median eminence and pituitary (Rosenblum et al., 1994), while in the chicken and quail immunoreactivity in the median eminence was detected, and sequence verification was performed by peptide sequencing using mass spectrometry (Clerens et al., 2003; van Gils et al., 1993). Interestingly, in mammals,

chicken GnRH-II has been cloned from the tree-shrew (Kasten et al., 1996), monkey (Urbanski et al., 1999), and human (White et al., 1998), but has not been identified in the rat or mouse. Additionally, the chimp chicken GnRH-II gene is considered a pseudogene due to a premature stop codon, and the human chicken GnRH-II gene polyadenylation sequence lies in-frame prior to the stop codon, which suggests the possibility of an unstable mRNA population (Morgan and Millar, 2004). Taken together, these data could be used to infer an optimization of the GnRH system has occurred, whereas the function of chicken GnRH-II may no longer be useful in more derived tetrapods (Morgan and Millar, 2004).

A third population of GnRH neurons exists in the teleost radiation of the osteichthyes, which in all species identified to date is salmon GnRH (Fernald and White, 1999; Parhar, 2002). Like chicken GnRH-II, salmon GnRH is generally extra-hypothalamic and is neuromodulatory in function. Although salmon GnRH is expressed in the hypothalamus and regulates the pituitary-gonadal axis in goldfish (Parhar, 2002), in most cases it is expressed in the ventral telencephalon/terminal nerve region of the brain (Fernald and White, 1999; Parhar, 2002). The extrahypothalamic function of salmon GnRH is not understood, however it is believed, like chicken GnRH-II, to function in reproductive behavior (for example nest building). Since salmon GnRH is only found in teleosts, it is thought to be lineage specific, although whether its gene arose prior to the teleosts or early in the teleost lineage is unknown (Okubo and Aida, 2001; Okubo et al., 1999).

GnRH Development: Embryonic Origin and Cellular Migration

The development of the GnRH system in vertebrates from fish to mammals has remained highly conserved (Muske, 1993; Parhar, 2002; Tobet et al., 2001). As to be expected based on the three different GnRH populations described above, there are three different major embryonic origins and migratory pathways that are seen in vertebrates. The migration of the hypothalamic GnRH producing neurons begins in the olfactory placode in the nasal cavity, migrates through the cribiform plate and finally rests in the preoptic anterior hypothalamus, where axons extend to the median eminence (Muske and Moore, 1988; Schwanzel-Fukuda and Pfaff, 1989; Tobet et al., 2001; Wray et al., 1989). As a note, the olfactory placode is a thickening of the ectoderm that develops as a result of cell division during the formation of the neural tube. The olfactory placodes develop into the primarily sensory neurons, support cells and basal cells of the olfactory system (Whitlock, 2004). The cues involved in this migration of the hypothalamic GnRH producing neurons have been investigated in many different species, however the exact nature of this process has not been fully described due to the complexity of the system, which is a result of the appearance of subsets of populations that results from differences in cell surface molecule expression (Tobet et al., 2001). The factors involved in this migration can be divided into two main categories, including components that interact at the cell surface (for example neural cell adhesion molecules (NCAM) and nasal embryonic luteinizing hormone-releasing hormone factor (NELF)) and informational molecules (GABA, peptide hormones) (Dellovade et al., 2001; Tobet et al., 2001; Wray, 2002). The olfactory placodal origin of the hypothalamic GnRH producing neurons was initially described based on ICC studies of mice (Schwanzel-Fukuda and Pfaff, 1989; Wray et al.,

1989) and amphibians (Muske and Moore, 1988) during embryonic development. When the olfactory placode is removed from the axolotl (amphibian) embryo for example, the adult does not produce hypothalamic GnRH, as examined by immunocytochemical analysis (Northcutt and Muske, 1994). Additional support of the olfactory origin of hypothalamic GnRH neurons hypothesis came from Kallmann syndrome research, which is characterized by anosmia and hypogonadotropic-hypogonadism (Rugarli, 1999; Schwanzel-Fukuda et al., 1989). Kallmann syndrome is caused by a genetic mutation in the Kal-1 gene, which encodes the extracellular matrix protein anosmin-1 (Legouis et al., 1991), and is necessary for olfactory bulb development (MacColl et al., 2002; Schwanzel-Fukuda et al., 1989). This link between olfactory function and GnRH ontogeny in Kallmann syndrome is considered a hallmark discovery and a proof of principle, however there is emerging data indicating alternative developmental schemes in certain vertebrates (Parhar, 2002; Parhar et al., 1998; Whitlock et al., 2003). First, the hypothalamic GnRH producing neurons in zebra fish were shown to originate in the adenohypophyseal regions of the anterior neural plate, which is flanked by the olfactory placode (Whitlock et al., 2003). Secondly, the origin of the hypothalamic GnRH producing neurons in seabream and medaka lies in the basal diencephalon, which notably is similar to the origin of the cells producing the hypothalamic GnRHs in the lamprey, which arise from the proliferative zones of the diencephalon (Tobet et al., 1995; Tobet et al., 1997). Additional investigation into the origin and migration of hypothalamic GnRH producing neurons from basal vertebrates, such as hagfish, and more derived vertebrates, such as mice, is needed in order to accurately describe this developmental process.

The development and migration of the cells producing the neuromodulatory GnRHs, chicken GnRH-II in the midbrain and salmon GnRH in the ventral telencephalon of the advanced teleosts, have been investigated, however the mechanisms regulating these processes are not as well understood compared to their hypothalamic GnRH neuron counterpart. Chicken GnRH-II producing neurons originate in the midbrain, not the olfactory placode, which was demonstrated in the aforementioned study by Northcutt and Muske. The ablation of the olfactory placode in axolotl abolished hypothalamic GnRH expression, but had no effect on chicken GnRH-II expression in the midbrain (Northcutt and Muske, 1994). In advanced teleosts, such as the seabream and medaka, the nucleus olfactoretinalis, which lies at the olfactory bulb/telencephalon junction, expression of salmon GnRH, which is distinct from the hypothalamic GnRH producing neurons, arises in the olfactory placode during early development (Parhar, 2002; Parhar et al., 1998). These two GnRH populations, as inferred based on the work of Whitlock's group, likely ultimately arose from the cranial neural crest.

Phylogeny of the GnRH Family

A proposed relationship of the GnRH family was recently described based on phylogenetic analysis, location of expression within the brain and general associated function (Fernald and White, 1999) (Figure 3). In this model, the GnRH family was divided into three paralogous lineages. GnRH-I (also known as mammalian GnRH and its orthologs) is expressed in the hypothalamus and is the central regulator of the pituitary gonadal axis. GnRH-II (also known as chicken GnRH-II) is expressed in the midbrain and is generally considered to have a neuromodulatory function

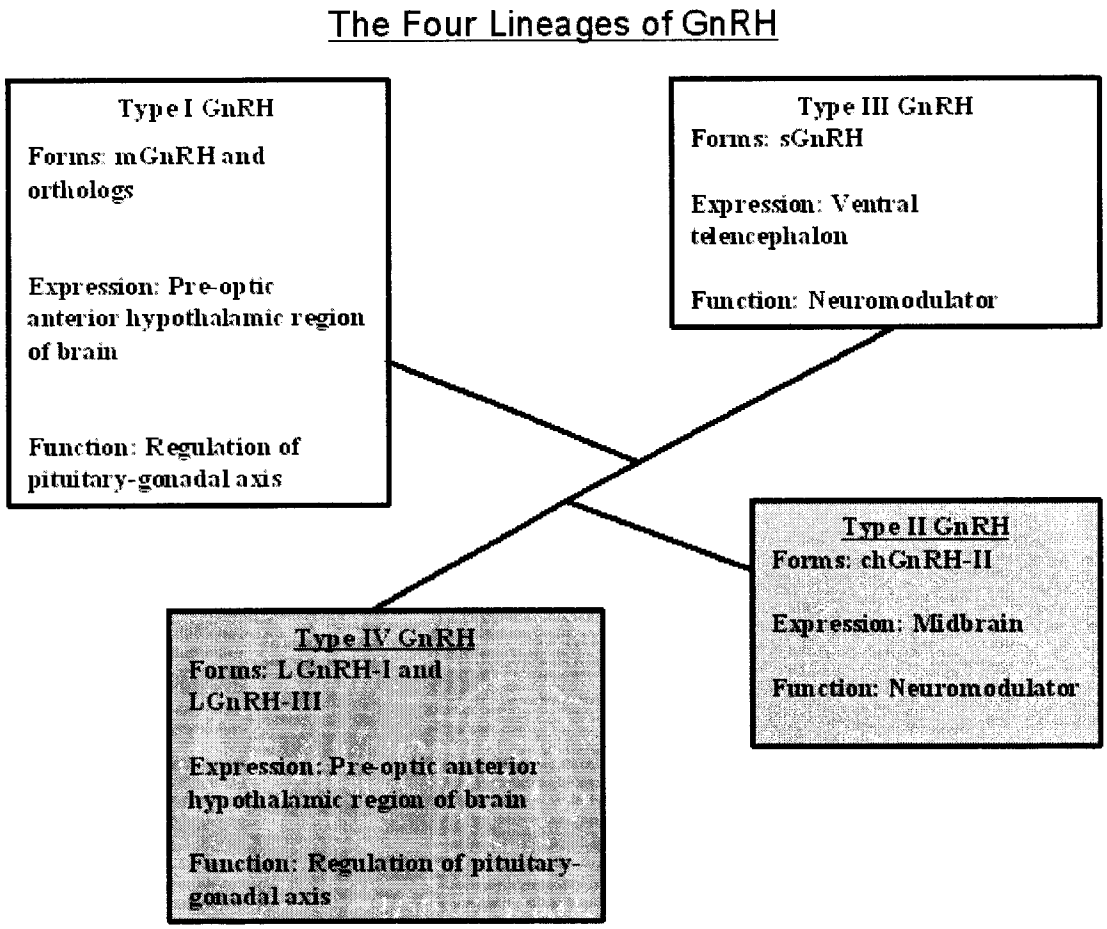

Figure 3. The Lineages of GnRH. The GnRH family is divided into four paralgous lineages based on phylogenetic analysis, location of expression within the brain and function. Modified from Gorbman and Sower,2003 and Silver et al., 2004.

(Fernald and White, 1999; Parhar, 2002). Finally, GnRH-III (or salmon GnRH), which is only found in the teleosts, is expressed in the telencephalon and is also believed to have a neuromodulatory function (Fernald and White, 1999; Parhar, 2002). Parhar recently modified this scheme by the addition of a fourth family that consists of medaka and seabream GnRH, which formerly would have been considered GnRH-I (Parhar, 2002). This modification is supported by the fact that the cells producing medaka and seabream GnRH do not share the same developmental origin as the other hypothalamic forms,

although they do function in a similar manner and group together phylogenetically. These models that describe the molecular phylogeny of the GnRH family are incomplete since only a limited number of the known GnRH sequences were used in these analyses. Based on my thesis research (Chapter II; (Silver et al., 2004)), we have proposed a new scheme, in which our phylogenetic analysis confirms Fernald and White's division of the GnRH family, but shows the medaka and seabream forms of GnRH grouping with the type I GnRHs, which conflicts with Parhar's model. Additionally the lamprey GnRH forms group together separately from the three previously described lineages of GnRH, and as such we suggest that they, and not medaka and seabream GnRH, form the 4th lineage of GnRH (See Table 3). Our assertion is based directly on our phylogenetic analysis, which is supported by immunocytochemical and functional data on lamprey GnRH (Deragon et al., 1994; Nozaki et al., 2000). These schemes will ultimately need to be revised when additional sequence, function and expression data are available.

GnRH: From Mature Peptide to Gene

Consistent with other neuropeptides, GnRH is synthesized as part of a larger protein precursor, prepro-GnRH, which upon post-translational modification yields the mature decapeptide (Klungland et al., 1992). Prepro-GnRH consists of a tripartite structure, including a N-terminal hydrophobic signal domain, followed by the mature decapeptide sequence and the Gly-Lys-Arg dibasic cleavage site, and finally the C-terminal GnRH associated peptide (GAP) (Figure 4). The functional significance of the tripartite structure relates to intracellular trafficking and processing. The signal peptide is essential for entry of the prepro-hormone into the endoplasmic reticulum (ER) during the

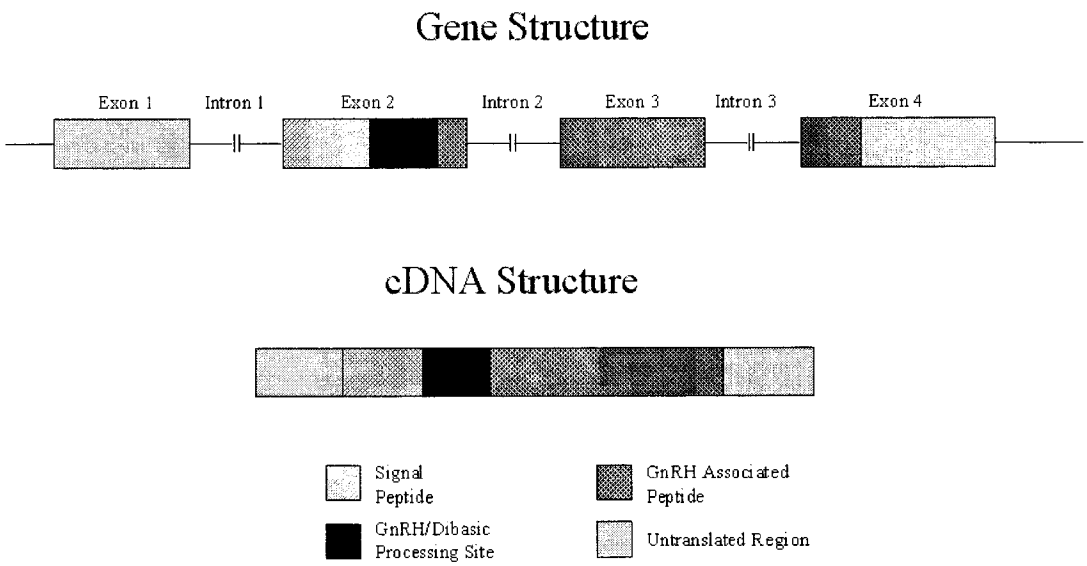

Figure 4. GnRH Gene and cDNA Structures. The conserved GnRH gene configuration consists of 4 exons and 3 introns. Exon 1 encodes the 5' UTR, exon 2 encodes the signal peptide, mature decapeptide and N-terminal end of the GAP, Exon 3 encodes the central region of the GAP while Exon 4 encodes the C-terminal end of the GAP and the 3' UTR.

translational process via its interaction with the signal recognition particle (SRP). The SRP then docks to the surface of the ER and the prepro-hormone is funneled into the lumen. The significance of the GAP region is less well understood, and may simply exist to provide the necessary length to bridge the space between the SRP and the ER surface, which was shown to require a minimum of 50 amino acids (Dores et al., 1996; Wolin and Walter, 1993). The extremely low amino acid sequence conservation of the GAP across vertebrates would suggest that there are no specific functional constraints, however it has been shown to be released with the mature GnRH decapeptide and to have some LH, FSH and prolactin regulatory properties in rat pituitary cell cultures (Nikolics et al., 1985).

In vertebrates, the conserved GnRH gene structure is composed of four exons, with three introns, which produces a mature mRNA that encodes the prepro-GnRH (see

Figure 4) (Fernald and White, 1999). The first exon encodes the 5'-untranslated region (UTR), the second exon encodes the signal peptide, GnRH decapeptide with dibasic cleavage site and the N-terminal portion of the GAP. The third exon encodes the middle potion of the GAP while the fourth exon encodes the C-terminal portion of the GAP and the 3'-UTR. The major difference seen between different GnRH genes lies within the size of the introns, which can range from between a few hundred bases to a few thousand bases (Fernald and White, 1999). Based on the high level of conservation of the GnRH gene organization, which has been described across vertebrates from lamprey (Suzuki et al., 2000) to humans (Adelman et al., 1986; Hayflick et al., 1989) it is likely that this organization represents the ancestral structure.

The upstream regulatory elements of the GnRH gene have been identified in a limited number of species, and have been characterized in the greatest detail in human (Belsham and Mellon, 2000; Kepa et al., 1996; Wolfe et al., 2002b), mouse (Givens et al., 2004; Lawson et al., 2002), and rat (Whyte et al., 1995). The GnRH gene has been shown to be regulated through both a promoter region, -173 to +1, and enhancer region -1863 to -1571 (Lawson et al., 2002), while more recently, using a phylogenetic footprinting analysis, Givens et. al. identified a conserved enhancer region, at ~3500 bp upstream of the transcriptional start site (Givens et al., 2004). This upstream enhancer was shown to contain nine Octamer-binding transcription factor 1 (Oct-1) binding sites as well as nuclear factor 1 (NF-1) binding sites, both of which have been previously shown to regulate GnRH expression (Belsham and Mellon, 2000; Wolfe et al., 2002b). Analysis of mature GnRH cells (GT1) transfected with luciferase reporter constructs containing variations of the mouse 5' GnRH gene regulatory sequence indicated that the promoter,

enhancer and upstream enhancer are all required for efficient GnRH expression, and that Oct-1 and NF1 interaction is also necessary for activation of transcription (Givens et al., 2004). The human GnRH gene regulatory region has also been shown to contain Brn-2 binding sites, in addition to Oct-1 binding sites, within a promoter region located -992 to -795 bases upstream of the transcriptional start site, which are necessary for GnRH neuron specific expression, as demonstrated using transgenic mouse lines (Wolfe et al., 2002a). Additional information is needed in order to identify, with more detail, all of the promoter/enhancer regions involved in GnRH transcriptional regulation, which would require sequencing and analysis of 5' sequences from representatives of all classes of vertebrates.

GnRH Neuron Regulation

Numerous internal and external factors are involved in the control of GnRH synthesis and secretion, which operate at all levels of cellular function including gene expression, transcript processing, prepro-peptide metabolism, vesicular packaging and release (Givens et al., 2004; Lawson et al., 2002; Martinez de la Escalera and Clapp, 2001; Wetsel et al., 1991; Wolfe et al., 2002b) (see Figure 5). Both *in vivo* (examples such as fish, rat and rabbit) and *in vitro* (immortalized cell lines such as GT1 (Mellon et al., 1990)) systems have been developed in order to probe these mechanisms. These experimental systems have provided invaluable insight into the physiology of GnRH neurons, despite their respective shortcomings. GnRH neurons receive input from a variety of sources, including amines, peptide hormones and gonadal steroids, which are thought to, in part, lead to the pulsatile nature of GnRH release (Martinez de la Escalera

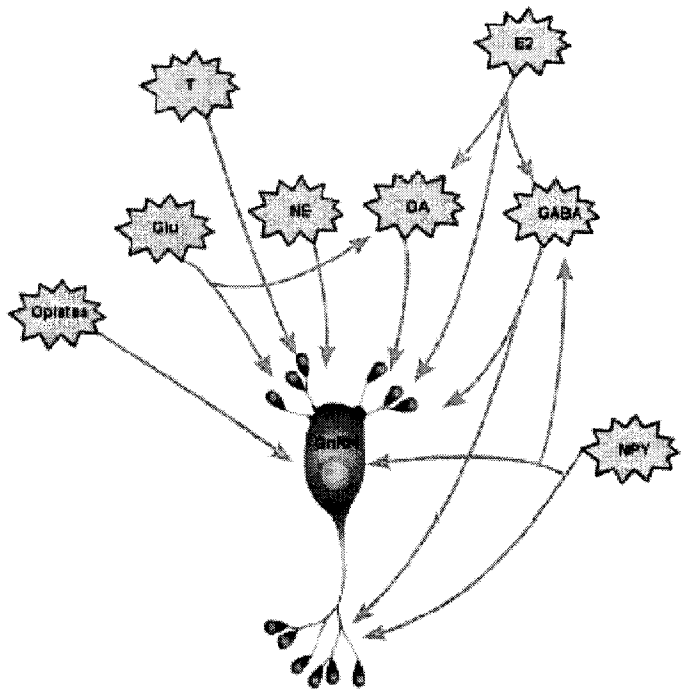

Figure 5. GnRH Neuron Regulation. GnRH neurons are regulated by several factors. The nature of the input for each component is variable between different reproductive states and species. NPY, neuropeptide Y; GABA, γ-aminobutyric acid; E2, 17-β estradiol; DA, dopamine; NE, norepinephrine; T, testosterone; Glu, glutamate.

and Clapp, 2001). The exact nature of the effect of the aforementioned signals is fairly well described (i.e. functioning through an inhibitory or excitatory post-synaptic potential (IPSP or EPSP), or through cellular modulation), however due to the complexity of these systems and the variations seen between different species, a single overriding model has yet to be established.

Regulation of GnRH neurons by amino acid or amine derivative neurotransmitters such gamma-amino butyric acid (GABA), glutamate (GLU), norepinephrine (NE), and dopamine (DA) has been well documented (Jennes and Conn, 2002; Levine, 1997). GABA, the primary inhibitory neurotransmitter of the central nervous system, has been shown to regulate the HPG axis in representative species across the vertebrate line

(Anglade et al., 1999; Root et al., 2004). The exact nature of the effect of GABAergic innervation is still in question as GABA has been shown to act primarily as a GnRH neuron inhibitor (Herbison and Dyer, 1991; Jarry et al., 1995; Terasawa et al., 1999), however GABA has been shown to elicit a stimulatory response in the rat embryonic (Kusano et al., 1995) and neonatal (Feleder et al., 1996) hypothalamus, in seabream preoptic-anterior hypothalamic tissue slices (Senthilkumaran et al., 2001), and in the GT-1 cell line (Martinez de la Escalera et al., 1992b; Martinez de la Escalera and Clapp, 2001). More recently GABA has been shown to increase brain GnRH content in the sea lamprey (Root et al., 2004). Similarly, DA and NE have been shown to regulate GnRH neurons directly, through the dopamine receptor and α/β-adrenergic receptor, receptively, leading to a variety of experimental results showing both a stimulatory or inhibitory response, based on species, laboratory and preparative differences (Kordon et al., 1994; Martinez de la Escalera et al., 1992a; Martinez de la Escalera et al., 1992c). Glutamate, on the other hand has been definitively shown to stimulate both GnRH secretion and increase plasma LH levels (Brann and Mahesh, 1997; Martinez de la Escalera and Clapp, 2001), however these effects are thought to be partially indirect due to the apparent absence or low level of expression of glutamate receptors in GnRH neurons (Mahesh et al., 1999; Martinez de la Escalera and Clapp, 2001). At this time there is no evidence to support the idea that one neurotransmitter is the primary regulator of GnRH neurons, but rather it is thought that a redundant, multi-neuromodulatory network is in place to regulate not only these neurons directly, but also to facilitate modulation through coordination of signals from other systems (Jennes and Conn, 2002).

An ever growing number of peptides have been shown to directly and indirectly regulate GnRH neurons, forming the link between reproduction and other physiological systems such as stress, energy balance, appetite, and the immune system. The most relevant of these peptides include neuropeptide Y (NPY), the opiates, interleukin-1 (IL1) and vasoactive intestinal polypeptide (VIP) (Jennes and Conn, 2002; Levine, 1997; Pau and Spies, 1997; Plant and Shahab, 2002). The role of NPY on GnRH regulation has been investigated extensively, and has been shown to stimulate or inhibit GnRH neurons in a wide range of vertebrates, depending on the particular network that is activated, i.e. innervating GnRH neurons directly (axo-somal or axo-axonal) or indirectly (through GABAergic neurons) and which NPY receptor subtype is involved (Y1-Y5) (Ichimaru et al., 2001; Knauf et al., 2001; Sainsbury et al., 2002; Senthilkumaran et al., 2001). In primates, intracerebroventricular (ICV) injection of NPY has been shown to suppress GnRH release in adults (Kaynard et al., 1990; Plant, 2001), while NPY mRNA levels were shown to be inversely related to GnRH synthesis and secretion during the juvenile-pubertal transition (El Majdoubi et al., 2000). In goats, ICV injection of NPY was also shown to decrease GnRH neurons pulsatility (Ichimaru et al., 2001). Furthermore, it has been well established that during periods of starvation, the hypothalamus-pituitary-gonadal axis is down regulated, primarily through NPY mediated inhibition of GnRH neurons (Wade and Jones, 2004). However, NPY has been shown to stimulate GnRH mediate LH release in rats (Crowley et al., 1987), and GnRH release in seabream hypothalamic tissue sections (Senthilkumaran et al., 2001), while arcuate nucleus NPY mRNA levels have been shown to increase just prior to the LH surge (Jennes and Conn, 2002; Sahu et al., 1994), illustrating a possible stimulatory effect on the GnRH system.

What is unknown, and therefore confounding, is in which of these studies NPY is acting directly or indirectly and through which receptor. It is likely that NPY acts through a variety of different mechanisms, which impart this differential response. For example NPY may act via inhibition of GABAergic neurons that innervate GnRH neurons, thereby inhibiting an inhibitor, and stimulating a response. Alternatively, NPY may deliver an IPSP through Y_x receptors at an axon terminal of a GnRH neuron (Knauf et al., 2001; Sainsbury et al., 2002). During these times it is clearly beneficial to the individual to focus energy on survival, and therefore shut down non-essential systems, such as reproduction. The hormones involved in these systems, interleukin-1 and the opiates (endorphins and enkephalins) have been shown to inhibit GnRH production and release both directly and indirectly (Jennes and Conn, 2002).

Not surprisingly, the role of sex steroids on GnRH neurons depends on species dependant physiological variations and the temporally dependent hormonal milieu (Hrabovszky et al., 2000; Moenter et al., 2003; Skynner et al., 1999). For example, 17β-estradiol (E2) is known to have an inhibitory effect on both the pituitary and hypothalamus, however it is also the trigger driving the pre-ovulatory GnRH, and hence LH, surge, which drives ovulation (Shirley et al., 1968). The failure of early attempts to identify estrogen receptors within GnRH neurons (Herbison and Theodosis, 1992; Navas et al., 1995) led to the hypothesis that E2 regulation of GnRH neurons occurs indirectly, for example through modulation of GABAergic or dopaminergic pathways. Recently, through mRNA cloning and immunocytochemistry, estrogen receptors have been identified in mouse and rat GnRH neurons (Hrabovszky et al., 2000; Skynner et al., 1999) and in GT1 cells (Butler et al., 1999; Roy et al., 1999), indicating E2 may assert its

function, in part, directly. E2 has been shown to function through a variety of mechanisms, including pulse frequency alteration, transcriptional activation/inhibition, sensitization and ion flux (Kelly et al., 2003; Moenter et al., 2003; Temple et al., 2004). Progesterone has also been shown to influence GnRH neuron activity directly in some cases, however less is known about the mechanisms involved, and progesterone receptors have only been seen in a limited number of GnRH neurons (King et al., 1995; Skinner et al., 2001). Androgens and glucocorticoids have been shown to regulate GnRH neurons, however far less attention has been given to the description of these mechanisms. *In vitro* analysis has shown that both testosterone and glucocorticoids decrease GnRH transcription and secretion in GT1 cells (Attardi et al., 1997; Belsham et al., 1998; Chandran et al., 1994). In teleosts, treatment with testosterone in a variety of species including salmonids and cichlids has been shown to both increase and decrease brain GnRH immunoreactivity (Francis et al., 1993; Grober et al., 1991), which is difficult to interpret, as it suggests either an inhibitory or stimulatory response. Steroid hormone regulation of GnRH neurons is heavily intertwined with other neuromodulatory systems and is therefore a complicated system to model, however recent research has led to the dissection of this system including the isolation and description of these vastly different mechanisms of action, from the traditional theory of steroid action through modulation of gene expression to the more recent observations of fast acting membrane associated steroid receptors.

Clearly GnRH neurons are highly regulated through a diverse set of pathways, which reflects the importance of proper interaction between the hypothalamus, pituitary and gonads. It is no surprise considering the central location of reproduction in selective

pressure and evolution; a species will be unfit despite any advantage, given a faulty reproductive axis. Proper maintenance of GnRH neuron function and coordination with the other physiological systems is absolutely necessary for a species to reproduce, and therefore propagate.

Clinical Application of GnRH and its Analogs

The clinical application of GnRH analogs as therapeutic drugs generates over 2 billion dollars per year in sales, most notably in the treatment of sex steroid driven cancers such as certain prostate and breast cancers; hence there is considerable interest in the function of each residue in the GnRH so that analogs can be designed with maximum efficiency as agonists or antagonists to the GnRH receptor to be used as drugs (Millar et al., 2000). The structure of the functional conformation of the GnRH decapeptide was first described as a β-turn using NMR in the 1970's (Deslauriers et al., 1975; Smith et al., 1973), and later using knowledge based computer modeling (Gupta et al., 1993). In order to determine the function of the ten residues in GnRH, thousands of analogs have been constructed and tested for pharmacological divergence (Karten and Rivier, 1986; Sealfon et al., 1997). This information has been used to construct a knowledge base to identify functional characteristics of each residue with respect to the mammalian GnRH decapeptides interaction with its receptor. It is understood that there are a number of complicating factors involved in trying to apply the results from the analogs pharmacological profiles to describe the function of the ten residues in the GnRH (Sealfon et al., 1997). For example, it is difficult to determine whether an altered residue is affecting the binding of the analog or its ability to activate the receptor. Furthermore, the change could alter the structure of the peptide thereby changing the three dimensional

orientation of functional groups from other residues that are involved in receptor interaction, although, the altered conformation could possibly facilitate new contact sites that could compensate and mask the effect of the altered residue. With these drawbacks in mind, Sealfon et al. in 1997 described the role of each residue of the GnRH decapeptide (see Figure 6). The general conclusions that they made were that there was no single amino acid that is critical for GnRH activity, although the amino and carboxyl terminal domains are predominantly involved in receptor binding, and the amino terminal residues function in the activation of the receptor. The 6th position is important in the folding of the peptide, while the function of the 8th position is most likely to confer specificity with respective receptors (Sealfon et al., 1997).

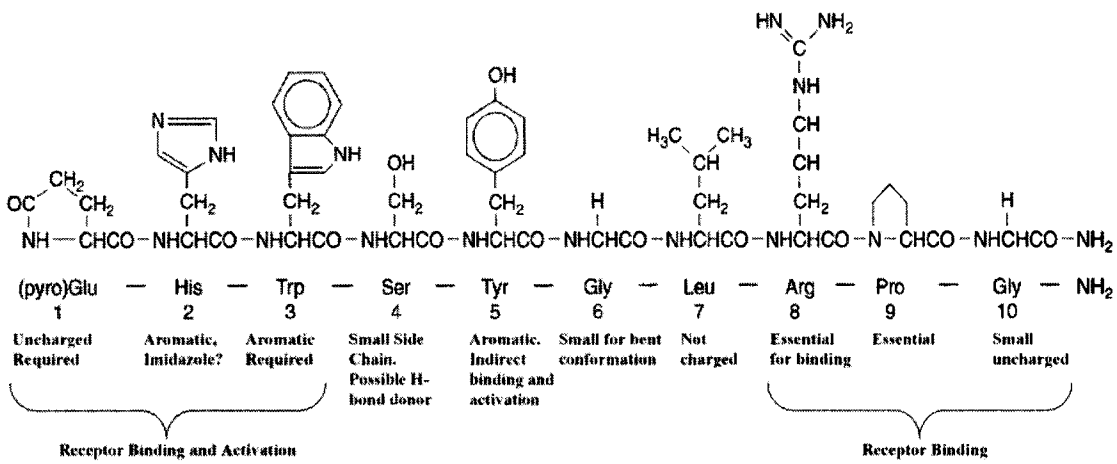

Figure 6. Function of Mammalian GnRH Residues. The function of each residue of mGnRH has been analyzed using analogs and computer modeling. This figure was adapted from Sealfon et al., 1997.

Gonadotropin-Releasing Hormone Receptor

The GnRH receptor is a member of the class A, or rhodopsin-like subfamily of the 7-transmembrane G-protein coupled receptor (GPCR) super family (Millar et al., 2004; Sealfon et al., 1997). The three families of GPCRs: the class A receptors, metatropic glutamate receptors and secretin-calcitonin-PTH receptors, are all seven transmembrane proteins, however they do not share an apparent global sequence identity (Sealfon et al., 1997). The basic GPCR structure consists of seven hydrophobic α-helical transmembrane domains, which are connected by intra- and extracellular loops with an extracellular N-terminal domain and an intracellular C-terminal tail (See Figure 7). Members of the class A family of GPCRs, such as the GnRH receptor family, are identified through several conserved amino acid motifs within the transmembrane domains, including an N in position 21 of transmembrane I, a D in position 11 in transmembrane II, a DRY motif in the basal region of transmembrane III, a W in position 12 of transmembrane IV, a P in position 16 in transmembrane V, a CWXP motif in the middle of transmembrane VI and an NPXXY motif in the basal portion of transmembrane VII (Millar et al., 2004; Sealfon et al., 1997). The GnRH receptors have several unique variations relative to other Class A GPCRs; most notably, the type I GnRH receptors, found in certain mammals, lack a C-terminal tail, which is found in all other known GPCRs (the human genome encodes approximately 1000 GPCRs (Fredriksson et al., 2003)). The functional significance of this "natural mutation" is relevant to both GPCR evolution and reproductive medicine since the C-terminal tail plays an important role in cell signaling, ligand dependant internalization (desensitization) and cell surface expression (Blomenrohr et al., 1999; Heding et al.,

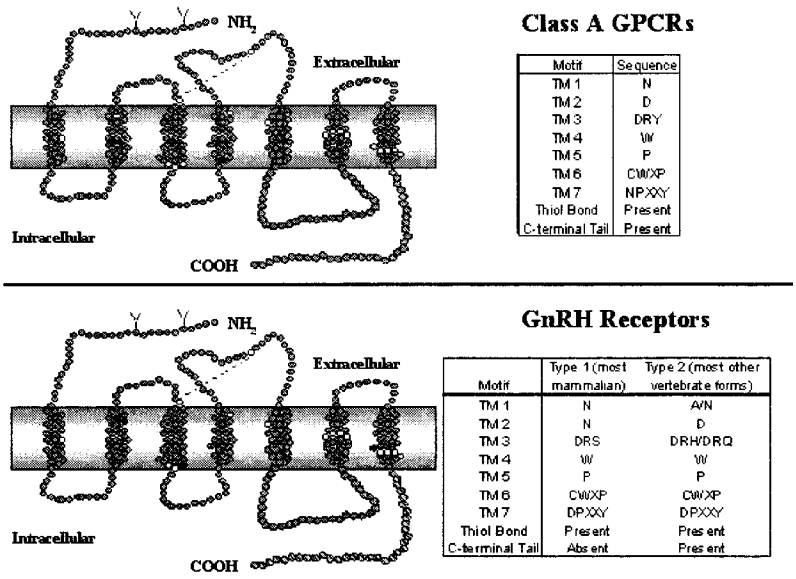

Figure 7. GPCR and GnRH Receptor Schematic. Schematic of GPCR structure, which consists of the characteristic 7 transmembrane spanning domains. Conserved Class A GPCR motifs are shown in the upper panel, while GnRH receptor unique motifs are indicated in the lower panel. This Figure was designed by Nathaniel V. Nucci (Nucci 2004, MS Thesis).

1998; Heding et al., 2000; Hislop et al., 2001; Lin et al., 1998; McArdle et al., 2002; Pawson et al., 1998; Pawson et al., 2003; Vrecl et al., 2000; Willars et al., 2001). Also of importance are the conserved D in transmembrane II and N in transmembrane VII, which are thought to interact in the membrane and are necessary for receptor function. These regions have undergone a reciprocal mutation in the type I GnRH receptors, however they are both D's in type II GnRH receptors (Sealfon et al., 1997).

The first GnRH receptor was cloned from a mouse cDNA library (Reinhart et al., 1992; Tsutsumi et al., 1992), shortly after which the human GnRH receptor was cloned (Chi et al., 1993; Kakar et al., 1992), both of which lacked a C-terminal tail (referred to as tail-less). GnRH receptors from rat (Eidne et al., 1992), sheep (Campion et al., 1996; Illing et al., 1993) and pig (Weesner and Matteri, 1994), which are also tail-less, were cloned over the next several years, giving rise to the belief that all GnRH receptors lack

C-terminal tails. In 1997 the first non-mammalian GnRH receptor was cloned from the catfish, which encoded a protein with the conserved GPCR structure including a C-terminal tail (Tensen et al., 1997). Since this time, over 36 GnRH receptors have been cloned, from which only 11 forms, all mammalian, lack the C-terminal tail (Millar et al., 2004).

Based simply on the gross structural distinction of tailed or tail-less, the GnRH receptors can be divided into two basic sub-groups: the type I receptors, which lack C-terminal tails and have only been found in mammals, and the type II receptors, which contain C-terminal tails and have been found across vertebrates (mammal and non-mammal species). An analysis of the phylogeny of the GnRH receptor family by Troskie *et al* in 1998 was performed based on conserved structural motifs in the third extracellular loop (Troskie et al., 1998). GnRH receptors with the conserved PXML/IXXXXE/D sequence motif, including the mammalian tail-less receptors and some teleost tailed receptors, were classified as subtypes Ia and Ib. Alternatively, receptors with the conserved PEMLTXXVRRXLSHIL sequence motif in extracellular loop 3 were classified as type II (Troskie et al., 1998). A more robust analysis was presented by Okubo et al., in which the full amino acid sequence of the known GnRH receptors and intron structure of the type I and type II division was suggested to correlate with the presence or absence of the C-terminal tail (Okubo et al., 2001). Later, Millar et al,. suggested yet another classification based on the phylogenetic analysis of the full length receptor sequences, in which the family was divided into 3 groups, which mixed tailed and tail-less receptors together (Millar et al., 2004). More recently, a phylogenetic analysis of nearly all known GnRH receptor amino acid sequences, including

representation from the agnathans, produced trees divided into three major clades, which represent two main groupings divided based on the presence or absence of a C-terminal tail (Silver et al., 2005), which agrees well with Troskie et al., and Okubo et al.. Ultimately, this naming and grouping system has become overly complicated, and likely inaccurate, and therefore, until more sequence information is available the most reasonable classification would likely come from the simple division based on phylogenetic analysis and the presence or absence of the C-terminal tail.

Vertebrates Express multiple GnRH receptors

Like the GnRH peptide system, it has now been established that most vertebrate species express multiple GnRH receptors. This concept was first confirmed by the identification of two GnRH receptors from the goldfish, termed goldfish GnRH receptor-a and GnRH receptor-b (Illing et al., 1999). Both receptors were shown to have a similar pharmacological profile, and were both shown to be expressed in the pituitary and brain, while only GnRH receptor-a was expressed in the ovary and liver. In 2001, Wang et al., described the cloning of three distinct GnRH receptors in the bullfrog, of which only bullfrog GnRH receptor-1 was shown to be expressed in the pituitary, while bullfrog GnRH receptor-2 and -3 were expressed in the brain (Wang et al., 2001). To date, multiple GnRH receptors have been identified in numerous species, including, as examples, two forms in the catfish (Bogerd et al., 2002; Tensen et al., 1997), three forms in the medaka (Okubo et al., 2003; Okubo et al., 2001), five forms in the salmon (Jodo et al., 2003), European sea bass (Moncaut et al., 2005), while only two forms have been

identified in monkey (Neill et al., 2001; Santra et al., 2000) and human (Chi et al., 1993; Millar et al., 1999).

The location of expression of the GnRH receptors varies widely among receptor subtypes and species. Unlike the GnRH peptide family, the GnRH receptors cannot be to this date divided neatly based on phylogenetic grouping, function or location of expression. The GnRH receptor is primarily expressed in the pituitary, where it functions in the regulation of the reproductive axis. Extra-pituitary GnRH receptor expression is typical, however to what extent and for what function varies from species to species, and is not currently well understood (Millar et al., 2004). However, it is thought that the type II GnRH receptors are more widely expressed than the type I receptors. For example, in primates, using a human tissue array, human type II GnRH receptor RNA was shown to be expressed in 21 different tissues, including the brain, pituitary, heart, stomach, spleen, ovary and testis (Neill et al., 2001) [NOTE the function of the human type II GnRH receptor is still in debate (Faurholm et al., 2001; Morgan and Millar, 2004)]. Conversely, the human type I GnRH receptor is expressed in the pituitary, liver, heart and kidney (Kakar and Jennes, 1995), while the guinea pig type I GnRH receptor was only shown to be expressed in the pituitary with no detectable transcript in the brain, spinal cord, heart, liver, kidney, adrenal gland, spleen, lung, thyroid gland, epididymis or ovary (Fujii et al., 2004). Less data has been collected in reptiles, where transcriptional analysis using RT-PCR in Xenopus indicates expression of the type II GnRH receptor is confined to the pituitary and hind brain, however no expression was detected in the forebrain, kidney, liver, heart or testis (Troskie et al., 2000). Bullfrogs express three distinct GnRH receptor subtypes; using RT-PCR bullfrog GnRH receptor-1 expression was detected in the

pituitary and bullfrog GnRH receptor-2 and -3 were detected in the brain (Wang et al., 2001). In contrast, the type II GnRH receptor from the leopard gecko was shown to be expressed in all tissues examine, including the pituitary, brain, eye, male reproductive components, female reproductive components, kidney, adrenal gland and liver, based on RT-PCR analysis (Ikemoto et al., 2004). There is a wealth of expression data from teleost fishes, from which only a few examples will be drawn. In the goldfish, the two GnRH receptors that have been identified, goldfish GnRH receptor-a and –b, have both been shown to be expressed in the pituitary and brain using *in situ* hybridization, but only goldfish GnRH receptor-a was shown to be expressed in ovary and liver (Illing et al., 1999). Catfish express two type II GnRH receptors, catfish GnRH receptor-1 and -2, which share a similar trend in expression compared to mammals. Catfish GnRH receptor-1, the primary pituitary form, is only expressed outside the pituitary in low levels in the brain. Conversely, the primary brain form, catfish GnRH receptor-2 is more widely expressed, including testes and ovaries. Five GnRH receptors have been identified in the masu salmon (Jodo et al., 2003) and European sea bass (Moncaut et al., 2005), however no activity data were reported to distinguish the functional receptors from possible pseudogenes. In the salmon, all five GnRH receptors are expressed in the brain and only three of these receptors were shown to be expressed in the pituitary. Additional, GnRH receptors were detected in the kidney, ovary and testis, but not muscle, heart, gill or liver. A similar trend was seen in the sea bass. On the other hand, the GnRH receptor from the Japanese eel was only shown to be expressed in the brain, pituitary and testis (Okubo et al., 2000). Lastly, in the most ancient vertebrates, the agnathans, the sea lamprey GnRH receptor is expressed in both the pituitary and testes

using RT-PCR, while *in situ* hybridization showed expression and localization of the transcript in the proximal pars distalis of the pituitary (Silver et al., 2005). The question is what, if anything, does all this expression data mean? Clearly the GnRH receptor is functional in a wide variety of tissues, likely regulating various paracrine / autocrine functions. The exact nature of these systems has yet to be described in a global sense, or in most cases even in each individual local environment. One outstanding interest that comes from these observations is that GnRH receptors are expressed in various cancers, such as prostate (Limonta et al., 1999), ovary (Emons et al., 1993) and breast (Eidne et al., 1985) cancers. A better description of how the GnRH receptor functions in extra-pituitary environments may provide clues to better understand these cancers and may shed light onto new approaches to develop novel therapeutics.

Structure and Regulation of the GnRH Receptor Gene

One potential means to organize the GnRH receptors into subdivisions could be based, in part, on gene structure. There have been several different GnRH receptor gene structures reported to date, from which several conserved introns have been reported, with several unique variations. The typical, perhaps basal gene structure consists of three exons divided by two introns located in transmembrane-4 and intracellular loop-3 (See Figure 8) (Millar et al., 2004). GnRH receptor genes with this structure have been identified in several vertebrates including human (Fan et al., 1994), mouse (Zhou and Sealfon, 1994), sheep (Campion et al., 1996), rat (Reinhart et al., 1997), dog (Cui et al., 2000), Japanese eel (Okubo et al., 2000) and medaka (receptor-1) (Okubo et al., 2001). A third intron is present in both the medaka GnRH receptor-2 gene and chicken GnRH receptor gene,

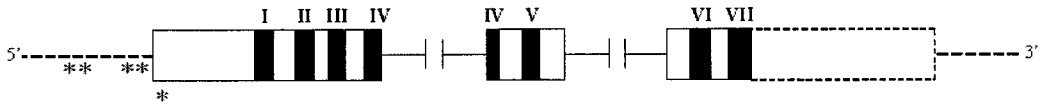

Figure 8. GnRH Receptor Gene Structure. The conserved GnRH receptor gene structure consists of three exons and two introns, located within the coding sequence of the 4th transmembrane domain and 3rd intracellular loop. Unique intron locations in the N-terminus of the coding sequence (*) or within the 5' UTR (**) are demarcated. Region encoding the C-terminal tail is dashed as it is not present in all GnRH receptors.

located in the very N-terminus of the open reading frame (Okubo et al., 2001; Sun et al., 2001), while a similar intron is also located in the -3 or -1 position relative to the start codon in Xenopus (Troskie et al., 2000) and trout (Madigou et al., 2002), respectively. Recently, the gene structure of the type II GnRH receptor from the gecko was described, which contained the conserved introns located in transmembrane-4 and intracellular loop-3, however two introns are located in the 5' untranslated region (Ikemoto et al., 2004). The applicability of this information to the description of the molecular evolution of the GnRH receptor family has promise, however there is not enough gene sequence data at this time to construct a full, robust description. Based on the available information, the presence of the third intron may be useful in distinguishing the subdivision of the tailed type II GnRH receptors, which may help delineate the difference in function of the multi-type II GnRH receptor expressing systems.

The *cis*-acting regulatory elements of the GnRH receptor gene have been characterized primarily using the mouse GnRH receptor gene as a model (Albarracin et al., 1994; Duval et al., 1997a; Duval et al., 1997b; Kam et al., 2005; McGillivray et al., 2005; Norwitz et al., 1999a; Norwitz et al., 1999b) (see Figure 9). The 1.2 kb flanking region of the GnRH receptor gene was initially shown to contain regulatory elements that were involved in gonadotrope specific expression (Albarracin et al., 1994). The 1.2 kb

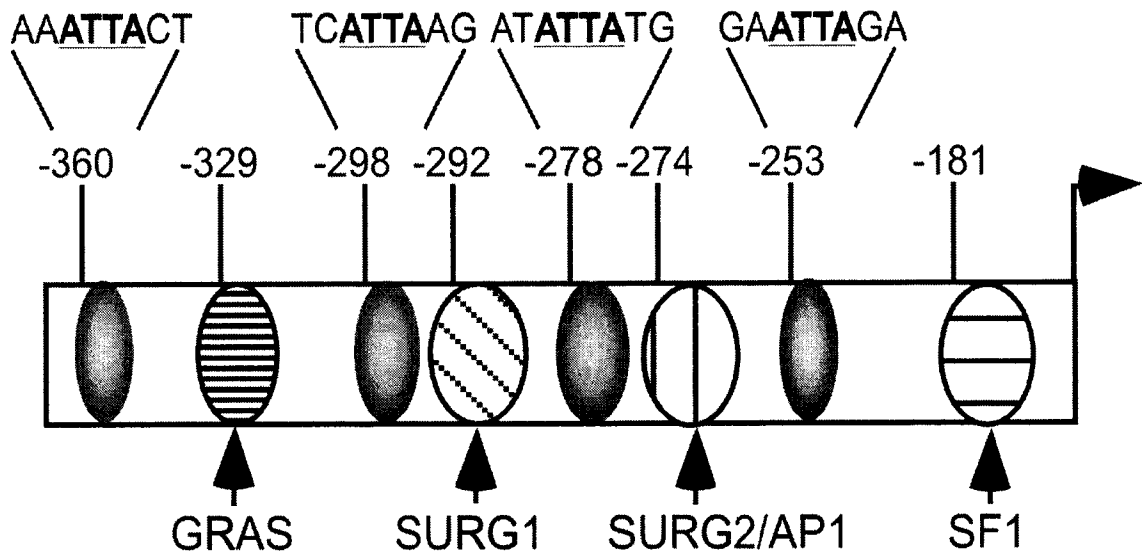

Figure 9. GnRH Receptor Gene 5' Regulatory Elements. Several regulatory elements have been identified in the GnRH receptor gene, as shown in this schematic from the mouse (McGillivray et al., 2005). GRAS, GnRH receptor activating sequence; SURG, sequence underlying responsiveness to GnRH; AP1, activator protein-1; SF1, steroidogenic factor-1.

promoter region of the GnRH receptor gene was shown to be transcriptionaly active, and to have several conserved regulatory elements, including an activator protein 1 (AP1) response element at -274 to -267 relative to the start codon, and a GnRH response element like sequence from position -354 to -335 (Albarracin et al., 1994). The region in between -492 to -235 of the GnRH receptor gene was shown to be sufficient to confer full basal activity, and was shown to not only contain the previously described AP-1 element, but an SF-1 binding site and a unique GnRH receptor activating sequence (GRAS) were identified between -393 and -330 (Duval et al., 1997b). In 1999, the promoter region was re-characterized and was described relative to two sequences underlying responsiveness to GnRH, or SURG elements, which were shown to be regulated via PKC and the activation of the cFos/cJun heterodimer family (AP1) (Norwitz et al., 1999a; Norwitz et al., 1999b). SURG-2, which contains the AP-1 binding site, is located at -276- to -269,

while SURG-1, which interacts with nuclear factor Y and Oct-1 is located between -292 to -285 (Kam et al., 2005; Norwitz et al., 1999a; Norwitz et al., 1999b). Further studies, especially using non-mammalian species, will provide insight into the exact mechanisms involved in tissue specific expression of the GnRH receptor.

GnRH Receptor Structure

Three dimensional structural information is key to the understanding of receptor function as it provides invaluable insight into the mechanisms of ligand interaction and receptor activation, however little information is available about GPCR structure to date. The seven transmembrane motif was first identified in bacteriorhodopsin using electron microscopy (Henderson and Unwin, 1975; Unwin and Henderson, 1975), while more recently the crystal structure for rhodopsin was resolved (Palczewski et al., 2000) (See Figure 10). Several studies have been performed to determine the relationship between structure and function of the GnRH receptor based on sequence conservation analysis, and biochemical and biophysical studies (Sealfon et al., 1997); however the actual three dimensional structure of the GnRH receptor has not been resolved. A model of the mammalian type I GnRH receptor has been proposed based on these studies and the projection map of the electron density of rhodopsin (Millar et al., 1999; Sealfon et al., 1997; Zhou and Sealfon, 1994).

Additional structural elements within the GnRH receptors include glycosylation, phosphorylation and disulfide bridging sites (Sealfon et al., 1997). The human, cow, sheep and pig GnRH receptors contain two putative glycosylation sites, one in the N-terminal domain and one in the first extracellular domain, while the mouse and rat GnRH

Figure 10. Bovine Rhodopsin Crystal Structure. Crystal structure of bovine rhodopsin, looking laterally along the membrane (left) and downward toward the membrane from the extracellular side (Palczewski et al., 2000).

receptors have an additional site in the N-terminal domain (Sealfon et al., 1997). Of these sites, the GnRH receptors have only been shown to be glycosylated on the N-terminal domain, which is thought to be involved in receptor expression and has been shown not to effect the affinity of ligand binding (Hazum and Keinan, 1984; Schvartz and Hazum, 1985). Many GPCRs are regulated through phosphorylation of intracellular serine and threonine residues, most particularly located in the C-terminal tail. GnRH receptors contain several putative phosphorylation sites, however due to the lack of a C-terminal tail in the type I receptors, there is a marked reduction in rapid internalization, which is seen in the tailed type II receptors (See below). A conserved disulfide bridge between the first and second extracellular loops has been shown to be required for receptor stability and ligand binding (Sealfon et al., 1997). The mouse GnRH receptor was shown to contain this conserved disulfide bridge, as demonstrated using mutation analysis, while the human GnRH receptor may have a second disulfide bridge between the N-terminal tail and the second extracellular loop (Sealfon et al., 1997).

GPCR Activation

Activation of GPCR signaling systems is a complex cascade of events that starts with ligand binding to its receptor, which leads to a conformational change that stabilizes the interaction between the receptor and a heterotrimeric G-protein. The exact changes in GPCR conformation and its mechanism of interaction with G-proteins are still not well understood due to the lack of structural data. However, based on the rhodopsin model, it is believed that receptor activation may cause a shift in transmembranes 3 and 6, which may result in the proper intracellular conformation for G-protein activation (Sealfon et al., 1997). The initial theory of receptor activation, referred to as the ternary complex model (De Lean et al., 1980) described a system where an agonist was required to activate a receptor, which would lead to the activation of a G-protein. Alternatively, as described by the two-state model (Samama et al., 1993), the receptor would naturally be in a state of equilibrium between the inactive state (R) and active state (R*), and G-proteins could only interact with R*. Based on this model, the function of an agonist could be described as having a higher affinity for R*, and therefore agonist stabilize the R* conformation, and effectively shift the equilibrium to a higher proportion of R* (Samama et al., 1993). Receptor activation is likely more complicated and may actually result in multiple active state conformations depending on the agonist, which is described by the three-state model (Leff et al., 1997). The three state model describes the receptor function as being in the R and R* states as well as a possible R** state, which describes how a receptor could activate different signaling systems when treated with different agonists, as seen in numerous systems, including the pituitary adenylate cyclase-

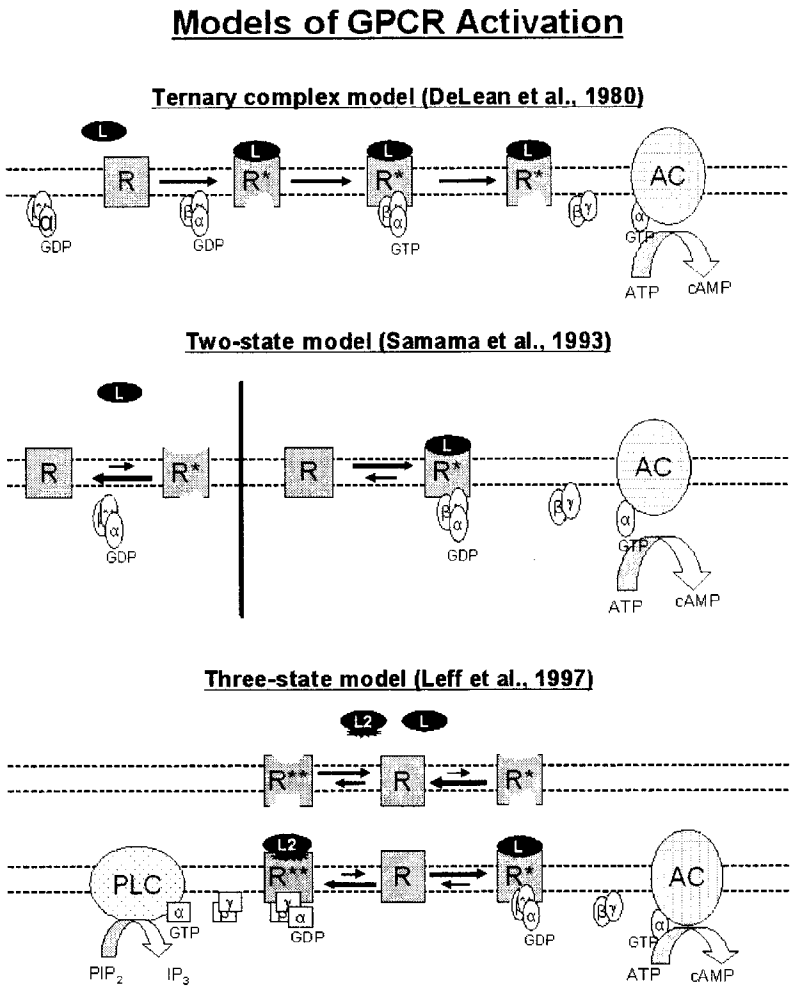

Figure 11. Models of GPCR Activation. These schematics represent the three models of GPCR activation, including the ternary complex, two-state and three-state models. R, inactive receptor; R* receptor in active state; R** receptor in alternative active state; AC, adenylate cyclase; PLC, phospholipase C.

activating polypeptide receptor (Spengler et al., 1993) and serotonin receptor (Berg et al., 1998) (See Figure 11).

After GPCR activation, system specific signaling occurs through the activation of specific G-proteins. G-proteins are heterotrimeric, consisting of a Gα subunit and a Gβγ subunit. There are 16 different Gα subunits, which are grouped into four families: $G\alpha_s$, which activates adenylate cyclase; $G\alpha_i$, which inhibits adenylate cyclase; $G\alpha_q$, which

activates phospholipase C; and $G\alpha_{12/13}$, which activates RhoA, a member of the Ras homology family of small GTPases. Additionally there are five Gβ subunits and 12 Gγ subunits (Bockaert et al., 2003). Exactly how system specific signaling occurs with such a limited number of G-proteins is currently a topic of intense research. In addition to stimulating second messenger production (such as cyclic adenosine monophosphate (cAMP) through adenylate cyclase (AC) or idenosine-1,4,5-triphosphate (IP_3) through phospholipase C (PLC)), GPCR and G-protein activation initiates the mitogen activated protein kinase (MAP kinase) signaling system as well (Kraus et al., 2001). The MAP kinase family consists of four subgroups, including extracellular signal-regulated kinase (ERK), Jun N-terminal kinase (JNK), P38 and Big MAP kinase (BMK) (Kraus et al., 2001). GPCR activation results in MAP kinase activation through several mechanisms. For example, activation of Gαs leading to cAMP accumulation results in the activation of the cAMP dependant protein kinase (PKA), which ultimately results in ERK activation (Birnbaumer, 1992). Alternatively, Gαq activation results in the conversion of phosphotidyl inositide-4,5-bisphosphate (PIP2) to IP3 and diacyl glycerol (DAG), which can link to the MAP kinase cascade in several ways, one of which is through the activation of protein kinase C by DAG, which activates pyk2, which in turn leads to the activation of ERK (Kraus et al., 2001). This multiplicity of GPCR signaling leads to a diverse response ranging from simple signal propagation and hormone release to mRNA transcription and translation as well (Nguyen et al., 2004).

Signaling Systems of the GnRH Receptor

Pituitary GnRH receptors are thought to primarily signal through Gαq11, resulting in the stimulation of the IP3 second messenger system, however Gs activation and cAMP signaling has been reported as well (Grosse et al., 2000; Stanislaus et al., 1998). In αT3-1 cells, a mouse gonadotrope cell line, the GnRH receptor was shown to exclusively couple to Gαq (Grosse et al., 2000), while in LβT-2 cells, a related cell line, both Gαq and GαS coupling was reported (Liu et al., 2002). Furthermore, functional distinction has been made between the signaling systems involved in the activation of type I and type II receptors, as in the case of the latter, Gαs coupling has been well documented (Oh et al., 2005). The presence or absence of the C-Terminal tail in the type II and type I GnRH receptors could possibly explain the signaling disparity between the two groups. Recently, the three bullfrog type II GnRH receptors were shown to activate both IP3 and cAMP systems (Oh et al., 2005). The tail-less mutant forms of the three receptors showed no significant reduction in IP3 signaling after activation of receptor-1 and receptor-3, however all three of the tail-less mutant forms had a significant decrease in cAMP signaling, which was brought to near basal levels in the case of receptor-1 and receptor-2. An HFRK motif in the membrane proximal region of the C-terminal tail was shown to confer cAMP signaling capability in type II GnRH receptors. Addition of this motif to the rat type I GnRH receptor drastically increased the level of cAMP signaling, which suggests this HFRK motif may be responsible for the differential signaling between type I and type II GnRH receptors (Oh et al., 2005).

GnRH receptor signaling through the MAP kinase cascade leading to the regulation of several gonadotrope specific transcripts, such as LH, FSH and the GnRH

receptor itself, has been well documented (Naor et al., 2000). Studies investigating the link between the GnRH receptor, MAP kinase signaling and transcriptional activation have been predominately performed using pituitary cell lines from the mouse (αT3-1 and LβT2), from which it has been shown that GnRH receptor signaling involves all four of the MAP kinase groups, however ERK and JNK activation was most significant (Gur et al., 2001; Levi et al., 1998; Reiss et al., 1997; Sim et al., 1993). In general, the cascade leading to ERK activation begins with Gαq, PLC and PKC, which lead to the activation of RAF, which in turn activates the MAP Kinase-ERK kinase (MEK) which ultimately leads to the activation of ERK (Naor et al., 2000). This cascade of events has been probed using various GnRH independent stimulants or inhibitors, such as tetradecanoyl phorbol acetate (TPA), an activator of PKC, which was shown to mimic GnRH stimulation, the effects of which, however were shown to be blocked by GF 109203X, a PKC inhibitor (Reiss et al., 1997). Furthermore, the MEK inhibitor PD-98059 was shown to eliminate ERK activation in GnRH stimulated αT3-1 cells (Weck et al., 1998). Similar results have been reported using tilapia pituitary cells, where GnRH stimulation of glycoprotein-α and LHβ expression was shown to be inhibited by both PD-98059 and GF 109203X (Gur et al., 2001). These studies indicate that MAP kinase signaling is an important mechanism of gonadotrope functioning that has been conserved across vertebrates.

An alternative mechanism linking the activation of the GnRH receptor to gonadotropin synthesis through a direct link to translational machinery has recently been reported (Nguyen et al., 2004). This mechanism is commonly seen in receptor tyrosine kinase systems, such as in the case of insulin (Mendez et al., 1996), however it has only

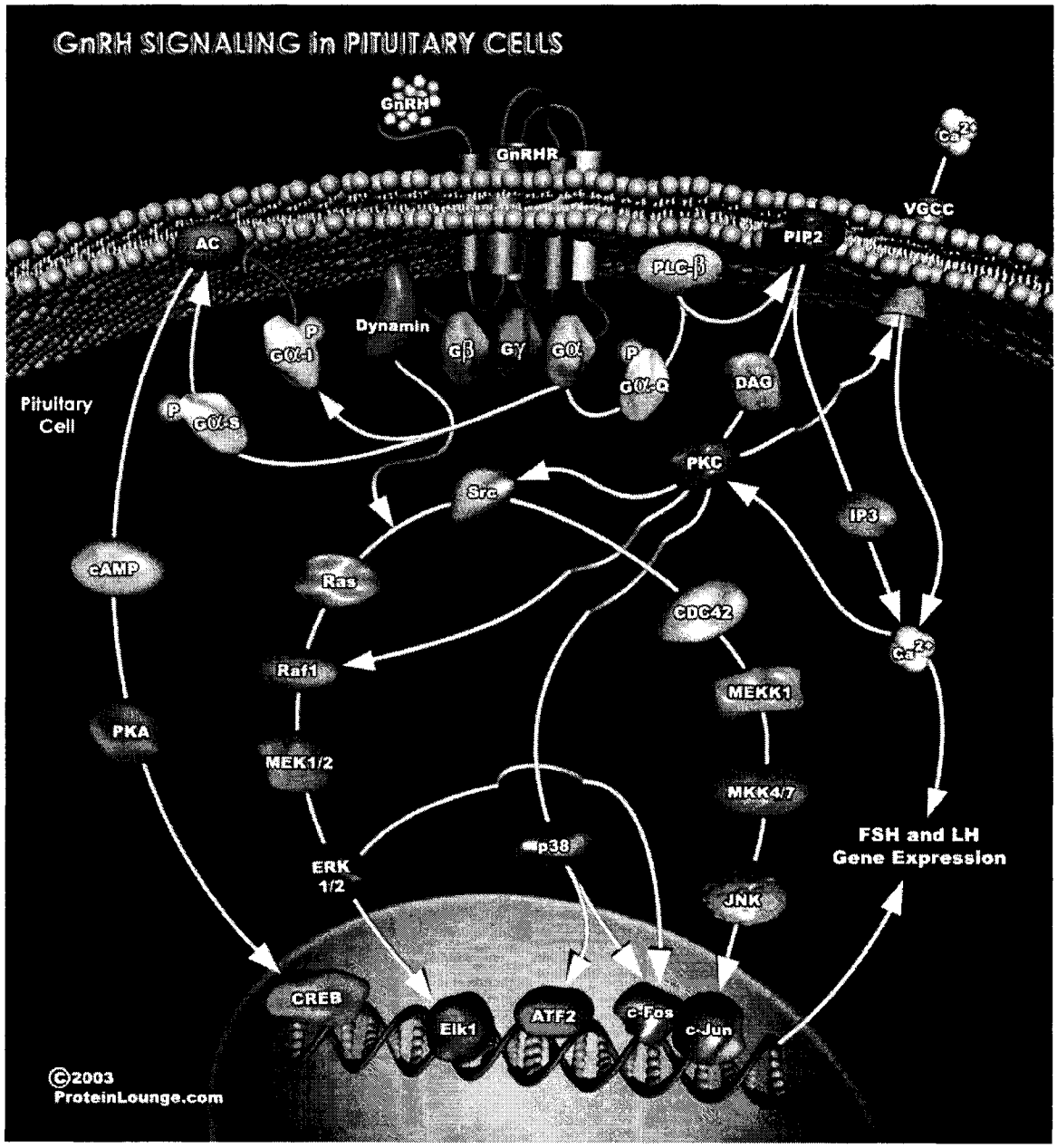

Figure 12. GnRH Receptor Signaling. GnRH receptor activation has been shown to lead to the activation of both IP_3 and cAMP as well as the MAPK signaling systems. (Source: Protein Lounge).

been reported in a handful of GPCR systems, including the μ-opiod receptor, endothelin and angiotensin receptors (Polakiewicz et al., 1998; Wang and Proud, 2002). Stimulation of LβT2 cells with GnRH in the presence of either actinomycin D, an inhibitor of transcription, or cyclohexamide, an inhibitor of translation, revealed a strong

transcriptionally-independent stimulation of LHβ synthesis. GnRH treatment led to a time and dose dependant activation of 4E-binding protein 1, eukaryotic initiation factor 4E and eukaryotic initiation factor 4G, all of which are involved in ribosome assembly during the translational process. This response, however, was abolished by PD-98059, indicating the role of MEK and ERK in this alternative GnRH receptor signaling system (Nguyen et al., 2004).

To summarize, as shown in Figure 12, GnRH receptors predominantly couple with $G\alpha_{q/11}$, leading to the activation of PLC and subsequently IP_3 and DAG production (Grosse et al., 2000). $G\alpha_s$ coupling to GnRH receptors occurs in many instances as well, resulting in AC activation and cAMP production. In addition to second messenger driven GTH release, GnRH receptor activation has been shown to lead to MAP kinase signaling resulting in both translational and transcriptional modulation (Nguyen et al., 2004). GnRH receptor signaling is clearly a multifaceted cascade of events that leads to a diverse level of cellular regulation likely to reflect the various reproductive strategies found across vertebrates.

Regulation of the GnRH Receptor: Functional Significance of the C-Terminal Tail

The regulation of GPCR activity is a critical check point in cellular function that involves multiple levels of control. Unchecked, as demonstrated by both naturally occurring and laboratory constructed mutants, constitutively active GPCRs can lead to a variety of cellular abnormalities, ranging from apoptosis to cancer (Bockaert et al., 2003; Lefkowitz, 2004). Early studies described receptor desensitization (defined as a waning of responsiveness in response to continuous stimulation) by monitoring cellular signaling

events, such as second messenger activation in treated tissues over time, or signal output response, such as hormone release over time (Belchetz et al., 1978; Heding et al., 1998). It wasn't until the first GPCRs were cloned, including the α and β-adrenergic receptors (Dixon et al., 1986; Kobilka et al., 1987), muscerinic receptors (Fukuda et al., 1987) and NK1 tachykinin receptor (Masu et al., 1987) in the mid 1980's, and subsequent mutational analysis studies that elucidated several key mechanisms that lead to desensitization of activated GPCRs through covalent modification, complex formation resulting in steric hindrance and finally receptor relocation. It should be noted that other non-GPCR related mechanisms are involved in the overall desensitization of cellular response, including alteration of transcription and/or translation, degradation, cellular transport and storage, however these pathways lay beyond the scope of this dissertation (Bockaert et al., 2003; Lefkowitz, 2004).

Receptor desensitization (Figure 13) begins through phosphorylation of specific Ser/Thr residues, typically in the C-terminal tail, by either a member of the G-protein coupled receptor kinases (GRK) or a second messenger dependant protein kinase, such as protein kinase A (PKA), protein kinase C (PKC) or casein II. Arrestins (arrestin1 – rhodopsin specific; arrestin 2 (β-arrestin1) and arrestin 3 (β-arrestin2) –general) bind the phosphorylated GPCR, which interferes with association of G-proteins through steric hindrance, leading to a first level of down regulation (McArdle et al., 2002). Furthermore, β-arrestin acts as a scaffold linking the phosphorylated active receptor/β-arrestin complex to clathrin coated pits, which are internalized in a dynamin dependant manor (McArdle et al., 2002). This internalization furthers the level of desensitization through relocation of the receptor, which can no longer interact with extracellular ligands nor can it activate

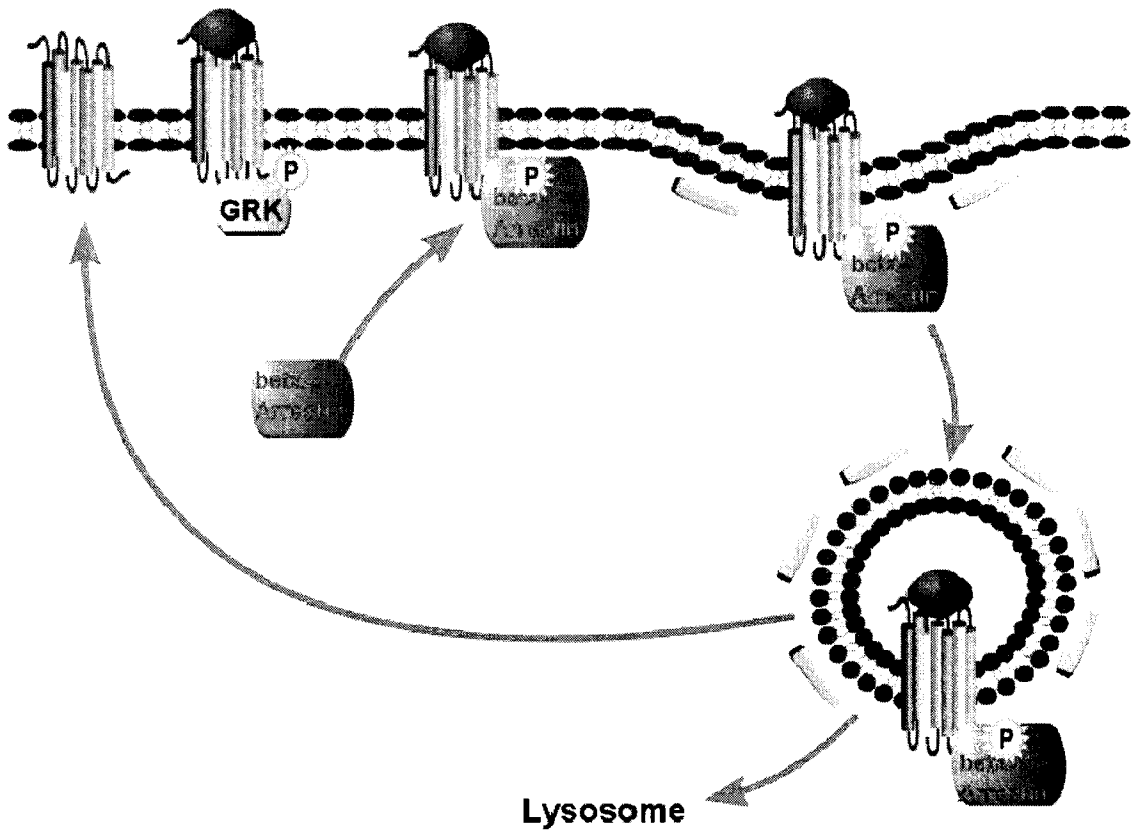

Figure 13. GPCR Internalization. GPCR activation leads to phosphorylation by G-protein coupled receptor kinase (GRK), which stabilizes β-arrestin/GPCR complex formation. This complex is targeted for internalization via dynamin dependant clathrin coated vesicles. When internalized, the receptor complex is either degraded or recycled back to the plasma membrane.

additional G-proteins. Simultaneously, the signaling cascade is deactivated through regulators of G-protein signaling (RGS), which enhances Gα GTPase activity, while second messengers are metabolized, for example through the activity of cAMP specific phosphodiesterases, which convert cAMP to 5' AMP (McArdle et al., 2002; Millar et al., 2004). This rapid (minutes) orchestration of events is an absolutely critical regulatory process, which is still far from being fully understood. It should be noted that there are a number of internalization mechanisms that are currently trying to be elucidated. Simply, these mechanisms can include internalization through β-arrestin independent mechanisms,

or can be β-arresting dependent but dynamin independent or through non-clathrin coated vesicles, such as caveolae (Claing et al., 2000). Numerous studies have been performed identifying these different means of internalization using inhibitors, mutagenesis and dominant negative co-transfections, however the exact mechanisms or molecular markers driving these processes are yet to be established (Claing et al., 2000; Hislop et al., 2005; Ronacher et al., 2004). As a final note, it is important to recognize the potential for receptor internalization through β-arrestin to be a signaling switch, where G-protein mediated second messenger signaling is down regulated however MAPK signaling is activated. The internalized GPCR is linked to the MAPK cascade through the multi-protein complex associated with β-arrestin, which includes a SRC homology domain, kinases and adapter protein 2 (McArdle et al., 2002).

Activation of type II GnRH receptors has been extensively shown to lead to rapid internalization that can be β-arrestin dependent or independent, dynamin dependent or independent and through either clathrin coated or caveolae (Acharjee et al., 2002; Hislop et al., 2005; Pawson et al., 2003; Ronacher et al., 2004). Type I GnRH receptors have been shown to be internalized, however at a significantly slower rate and to a lesser extent when compared to type II GnRH receptors (Hislop et al., 2005; Pawson et al., 1998; Willars et al., 1999). Addition of the C-terminal tail from the type II catfish GnRH receptor to the rat GnRH receptor was shown to result in several functional aberrations, most notably induction of rapid ligand dependant internalization, which was shown to be reversible through C-terminal tail truncations (Lin et al., 1998). Furthermore, removal of the C-terminal tail from the type II chicken GnRH receptor was shown to result in an internalization profile similar to the naturally tail-less human type I GnRH receptor

(Pawson et al., 1998). These studies provided the impetus to further define the motifs involved in this rapid internalization, leading to a series of studies indicating specific Ser/Thr moieties located in both the membrane proximal or distal regions of the C-terminal tail (Blomenrohr et al., 1999; Millar et al., 2004; Pawson et al., 2003; Ronacher et al., 2004). For example, Ser^{363} of the catfish GnRH receptor is a major ligand induced phosphoaccepter, which, through site directed mutagenesis (S363A), was shown to be required for rapid internalization. Mutation of a Thr doubled ($Thr^{369}Thr^{370}$) in the distal region of the chicken GnRH receptor C-terminal tail led to a 70% reduction in ligand dependent internalization compared to wild-type, which was shown to internalize in a β-arrestin independent, dynamin dependant manner through caveolae (Pawson et al., 2003). More recently, Ronacher et al., identified a Ser doublet ($Ser^{338}Ser^{339}$) in the membranes proximal region of the human type II GnRH receptor, which when mutated to Ala led to a 75% reduction in internalization compared to wild-type, which was also shown to require both GRK and dynamin, but not β-arrestin, and to proceed through both clathrin coated pits and caveolae (Ronacher et al., 2004).

To summarize, rapid ligand dependant internalization is a critical distinguishing characteristic between the regulation of tailed type II GnRH receptors and tail-less type I GnRH receptors. Activated type II GnRH receptors can be phosphorylated at Ser/Thr residues within the C-terminal tail, which leads to an interaction with β-arrestin. β-arrestin can block G-protein coupling, link the receptor to G-protein independent signaling mechanisms and also lead to dynamin dependant or independent internalization through clathrin coated vesicles. The lack of rapid internalization of the type I GnRH receptors may reflect the various actions of GnRH in controlling pituitary gonadotropins through diverse regulatory mechanisms.

Agnathans

Lamprey (Petromyzoniformes) and hagfish (Myxiniformes) are the only living representatives of the agnathans, the oldest class of vertebrates, which diverged from the main line of vertebrate evolution approximately 500-550 million years ago (Janvier, 1981). The relationship between lamprey and hagfish is unclear, particularly whether their divergence is monophyletic (lamprey and hagfish form a natural group) or paraphyletic (lamprey being more closely related to the gnathostome (jawed vertebrates) (Forey, 1984; Forey and Janvier, 1993). Lamprey and hagfish have many similarities including the lack of a jaw, internal ossification, scales and paired fins, and each has a single nostril, pore like gill openings, and multicuspid lingual lamina (Forey, 1984; Forey and Janvier, 1993; Hubbs and Potter, 1971). These traits may represent a close relation or rather could be the product of convergent evolution. On the other hand, there are many differences between lamprey and hagfish. For example hagfish are exclusively marine fish, where as lampreys are anadromous. There are also anatomical and physiological differences as well, as shown in Table 1, which is a summarization from Hubbs and Potter, 1971. Using morphology and physiology as a bases for construction of the early vertebrate phylogeny is difficult because of the uncertainty as to which traits are ancestral, and therefore uninformative, and which are derived (Stock and Whitt, 1992). Molecular analysis has shed light onto the problem, where initial studies investigated the molecular evolution of 18S and 28S ribosomal RNA (rRNA), indicating lamprey and hagfish form a monophyletic group, separate from the gnathstomes (Mallatt and Sullivan, 1998; Stock and Whitt, 1992). Furthermore, analysis of protein tyrosine kinase cDNAs (Suga et al., 1999), mitochondrial DNA (Delarbre et al., 2002) and a reconcile tree

Trait	Lamprey	Hagfish
Dorsal Fin	One or Two	None
Eyes	Moderately developed	Very Degenerate
Lingual Lamina	One transverse and two longitudinal	Two longitudinal pairs
Oculomotor Muscles	Yes	No
Teeth	On oral disc and tongue	Tongue and palate
Nasohypophysial opening	On top of the head	In front of the head
Number of Gills	7	5 to 14
Skull	Mostly cartilaginous	Membranous
Eggs	Small and unkeratinized	Large karatinized

Table 1. Distinguishing Characteristics of Lamprey and Hagfish. Examples of differences between the morphology and physiology of the adult lamprey and hagfish, adapted from Pottter et al., 1971.

analysis of 118 gene families (Page, 2000) supports the monophyletic divergence of the agnathans.

Petromyzoniformes

The phylogeny of Petromyzoniforms is less controversial than that of the agnathans as a whole, however a considerable amount of further research in the area would be warranted. The lamprey lineage is divided into three families: the Petromyzonidae, or Northern Hemisphere lamprey, also referred to as the Holarctic species, and the two Southern Hemisphere families, Geotriidae and Mordaciidae (Hubbs and Potter, 1971; Potter, 1980; Potter and Hilliard, 1987), although there are some taxonomists that believe that the *Geotria* and *Mordacia* belong to a single family (Eigenman, 1928; McCulloch, 1929; Scott, 1962). The lamprey lineage will be discussed here forth using this tripartite division, as it is the consensus view in the field.

The Petromyzonidae consists of 6 genera: *Ichthyomyzon, Petromyzon, Caspiomyzon, Eudontomyzon, Tetrapleurodon*, and *Lampetra*, which is further divided

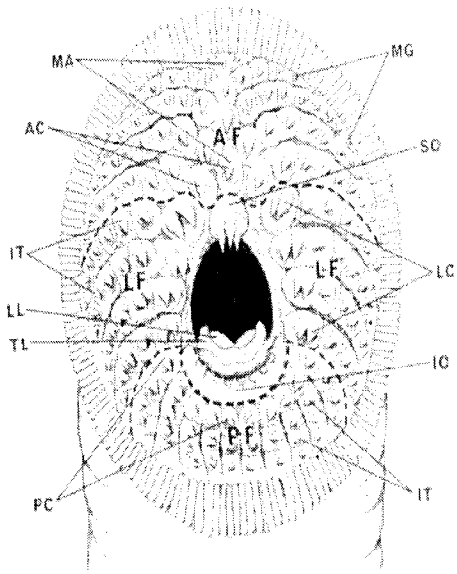

Figure 14. Dentition of the Lamprey. Diagram and key to the terminology used to describe the phylogeny of the lamprey lineage. This is a picture of *Ichthyomyzon bdellium.* This figure has been adapted from Hubbs and Potter, 1971.

Lingual Lamina-multicuspid teeth of the tongue. Consists of the lingual lamina (LT) and longitudinal lingual laminae (LL)
Supraoral (SO)-anterior tooth to the front edge of the oesophageal opening-directly behind the anterior circumorals
Infraoral (IO)-tooth distal to the posterior edge of the oesophageal opening
Circumoral row-the row of teeth, continuous or not, around the oesophageal opening not including the IO of SO. Consisting of the anterior (AC), lateral (LC), and posterior (PC) circumorals
Intermediate Disc Teeth (IT)-teeth between the circumorals and the marginals
Median Anterior Tooth Row (MA)-the row of teeth extending from the suproral on the midline of the disc. The first tooth is referred to as the anteromedian circumoral (AC)
Anterior Field (AF)-the anterior portion of the oral disc, not including the supraoral.
Lateral Field (LF)-teeth within the area of the oral disc that between the anterior and posterior field
Posterior Field (PF)- the posterior portion of the oral disc, not including the infraoral.
Marginals (MG)-all teeth adjacent to the periphery of an alate rows

into the three sub-genera *Entosphenus*, *Lethenteron*, and *Lampetra* (Hubbs and Potter, 1971). The Geotriidae and Mordaciidae each consist of only one genus, *Geotria* and *Mordacia* respectively. This phylogeny of the lamprey lineage is based primarily on dentition (see Figure 14 for dental terminology) and is justified by other shared anatomical traits, such as the proportional measurements of body parts, size of the adult, snout shape, eyes and dorsal fins (Hubbs and Trautman, 1937; Vladykov and Follett, 1967). The *Ichthyomyzon* are thought to be the most ancient of the lamprey because their simple teeth arrangement into rows throughout the entire oral disc. Of the *Ichthyomyzon* species, the *I. Unicuspis* had been considered the most primitive, (Hubbs and Potter, 1971; Hubbs and Trautman, 1937) based on dentition, having mostly undifferentiated

teeth in the anterior, lateral, and posterior field of the circumoral row (Hubbs and Potter, 1971). The *Petromyzon* and *Caspiomyzon* have a similar pattern of undifferentiated teeth in the circumoral row and a small supraoral lamina, but both have slightly more differentiated lateral circumoral teeth. The remaining three genera, all having a larger supraoral lamina and lacking complete alate rows of teeth, are thought to have evolved along two different lines, although both are more similar to the *Petromyzon* then to the *Caspiomyzon* (Hubbs and Potter, 1971; Potter, 1980; Potter and Hilliard, 1987). The *Tetrapleurodon* and *Eudontomyzon* are distinguished from the ancestral stock by their increased number of teeth, although much smaller in size, and number of rows of teeth (Hubbs and Potter, 1971; Potter, 1980). The *Lampetra* are separated from the *Tetrapleurodon* and *Eudontomyzon* as they have much fewer, but larger, teeth and more differentiated teeth in the circumoral row (Hubbs and Potter, 1971; Potter, 1980).

The dentition of the Southern Hemisphere lampreys is considerably different from that of the Holarctic genera and to themselves. The supraoral lamina of the *Geotria* is a wide pronged tooth with four points and that of the *Mordacia* consists of two tricuspids, both markedly different then any of the Holarctic species (Hubbs and Potter, 1971; Potter, 1980). Their retention of the alate rows of teeth in the anterior, lateral, and posterior field suggests an ancient divergence of the Southern Hemisphere lamprey (see Figure 15). This system describing the lamprey phylogeny is unconvincing as it is based entirely on assumptions concerning what dentition patterns represent derived states.

This overall division into three families is supported by sequence analysis of the primary structure of insulin, where the *M. mordax* sequence is more similar to that of the holarctic sequences than to the *G. australis* sequence (Conlon, 2001; Conlon et al., 2001).

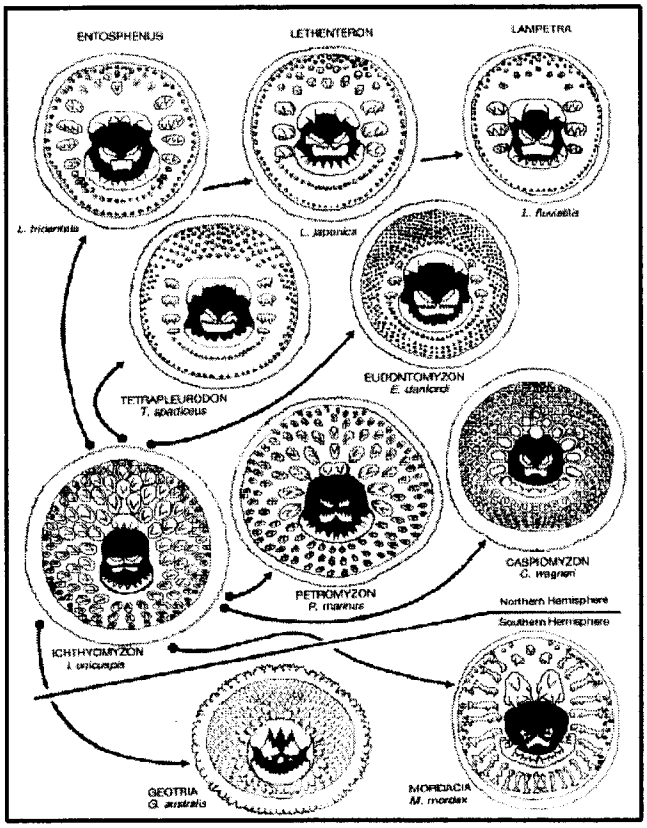

Figure 15. Lamprey Phylogeny Based on Dentition. The lamprey lineage has previously been described based on dentition. The scheme divides the lamprey into three families, including the Petromyzonidae, Geotriidae and Mordaciidae (Potter and Hilliard, 1987).

One of the divisions of lampreys has since been supported by an analysis of mitochondrial genes exclusively within the Lampetra, reconfirming the species designation within this family (Docker et al., 1999), however, further analysis of other characters is needed to better our understanding of the lamprey phylogeny.

Lamprey Ecology

The three families of lampreys are distributed antitropically (at latitudes greater than 30°N/S), except for the *tetrapleurodon*, which are found in high altitude lakes of Mexico (Hardisty and Potter, 1971). Prior to metamorphosis, all lamprey live in a fresh water environment, after which they divide into three classes: fresh water non-parasitic, fresh water parasitic, and anadromous parasitic. The holarctic *Petromyzon marinus* and

Lampetra tridentatus are anadromous parasitic lamprey of vast distribution. The *P. marinus* can be found along the entire eastern coast of the United States and part of Canada, as well as the coastal region of Western Europe, while the *L. tridentatus* is distributed along the western coast of North America, from Alaska to California. Of the two southern hemisphere species the *M. mordax* is the most restricted in distribution, found only in southeast Australia and Tasmania. The *G. australis* is of considerably larger distribution, being found around western and southern Australia, Victoria, Tasmania, New Zealand, Chile, and Argentina (Hubbs and Potter, 1971). The life cycle of anadromous parasitic lamprey begins as a blind, filter-feeding larva, called an ammocoete, burrowed in the sands of fresh water streams. The ammocoete remains burrowed in the stream for three to five years until undergoing metamorphosis to a sexually immature parasitic stage, where eyes begin to develop. The parasitic lamprey migrates to the ocean, where they latch onto, and feed off of, host fish. The parasitic stage lasts up to two years, during this period the lamprey begins sexual maturation. Finally, the lamprey returns to freshwater streams where they become fully mature, spawn, and die (Hardisty and Potter, 1971; Hubbs and Potter, 1971).

Lamprey GnRH

Two primary amino acid forms of GnRH have been identified in the sea lamprey, lamprey GnRH-I and lamprey GnRH-III, both of which were first discovered through protein purification (Sherwood et al., 1986; Sower et al., 1993). In addition, the cDNA and partial gene encoding lamprey GnRH-I (Suzuki et al., 2000) and cDNA encoding lamprey GnRH-III have been cloned (Silver et al., 2004). The amino acid sequence of

the mature lamprey GnRH-I (PGlu-His-Tyr-Ser-Leu-Glu-Trp-Lys-Pro-Gly-NH2) and lamprey GnRH-III (PGlu-His-Trp-Ser-His-Asp-Trp-Lys-Pro-Gly-NH2) decapeptides share the highly conserved N-terminus, C-terminus and Ser in the 4th position, when compared to other vertebrate forms. Interestingly, lamprey GnRH-I and lamprey GnRH-III have a Glu or Asp, respectively, in the 6th position, which is unique among all other vertebrate forms. In general, positions 5-8 are thought to be involved in a β-turn (Sealfon et al., 1997), which is interrupted by the 6th position substitutions in the lamprey GnRH forms, leading to an alteration in their three dimensional structure and therefore function (Sealfon et al., 1997). Indeed, when lamprey GnRH-I is cyclized thereby forming a β-turn conformation, it shows a reduced capacity to stimulate plasma estradiol levels compared to lamprey GnRH-I (Sower et al., 1995a).

Lamprey are the earliest evolved vertebrates for which two forms of GnRH have been clearly demonstrated as functional neurohormones mediating the pituitary-gonadal axis (Sower and Kawauchi, 2001). Both lamprey GnRH-I and lamprey GnRH-III were shown to be expressed in the preoptic anterior hypothalamus and the posterior hypothalamus of the adult sea lamprey using immunocytochemistry (Nozaki et al., 2000). Recently, using *in situ* hybridization, both lamprey GnRH-I and lamprey GnRH-III mRNAs were detected in the preoptic anterior hypothalamus, and furthermore, colocalization was observed in adult lamprey using a dual-label *in situ* approach (Root et al., 2005). Lamprey GnRH-III is considered the most active form during sea lamprey maturation based on the relative number of lamprey GnRH-III producing neurons which is larger than lamprey GnRH-I producing neurons during the larval and adult stages (Nozaki et al., 2000; Tobet et al., 1995) and that lamprey GnRH-III was more potent

compared to lamprey GnRH-I in inducing spermiation in male sea lamprey (Deragon and Sower, 1994; Sower, 2003). Neither lamprey GnRH-I or lamprey GnRH-III peptides have been isolated from any other species of lamprey, although both forms have been detected in the brain and hypothalamus using immunocytochemistry and HPLC coupled with radioimmunoassay or immunocytochemistry in the pouched lamprey (*Geotria australis*), Australian lamprey (*Mordacia mordax*), silver lamprey (*Ichthyomyzon unicuspis*) and western brook lamprey (*L. richardsoni)* (Eisthen and Northcutt, 1996; Sower et al., 2000) and the cDNA encoding lamprey GnRH-III has been cloned from *Petromyzon marinus, Lampetra tridentatus, Lampetra richardsoni, Lampetra appendix, Ichthyomyzon unicuspis, Ichthyomyzon fossor, Mordacia mordax* and *Geotria australis,* see chapter IV (Silver et al., 2004).

Sea Lamprey GnRH Receptor

The lamprey GnRH receptor was first described by means of quantitative *in vitro* autoradiography using ^{125}I-DAla^{6}Pro^{9}NEt mammalian GnRH (Knox et al., 1994). Two high affinity binding sites (Kd $1.5x10^{-12}$M and $5x10^{-9}$M) were identified using the Rosenthal analysis, which were shown to be located predominantly in the proximal pars distalis and to a lesser extent in the rostral pars distalis of the pituitary. These binding sites were shown to be saturable, reversible, tissue specific and time and temperature dependant (Knox et al., 1994). Moreover, lamprey GnRH-I, lamprey GnRH-III, mammalian GnRH, salmon GnRH, chicken GnRH-I, chicken GnRH-II and DPhe^{2,6}Pro^{3} lamprey GnRH were able to compete for binding, while DAla^{6}Pro^{9}-OH lamprey GnRH and TRH were not (Knox et al., 1994). Two high affinity GnRH binding sites in the

pituitary were characterized during the reproductive development of the lamprey (Materne et al., 1997). GnRH binding capacity at both sites increased during the development of the lamprey and correlated with gonadal maturation and brain GnRH content (Materne et al., 1997). Extra-pituitary GnRH binding has been demonstrated as well; one high affinity GnRH binding site was characterized in both the testes and ovary (Gazourian et al., 1997; Materne et al., 1997) and GnRH binding has been shown in choroid plexus within the lamprey brain (Rosen and Sower 1996, unpublished). The presence of a high affinity lamprey GnRH binding site and lack of GnRH in circulation suggests the gonad may produce a GnRH-like factor which serves as an autocrine/paracrine regulator (Gazourian et al., 1997). Finally, the GnRH binding sites in the choroids plexus provides evidence of GnRH action in the brain or alternative transportation mechanisms, where GnRH may be transported throughout the brain by means of the ventricular system (Rosen and Sower 1996, unpublished). Most recently, a cDNA encoding a type II GnRH receptor with a lengthy C-terminal tail was cloned from the sea lamprey (Silver et al., 2005). Lamprey GnRH receptor transcript was detected in the proximal pars distalis using *in situ* hybridization, which correlated well with the previously described high affinity binding sites (Knox et al., 1994), while expression was detected in the testes using RT-PCR (Silver et al., 2005). The functional characterization and binding kinetics of this receptor constitutes the majority of this dissertation, and will be described in detail in subsequent chapters.

Opening the Black Box: Initial Isolation of lamprey GTHβ

The years of exhaustive attempts to isolate GTH from lamprey, however unsuccessful, bore unexpected fruit. Through a series of biochemical, immunological and molecular experiments, numerous lamprey pituitary hormones have been discovered, including adrenocorticotropin (ACTH) (Takahashi et al., 1995), melanotropin (MSH-A and MSH-B) (Takahashi et al., 1995), nasohypophysial factor (NHF) (Sower et al., 1995b), AVT (Lane et al., 1988; Suzuki et al., 1995), proopiocortin (POC) (Heinig et al., 1995), proopiomelanotropin (POM) (Takahashi et al., 1995), and growth hormone (Kawauchi et al., 2002). Histological analysis revealed GTHβ-like cells in the proximal pars distalis of the sea lamprey pituitary (Nozaki et al., 1999), which overlapped with previously described GnRH binding sites (Knox et al., 1994). Screening was performed using a variety of antisera to gonadotropins from various species, however the strongest immunoreactivity was detected using the anti-ovine LH antisera (Nozaki et al., 1999). Using lectin screening, glycoconjugates were detected in the same cell population as GTHβ-immunoreactive cells (Nozaki et al., 1999). Recently, a large scale EST analysis was performed, in which 2208 clones were sequenced, yielding 3 encoding GTHβ (Sower et al., submitted). The lamprey GTHβ cDNA encodes the 134 amino acid mature GTHβ along with a 16 amino acid signal peptide. Lamprey GTHβ contains the conserved 12 Cys residues and 2 putative N-glycosylations sites common to LHβ and FSHβ. Based on phylogenetic analysis, where the lamprey GTHβ was a clear out-group, it was proposed to be closely related to the glycoprotein hormone ancestor (Sower et al., submitted). The existence of a GTHα is still in debate, however preliminary data indicate a GTHα in the lamprey genome (Scott Kavanaugh, personal communication).

Objectives

GnRH and the GnRH receptor are critical components of the vertebrate hypothalamic-pituitary-gonadal axis and hence reproduction. Significant research efforts have been applied to the understanding of how reproductive regulation has evolved across vertebrates, at a physiological and molecular level. The objectives of this dissertation are to address these very issues through the characterization of both the recently cloned lamprey GnRH receptor and lamprey GnRH-III cDNA. Initial characterization of cell culture and activity assays using COS7 cells and the pcDNA3.1 control vector is first described. Using these techniques, the lamprey GnRH receptor was used in a series of functional and binding kinetics studies using the wild-type and C-terminal tail mutants (80 amino acids, 40 amino acids and 0 amino acids). These constructs were used to characterize the lamprey GnRH receptor in regards to second messenger activation (IP_3 and cAMP), binding kinetics (Kd and BMax), pharmacological profile and internalization.

To address the phylogeny of both the GnRH family of peptides and the Petromyzoniform lineage, the cDNA encoding the lamprey GnRH-III was cloned from eight species of lamprey, representing the Petromyzonidae, Mordaciidae and Geotriidae. These sequences were used in a phylogenetic analysis with all known GnRH sequences, providing a more robust analysis of the GnRH family lineage. Analysis of the lamprey GnRH-III cDNAs on their own was used to describe the relationship of the three families of lamprey.

CHAPTER II

LAMPREY GnRH RECEPTOR FUNCTIONAL ASSAY DEVELOPMENT AND INITIAL CHARACTERIZATION

Introduction

The gonadotropin-releasing hormone (GnRH) is the central regulator of reproductive function in all vertebrates. The GnRH decapeptide is produced and released from the preoptic anterior-hypothalamic region of the diencephalon and stimulates the pituitary through its interaction with the gonadotropin-releasing hormone receptor. The GnRH receptor is a member of the class A, rhodopsin like family of 7-transmembrane G-protein coupled receptors (GPCRs), consisting of a core 7 hydrophobic α-helical transmembrane domains connected by three intra and extracellular loops. Activation of the GnRH-R has been shown to be linked to both $G_{q/11}$ and G_s signaling, which lead to the synthesis and secretion of the gonadotropins, luteinizing hormone (LH) and follicle-stimulating hormone (FSH).

The first GnRH-R was cloned from a mouse pituitary cDNA library in 1992, which was shortly followed by the cloning of a human GnRH-R (Kakar et al., 1992; Tsutsumi et al., 1992). Strikingly, both receptors lacked intracellular C-terminal tails, which is a unique feature among GPCRs. These tail-less receptors were of interest as the C-terminal tail is thought to have numerous functional roles, including coupling with

intracellular signaling components as well as desensitization and internalization pathways (McArdle et al., 2002). It was believed that all GnRH-Rs were tail-less until 1997 when a GnRH-R with a C-terminal tail was cloned from the catfish (Tensen et al., 1997). Since then, 40 more GnRH receptors have been cloned, including 11 tail-less receptors and 29 tailed receptors (Millar et al., 2004). Based on structural features (i.e. tailed or not) and phylogenetic analysis, it has been proposed that the GnRH receptors form two separate major groups, the type I receptors, which are tail-less and have only been identified in mammals, and the type II receptors, which have tails and have been identified in vertebrates from Osteichthyes to mammals. Insight into the relationship of the GnRH receptors could be gained through the analysis of an ancestral model, such as the sea lamprey, which could provide clues to better describe the evolutionarily conserved motifs.

The sea lamprey, *Petromyzon marinus*, whose lineage dates back approximately 550 million years, are members of the oldest lineage of vertebrates, the agnathans (Janvier, 1981). Two forms of GnRH have been identified in the sea lamprey, lamprey GnRH-I (Sherwood et al., 1986; Suzuki et al., 2000) and lamprey GnRH–III (Sower et al., 1993; Silver et al., 2004), both of which have been shown to be involved in regulation of the reproductive axis (Deragon and Sower, 1994; Sower, 2003). Two specific GnRH binding sites were demonstrated in the sea lamprey pituitary (Knox et al., 1994), which suggested the lamprey express one, if not two GnRH receptors. Recently, a cDNA was cloned that encoded a protein with seven hydrophobic domains which was identified as a GnRH receptor via homology search (Silver et al., 2005). The cloned sequence was shown to be expressed in the pituitary and testes using RT-PCR, while *in situ* hybridization localized the pituitary expression to the proximal pars distalis, the same

region previous GnRH binding sites where characterized by Knox et al. (Knox et al., 1994).

The aforementioned data suggest the putative lamprey GnRH receptor clone encodes a functional GnRH receptor, however, analysis in a heterologous expression system is absolutely necessary in order to fully describe and classify receptor encoding clones. The objective of this research is to develop and characterize the methodologies to specifically test the function of the cloned putative GnRH receptor, and then to use this heterologous expression system to characterize the response of the lamprey GnRH receptor through second messenger accumulation assays. Using these techniques, the cloned lamprey GnRH receptor was shown to be functional, and to respond to both lamprey GnRH-I and lamprey GnRH-III in a dose dependant manner.

Materials and Methods

Cells, Solutions and Reagents

COS7 cells were provided by Dr. Aniko Fejes-Toth (Dartmouth Medical School), or where purchased from American Type Culture Collection (ATCC). Cells from Dr. Fejes-Toth were used for technical development, while the ATCC cells were used for the functional assay. Cell cultures were maintained using fetal bovine serum (FBS) and Dulbecco's Modified Eagle's Medium, and were passaged using 1X Trypsin-EDTA, all of which were purchased from Invitrogen. Lamprey GnRH-I and lamprey GnRH-III were purchased from American Peptide, and were dissolved in MQ H_2O in a final concentration of 5 mg/ml (4.07 mM lamprey GnRH-I and 3.97 mM lamprey GnRH-III).

Cell line origination and maintenance

Prior to seeding, stocks were removed from cryo-storage and were quickly thawed in a room temperature water bath. Stocks were then centrifuged for 5 minutes at low rpm, and medium containing DMSO was aspirated. Cells were then resuspended in 5 mL in 10% FBS in DMEM. Two T_{75} culture flasks were seeded with 2.5 mL of diluted cells and 12.5 mL of 10% FBS in DMEM. All culture incubations were carried out at 37°C in 5% CO_2. At approximately 90% confluence, cells were passed to new culture flasks or were thinned out via treatment with 1X trypsin-EDTA. Cultures were first washed one time with 10 mL of phosphate buffered saline (PBS), pH 7.4, followed by application of trypsin-EDTA [in a container dependant volume] and were incubated at 37°C for 10 minutes. Cells were collected in 50 mL falcon tubes, and then redistributed into culture plates/flasks and filled to appropriate volume with 10% FBS in DMEM.

Lamprey GnRH receptor construct development

The coding region of the wild type and mutant lamprey GnRH-R was inserted into the pcDNA3.1 mammalian expression vector (Invitrogen) via topo cloning. The lamprey GnRH-R open reading frames were amplified via PCR using the Advantage2 PCR system (CLONTECH) with the LGnRH-R ORF 5' (5'-CAC CAT GGA ACC CAT CAA CAT GAA CAT GAC-3') combined with either the LGnRH-R ORF 3' (to produce the wild type ORF: 5'-TCA GAT GCAGCA GCT TTC AGG ACA TAC GAG AG-3'), LGnRH-R 80aa 3' (to produce the LGnRH-R with an 80aa C-terminal tail: TCA-TGC-CGC-TCT-GTT-CAC-GGG-GAC), LGnRH-R 40aa 3' (to produce the LGnRH-R with a 40aa C-terminal tail: TCA-ACT-CCG-CAC-GGA-CGA-GGC-CGA), or the LGnRH-R

0aa 3' (to produce the tail-less LGnRH-R: TCA-CGC-CGC-GAA-CAC-GCC-GTA-GAT), using the following cycling parameters: a 95°C initial denaturing step for 1 minute followed by 25 cycles of 95°C 15 sec, 62°C 1 minute and 72°C 1 min, followed by a final 5 minute 72°C extension and 10°C hold. 4 μL of PCR product was used in the topo cloning reaction, which was then used to transform Top10 *E. coli* cells (Invitrogen). Clones were screened by sequencing at the HSC Core Research Facility at the University of Utah in at least 3X coverage in order to identify the correct clone. Finally, the clone containing the proper sequence was used to inoculate 100 mL of LB, which was grown overnight prior to plasmid purification using the EndoFree MaxiPrep Plasmid Purification system (Qiagen).

Transfection Optimization

Transfection was performed using the Lipofectamine transfection reagent (Invitrogen) in 60 millimeter culture plates, which was optimized using a pcDNA3.1 vector containing the lacZ gene. Efficiency of transfection was monitored using the β-Gal Histochemical Staining Kit (Invitrogen). Optimization was performed by varying mass of vector used (2.5 μg-15 μg), volume of Lipofectamine (5 μL-15 μL), ratio of vector to Lipofectamine, and finally cell seed number ($5x10^5$ – $1x10^6$). Efficiency of transfection was estimated by comparing the number of blue cells (transfected) to clear cells (non-transfected) after β-Gal staining.

Inositol Phosphate Assay

The inositol phosphate (IP) stimulation and extraction protocol used was adapted from

previous studies (Berg et al., 1994; Ikemoto and Park, 2003; Okubo et al., 2001). Briefly, 24 hours after transfection, cells were trypsinized, and seeded in 12-well plates at approximately $1.5x10^5$ cells/well. At hour 72 cells were washed in PBS and incubated in 1 mL of 2% dialyzed fetal bovine serum and 2 μCi/mL myo[2-^{3}H]inositol (Amersham) in medium 199 (Invitrogen). At hour 96 cells were washed 2 times with IP buffer (20 mM HEPES, 20 mM LiCl in 1X HBSS), and were pre-incubated in IP buffer for 15 minutes at 37 °C, followed by stimulation with either lamprey GnRH-I (American Peptide Company, Sunnyvale, CA) or lamprey GnRH–III (American Peptide Company) in IP buffer (concentrations ranging from 10^{-6}M to 10^{-11} M for dose response analysis or 10^{-6}M for the C-terminal tail functional analysis), for 1 hour at 37 °C with gentle shaking. The reactions were stopped with the addition of 0.2 mL of pre-chilled 20% perchloric acid, and the plates were placed on ice for 30 minutes. The wells were scraped and the extracts were transferred to sterile 1.5 mL tubes and neutralized with 5M KOH, followed by a 1 hour incubation at 4°C. Tubes were centrifuged at 5,000 rpm at 4°C for 15 minutes, and 1.2 mL of supernatant was transferred to a new sterile 1.5 mL tube. IPs (IP, IP_2 and IP_3) were isolated by ion exchange chromatography using AG1X8-200 resin (Bio-Rad) in formate form. IPs were eluted with 1 M ammonium formate / 0.1 M formic acid, samples were counted by liquid scintillation and data were analyzed using Prism (GraphPad). Treatments were performed in triplicate in three independent experiments, and cells transfected with blank vector and non-transfected cells were used as negative controls.

Results

Construct Development

The wild type and mutant lamprey GnRH receptor open reading frames were amplified via PCR, and were verified by both gel electrophoresis (see Figure 16) and sequence analysis (see Figure 17). Restriction digest indicated the vectors contained an insert of appropriate size, however PCR confirmed that there was no trace level of contamination. Sequence analysis was ultimately used in order to verify that there were no errors introduced into the sequences during amplification steps. Vectors were confirmed to contain the appropriate ORF prior to use.

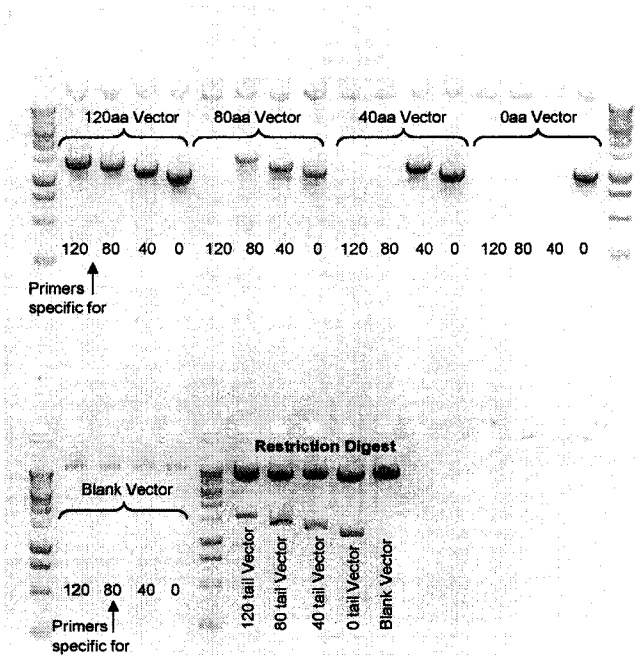

Figure 16. Mutant Construct Analysis. Both PCR and restriction digests were used to verify the lamprey GnRH receptor constructs. Each vector, containing the wild type (120 aa), 80aa, 40aa, 0aa (tail-less) or blank (control) was used as template for PCR using primers specific to each transcript (as noted at the bottom of each lane. Results from the restriction digest are shown in the bottom right of the gel, where each lane contains a digest of a different vector, as denoted in each lane.

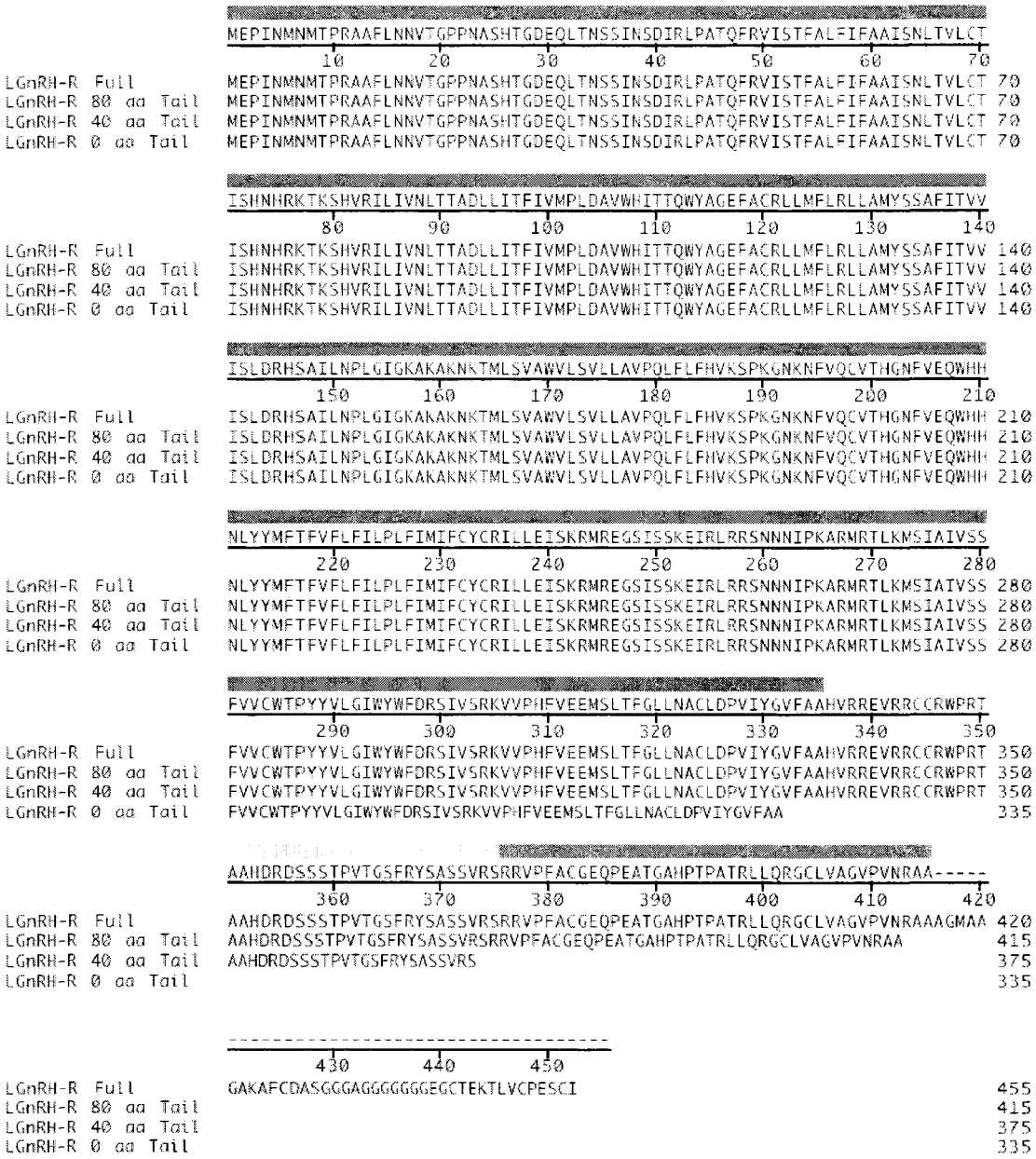

Figure 17. Lamprey GnRH Receptor C-Terminal Tail Truncations. Alignment of the lamprey GnRH receptor mutants.

Transfection Optimization

Transfection of COS7 cells with 5 μg of the pcDNA3.1 vector containing the lacZ gene using 15 μL of Lipofectamine was determined to be optimal. Increasing seed number from $1x10^5$ to $1x10^8$ was shown to increase the number of transfected cells, however the efficiency of transfection dramatically decreased (Figure 18).

Lamprey GnRH receptor Functional Analysis

Both lamprey GnRH-I and lamprey GnRH-III stimulated a significant response in IP accumulation, in a dose dependant manner, in COS7 cells that were transiently transfected with the lamprey GnRH receptor (Figure 19). The $LogEC_{50}$ (represented as mean ± SEM; n=3) of lamprey GnRH-III (-9.37 ± 0.243) was significantly ($P < 0.0002$) lower then the $LogEC_{50}$ of lamprey GnRH-I (-8.10 ± 0.150). This significant difference in IP activation suggests the presently cloned lamprey GnRH-R is lamprey GnRH-III selective. Cells transfected with blank pcDNA3.1 vector showed no response in IP accumulation following treatment with either lamprey GnRH-I or lamprey GnRH-III (data not shown).

Despite this difference in LogEC50, both lamprey GnRH-I and lamprey GnRH-III were shown to stimulate a statistically indistinguishable maximum level of IP accumulation when the wild type or mutant lamprey GnRH receptors were treated with a high dose (10^{-6}M) (Figure 20). Serial truncation of the lamprey GnRH receptor resulted in an initial decrease in magnitude of IP signaling. The 80aa C-terminal tail resulted in an approximately 43% reduction in signal relative to wild type. Further truncation of the

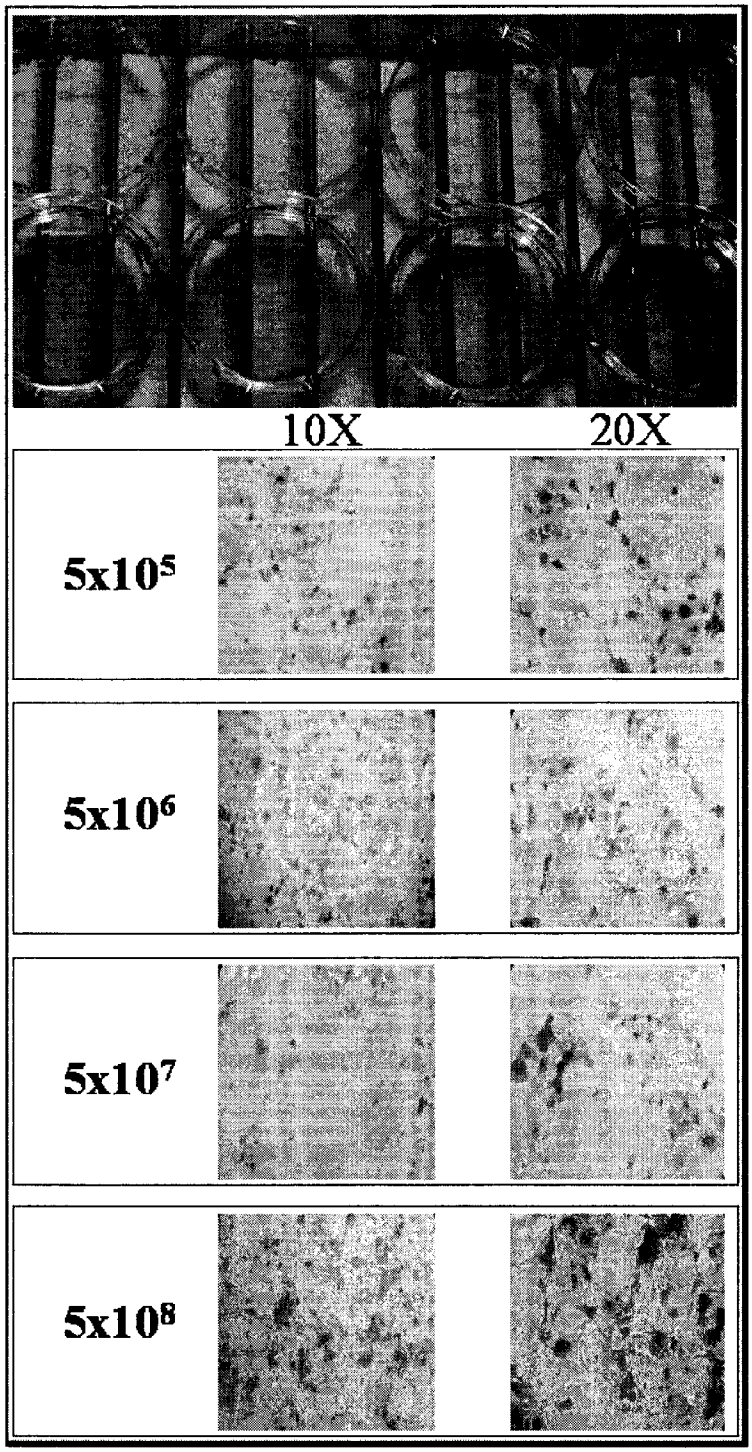

Figure 18. Transfection Optimization. Transfection was optimized using the lacZ gene. Shown here, increasing numbers of cells were seeded prior to transfection in order to optimize cell density. Percent transfection was determined based on the number of blue cells relative to the number of total cells (blue and clear). Top picture runs in decreasing order from left to right.

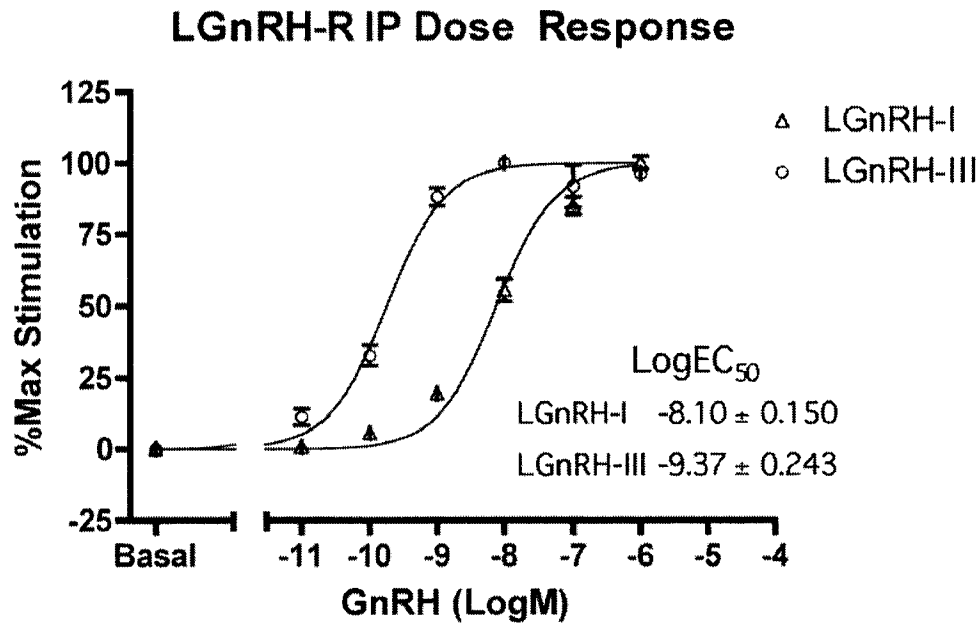

Figure 19. Lamprey GnRH Receptor IP Dose Response. The lamprey GnRH receptor was shown to activate the IP signaling system, in a dose dependant manner, in transiently transfected COS7 cells. Lamprey GnRH-III stimulated IP accumulation at a significantly lower $logEC_{50}$ when compared to lamprey GnRH-I ($P<0.002$). $LogEC_{50}$ shown as mean ± SEM; N=3.

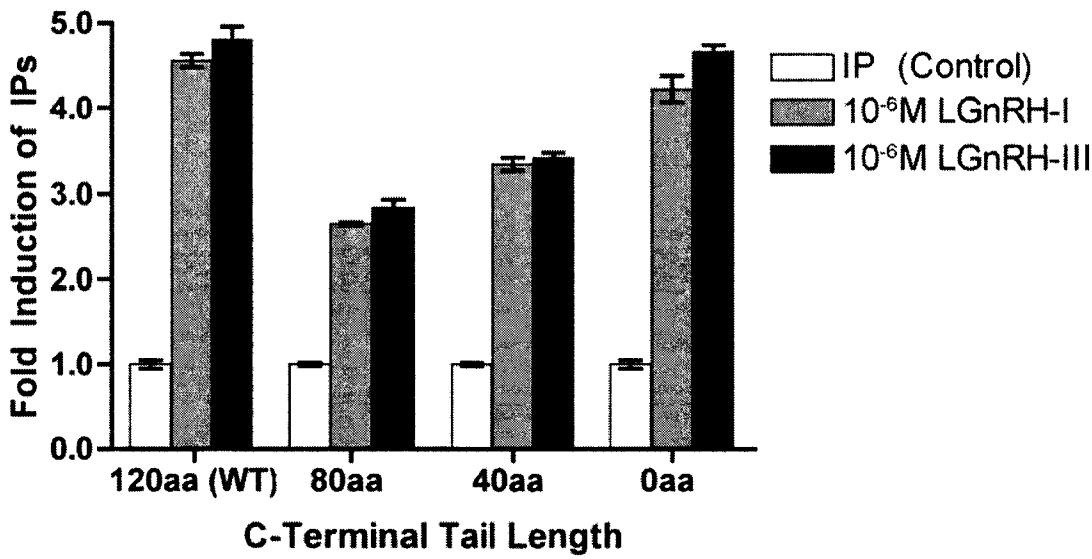

Figure 20. Efficacy of Lamprey GnRH Receptors with C-Terminal Tail Truncations. The wild-type lamprey GnRH receptor and mutants containing intracellular C-terminal tail truncations were expressed in COS7 cells and treated with a maximum dose (10-6 M) of lamprey GnRH-I, lamprey GNRH-III or control (IP buffer). Functional analysis was performed based on the fold induction of IP stimulation relative to control within each group. Truncations of the lamprey GnRH receptor results in a decrease in IP production, which is fully recovered in the tail-less mutant form.

C-terminal tail to 40aa also showed a decrease in signal relative to wild type (28% reduction), however to a lesser extent as compared to the 80aa C-terminal tail. Finally, the magnitude of signaling of the tail-less lamprey GnRH receptor mutant was shown to fully recover, producing IP levels comparable to wild type.

Discussion

Through the characterization and optimization of cell culture and transfection techniques, the cloned lamprey GnRH receptor has been shown to be functional. Optimal transfection efficiency was shown to be achieved when performed in 60 millimeter culture plates with $5x10^5$ cells using 5 μg vector and 15 μL of Lipofectamine. Stimulation of the transfected receptor with either lamprey GnRH-I or lamprey GnRH-III led to an accumulation of IP in a dose dependant manner. Lamprey GnRH receptor C-terminal tail truncations resulted in an initial decrease in IP production relative to wild type, which was fully recovered by the tail-less mutant. These data suggest the cloned lamprey GnRH receptor is lamprey GnRH-III selective.

The GnRH receptor family is unique among G protein-coupled receptors because a number of its members lack an intracellular C-terminal tail. All of these tailless receptors have been identified from mammalian species, and since the first six GnRH receptors identified were tailless, it was originally thought that all GnRH receptors lacked a C-terminal tail. In 1997, the first GnRH receptor with a C-terminal tail was identified in the African catfish (Tensen et al., 1997). The implications of this variation in presence or absence of the C-terminal tail remains an important subject of investigation. Various studies have shown that the C-terminal tail of GPCRs has an important role in controlling

expression, membrane cycling, desensitization, and G protein activation (Blomenrohr et al., 2002; Sealfon et al., 1997). Progressive truncation of the C-terminal tail of the catfish receptor 1 was shown to drastically and progressively reduce surface expression of the receptor (Blomenrohr et al., 1999; Bogerd et al., 2002), and addition of the catfish GnRH receptor 1 C-terminal tail to the rat GnRH receptor greatly increased the surface expression of the receptor construct (Lin and Conn, 1998). The known tailed GnRH receptors contain intracellular tails of varying sizes from 40 – 80 amino acids in length. The lamprey GnRH receptor includes a C-terminal tail of 120 amino acids; this is the longest C-terminal end identified to date in a GnRH receptor. An initial analysis of the functional significance of the lamprey GnRH receptor's lengthy C-terminal tail was performed based on the magnitude of IP signaling using mutants of the lamprey GnRH receptor containing serial truncations of the intracellular C-terminal tail (120aa (wild type), 80aa, 40 aa and 0aa (tail-less)), which were stimulated with a maximal dose (10^{-6} M) of either lamprey GnRH-I or lamprey GnRH-III. Partial truncations of the C-terminal tail resulted in a decrease in the magnitude of IP accumulation compared to wild type, however signaling was fully recovered by the tail-less mutant of the lamprey GnRH receptor, as shown in Figure 20. Interpretation of these data can be difficult, however in conjunction with previous studies of the function of other GnRH receptor C-terminal tails (Blomenrohr et al., 1999; Lin and Conn, 1998; Oh et al., 2005; Pawson et al., 1998) we suggest these findings indicate that truncation of the lamprey GnRH receptor C-terminal tail may cause a structural alteration which decreases ligand binding affinity and therefore decreases IP signaling. Alternatively, this structural alteration could lead to a disturbance in G protein binding to the activated receptor, however it may likely be a

combination of both (Blomenrohr et al., 1999). Interestingly, the magnitude of IP signaling of the tail-less lamprey GnRH receptor is equivalent to the wild type, which we speculate to be due to a compensation resulting from a reduced level of ligand dependant internalization, which is typically seen in tail-less type I GnRH receptors and some tail-less type II GnRH receptor mutants (Blomenrohr et al., 1999; Lin and Conn, 1998; Oh et al., 2005). This suggestion that the tail-less mutant may be subjected to a reduced level of ligand dependant internalization is supported by the fact that all of the phosphoacceptor sites located within the lamprey GnRH receptor's C-terminal tail are located within the first 40 amino acids, as predicted by the CBS Prediction Server at the Technical University of Denmark (Figure 21). Additionally, the long C-terminal tail of the lamprey GnRH receptor may function, in part, to produce the high levels of surface expression demonstrated in our previous GnRH binding studies (Knox et al., 1994; Materne et al., 1997). This analysis indicates the lamprey GnRH receptor possesses several of the conserved structural and functional attributes of both type I and type II GnRH receptors, and is therefore likely a representative of the ancestral form. Further analysis of the function of the lamprey GnRH receptor's lengthy C-terminal tail on the level of cell surface expression, ligand binding affinity and internalization is the subject of ongoing research in our laboratory, which will be necessary to fully describe this unique system.

In conclusion, cell culture and transfection techniques were established, which were used to show that stimulation of the cloned lamprey GnRH receptor led to a dose dependant response in accumulation of IP when treated with either lamprey GnRH-I or lamprey GnRH-III. Functional analysis of the lamprey GnRH receptor C-terminal tail

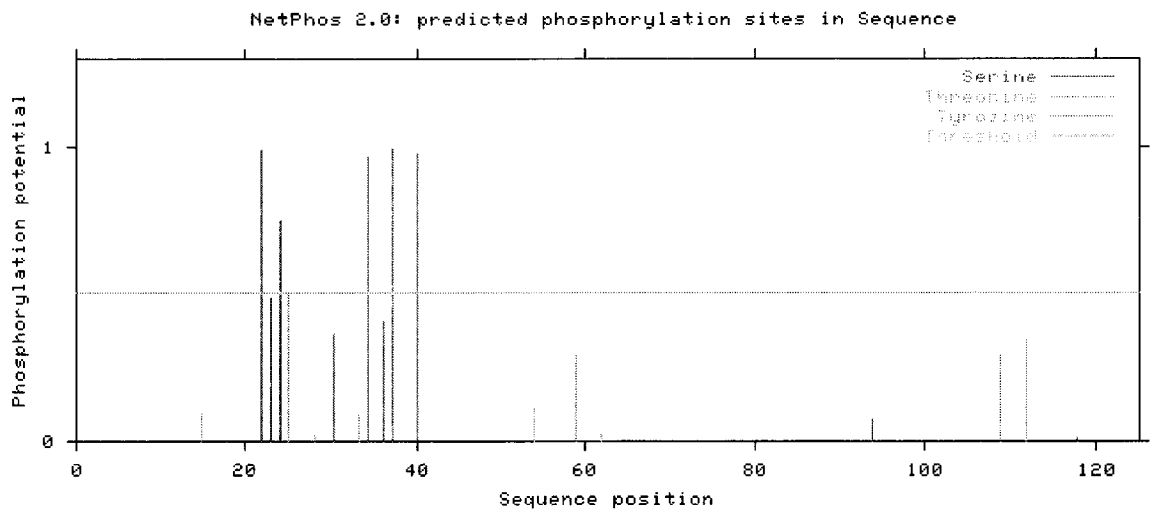

Figure 21. Predicted Phosphoacceptor Sites Located Within the Lamprey GnRH Receptor C-Terminal Tail. The lamprey GnRH receptor C-terminal tail amino acid sequence was used to search for predicted phosphoacceptor sites using the NetPhos 2.0 search tool through the Technical University of Denmark's Center for Biological Sequence Analysis. Sequence position referes to amino acid number within the C-terminal tail.

indicates it's significant role in IP signaling and internalization. In addition to having an important role in lamprey reproductive processes, the extensive C-terminal tail of the lamprey GnRH receptor has great significance for understanding the evolutionary change of this vital structural feature within the GnRH receptor family.

CHAPTER III

FUNCTIONAL CHARACTERIZATION AND KINETIC STUDIES OF AN ANCESTRAL LAMPREY GnRH-III SELECTIVE TYPE II GnRH RECEPTOR FROM THE SEA LAMPREY, *PETROMYZON MARINUS*

Introduction

The vertebrate hypothalamic-pituitary-gonadal (HPG) axis is regulated by the gonadotropin-releasing hormone (GnRH), a decapeptide hormone that is produced and released from the hypothalamus. At the anterior pituitary, GnRH action is mediated through high affinity binding with the GnRH receptor, a class A, or rhodopsin-like seven transmembrane G-protein coupled receptor (GPCR) (Millar et al., 2004). The GnRH receptor is unique among all GPCRs in that the type I mammalian GnRH receptors lack the highly conserved intracellular carboxy-terminal (C-terminal) tail, which has been shown to be a vital structural element required for several key functions, such as G-protein coupling and second messenger activation, ligand binding, cell surface expression and ligand dependant internalization (Blomenrohr et al., 1999; Bockaert et al., 2003; Heding et al., 1998; Koenig and Edwardson, 1997; Ronacher et al., 2004). Based simply on the presence or absence of a C-terminal tail, the GnRH receptors can be divided into two groups; the type I tail-less GnRH receptors, which have only been identified in mammals and the C-terminal tail containing type II GnRH receptors, which have been

identified across the vertebrate lineage (Ikemoto et al., 2004; Okubo et al., 2001; Silver et al., 2005).

GnRH receptor signaling has been characterized in several systems, and is primarily thought to function through the IP_3 second messenger pathway, however type II GnRH receptors and in some cases type I GnRH receptors have been shown to also activate cAMP signaling (Arora et al., 1998; Grosse et al., 2000; Liu et al., 2002; Oh et al., 2005; Stanislaus et al., 1998). The presence or absence of the C-terminal tail in the type II and type I GnRH receptors, respectively, could possibly explain the signaling disparity between the two groups, where, for example, an HFRK motif in the membrane proximal region of the bullfrog type II GnRH receptors was recently shown to be required for cAMP signaling (Oh et al., 2005). Rapid, ligand dependant internalization resulting in desensitization and/or signal switching is another key functional difference between type I and type II GnRH receptors, which has been described and attributed to the presence or absence of specific Ser/Thr residues located throughout the C-terminal tail (Blomenrohr et al., 1999; Pawson et al., 2003; Ronacher et al., 2004; Willars et al., 1999).

As an agnathan, the oldest class of vertebrates, the sea lamprey has become a model system for the analysis of the evolution of the neuroendocrine regulation of reproduction (Sower, 2003). Lamprey express two forms of GnRH, lamprey GnRH-I and lamprey GnRH-III, both of which are produced in the hypothalamus and have been shown to regulate the reproductive axis (Deragon and Sower, 1994; Sherwood et al., 1986; Silver et al., 2004; Sower et al., 1993; Suzuki et al., 2000). Recently, a GnRH receptor cDNA was cloned from the sea lamprey, Petromyzon marinus, which contained

a C-terminal tail of 120 amino acids, the longest of any previously described GnRH receptor (Silver et al., 2005). To better describe the lamprey GnRH receptor a series of functional and pharmacological assays were performed in the current study. The lamprey GnRH receptor was shown to stimulate the cAMP signaling system, in a dose dependant manner, which, through mutagenesis studies, was shown to depend on the presence of the C-terminal tail. The C-terminal tail was also shown to be required for rapid ligand dependant internalization, binding affinity and to some degree, cell surface expression. Finally, pharmacological profiling, in conjunction with these and previous efficacy data confirms that the lamprey GnRH receptor is lamprey GnRH-III selective. These data indicate that the lamprey GnRH receptor shares several characteristics of both type I and type II GnRH receptors, and may represent an ancestral form, which provides insight into the evolution and function of the GnRH receptor family.

Materials and Methods

Cell Culture, Construct Development and Transfection

COS7 cells (American Type Culture Collection, Manassas, VA) were maintained in 10% fetal bovine serum (FBS) in Dulbecco's Modified Eagle's Medium (DMEM) at 37°C in 5% CO_2. The coding regions of the wild type and mutant lamprey GnRH receptor (GenBank Accession Number AF439802) (See Figure 17) were inserted into the pcDNA3.1 mammalian expression vector (Invitrogen, Carlsbad, CA). The lamprey GnRH receptor open reading frames were amplified via PCR using the Advantage2 PCR system (CLONTECH, Mountain View, CA) with the LGnRH-R ORF 5' (5'-CAC CAT

GGA ACC CAT CAA CAT GAA CAT GAC-3') combined with either the LGnRH-R ORF 3' (to produce the wild type ORF: 5'-TCA GAT GCAGCA GCT TTC AGG ACA TAC GAG AG-3'), LGnRH-R 80aa 3' (to produce the lamprey GnRH receptor with an 80aa C-terminal tail: TCA-TGC-CGC-TCT-GTT-CAC-GGG-GAC), LGnRH-R 40aa 3' (to produce the lamprey GnRH receptor with a 40aa C-terminal tail: TCA-ACT-CCG-CAC-GGA-CGA-GGC-CGA), or the LGnRH-R 0aa 3' (to produce the tail-less lamprey GnRH receptor: TCA-CGC-CGC-GAA-CAC-GCC-GTA-GAT). The day prior to transfection, $5x10^5$ cells were seeded in 60-millimeter culture plates. Transfection was performed using 5 μg of vector and 15 μL of Lipofectamine (Invitrogen) in 2.4 mL total volume in Opti-MEM-I (Invitrogen) per culture.

LGnRH Receptor cAMP Assay

The day after transfection, cells were trypsinized and 96-well plates were seeded with $5x10^4$ cells/well, and cultures were grown overnight. On day 3, cells were stimulated with either control (ID buffer - 1.0 mM 3-isobutyl-1-methylxanthine in DMEM), LGnRH-I (American Peptide Company, Sunnyvale, CA) or LGnRH-III (American Peptide Company) in ID buffer (concentrations ranging from 10^{-5} M to 10^{-10} M for dose response analysis or 10^{-5} M for the C-terminal tail functional analysis) for 1 hr at 37°C. Treatments were performed in triplicate and cells transfected with blank vector were used as negative controls. cAMP assays were performed using the BioTrak Enzymeimmunoassay system (Amersham), per the manufacturers instructions. Analyses were performed using Prism (GraphPad San Diego, CA).

Whole Cell LGnRH Receptor Saturation Binding Assay

Saturation binding assays were performed using adherent, intact cells with ^{125}I-lamprey GnRH-I. ^{125}I-lamprey GnRH-I was iodinated using a modification of the chloramine-T method, which was purified as described by Stopa et al.(Stopa et al., 1988). As a note, lamprey GnRH-III was not used as the radioligand since it cannot be iodinated due to the lack of a Tyr residue. For saturation binding assays, the day after transfecting COS7 cells with either the wild type of mutant lamprey GnRH receptors, $1x10^5$ cells were seeded into 24 well plates in 500 µL of medium, which were grown for 2 days at 37°C in 5% CO_2. Cells were then washed one time in 500 µL of assay buffer (25 mM HEPES modified DMEM with 0.1% BSA), followed by incubation with increasing concentrations of ^{125}I-lamprey GnRH-I (1nM to 100nM), in 200 µL total volume of assay buffer, for 3.5 hrs on ice in the 4°C cold room. Non-specific binding was determined using cells incubated with both ^{125}I-lamprey GnRH-I and 10 µM cold lamprey GnRH-III. After 3.5 hrs, cells were quickly washed two times with 500 µL of ice cold PBS and cells were examined using an inverted microscope to insure no cells were lost. Cells were solubilized with 300 µL of 0.5 M NaOH, 0.1% BSA, and bound ^{125}I-lamprey GnRH-I was counted using a γ-counter. All total binding samples were run in triplicate, while NSBs were run in duplicate, and each independent experiment was repeated 2-3 times. Data were analyzed using Prism (GraphPad).

Competitive Binding Analysis

Competitive binding properties of lamprey GnRH-I, lamprey GnRH-III, chicken GnRH-II (Peninsula Laboratories, San Carlos, CA), mammalian GnRH (Peninsula Laboratories)

and DAla^6-Pro^9-NEt mGnRH (Peninsula Laboratories) were performed using COS7 cells transfected with the wild type lamprey GnRH receptor. Cells were prepared as described above, however the ^{125}I-lamprey GnRH-I concentration was held constant at 10 nM, with either assay buffer (total binding) or increasing concentrations of cold competing ligand in assay buffer (ranging from 10^{-13} M to 10^{-6} M) in 200 µL total volume for 3.5 hrs on ice in the 4°C incubator. Cells were washed and processed as described above. All samples were run in triplicate, in three independent experiments. Data were analyzed using Prism (GraphPad).

LGnRH Receptor Internalization Assay

Internalization of ^{125}I-LGnRH-I was performed based on the acid-wash method, as previously described (Hazum et al., 1983; King et al., 2000; Pawson et al., 1998). Briefly, COS7 cells transfected with the wild type lamprey GnRH receptor or C-terminal tail truncated mutants were seeded ($1x10^5$ cells) in 24 well plates in 500 µL of medium, and were grown for 48 hours. Cells were incubated with 10 nM ^{125}I-LGnRH-I on ice at 4°C for 3.5 hours. Cells were then immediately brought to 37°C for increasing periods of time. At the end of each time point, cells were placed on ice and were washed two times with 500 µL of ice cold PBS. Acid sensitive (surface bound) ^{125}I-LGnRH was washed away by the addition of 0.3 mL of acid solution (150 mM NaCl, 50 mM acetic acid, pH 2.8) for 12 min. The acid wash was removed, and acid insensitive (internalized) ligand was recovered using solubilizing reagent (0.5 M NaOH with 0.1% SDS). Both acid sensitive and insensitive binding was quantified via γ-counter, and percent internalization was determined based on comparison of internalized ligand to total cell associated ligand

(internalized + surface bound). NSBs were determined using non-transfected cells. Treatments were performed in triplicate, and independent experiments were run 3 times for each receptor construct. Data were analyzed using Prism (GraphPad).

Results

Lamprey GnRH receptor cAMP activation

Both lamprey GnRH-I and lamprey GnRH-III stimulated a significant response in cAMP accumulation, in a dose dependant manner, in COS7 cells that were transiently transfected with the lamprey GnRH receptor (Figure 22). The $LogEC_{50}$ (represented as mean ± SEM; n=3) of lamprey GnRH-III (-8.29 ± 0.090) was significantly ($P < 0.0001$) lower then the $LogEC_{50}$ of lamprey GnRH-I (-6.57 ± 0.150). This approximately 50-fold difference suggests that the presently cloned lamprey GnRH-R is lamprey GnRH-III selective. Cells transfected with blank pcDNA3.1vector did not respond to treatment with ether lamprey GnRH-I or lamprey GnRH-III (data not shown).

Consistent with this difference in $LogEC_{50}$, lamprey GnRH-III stimulated a larger magnitude of cAMP accumulation, compared to lamprey GnRH-I, when the wild type or mutant lamprey GnRH receptors were treated with a maximum dose (10^{-5}M) (Figure 23). Stimulation of the wild type lamprey GnRH receptor led to a 1.68 ± 0.073 fold increase in response to lamprey GnRH-I and a 2.11 ± 0.162 fold increase in response to lamprey GnRH-III. Serial truncation of the lamprey GnRH receptor led to a drastic decrease in the magnitude of cAMP accumulation, whereas the stimulation of the 80aa C-terminal tail resulted in 1.26 ± 0.204 and 1.36 ± 0.117 fold increases in response to lamprey

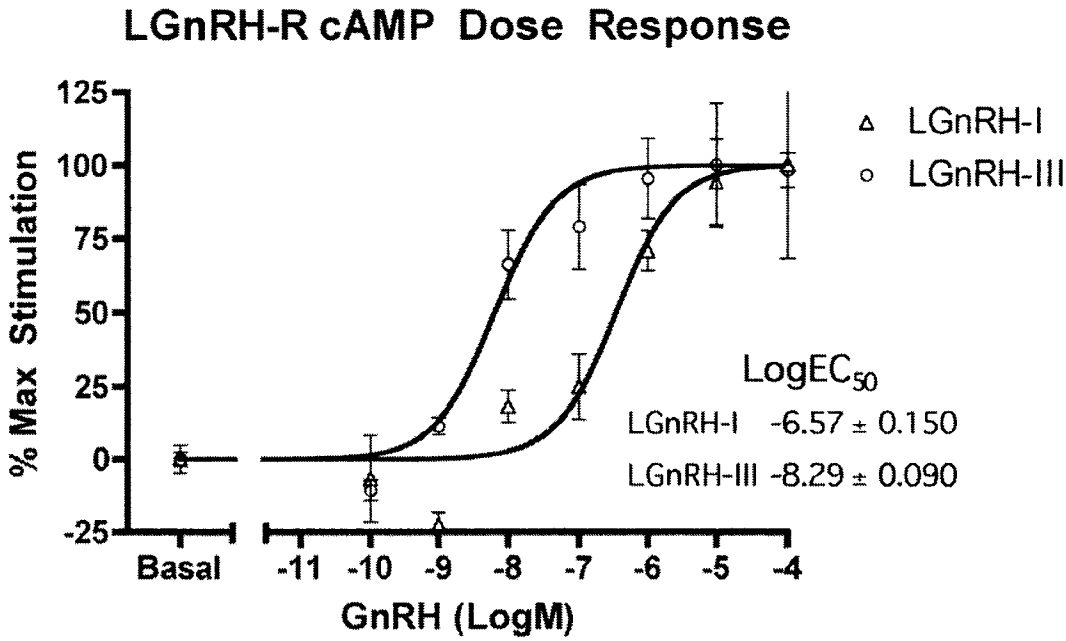

Figure 22. Lamprey GnRH Receptor cAMP Dose Response. The lamprey GnRH receptor was shown to activate the cAMP signaling system in a dose dependant manner. Lamprey GnRH-III ($LogEC_{50}$ -8.29 ± 0.090) was a significantly more potent activator of the lamprey GnRH receptor relative to lamprey GnRH-I ($LogEC_{50}$ -6.57 ± 0.150). Representative curve from 3 independent experiments, $LogEC_{50}$ shown as mean SEM; n=3.

GnRH-I and lamprey GnRH-III, respectively. Further truncation of the C-terminal tail to 40aa resulted in a recovery of cAMP accumulation (1.77 ± 0.242 fold for lamprey GnRH-I and 2.19 ± 0.262 fold increases for lamprey GnRH-III), however the tail-less mutant showed a drastic reduction in ability to activate the cAMP system (1.26 ± 0.056 fold increase in response to lamprey GnRH-I and 1.30 ± 0.059 fold increase in response to lamprey GnRH-III).

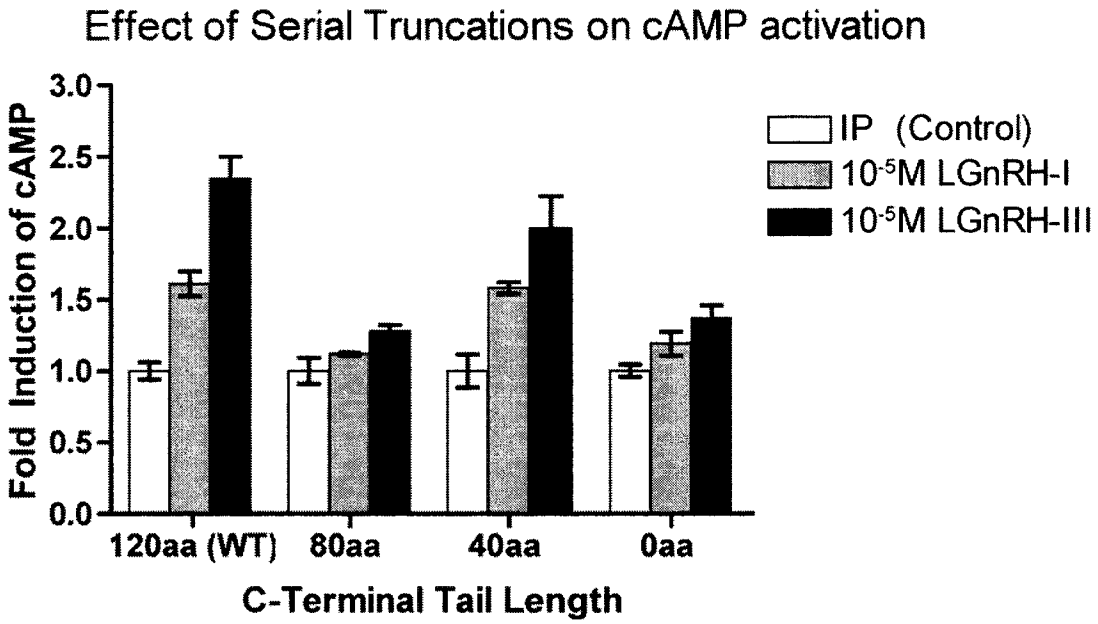

Figure 23. Efficacy of Lamprey GnRH Receptors with C-Terminal Tail Truncations. cAMP accumulation assay using Wild type and mutant lamprey GnRH receptors containing C-terminal tail truncations treated with a maximum dose (10^{-5} M) of either lamprey GnRH-I or lamprey GnRH-III are shown relative to fold stimulation. Lamprey GnRH-III simulates a greater magnitude of cAMP signaling compared to lamprey GnRH-I in the wild type receptor. Truncation of the C-terminal tail reduced cAMP accumulation, which was not recovered in the tail-less mutant form.

Pharmacological Characterization

Binding of ^{125}I-lamprey GnRH-I to intact, adherent COS7 cells transfected with the lamprey GnRH receptor was saturable with a B_{max} of 394.6 fMol/well and a K_d of 31.1 nM. This relatively high K_d was expected, given that this receptor is likely lamprey GnRH-III selective. Binding of ^{125}I-lamprey GnRH-I to the C-terminal tail truncated mutant lamprey GnRH receptors was also saturable (see Figure 24), whereas the 80aa tail mutant had a Bmax of 276 fMol/well and a K_d of 51.7 nM, the 40aa tail mutant had a Bmax of 1906 fMol/well and a K_d of 85 nM, while the 0aa tail-less mutant had a Bmax of 775 fMol/Well and a K_d of 72 nM (See table 2). C-terminal tail truncations resulted in an

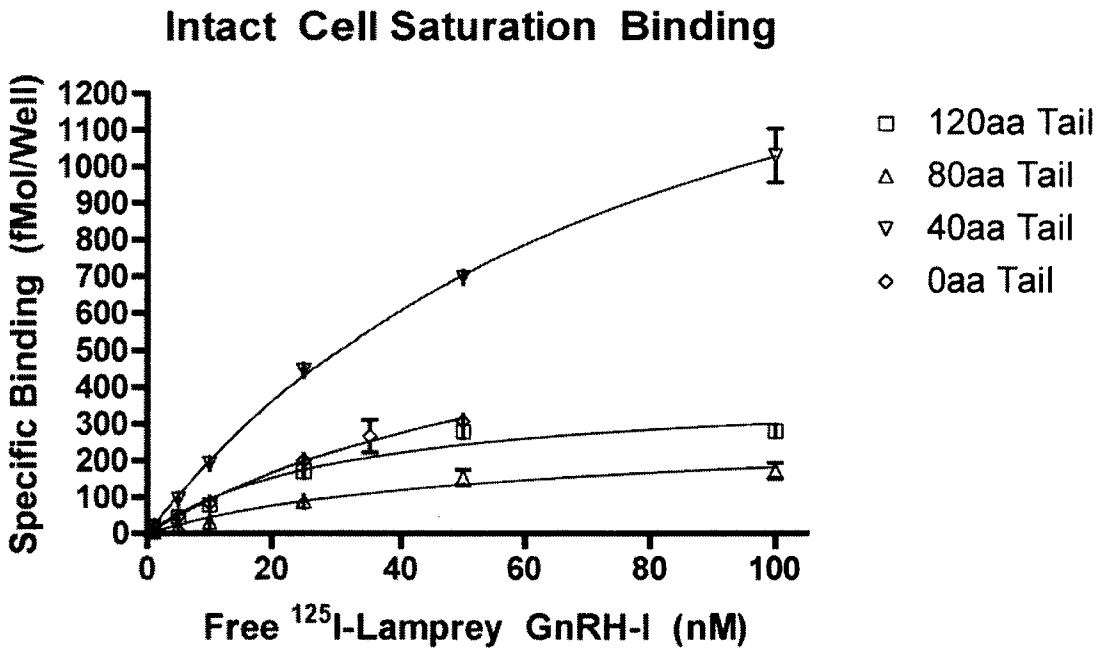

Figure 24. Saturation Binding Analysis. Saturation binding analysis of ^{125}I-lamprey GnRH-I using adherent, intact COS7 cells transfected with the wild type lamprey GnRH receptor (□), 80aa tail (Δ), 40aa tail (▽), or tail-less (◊) mutants. Data shown as mean ± SEM (n=3), representing 2-3 independent experiments.

increase in cell surface expression of the 40 aa C-terminal tail (483.0% of wild-type) and tail-less mutants (196.4% of wild-type), while the 80aa C-terminal tail mutant expression decreased (69.9% of wild-type). In all cases the truncations resulted in an increase in Kd, 80aa tail (166.2% of wild-type), 40aa tail (273.3% of wild-type) and tail-less (231.5% of wild-type).

COS7 cells transfected with the wild-type lamprey GnRH receptor were used in a series of competitive binding assays in order to describe the binding affinity of different GnRH isoforms. Assays using intact, adherent cells were performed holding ^{125}I lamprey GnRH-I constant at 10 nM with increasing concentrations of cold competitor, ranging from 10^{-13} M to 10^{-6} M. These competitive binding analyses indicate that the wild type

Lamprey GnRH Receptor C-terminal Tail Truncations: Function and Pharmacology								
	cAMP Accumulation				Binding Kinetics			
Construct	LGnRH-I		LGnRH-III		Kd (nM)		Bmax (fMol/Well)	
Wild Type	1.68	± 0.073	2.11	± 0.162	31.1	± 10.3	394.6	± 52.1
80aa Tail	1.26	± 0.204	1.36	± 0.117	51.7	± 18.4	276.1	± 46.9
40aa Tail	1.77	± 0.242	2.19	± 0.262	85.4	± 5.3	1906	± 67.1
Tail-Less	1.26	± 0.056	1.30	± 0.059	72.3	± 19.8	774.5	± 140.9

Table 2. Summary of Lamprey GnRH Receptor C-Terminal Tail Truncation Analysis. Note, mGnRHa is DAla^{6}Pro^{9}NEt mammalian GnRH.

lamprey GnRH receptor is lamprey GnRH-III selective (Figure 25), with a pharmacological profile of lamprey GnRH-III (K_i = 0.708 ± 0.245 nM) = chicken GnRH-II (K_i = 0.765 ± 0.160 nM) > mammalian GnRH (K_i = 12.9 ± 1.96 nM)> DAla6-Pro9 NEt mammalian GnRH (K_i= 21.6 ± 9.68 nM) > lamprey GnRH-I (118.0 ± 23.6) (see table 3).

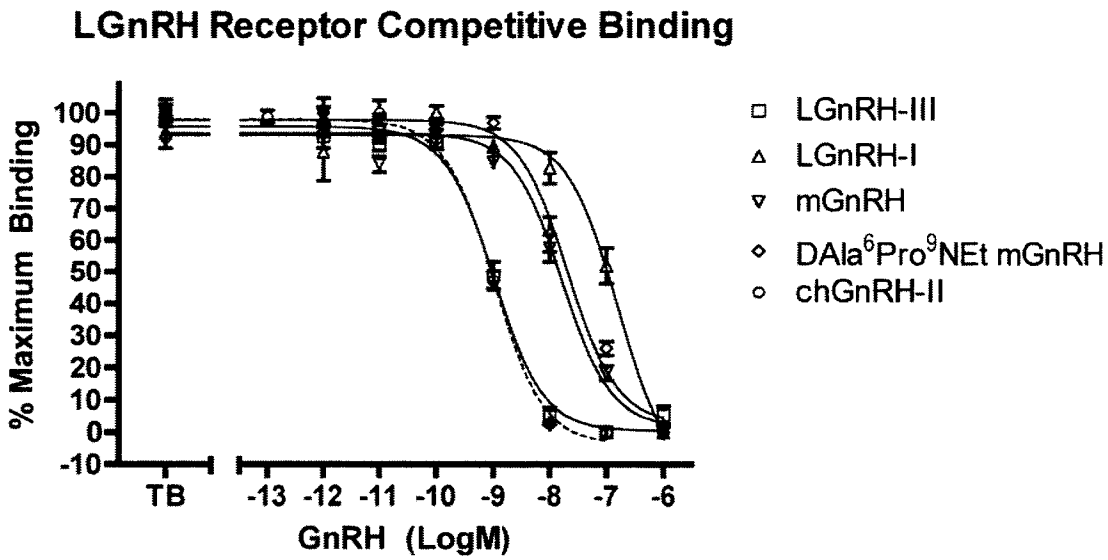

Figure 25. Lamprey GnRH Receptor Competitive Binding Analysis. Competitive binding analysis of ^{125}I-lamprey GnRH-I using intact adherent COS7 cells transfected with the wild type lamprey GnRH receptor incubated with increasing concentrations of lamprey GnRH-III (□), lamprey GnRH-I (Δ), mammalian GnRH (▽), DAla6Pro9NEt mammalian GnRH (◊) or chicken GnRH-II (○). Data shown as mean ± SEM (n=3) of % maximum binding demonstrates a binding preference for lamprey GnRH-III, which was equal to chicken GnRH-II > mammalian GnRH > DAla6Pro9NEt mammalian GnRH > lamprey GnRH-I.

Lamprey GnRH Receptor Characterization						
GnRH Form	LogEC50		LogIC50		Ki (nM)	
lGnRH-III	-8.29	± 0.09	-8.99	± 0.0922	0.708	± 0.245
lGnRH-I	-6.57	± 0.15	-6.81	± 0.116	118.0	± 23.6
chGnRH-II	---	---	-8.96	± 0.0713	0.765	± 0.16
mGnRH	---	---	-7.79	± 0.147	12.9	± 1.96
mGnRHa	---	---	-7.68	± 0.116	21.6	± 9.68

Table 3. Summary of lamprey GnRH receptor efficacy and pharmacological profiling. Note, mGnRHa is DAla^{6}Pro^{9}NEt mammalian GnRH.

Lamprey GnRH Receptor Internalization

Internalization of ^{125}I-lamprey GnRH-I was used to characterize the effect of C-terminal tail length on the rate of ligand dependant internalization, which is described as the percent of total cell associated ligand, and fit using a single component exponential equation ($Y=Y_{max}(1-e^{kt})$; y= % internalized, k = % internalized/min and t=time in minutes (see Figure 26). The wild-type lamprey GnRH receptor was rapidly internalized in response to treatment with 10 nM ^{125}I-lamprey GnRH-I. Within the first 10 min at 37°C, approximately 63% of the cell associated radioligand was found in the intracellular fraction. Lamprey GnRH receptor mutants with C-terminal tail truncations were used to identify regions containing motifs that are required for rapid ligand dependant internalization. Truncation of the C-terminal tail to 80aa or 40aa had no effect on either the rate or extent of internalization when compared to wild-type. The tail-less lamprey GnRH receptor, however, showed a marked reduction in ligand dependent internalization, with a Ymax of 28.9%, a drastic reduction compared to the wild-type or other truncated mutants.

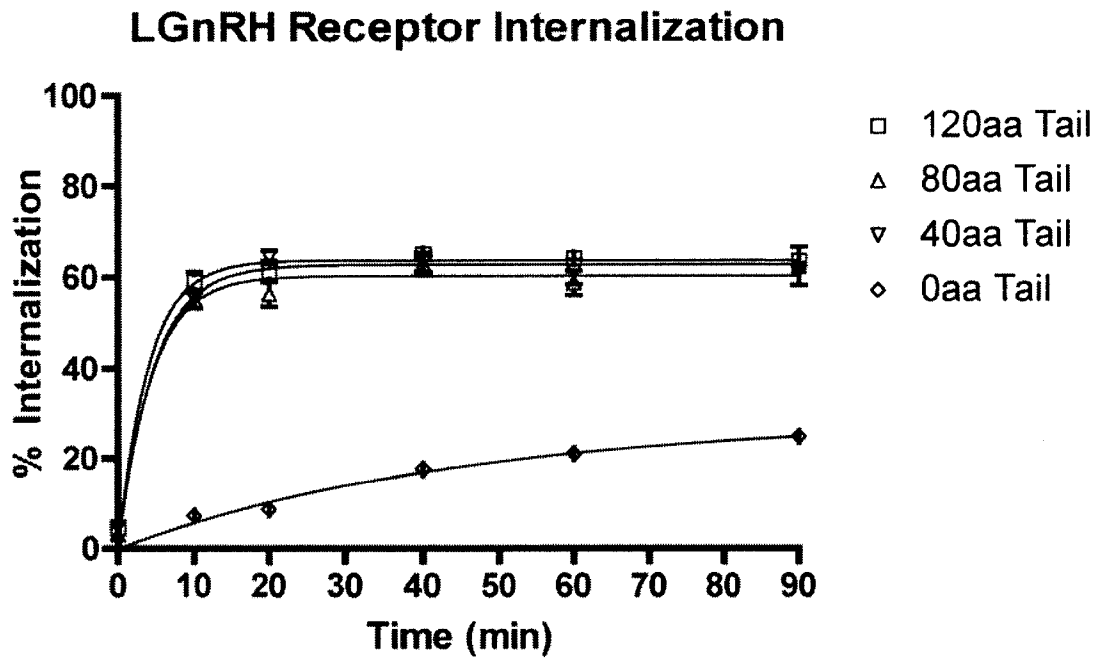

Figure 26. Internalization Profiles of the Wild-Type and Mutant Lamprey GnRH Receptors. ^{125}I-lamprey GnRH-I mediated internalization of the wild type lamprey GnRH receptor (□), 80aa tail (Δ), 40aa tail (▽), or tail-less (◊) mutants in transiently transfected COS7 cells. Treatments were brought to steady state on ice and then rapidly brought to 37°C for increasing periods of time. Percent internalization was calculated as a measurement of internalized radioligand relative to the total cell associated radioligand. Data shown as mean ± SEM (n=3), representing 2-3 independent experiments.

Discussion

The GnRH receptor from the sea lamprey (*Petromyzon marinus*), a member of the oldest class of vertebrates, the agnathans, was used in a series of functional and pharmacological experiments in order to investigate and better define how the function of the GnRH receptor family has evolved across vertebrates. Stimulation of the lamprey GnRH receptor was shown to activate cAMP production, however this activation was shown to require the first 40aa of the C-terminal tail. Since the tail-less lamprey GnRH receptor mutant was capable of ligand binding we speculate that $G\alpha_s$ coupling requires a

motif within the first 40aa of the C-terminal tail. Pharmacological profiling indicates the lamprey GnRH receptor is lamprey GnRH-III selective, with an equal affinity for lamprey GnRH-III (Ki = 0.708 ± 0.245nM) and chicken GnRH-II (Ki = 0.765 ± 0.16 nM). The wild-type, 80aa C-terminal tail and 40aa C-terminal tail lamprey GnRH receptors were shown to undergo rapid, ligand dependant internalization, however the tail-less mutant was not, indicating a motif within the first 40 amino acids that is required for this process. This unique lamprey GnRH receptor, with both high affinity for lamprey GnRH-III and chicken GnRH-II and ligand binding activity in the tail-less form may represent an important ancestral state which provides insight into the function and evolution of the vertebrate GnRH receptor family.

Unique among the approximately 1000 GPCR encoding genes in the human genome (Fredriksson et al., 2003), the type I GnRH receptor lacks an intracellular C-terminal tail, which is thought to be involved in G protein coupling, cell surface expression and internalization (Blomenrohr et al., 1999; Ronacher et al., 2004; Vrecl et al., 2000). Interestingly, tail-less, or type I GnRH receptors, have only been identified in mammals, while type II GnRH receptors, which contain C-terminal tails, have been cloned from species across the vertebrate lineage, suggesting a recent, rapid evolutionary history (Millar et al., 2004; Sealfon et al., 1997). A comparative analysis of GnRH receptors across the vertebrate lineage can provide significant insight into the molecular evolution of this receptor family. In this light, the GnRH receptor from the sea lamprey, Petromyzon marinus (Silver et al., 2005), provides an ideal model to analyze basal, or ancestral-like functions and functional elements that are involved in ligand binding, signaling and internalization.

Pituitary GnRH receptors are thought to primarily signal through $G\alpha_{q/11}$, resulting in the stimulation of the IP_3 second messenger system, however $G\alpha_s$ activation and cAMP signaling has been reported as well (Arora et al., 1998; Grosse et al., 2000; Liu et al., 2002; Oh et al., 2005; Stanislaus et al., 1998). G-protein coupling to type I GnRH receptors clearly occurs within the intracellular loops, where several motifs have been identified that may be involved in G-protein coupling (See table 4). For instance, the DRxxxI/VxxPL motif in IL2 and a conserved Ala residue in IL3 have been linked to Gaq/11 coupling (Arora et al., 1995; Myburgh et al., 1998), while a BBxxB (where B is any basic amino acid) in IL1 was shown the be required for $G\alpha_s$ coupling (Arora et al., 1998). Furthermore, the presence or absence of the C-terminal tail in the type II or type I GnRH receptors could possibly explain the signaling disparity between the two groups, whereas an HFRK motif in the membrane proximal region of the bullfrog type II GnRH receptor-1 was recently shown to be required for cAMP signaling, but not for IP signaling (Oh et al., 2005). In the present study, lamprey GnRH receptor was shown to activate the cAMP signaling system, in a dose dependent manner, in transiently transfected COS7 cells. Lamprey GnRH-III ($LogEC_{50}$ -8.47 ± 0.046) was a more potent activator of this system compared to lamprey GnRH-I ($LogEC_{50}$ -6.59 ± 0.082), which supports the previous hypothesis, based on IP activation (Silver et al., 2005) that the lamprey GnRH receptor is lamprey GnRH-III selective. These data have several interesting implications. The lamprey GnRH receptor activates both the cAMP and IP signaling systems, however the IP system is activated at an approximately 10 fold lower concentration of both lamprey GnRH-I and lamprey GnRH-III, and is also activated to a greater magnitude of approximately 4.5 fold, compared to ~1.7 fold (lamprey GnRH-I) or

Receptor	G-Protein	Region Involved in G-Protein Coupling	Reference
Mouse GnRH-R	$G\alpha_S$	IL1 ($K^{71}LKSR^{75}$; L^{58}; L^{80})	Arora et al., 1998
Mouse GnRH-R	$G\alpha_{q/11}$	IL2 (L^{147})	Arora et al., 1995
Mouse GnRH-R	$G\alpha_{q/11}$	IL2 (DRS)	Kitanovic et al., 2001; Arora et al., 1997
Mouse GnRH-R	$G\alpha_{q/11}$	IL3 (Leu^{237})	Chung et al., 1999
Rat GnRH-R	$G\alpha_{q/11}$ & $G\alpha_S$	IL3	Ulloa-Aguirre et al., 1998
Rat GnRH-R	$G\alpha_{q/11}$	C-terminus ($F^{325}S^{326}L^{327}$)	Brothers et al., 2002
Human GnRH-R	$G\alpha_{q/11}$	IL3 (A^{261})	Myburgh et al., 1998
Bullfrog GnRH-R1	$G\alpha_S$	C-T (membrane proximal HFRK)	Oh et al., 2005
Lamprey GnRH-R	$G\alpha_{q/11}$	IL1, 2 or 3 (Activated by tail-less mutant)	Silver et al., 2005
Lamprey GnRH-R	$G\alpha_S$	C-T (within first 40 amino acids)	Presented Here

Table 4. GnRH Receptor Domains Involved in G-Protein Coupling. IL, intracellular loop; C-T, C-terminal tail; C-terminus refers to the C-terminal amino acids of the tail-less type GnRH receptors.

~2.1 fold (lamprey GnRH-III) (Figure 23) accumulation of cAMP. Not unexpectedly, truncation of the lamprey GnRH receptor C-terminal tail interfered with cAMP signaling, which is partially recovered by the 40aa tail mutant, and lost again in the tail-less mutant form. The exact nature of GPCR/G-protein coupling is still in question since no conserved motifs that can be generally used to define G-protein specificity have been identified, nor has any particular domain been shown to be required. These current data indicate that a motif within the first 40aa of the lamprey GnRH receptor is involved in the $G\alpha_s$ coupling, which we speculate to be the "HFRK" like motif (HVRR in lamprey) located within the membrane proximal region of the C-terminal tail. Furthermore, this region contains a BBxxB (B-any basic amino acid), which has been shown to be involved in $G\alpha_s$ coupling in type I GnRH receptors, however in which case this motif is located in the first intracellular loop (Arora et al., 1998).

The pharmacological profile of the lamprey GnRH receptor, as shown in Figure

25 and Table 1, confirms the hypothesis that the lamprey GnRH receptor is lamprey GnRH-III selective, and furthermore supports the hypothesis that lamprey express at least one additional, lamprey GnRH-I selective receptor, as previously described based on quantitative in vitro autoradiography (Knox et al., 1994) and IP signaling efficacy (Silver et al., 2005). The equivalent affinity for lamprey GnRH-III and chicken GnRH-II for this ancestral vertebrate GnRH receptor implies that perhaps they represent ancestral forms of GnRH, as previously suggested (Sherwood et al., 1997; Sower, 1997); nonetheless more data would be required to confirm this hypothesis.

Efficacy data on their own can be difficult to interpret and misleading, however in conjunction with binding affinity studies they can provide invaluable insight into the molecular mechanisms of GPCR function. The effects of lamprey GnRH receptor C-terminal tail truncations on binding affinity and cell surface expression showed that perturbation of the C-terminal tail increases Kd (reduces binding affinity) and increases the level of cell surface expression, except in the case of the 80aa C-terminal tail mutant, in which case the level of surface expression decreased . These data may explain the drastic decrease in signaling of the 80aa C-terminal tail mutant, which likely results from a combination of decreased binding affinity (from 31.1 ± 10.3 nM to 51.7 ± 18.4 nM) and surface expression (from 394.6 ± 52.1 fMol/well to 276.1 ± 46.9 fMol/well). Alternatively, the recovery of cAMP accumulation that was seen when the lamprey GnRH receptor with 40aa tail was activated may be due to the increase in cell surface expression (1906 ± 67.1 fMol/well), which compensates for the reduction in binding affinity (85.4 ± 5.3 nM). Lastly, the tail-less mutant was shown to maintain ligand binding capability at a diminished binding affinity (72.3 ± 19.8 nM), which, despite an

increase in surface expression (774.5 ± 140.9 fMol/well), led to the accumulation of near basal levels of cAMP (1.26 ± 0.056 fold and 1.30 ± 0.059 fold, when stimulated with lamprey GnRH-I or lamprey GnRH-III, respectively). Due to the fact that the tail-less lamprey GnRH receptor mutant is known to be active in stimulating IP_3 at an equivalent magnitude compared to wild-type type (Silver et al., 2005), these data support the hypothesis that $G\alpha_s$ coupling occurs within the first 40 amino acids of the C-terminal tail.

Rapid, ligand dependant GPCR internalization is a well established regulatory mechanism that results in receptor desensitization or alternatively facilitates signal switching from G-protein mediated second messaging to MAP kinase signaling (McArdle et al., 2002). Activation of type II GnRH receptors has been extensively shown to lead to rapid internalization that can be β-arrestin dependent or independent, dynamin dependent or independent and through either clathrin coated or caveolae (Acharjee et al., 2002; Hislop et al., 2005; Pawson et al., 2003; Ronacher et al., 2004). Type I GnRH receptors have been shown to be internalized, however at a significantly slower rate and to a lesser extent when compared to type II GnRH receptors (Hislop et al., 2005; Pawson et al., 1998; Willars et al., 1999). Addition of the C-terminal tail from the type II catfish GnRH receptor to the rat GnRH receptor was shown to result in several functional aberrations, most notably induction of rapid ligand dependant internalization, which was shown to be reversible through C-terminal tail truncations (Lin et al., 1998). Furthermore, removal of the C-terminal tail from the type II chicken GnRH receptor was shown to result in an internalization profile similar to the naturally tail-less human type I GnRH receptor (Pawson et al., 1998). These studies provided the impetus to further define the motifs involved in this rapid internalization, leading to a series of studies indicating specific

Ser/Thr moieties located in both the membrane proximal or distal regions of the C-terminal tail (Blomenrohr et al., 1999; Millar et al., 2004; Pawson et al., 2003; Ronacher et al., 2004). For example, Ser^{363} of the catfish GnRH receptor is a major ligand induced phosphoaccepter, which, through site directed mutagenesis (S363A), was shown to be required for rapid internalization (Blomenrohr et al., 1999). Mutation of a Thr doubled ($Thr^{369}Thr^{370}$) in the distal region of the chicken GnRH receptor C-terminal tail led to a 70% reduction in ligand dependent internalization compared to wild-type, which was shown to internalize in a β-arrestin independent, dynamin dependant manner through caveolae (Pawson et al., 2003). More recently, Ronacher et al. identified a Ser doublet ($Ser^{338}Ser^{339}$) in the membranes proximal region of the human type II GnRH receptor, which when mutated to Ala led to a 75% reduction in internalization compared to wild-type, which was also shown to require both GRK and dynamin, but not β-arrestin, and to proceed through both clathrin coated pits and caveolae (Ronacher et al., 2004). The 120aa C-terminal tail of the lamprey GnRH receptor, the longest of any known GnRH receptor (Silver et al., 2005), contains several Ser/Thr residues located throughout the entire sequence, however they are concentrated within the first 40aa (Figure 27). The lamprey GnRH receptor was shown to be rapidly internalized in response to stimulation, whereas approximately 60% of the ligand bound receptors were located in the intracellular space. Truncation of the C-terminal tail to 80aa or 40aa had no effect on internalization (rate or maximum level), however the tail-less mutant showed a drastic reduction in internalization, similar to other previously described tail-less receptor internalization profiles (Heding et al., 1998; Pawson et al., 1998).

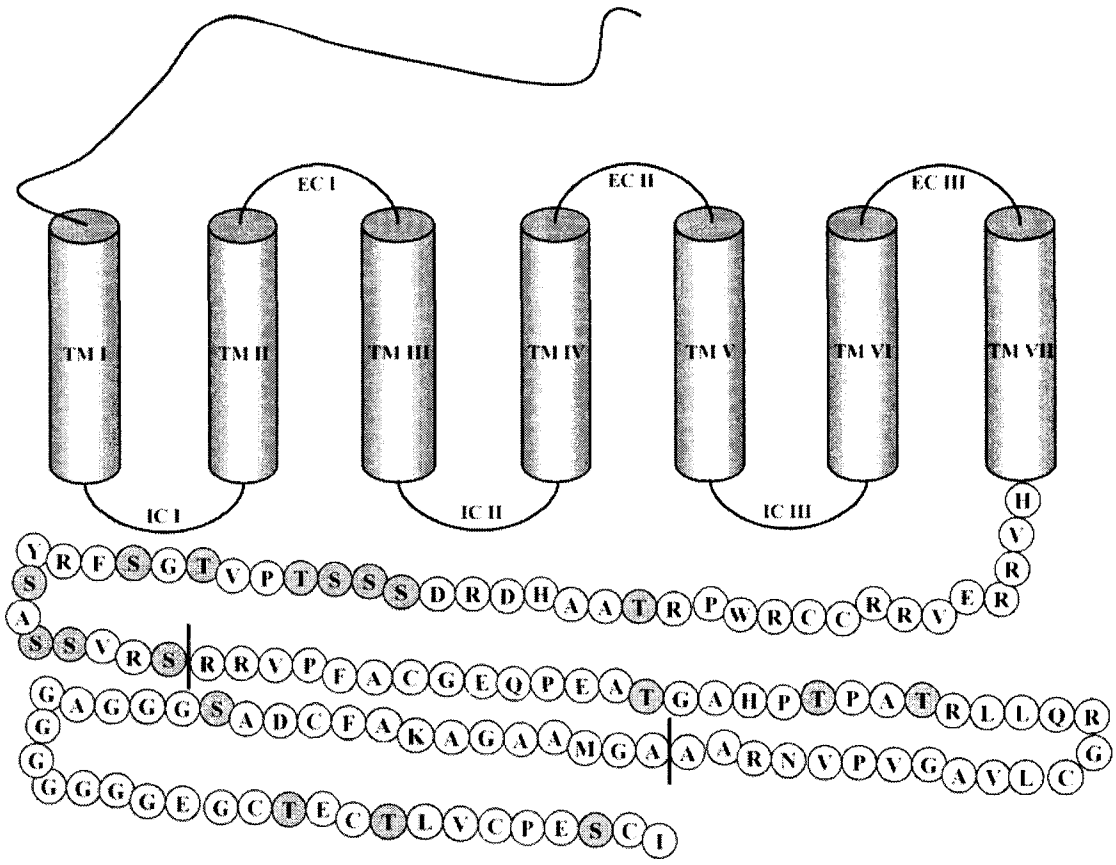

Figure 27. GnRH Receptor Schematic II: Putative C-Terminal Tail Phosphoacceptor Sites. The lamprey GnRH receptor C-terminal tail of 120aa is the longest of any known GnRH receptor. Several putative phosphorylation sites are located within the C-terminal tail, which are shown highlighted in gray. Black lines represent cutoff points within the sequence used to develop vectors containing C-terminal tail truncations to include the wild type (120aa tail), 80aa tail, 40aa tail or tail-less (0aa tail) mutants.

In summary, the lamprey GnRH receptor was shown to activate the cAMP signaling system, which required the first 40aa of the C-terminal tail. Pharmacological profiling, in conjunction with efficacy data, provided evidence that the lamprey GnRH receptor is lamprey GnRH-III selective, which supports the hypothesis that lamprey express a second, lamprey GnRH-I selective receptor. Truncations of the lamprey GnRH receptor's C-terminal tail were shown to reduce binding affinity, which explains their reductions in signaling capacity. Finally, the lamprey GnRH receptor underwent rapid

ligand dependant internalization, which was drastically reduced in the tail-less mutant form, suggesting that putative phosphoacceptor sites located within the first 40aa of the C-terminal tail are required for this regulatory mechanism. Since the 40aa C-terminal tail lamprey GnRH receptor mutant is capable of stimulating both IP_3 and cAMP accumulation and undergoes rapid ligand dependant internalization, we speculate that the extensive length of the lamprey GnRH receptor C- terminal tail may not have a functional significance with these signaling systems. However, the intact lamprey GnRH receptor C-terminal tail likely is required for structural stability and therefore efficient ligand binding, or possibly for some unknown signaling mechanism. The loss of amino acids in the C-terminal tail of GnRH receptors during the subsequent evolution of vertebrates to the tail-less form in mammals may reflect the various actions of GnRH in controlling pituitary gonadotropins through diverse regulatory mechanisms. These data support the evidence that the lamprey GnRH receptor shares several characteristics of both type I and type II GnRH receptors, and may represent an ancestral form, which provides insight into the evolution and function of the GnRH receptor family.

CHAPTER IV

CLONING AND ANALYSIS OF THE LAMPREY GnRH-III cDNA FROM EIGHT SPECIES OF LAMPREY REPRESENTING THE THREE FAMILIES OF PETROMYZONIFORMES

Introduction

Gonadotropin-releasing hormone (GnRH) is the central regulator of the hypothalamic-pituitary-gonadal axis, and therefore reproductive function, in all vertebrates. To date twenty-four isoforms of GnRH have been identified, fourteen from vertebrates and ten from invertebrates, two of which were identified in the sea lamprey (*Petromyzon marinus*), lamprey GnRH-I and –III (Sherwood et al., 1986; Sower et al., 1993). In addition, the cDNA encoding lamprey GnRH-I has been identified in the sea lamprey (Suzuki et al., 2000). Lamprey are the earliest evolved vertebrates for which two forms of GnRH have been clearly demonstrated as functional neurohormones mediating the pituitary-gonadal axis (Sower and Kawauchi, 2001). Both lamprey GnRH-I and –III were shown to be expressed in the preoptic anterior hypothalamus and the posterior hypothalamus of the adult sea lamprey (Nozaki et al., 2000). Lamprey GnRH-III is considered the most active form during sea lamprey maturation based on the relative number of lamprey GnRH-III producing neurons which is larger than lamprey GnRH-I producing neurons during the larval and adult stages (Nozaki et al., 2000; Tobet et al.,

1995) and that lamprey GnRH-III was more potent compared to lamprey GnRH-I in inducing spermiation in male sea lamprey (Deragon and Sower, 1994; Sower, 2003). Neither lamprey GnRH-I or –III peptides have been isolated from any other species of lamprey, although both forms have been detected in the brain and hypothalamus using immunocytochemistry and HPLC coupled with radioimmunoassay or immunocytochemistry in the pouched lamprey (*Geotria australis*), Australian lamprey (*Mordacia mordax*), silver lamprey (*Ichthyomyzon unicuspis*) and western brook lamprey (*L. richardsoni)* (Eisthen and Northcutt, 1996; Sower et al., 2000).

In vertebrates it is now known that two or three forms of GnRH can be expressed within the brain of a single species (Dubois et al., 2002; Miyamoto et al., 1984; Wada et al., 1998; White and Fernald, 1998; White et al., 1994). A proposed relationship of these GnRH forms was recently described based on phylogenetic analysis, location of expression within the brain and general associated function (Fernald and White, 1999). In this model, the GnRH family was divided into three paralogous lineages, starting with GnRH-I (also known as mammalian GnRH and its orthologs), which is expressed in the hypothalamus and is the central regulator of the pituitary gonadal axis. GnRH-II (also known as chicken GnRH-II) is expressed in the midbrain and is generally considered to have a neuromodulatory function (Fernald and White, 1999; Parhar, 2002). Finally, GnRH-III (also known as salmon GnRH), which is only found in the teleosts, is expressed in the telencephalon and is also believed to have a neuromodulatory function (Fernald and White, 1999; Parhar, 2002). Parhar recently modified this scheme by the addition of a fourth family that consists of medaka and seabream GnRH, which formerly would have been considered GnRH-I (Parhar, 2002). This modification is supported by

the fact that the cells producing medaka and seabream GnRH do not share the same developmental origin as the other hypothalamic forms, although they do function in a similar manner and group together phylogenetically. These models that describe the molecular phylogeny of the GnRH family are incomplete since only a limited number of the known GnRH sequences were used.

Interest in the evolution of reproductive mechanisms has led researchers to study lamprey, which are one of the two extant representatives of the most ancient class of vertebrates, the agnathans, which diverged from the main line of vertebrate evolution approximately 450 million years ago. The divergence of the lamprey lineage is believed to have occurred between two proposed early genome duplications (Ohno, 1970), making the lamprey an important model for evolutionary biology (Kuratani et al., 2002). Despite this interest in the lamprey, very little is known about the lamprey phylogeny. In the 1970s a lamprey phylogeny was proposed based primarily on the size, shape and distribution of dentition (Hubbs and Potter, 1971; Potter and Hilliard, 1987). Based on the morphology of their teeth, the lamprey were divided into three families: the Petromyzonidae, which are found in the northern hemisphere, also referred to as the holarctic species, and the two southern hemisphere families, Geotriidae and Mordaciidae. The Petromyzonidae consists of six genera: *Ichthyomyzon, Petromyzon, Caspiomyzon, Eudontomyzon, Tetrapleurodon*, and *Lampetra*. Notably the *I. unicuspis* is believed to be the most closely related to the ancestral stock of lamprey due to its simple tooth shape and distribution (Hubbs and Potter, 1971). The Geotriidae and Mordaciidae each consist of only one genus, *Geotria* and *Mordacia* respectively (Hubbs and Potter, 1971). This overall division into three families is supported by sequence analysis of the primary

structure of insulin, where the *M. mordax* sequence is more similar to that of the holarctic sequences than to the *G. australis* sequence (Conlon, 2001; Conlon et al., 2001). One of the divisions of lampreys has since been supported by an analysis of mitochondrial genes exclusively within the Lampetra, reconfirming the species designation within this family (Docker et al., 1999), however, further analysis of other characters is needed to better our understanding of the lamprey phylogeny.

To address the question of the molecular relationship within the GnRH family, as well as the phylogeny of the lamprey lineage we have isolated the cDNA encoding the prepro-lamprey GnRH-III from eight different species of lamprey representing all three families of the Petromyzoniformes. These sequences were used in a phylogenetic analysis with other known prepro-GnRH sequences published on GenBank, or solely with each other to address each question respectively. Our analysis suggests that the lamprey GnRH forms are unique as they grouped together, yet separate from the three previously described paralogous lineages of GnRH. The analysis within the lamprey lineage supports the phylogeny based on dentition at the family level, although ultimately additional traits need to be considered.

Materials and Methods

Animal Collection and Handling

Eight species of lamprey were used in this study, which were collected from both North America and Australia in accordance to UNH IACUC animal care guidelines. Experts in the field, using local fish guides, verified the identity of each species. Species collection sites and tissues dissected are described in Table 1. In all cases except one, the dissected

tissue was immediately frozen in either liquid nitrogen or dry ice, then stored at –80°C. The tissues collected from *G. australis* were immediately submersed in 1 mL of RNA*later* (Ambion, Austin, TX) at room temperature. Samples were then stored at –20°C until used, at which point the RNA*later* was poured off the tissue.

RNA Isolation

Total RNA was isolated using the Tri-Reagent (Molecular Research Center, Inc., Cincinnati OH), an isothyocyanate phenol chloroform extraction method, with a glass homogenizer. RNA was isolated using 1 mL Tri-Reagent per 100 mg of tissue. Yield was determined by spectrophotometry in MilliQ H2O or phosphate buffer at pH 8.1.

cDNA synthesis

First strand cDNA synthesis was done using the First Strand cDNA Synthesis kit by Amersham Pharmacia Biotech (Buckinghamshire, England). First strand synthesis uses 5 μg of total RNA and is catalyzed using the Moloney Murine Leukemia Virus (M-MuLV) reverse transcriptase with a Not1-dT_{18} primer. The RNA was denatured via a 10-minute incubation at 65°C before being used. Denatured RNA was then mixed with 11uL of kit reaction mixture (M-MuLV reverse transcriptase, porcine RNAguard, RNase/DNase-free BSA, dATP, dCTP, dGTP, and dTTP in buffer), 1 μL of 200mM dithiothreithol (DTT), and 1 μl of 5 μg/μl Not1-dT_{18} primer followed by incubation for 1 hour at 37°C. First strand cDNA was stored at –20°C. The single strand cDNA can be amplified via the polymerase chain reaction (PCR), as described below, or used to make second-strand cDNA via the Gubler-Hoffman technique (Gubler and Hoffman, 1983), as done using the

Species	Collection Location	Collectors	Tissues Collected
P. marinus	Cocheco River, NH	Sower Lab	Brain, pituitary, testis, ovary, liver, kidney, heart, muscle and eye
L. appendix	Oyster River, NH	Sower Lab	Brain
L. tridentatus	Washington	Craig Robinson (USGS)	Brain, testis, ovary, liver and muscle
L. richardsoni	Washington	Craig Robinson (USGS)	Brain, ovary, liver and muscle
I. Unicuspis	Great Lakes Region	Sid Morkert (US FWLS)	Brain and pituitary
I. Fossor	Great Lakes Region	Paul Sullivan (DFO)	Brain, pituitary, testis, ovary, kidney and liver
G. australis	Tasmania, Australia	Sower and Colleagues	Brain, pituitary, testis, kidney, liver, muscle, heart and eye
M. mordax	Tasmania, Australia	Sower and Colleagues	Brain and ovary

Table 5. Summary of Lamprey and Tissue Collection. Tissues were collected from both North America, including six species from the Petromyzonidae, and Australia, including one species from both the Geotriidae and Mordaciidae. Obtained tissues were subject to availability.

Marathon™ cDNA Amplification Kit (Clontech, Palo Alto, CA, USA). Second-strand cDNA was synthesized from single stranded cDNA mixed with 48.4 µl sterile water, 16 µl 5X second-strand buffer (500 mM KCl, 50 mM ammonium sulfate, 25 mM $MgCL_2$, 0.75 mM β-NAD, 100 mM Tris (pH 7.5), and 0.25 mM BSA), 1.6 µl 10mM dNTPs, and 4 µl of 20X second-strand Enzyme cocktail (6 units/µl E. *coli* DNA polymerase I, 1.2 units/µl E. *coli* DNA ligase, and 0.25 units/µl E. *coli* RNase H) followed by incubation at 16°C for 1.5 hours. The second-strand cDNA was isolated via rounds of extraction using 25:24:1 phenol:chloroform:isoamyl alcohol followed by 24:1 chloroform:isoamyl-alcohol, and was finally precipitated using 4 M ammonium acetate and ethanol.

Degenerate PCR

Amplification of the 3' end of the lamprey GnRH-III cDNA from all eight species was performed by PCR using single stranded brain cDNA (ss cDNA) as template with the lamprey3-1 degenerate primer (GAR-CAY-TGG-TCN-CAC-GAT-TGG) paired with the

NotI primer. The lamprey3-1 primer was designed based on the known decapeptide sequence of lamprey GnRH-III, with consideration of the specific differences to lamprey GnRH-I decapeptide. Promega PCR components were used and the cycling was performed with an Eppendorf Master Gradient thermal cycler under the following PCR parameters: 95°C for 2 min followed by 35 cycles of 95°C 30 sec, 60 °C for 1 min, and 72 °C for 1min, and finished with a 5 min 72 °C incubation and 4 °C hold. Samples were analyzed by electrophoresis using 2% agarose gels stained with ethidium bromide. The pGEM-T Easy Vector system (Promega, Madison, WI) was used to clone amplified products, which was then sequenced at the Huntsman Cancer Institute DNA Sequencing Facility at the University of Utah.

5' Rapid Amplification of cDNA Ends (RACE)

5'-RACE was performed using the Marathon cDNA Amplification Kit (Clontech). A partially double stranded DNA adapter was ligated onto both ends of the synthesized double stranded cDNA (see above), to which a specific primer was used in combination with a gene specific sense or anti-sense primer [*P. marinus:* GGCGCTCTCGAGGAACTTCTCG; *I. unicuspis:* CGCGTG CCCTGTTCGTGACCAATAA; *I. fossor:* CAGGGTTCGTGTCACGTGGCGCTCT; *L. tridentatus:* CCTACACACAGCCACTCTGGGACACGC; *L. richardsoni:* CGTCAC AGACCACAGCGAGGGCATT; *L. appendix:* GACCCCTGCTGGAGGAGCTTGAGG C; *G. australis:* GGCTCTCGCTGGACGGGTTCG; *M. mordax*: CTGCGAGAGGTA ACTGAGGAGGTC]. All reactions were cycled using the Eppendorf Master Gradient thermal cycler under the following parameters: 95°C 2 min followed by 5 cycles of 95 °C

for 15 sec, 2 °C above primer specific annealing temp 5 min, 5 cycles of 95 °C for 15 sec, primer specific annealing temp, 10 cycles of 95 °C for 15 sec, primer specific annealing temp less 2 °C for 5 min, 15 cycles of 95 °C for 15 sec, primer specific annealing temperature less 4 °C for 5 minutes, and finished with a final 5 minutes 72 °C incubation and 4 °C hold. Amplified 5' ends were cloned and sequenced as described above.

Phylogenetic Analysis

The DNAStar suite by Lasergene was used for sequence analysis (EditSeq) and alignments (MegAlign). Two alignments of the GnRH deduced amino acid precursors (including the signal peptide, GnRH decapeptide, dibasic cleavage site and GnRH associated peptide) were constructed using ClustalW. The first alignment contained the 8 lamprey GnRH-III precursors (or prepro GnRH) with 64 GnRH precursors representing type I, II and III GnRHs as well as the octopus GnRH and 2 tunicate prepro-GnRHs. This analysis was rooted using the prepro-octopus GnRH protein; the tunicate forms were not used as the root due to alignment difficulties. The second alignment contained only the 8 lamprey GnRH precursors. Both were analyzed using Phylogenetic Analysis Using Parsimony (PAUP) version 4.0beta10 (Swofford, 2001), and trees were constructed using the neighbor joining method.

Reverse Transcriptase PCR (RT-PCR)

RNA extracts from all tissues collected were treated with RQ1-RNase free-DNase (Promega). 50 ng of DNase free RNA was used in each reaction using the AccessQuick RT-PCR System (Promega) with a 0.1 μM final primer concentration in a 25.5 μL final

reaction volume. The primers used were designed to amplify an approximately 400-500 base pair product [*P. marinus* 5': CTG GAATCATCACAGAAGCCACACT, 3': TCTAAGAGACGTCACAGACCACAGC; *I. unicuspis* 5': GTGTCGCTGACGCACACACAGCAGT, 3': TCATGTTGACGATAC GCTGAGCGGC; *I. fossor* 5': CACACTCGGCTGCTTGTAGACAT, 3': AGCGGC GATGAAGAATTAAATAAAC ; *L. tridentatus* 5': CGGTGGTTTATTTTCTCAACA GACC, 3': TCTAAGAGACGTCACAAACCAGAGC; *L. richardsoni* 5': GAAACA AACAGATTCCTCTCCGAGC, 3': CGTTGATTATCTTCGTCTGCAGCTT ; *L. appendix* 5': CTGGAATCATCACAGAAGCCACACT, 3': CTGAGCGGCGATGAA AAATTAAATA; *G. australis* 5': ACAACTTTATTCACGGACAACACCC, 3': AGA TTGTGAGCTACCTCTCGCAGAA; *M. mordax* 5': ACACGTGTTGAGACGATG GAGAAAT, 3': GATACACCTTGCAGGAATCATCACC]. Thermal cycling was performed using an Eppendorf Master Gradient thermal cycler using the following parameters: 48°C for 45 min, 95 °C for 2 min followed by 30 cycles of 95 °C for 15 sec, primer specific annealing temperature for 1 min and 72 °C for 1 min, and finishing with a 5 min 72 °C incubation and 4 °C hold. Samples were analyzed by electrophoresis using 2% agarose gels stained with ethidium bromide, and were visualized using the Molecular Imager FX (BioRad, Hercules, CA).

Results

Cloning of the Lamprey GnRH-III cDNAs

The initial products amplified by degenerate PCR encoded from the mature GnRH decapeptide through the poly-adenylation sequence. These sequences were then used to

develop the gene specific primers that were used for 5'RACE, which amplified products from the 5' untranslated region (UTR) through the mature GnRH decapeptide. In all, eight cDNAs were cloned that encoded the conserved tripartite structure of the prepro-GnRH protein, including a signal peptide (Sig), mature lamprey GnRH-III decapeptide and dibasic cleavage site, followed by the GnRH associated peptide (GAP), which are flanked by untranslated regions on either side.

The 8 lamprey GnRH-III cDNAs were used to construct a percent identity matrix (Table 6). This matrix shows the high percent identity (~90%) between the Petromyzonidae sequences, and most notably the relatively low percent identity between the two southern hemisphere sequences, the *G. australis* and *M. mordax* (48.5%), highlighted in grey. The encoded precursor proteins were compared by domain, which showed a high level of sequence conservation relative to the *P. marinus* (Figure. 28). The signal peptide and GAP region are highly conserved within the Petromyzonidae, but are less conserved compared between families.

cDNA Percent Identity Matrix

	If	*Iu*	*La*	*Lr*	*Lt*	*Pm*	*Ga*	*Mm*
I. Fossor	X	88.8	89.5	90.1	88.0	89.0	54.3	49.2
I. Unicuspis		X	95.4	90.8	91.6	95.4	59.2	53.9
L. appendix			X	92.3	93.1	93.9	57.3	52.6
L. richardsoni				X	91.5	90.7	56.3	54.2
L. tridentatus					X	92.8	59.2	57.1
P. marinus						X	60.9	53.6
G australis							X	48.5
M. mordax								X

Table 6. Percent Identity Matrix of the Cloned Lamprey GnRH-III cDNAs. The eight lamprey GnRH-III cDNAs were used to construct a percent identity matrix. As expected, there is a relatively high percent identity between the holarctic sequences (low 90's). Most notably, the percent identity between the sequences from the two southern hemisphere families is relatively low (48.5).

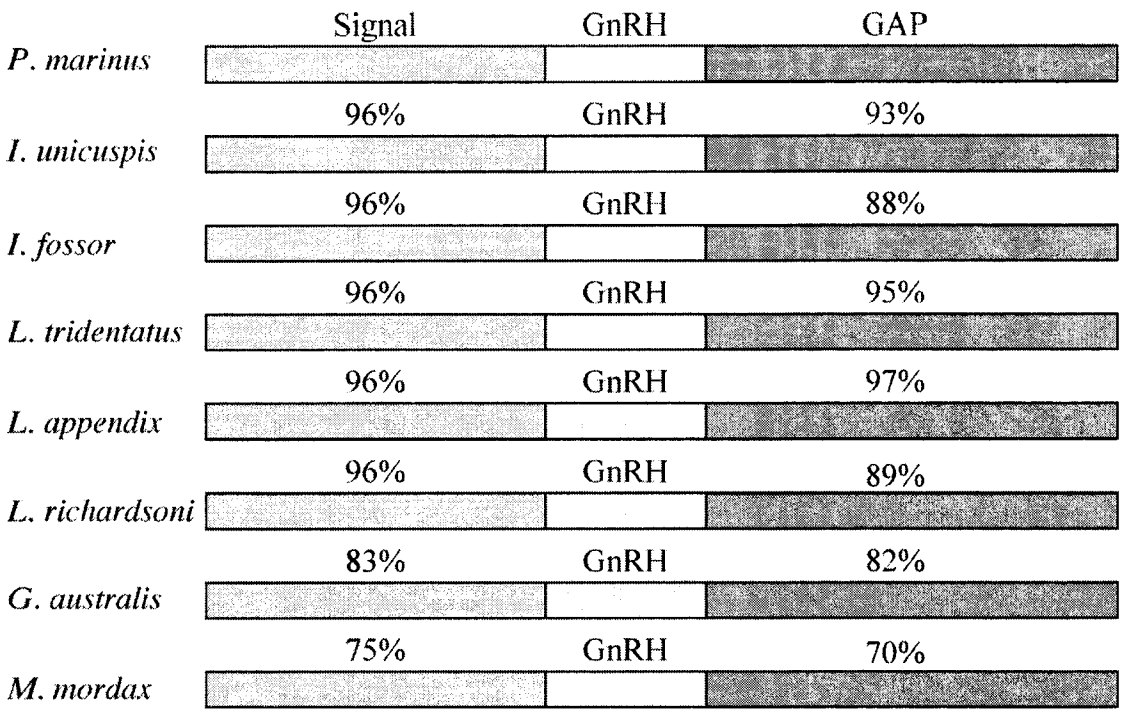

Figure 28. Lamprey GNRH-III Protein Precursor Domain Conservation. The signal peptide (mid-grey) and GAP (dark grey) regions of the lamprey GNRH-III precursors were compared relative to the *P. marinus*. Both regions are highly conserved within the Petromyzonidae, but are less conserved between the three families.

The *P. marinus* lamprey GnRH-III cDNA (AY052628) is 718bp in length and includes an open reading frame (ORF), $bp_{107\text{-}388}$, which encodes a 93 amino acid (aa) peptide (Sig_{24aa}, GAP_{56aa}). The *L. appendix* lamprey GnRH-III cDNA (AY307176) is 722bp in length and contains an ORF, $bp_{111\text{-}389}$, which encodes a 92aa peptide (Sig_{24aa}, GAP_{55aa}). The *L. tridentatus* lamprey GnRH-III cDNA (AY307178) is 732bp in length and contains an ORF, $bp_{121\text{-}399}$, which encodes a 92aa peptide (Sig_{24aa}, GAP_{55aa}). The *L. richardsoni* lamprey GnRH-III cDNA (AY307177) is 710bp in length and contains an ORF, $bp_{110\text{-}388}$, which encodes a 92aa peptide (Sig_{24aa}, GAP_{55aa}). The *I. unicuspis* lamprey GnRH-III cDNA (AY307176) is 723bp in length and contains an ORF, $bp_{114\text{-}392}$,

which encodes a 92aa peptide (Sig_{24aa}, GAP_{55aa}). The *I. fossor* lamprey GnRH-III cDNA (AY307175) is 728bp in length and contains an ORF, $bp_{108-386}$, which encodes a 92aa peptide (Sig_{24aa}, GAP_{55aa}). The *G. australis* lamprey GnRH-III cDNA (AY307172) is 774bp in length and contains an ORF, $bp_{124-408}$, which encodes a 94aa peptide (Sig_{25aa}, GAP_{56aa}). The *M. mordax* lamprey GnRH-III cDNA (AY307173) is 666bp in length and contains an ORF, bp_{85-363}, which encodes a 92aa peptide (Sig_{24aa}, GAP_{55aa}) (See Figures 29-32).

Phylogenetic Analysis

The first alignment used included the eight newly discovered deduced amino acid lamprey GnRH-III precursor (preproGnRH) sequences with 64 other sequences from GenBank. These additional sequences were selected such that representatives from the different classes of vertebrates where included for each GnRH type where possible. Trees were constructed using the neighbor joining method with the prepro-octopus GnRH (AB037165) sequence as an out-group (Figure. 33). The produced tree clearly shows the lamprey GnRH forms grouping together, yet outside of the other previously described groups of GnRH (I, II and III). Phylogenetic analysis of the 8 lamprey GnRH-III cDNAs shows the sequences are divided into 3 groups (Figure. 34), which is a neighbor joining tree overlaid with bootstrap values (1000 replicates). This analysis, where branch length represents sequence distance, clearly shows the sequences are grouped corresponding to the Petromyzonidae, Geotriidae or Mordaciidae, and furthermore, the separation of the Geotriidae and Mordaciidae into two distinct families is supported as the *G. australis* and *M. mordax* sequence are approximately equally removed from each other as they are from the Petromyzonidae sequences.

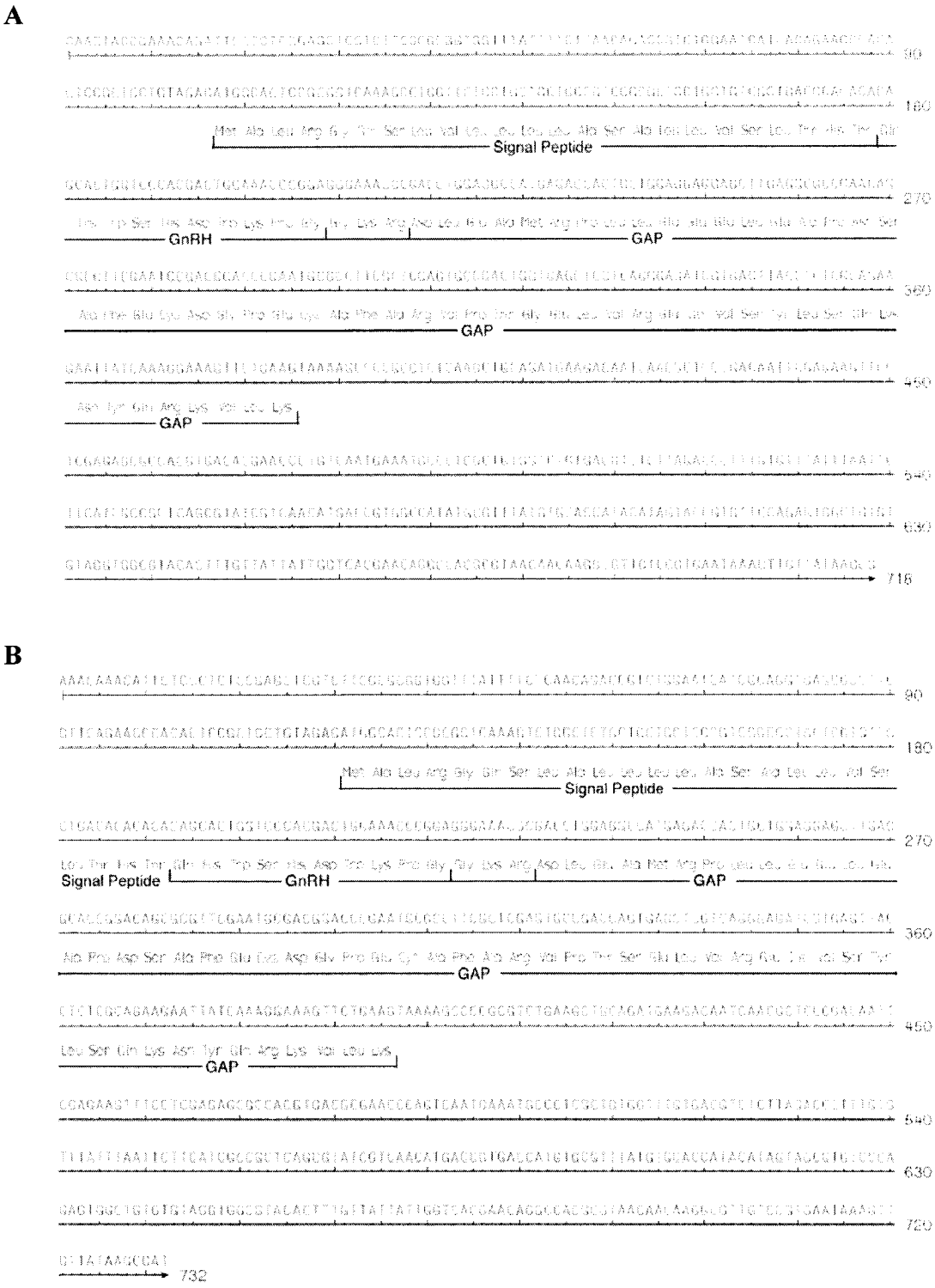

Figure 29 Lamprey GnRH-III cDNA Sequences I. Lamprey GnRH-III cDNA and encoded protein from the *Petromyzon marinus* (A) and *Lampetra tridentatus* (B).

Figure 30. Lamprey GnRH-III cDNA Sequences II. Lamprey GnRH-III cDNA and encoded protein from the *Lampetra appendix* (A) and *Lampetra richardsoni* (B).

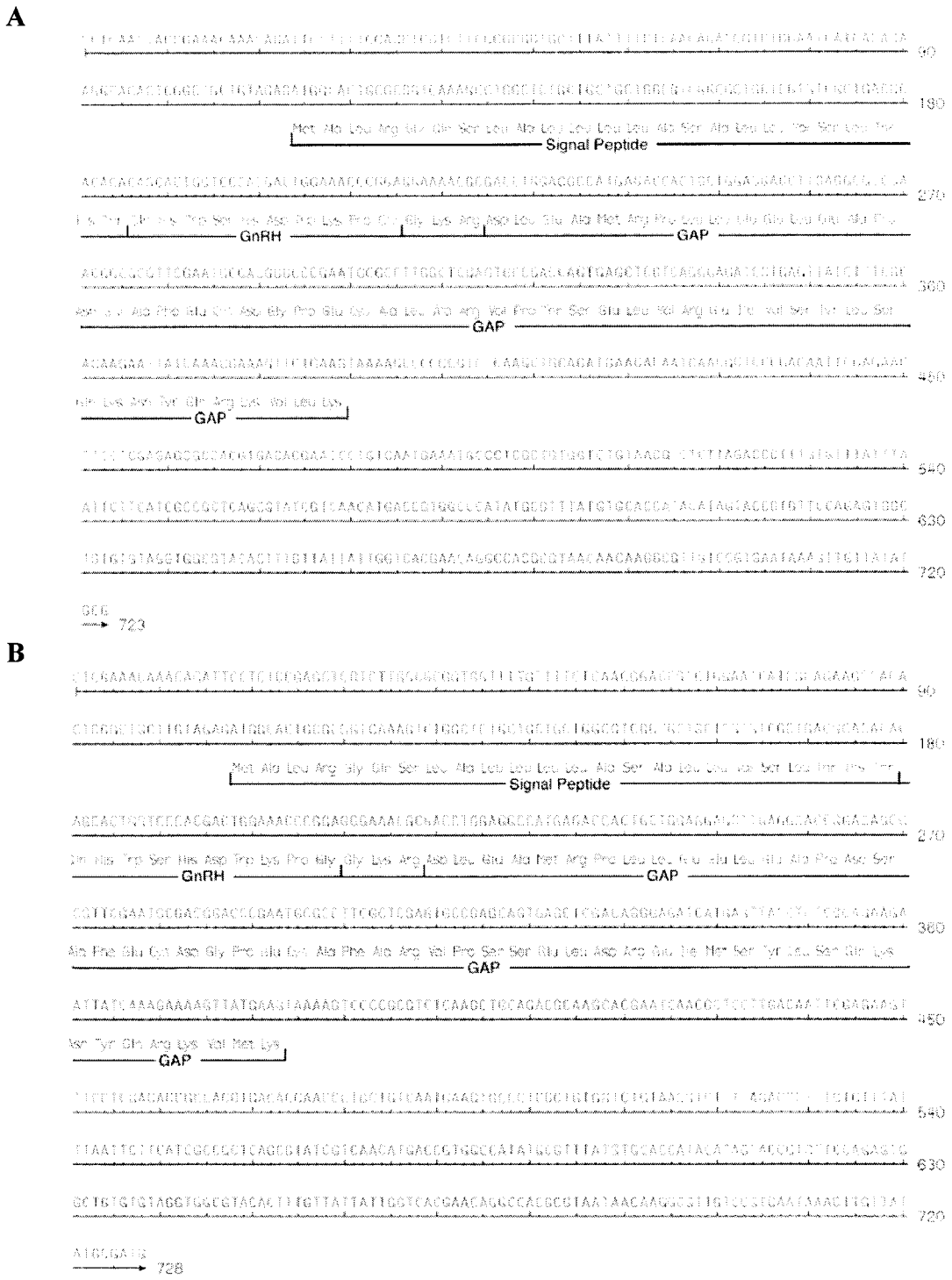

Figure 31. Lamprey GnRH-III cDNA Sequences III. Lamprey GnRH-III cDNA and encoded protein from the *Ichthyomyzon unicuspis* (A) and *Ichthyomyzon fossor* (B).

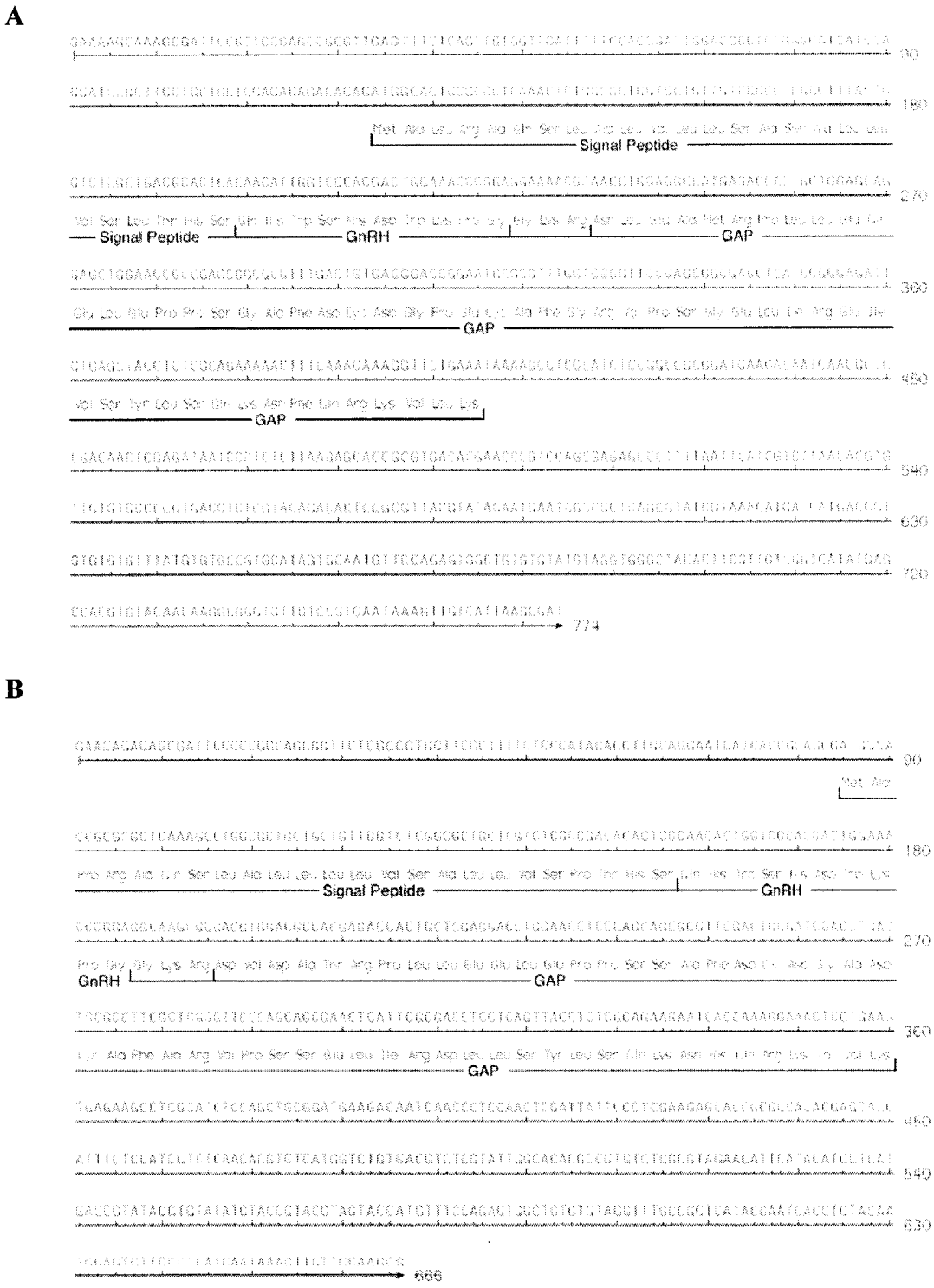

Figure 32. Lamprey GnRH-III cDNA Sequences IV. Lamprey GnRH-III cDNA and encoded protein from the *Geotria australis* (A) and *Mordacia mordax* (B).

RT-PCR

Analysis of tissue specific expression of lamprey GnRH-III was limited to the available tissues from each species. In all species examined the lamprey GnRH-III amplicon was visualized in the brain. Of the 4 pituitary samples, *P. marinus, I. fossor, I. unicuspis* and *G. australis*, lamprey GnRH-III expression was only visualized in the *P. marinus* and *I. unicuspis*. Lamprey GnRH-III expression in the gonads was only seen in the ovary of the *L. tridentatus*, which was verified by sequence analysis (Figure. 35). Expression was not seen in any of the other tissues examined. Negative controls, which were identical to the experimental reactions in setup and cycling but lacked RNA template, did not show any bands (data not shown).

Discussion

We have cloned the full-length cDNA encoding the deduced prepro-lamprey GnRH-III precursor from eight species of lamprey. Based on our phylogenetic analysis of 72 GnRH precursors, including the lamprey GnRH-III precursor sequences, along with data from previous immunocytochemical and functional studies, we propose that the lamprey GnRH isoforms constitute a fourth group of the GnRH family of peptides. In addition, our data showed that the lamprey GnRH-III precursors of the two southern hemisphere species are highly divergent from the lamprey GnRH-III of the holarctic species, and to each other, which supports the lamprey phylogeny based on dentition (Hubbs and Potter, 1971).

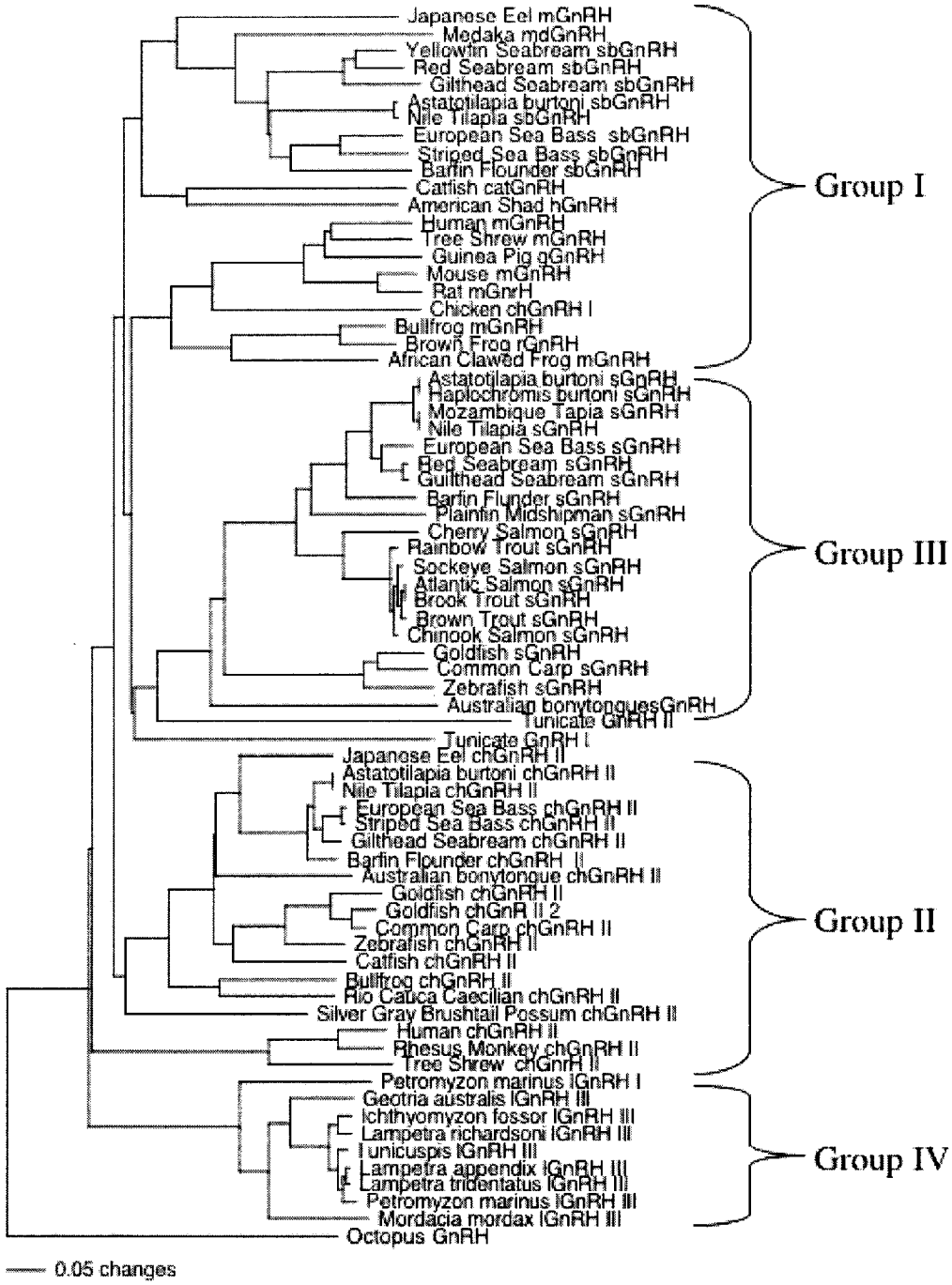

Figure 33. Phylogenetic Analysis of GnRH Precursors. The neighbor joining method was used to construct this phylogenetic tree of the deduced amino acid prepro-GnRH precursors (Signal peptide, GnRH, dibasic cleavage site and GAP), which is rooted with octopus GnRH. The lamprey GnRH forms group separately from the previously described type I, II and III GnRHs, which suggests they form a unique lineage of the GnRH family. M, mammalian; cat, catfish; h, herring; g, guinea pig; ch, chicken; r, rana; md, medaka; sb, seabream; s, salmon; l, lamprey.

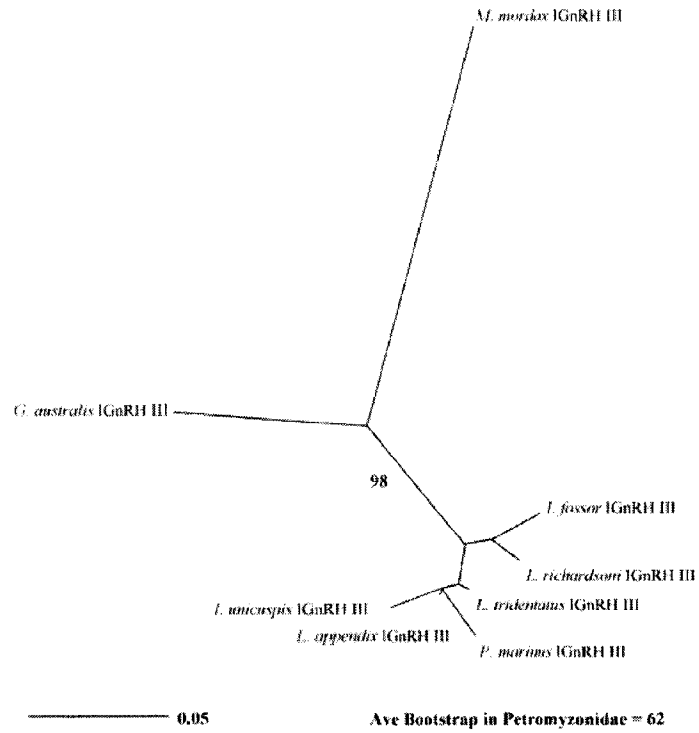

Figure 34. Phylogenetic Analysis of the Lamprey GnRH-III Precursors Proteins. The neighbor joining method was used to construct this unrooted phylogenetic tree, where branch length represents sequence dissimilarity, of the eight prepro-lamprey GNRH-III precursors (signal peptide, GnRH, dibasic cleavage site and GAP). The topology of the tree is divided into three groups corresponding to the three families of lamprey, the Petromyzonidae, Geotriidae and Mordaciidae. The analysis was resampled with 1000 bootstrap replicates, which were averaged for the Petromyzonidae group for logistical purposes. This tree supports the lamprey phylogeny based on dentition at the family level, which is a strong division as the bootstrap value of the internal branch between the three groups is 98.

Although the application of molecular phylogenetic analysis to reconstruct species phylogeny is prone to conflicting results, it is widely accepted as an invaluable addition to anatomical, physiological, and behavioral analyses (Brocchieri, 2001; Lio andGoldman, 1998; Slowinski and Page, 1999). In our analysis a molecular phylogenetic approach was used to assess the previously proposed phylogeny of Petromyzoniformes (Hubbs and Potter, 1971), which was based primarily on the size, shape and distribution of dentition.

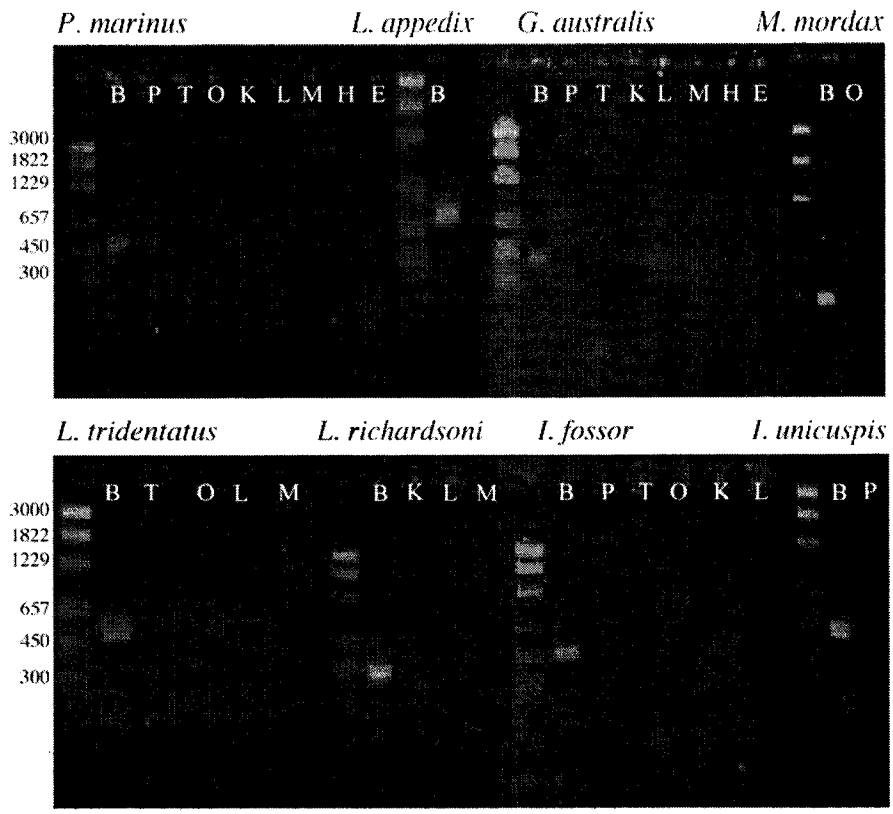

Figure 35. Tissue Specific Expression of the Lamprey GnRH-III Transcript. Tissue specific expression of the lamprey GNRH-III transcript was determined using RT-PCR. Products were analyzed by gel electrophoresis using the PGem7 ladder (in lane one of each gel), which contains molecular weight markers of 3000, 1822, 1229, 657, 450 and 300 bp. B, brain; P, pituitary; T, testes; O, ovary; K, kidney; L, liver; M, muscle; H, heart; E, eye.

Based on this scheme, it is proposed that the lamprey lineage is divided into three families, including the Petromyzonidae, or holarctic species, and the two southern hemisphere families, which include the Geotriidae and Mordaciidae. This model has been supported by sequence analysis of the primary structure of insulin, which suggested that the *M. mordax* insulin was more closely related to the insulin of the holarctic species than to the *G. australis* insulin, although no molecular phylogenetic analysis has been performed to date to reexamine this relationship (Conlon, 2001; Conlon et al., 2001). Our phylogenetic analysis of the cloned lamprey GnRH-III precursors, using the neighbor

joining method, in which the tree is divided into 3 groups corresponding to the Petromyzonidae, Geotriidae and Mordaciidae, supports the previously described lineage based on dentition at the family level. Furthermore, this tree, which shows the distances between taxa as a reflection of dissimilarity, confirms the tripartite division of the Petromyzoniformes in which *M. mordax* lamprey GnRH-III and *G. australis* lamprey GnRH-III sequences are approximately equally removed from holarctic clade as they are from each other. However, the internal grouping within the Petromyzonidae did not match the arrangement of the phylogeny based on dentition, in which the *Ichthyomyzon* lamprey GnRH-III sequences and *Lampetra* lamprey GnRH-III sequences would be expected to form groups together, but were rather mismatched in our analysis. Nonetheless, our analysis has provided the first supporting evidence of the phylogeny based on dentition at the family level. Further analysis on the internal grouping would need to be verified using additional traits, such as a more standard molecular marker and/or additional anatomical / physiological / behavioral traits. Concerning the bootstrap values, although there was an average bootstrap value of 62 within the Petromyzonidae sequences, the most critical internal branch, which divides the three groups of sequences into Geotriidae, Mordaciidae and Petromyzonidae was 98, and therefore we are only drawing conclusions at the family level.

The GnRH family of peptides is a highly conserved group of neurohormones that has been subject to intense investigation since the first primary structure of GnRH was identified in the early 1970s (Burgus et al., 1972; Matsuo et al., 1971). As more sequences have been identified and more species representing different classes of vertebrates have been investigated it has become clear that multiple forms of GnRH are

expressed within the brain of a single species (Dubois et al., 2002; King and Millar, 1995; White et al., 1998; White and Fernald, 1998). An earlier analysis considering phylogenetic analysis of 23 GnRH transcripts, location of expression within the brain, and function demonstrated that the GnRH family was composed of 3 paralgous lineages (Fernald and White, 1999), which included GnRH-I (hypothalamic releasing form: mammalian GnRH and orthologs), GnRH-II (midbrain neuromodulatory form: chicken GnRH-II in all vertebrates but lamprey) and GnRH-III (telencephalic neuromodulatory form: salmon GnRH, found only in teleosts). It was later suggested that a 4th lineage of GnRH consisted of medaka and seabream GnRH, which is based exclusively on the difference in origin of the cell bodies that produce these forms of GnRH compared to the other type I GnRHs (Parhar, 2002). Neither of these analyses considered the lamprey GnRH forms of GnRH in their models, and as such we have re-evaluated these groupings using the deduced amino acid sequences from the eight cloned lamprey GnRH-III cDNAs, the previously described prepro lamprey GnRH-I protein (Suzuki et al., 2000), and the known distribution and origin of the GnRH lineages. Our phylogenetic analysis confirms Fernald and Whites division of the GnRH family, but shows the medaka and seabream forms of GnRH grouping with the type I GnRHs, which conflicts with Parhar's model. Additionally the lamprey GnRH forms group together separately from the three previously described lineages of GnRH, and as such we suggest that they, and not medaka and seabream GnRH, form the 4th lineage of GnRH (Table 7). Our assertion is based directly on our phylogenetic analysis which is supported by immunocytochemical and functional data on lamprey GnRH. The origin of the cells that produce the lamprey GnRH forms, which were shown to arise from cells in the proliferative zones of the

GnRH	Distribution/Origin	Primary GnRH Structures Identified in Vertebrates
GnRH-I	Hypothalamus, diencephalon/ Olfactory origin	Mammal GnRH in *mouse, primate, human, sheep, pig, eel, newt, frog*; chicken GnRH-I in *chicken, lizard*; salmon GnRH in *goldfish, salmon*; catfish GnRH in *catfish*; dogfish GnRH in *dogfish*
GnRH-II	Midbrain/ ventricular ependyma	Chicken GnRH-II in *primate, human, chicken, lizard, frog, newt, eel, goldfish, catfish, salmon, medaka, red seabream, tilapia, ratfish*
GnRH-III	Telencephalon/ Olfactory	Salmon GnRH in *medaka, red seabream, tilapia*
GnRH-IV	Hypothalamus, diencephalons/ ventricular	Lamprey GnRH-I and lamprey GnRH-III in *lamprey*

Table 7. The Four Putative Paralogous Lineages of GnRH. The GnRH family is proposed to be divided into four paralogous lineages based on phylogenetic analysis, function, neural distribution and developmental origin.

diencephalon (Tobet et al., 1993; Tobet et al., 1997), differs from the origin of the type I GnRHs, which arise from cells in the olfactory placode (Norgren and Gao, 1994; Schwanzel-Fukuda, 1999; Schwanzel-Fukuda et al., 1989; Wray et al., 1989). In addition, both lamprey GnRH-I and lamprey GnRH-III differ from the type I GnRH lineage as they are both distributed within the pre-optic-neurohypophyseal system of the lamprey as demonstrated using immunocytochemistry in the *P. marinus* (Nozaki et al., 2000), *G. australis* (Sower et al., 2000), *I. unicuspis* (Eisthen and Northcutt, 1996), *L. richardsoni* (Crim, 1985) *and L. tridentatus* (Crim et al., 1979), and both are active in the regulation of the reproductive axis in the *P. marinus* (Deragon and Sower, 1994). In order to ultimately understand the molecular evolution of the GnRH family additional sequence data, distribution, functional and physiological data are needed across vertebrates and invertebrates.

Expression of the lamprey GnRH-III mRNA was detected in the brain of all 8 species, as well as the pituitary of the *P. marinus* and *I. unicuspis*. Expression was also detected in the ovary of the *L. tridentatus*, which is unique among all of the species tested.

The expression pattern of lamprey GnRH-III in the *P. marinus* differs to that of the previously described lamprey GnRH-I pattern, where expression was only seen in the brain and faintly in the testis (Suzuki et al., 2000), although pituitary expression was not investigated in this study. GnRH expression in non-neural tissues is typical (Azad et al., 1991; Dong et al., 1996; White and Fernald, 1998; Yoo et al., 2000), although it's function in tissues other than the gonad is unclear. In *P. marinus* it has been demonstrated that both lamprey GnRH-I and -III stimulates steroidogenesis at the level of the gonads, suggesting a possible paracrine and/or autocrine regulatory mechanism (Gazourian et al., 1997; Gazourian et al., 2000), although there was no lamprey GnRH-III expression detected in the *P. marinus* testis or ovary in this study.

In summary, the cDNA encoding the lamprey GnRH-III was cloned from eight species of lamprey, which was used in a series of phylogenetic analyses to address questions concerning the molecular phylogeny of the GnRH family and the lineage of the Petromyzoniformes. Based on our phylogenetic analysis using the deduced prepro lamprey GnRH-III with 64 other GnRH precursors, along with data from previous immunocytochemical and functional studies, we propose that the lamprey forms of GnRH constitute a unique lineage within the GnRH family. The phylogenetic analysis of the lamprey GnRH-III precursors supports the phylogeny based on dentition at the family level, dividing into the Petromyzonidae, Geotriidae and Mordaciidae. The information derived from this study provides critical information on the molecular evolution of GnRH in vertebrates.

CHAPTER V

SUMMARY

The field of reproductive neuroendocrinology has grown immensely during the past 30 years, however the ancestral origin of the hypothalamic-pituitary system within vertebrates is still in part undefined, although it has been actively studied by Dr. Sower, her collaborators and students. Previous studies investigating fish of ancient origin have identified many of the components of the HPG axis in lamprey, including the peptide and cDNA encoding lamprey GnRH-I (Sherwood et al., 1986; Suzuki et al., 2000) and the lamprey GnRH-III peptide (Sower et al., 1993), the cDNA encoding a putative type II GnRH receptor, and most recently, a putative gonadotropin (Sower et al., accepted) and gonadotropin receptor (unpublished). The functional role of the lamprey GnRHs has been established based on many physiological, biochemical and immunological studies, in which both lamprey GnRH-I and lamprey GnRH-III have been shown to regulate steroidogenesis and gametogenesis (Deragon and Sower, 1994; Sower, 2003). The lamprey GnRH receptor was first described using *in vitro* quantitative autoradiography, from which two high affinity binding sites were characterized in the proximal pars distalis (Knox et al., 1994). These studies led to the cloning of a type II GnRH receptor from the lamprey pituitary followed by phylogenetic analysis and *in situ* hybridization studies by Nathaniel Nucci, a previous graduate student from Dr. Sower's laboratory.

These studies provided the impetus for the research presented within this dissertation, which contributes to the field of neuroendocrinology through the analysis of the key molecular components involved in the regulation of the HPG axis of the sea lamprey. This dissertation is divided into two major components, the function characterization and kinetic studies of the lamprey GnRH receptor (described in chapters II and III), and the cloning and analysis of the lamprey GnRH-III cDNA (described in chapter IV). Separately, these data have resulted in three first author publications (one in preparation), a patent and two additional patent applications (in review). Together, these data provide further evidence to support the hypothesis that the HPG axis is highly conserved across vertebrates, and that the evolution of the molecular components and mechanisms most likely antedated the origin of all known vertebrates.

Interest in GnRH receptors has developed due to its dual significance in both medicinal therapy development and the understanding of GPCR molecular evolution. In a general sense, approximately 45% of all drugs on the market target GPCRs , while the GnRH receptor is a target for several pharmaceuticals, such as Lupron Depot (TAP Pharmaceutical Products Inc.), which is the number one prescription for the treatment of prostate cancer. The GnRH receptor is unique among the approximately 1000 GPCRs in the human genome as it lacks the highly conserved intracellular C-terminal tail, which is thought to be critical for G-protein coupling, structural stability, internalization and cell surface expression (Blomenrohr et al., 1999; Lin et al., 1998). The GnRH receptor has been the focus of many studies investigating reproduction in species ranging from mammals to teleosts, however little is known about these receptors in agnathans, the most ancient lineage of vertebrates. As mentioned above, GnRH binding sites have been

characterized in the lamprey pituitary and a type II GnRH receptor has recently been cloned. Interestingly, the lamprey GnRH receptor has the longest C-terminal tail of any vertebrate GnRH receptor which opens the question as to the role of this extra long tail.

The aims of the initial studies described in this dissertation were to determine whether the lamprey GnRH receptor cDNA encoded a functional protein, and to examine the function of the lengthy C-terminal tail. To accomplish these aims, cell culture, transfection and second messenger assays first needed to be developed and characterized. Analysis of IP_3 accumulation was initially chosen for characterization since it was thought to be the primary signaling pathway of GnRH receptors. Both lamprey GnRH-I and lamprey GnRH-III were found to activate the lamprey GnRH receptor in a dose dependant manner, however lamprey GnRH-III was shown to be more potent. Serial truncation of the C-terminal tail decreased the magnitude of IP accumulation, however the tail-less mutant showed a full recovery compared to wild-type levels, which was significant in that it showed that the tail-less mutant retained ligand binding activity and that $G\alpha_{q/11}$ coupling was not dependent on the C-terminal tail. The full recovery in IP accumulation of the tail-less mutant receptor also indicated that perhaps a decrease in the level of ligand dependant internalization compensated for a decrease in binding affinity. Ultimately, further studies were needed for evaluation of the lamprey GnRH receptor since efficacy data on their own are difficult to interpret and can be misleading.

Given that the lamprey GnRH receptor was functional and was activated by both lamprey GnRH-I and lamprey GnRH-III, a series of experiments were designed to further investigate its functional characteristics and ligand binding properties. The lamprey GnRH receptor activated cAMP accumulation in response to both lamprey GnRH-I and

lamprey GnRH-III, where again lamprey GnRH-III was more potent. Serial truncation of the C-terminal tail again led to a reduction in efficacy, however cAMP accumulation was not recovered by the tail-less mutant. These data suggested that a motif within the first 40 amino acids of the C-terminal tail is involved in $G\alpha_s$ coupling. Pharmacological profiling was used to verify that the lamprey GnRH receptor is lamprey GnRH-III selective. The Ki of lamprey GnRH-I was 390% higher then lamprey GnRH-III, indicating a lower binding affinity. Based on IP accumulation, cAMP accumulation and pharmacological profiling, the lamprey GnRH receptor is proposed to be lamprey GnRH-III selective. The wild-type and C-terminal tail mutant lamprey GnRH receptors were then used in saturation binding assays using intact adherent cells. Truncations of the lamprey GnRH receptor's C-terminal tail were shown to reduce binding affinity, which explains their reductions in signaling capacity. Furthermore, the saturation binding assays using the wild type and mutant lamprey GnRH receptors revealed that the tail-less lamprey GnRH receptor is capable of binding lamprey GnRH-I, and therefore further supports the hypothesis that $G\alpha_s$ couples to a motif within the first 40aa of the C-terminal tail. Finally, the lamprey GnRH receptor was shown to undergo rapid ligand dependant internalization, which was greatly diminished in the tail-less mutant form. This unique lamprey GnRH receptor, with both high affinity for lamprey GnRH-III and chicken GnRH-II and ligand binding activity in the tail-less form may represent an important ancestral state which provides insight into the function and evolution of the vertebrate GnRH receptor family.

These data indicate that the lamprey GnRH receptor activates IP_3 accumulation to a greater magnitude compared to cAMP, when stimulated with lower concentrations of agonist (Figure 36). These data thus suggest that IP_3 may be the predominant mechanism

of signal transduction associated with the lamprey GnRH receptor. However, it cannot be ruled out that in light of the extra length of the C-terminal tail of the GnRH receptor, another unknown signal transduction system may be used in lamprey pituitary gonadotrope cells in response to GnRH. All functional studies described within this dissertation were performed in mammalian cells which typically are tested for cAMP and IP_3. C-terminal tail truncations indicate that a motif located within the membrane proximal 40 amino acids is involved in $G\alpha_s$ coupling, however $G\alpha_{q/11}$ coupling likely takes place within one of the intracellular loops. Furthermore, an additional motif within the membrane proximal 40 amino acids is required for rapid ligand induced internalization. The C-terminal tail therefore is likely important for structural integrity, or rather the truncation or removal of which results in a structural perturbation leading to a decreased binding affinity. Since the 40 amino acid C-terminal tail lamprey GnRH receptor mutant is capable of stimulating both IP_3 and cAMP accumulation and undergoes rapid ligand dependant internalization, it is possible that the extensive length of the lamprey GnRH receptor C-terminal tail may not have a functional significance for these signaling systems. The loss of amino acids in the C-terminal tail of GnRH receptors during the subsequent evolution of vertebrates to the tail-less form in mammals may reflect the various actions of GnRH in controlling pituitary gonadotropins through diverse regulatory mechanisms. One major difference between lampreys and mammals is the transport system of neurohormones to the pituitary. In mammals, neurohormones are released into the median eminence and travel via the portal system to the anterior pituitary gonadotrope cells. In contrast, in lampreys, GnRH travels to the pituitary either

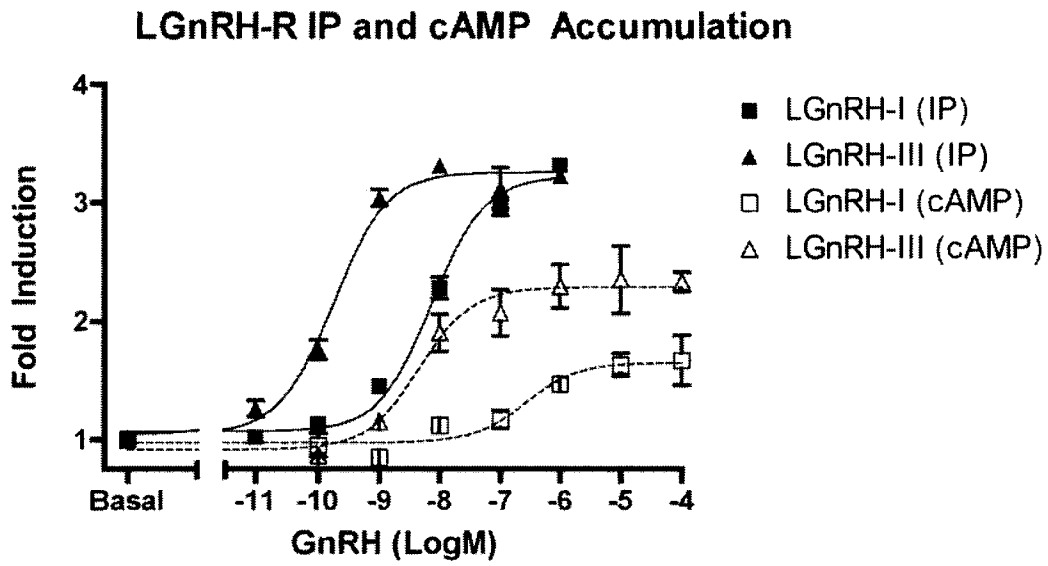

Figure 36. Lamprey GnRH Receptor Signaling Composite. The lamprey GnRH receptor activates both IP (line with filled in icon) and cAMP (dashed with outlined icon) accumulation when stimulated with either lamprey GnRH-I (square) or lamprey GnRH-III (triangle). These data are represented here relative to fold induction over basal levels. IP accumulation is occurs to a greater magnitude compared to cAMP, and is triggered at a lower concentration of agonist.

by diffusion and/or by transport from the third ventricle following release. Lampreys only spawn once in their life and die and the molecular mechanisms involved in GnRH-GnRH-R interactions likely differ between lampreys and later evolved vertebrates. Moreover, the longer transport time of GnRH to the pituitary (Nozaki et al., 1994) and higher concentrations of GnRH in lampreys compared to mammals may be reflected in some significant differences in the GnRH receptor in its binding and signaling properties.

The future directions of this project include several different lines of research. Most directly, further functional characterization would need to be performed to identify specific residues within the C-terminal tail that are involved in both the rapid internalization and cAMP signaling. This could be performed using site-directed mutagenesis to remove phosphoacceptor sites, one at a time or in multiples, within the first 40 amino acids of the C-terminal tail and perform internalization assays.

Characterization of cAMP signaling could start with mutation of the HFRK-like motif located in the membrane proximal region of the lamprey GnRH receptor C-terminal tail. Furthermore, characterizing MAPK signaling and the role of β-arrestin dependant internalization on this process would be of interest. Notably, all of these studies were performed using COS7 cells, a monkey kidney cell line typically used to characterize receptor function. It is very possible that the lamprey GnRH receptor may function differently within lamprey gonadotropes, and therefore it would be helpful to develop a lamprey pituitary cell line. Description of MAPK signaling may be most significant if performed using a lamprey gonadotrope cell line.

Cloning all other lamprey GnRH receptors would allow the development of specific receptor type agonists and antagonists. These analogs could be used to describe receptor specific signaling events *in vivo*, where for example microarray or proteomic analysis could be used to better understand the cellular response to activation of these putative receptors. Such analogs could also be applied to the lamprey population control initiative within the Great Lakes region, where a non-toxic GnRH receptor subtype specific antagonist could be used to induce infertility while not effecting behavior. These additional sequences would also contribute to our general understanding of the reproductive system within lamprey, and therefore the ancestral vertebrate state.

The second section of this dissertation describes the cloning and analysis of the lamprey GnRH-III cDNA from eight species of lamprey, representing the three lineages of the Petromyzonidae. The lamprey GnRH-III cDNA was cloned using a PCR based strategy, from which the deduced amino acid sequences of the prepro-proteins were used in a series of phylogenetic analyses to address both the molecular evolution of the GnRH

family and the lineage of the Petromyzonidae. The produced trees were divided into four groups, where the lamprey lineage grouped together separately from the three previously described groups. Furthermore, the lamprey GnRH-III sequences were used in an un-rooted phylogenetic analysis, which produced trees that were divided into three groups corresponding to the three families of Petromyzonidae. These data support the lamprey phylogeny based on dentition at the family level, however the groupings within the Petromyzonidae are not well resolved, indicated by the low bootstrap numbers, which likely results from the high level of sequence identity within the group. These data provide the first molecular analysis to address the lamprey phylogeny.

Further research on this topic is warrented; analysis of additional traits would greatly strengthen our understanding of the lamprey phylogeny. For example, analysis of the lamprey GnRH-I cDNAs, GnRH receptors or other sequences could be useful. Indeed, a reconcile tree analysis of several hundred sequences would be a powerful approach. Furthermore, a comparative analysis of genomes would be valuable, however at this time or in the near future it is unlikely that such information would be available for many species of lamprey (the sea lamprey genome is currently being sequence). Additionally, analysis of physiological or anatomical traits would be beneficial as well. Ultimately, a multi-trait approach is needed. In regard to the GnRH family, more data is key. For example, more sequence information from ancient fish, such as hagfish, and from the elasmobranches would provide invaluable insight into the molecular evolution of the GnRH family. Additionally, a better understanding of the expression and function of non-type I GnRHs would help classify novel GnRHs.

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APPENDICES

APPENDIX A

IACUC APPROVAL

UNIVERSITY OF NEW HAMPSHIRE

Office of Sponsored Research
Service Building
51 College Road
Durham, New Hampshire 03824-3585
(603) 862-3564 FAX

LAST NAME	Sower	FIRST NAME	Stacia
DEPT	Biochemistry & Molecular Biology	APP'L DATE	9/24/2001
OFF-CAMPUS ADDRESS (if applicable)	316 Rudman Hall	IACUC #	010902
		REVIEW LEVEL	B
		TODAY'S DATE	10/4/2001
PROJECT TITLE	Neuroendocrine Control of Reproduction in Fish		

All cage, pen or other animal identification records must include your IACUC Protocol # as listed above.

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved the protocol submitted for this study under Category B on Page 4 of the "Application for Review of Animal Use or Instruction Protocol" - the study involves either no pain or potentially involves momentary, slight pain, discomfort or stress.

Approval is granted for a period of three years from the approval date above. Continued approval throughout the three year period is contingent upon completion of annual reports on the use of animals. At the end of the three year approval period you may submit a new application and request for extension to continue this project. Requests for extension must be filed prior to the expiration of the original approval.

Please note: Use of animals in research and instruction is approved contingent upon participation in the UNH Occupational Health Program for persons handling animals. *Participation is mandatory* for all principal investigators and their affiliated personnel, employees of the University and students alike. A Medical History Questionnaire accompanies this approval; please copy and distribute to all listed project staff who have not completed this form already. Completed questionnaires should be sent to Dr. Gladi Porsche, UNH Health Services.

If you have any questions, please contact either Van Gould at 862-4629 or Julie Simpson at 862-2003.

For the Institutional Animal Care and Use Committee,

John A. Litvaitis, Ph.D.
Chair

cc: File

APPENDIX B

A BRIEF HISTORY OF NEUROENDOCRINOLOGY

The following is based primarily from the following sources:

Guillemin, R., 2005. Hypothalamic hormones a.k.a. hypothalamic releasing factors. J Endocrinol, 184(1): 11-28.
Wade, N., 1981. The Nobel Duel. Anchor Press/Doubleday, Garden City, New York.

The classical view of cellular regulation is divided into three isolated systems: neural, immune and endocrine; neurologists studied neural networks and transmitters, immunologists studied antibodies and lymphocytes, and endocrinologists studied the informational molecules released from glands, which traveled via blood to their target organ mediating some physiological response. A series of paradigm shifting events began in 1928 with Ernst Scharrer's concept of neurosecretion; the brain producing and releasing peptides that had extra-neural regulatory function (Scharrer, 1928). One such function of extraordinary interest was the regulation of the pituitary gland, or more specifically the hypophyseal portal vessel-chemotransmitter hypothesis of pituitary control, which was fully developed by the 1940's (Green and Harris, 1947). [NOTE-Gregory Popa published the first modern description of the portal system, but incorrectly postulated that the blood flowed from the anterior pituitary to the hypothalamus (Popa and Fielding, 1930), an idea that was corrected by Wislocki and King in 1936 (Wislocki and King, 1936). Harris was credited with developing the hypothesis that the portal system facilitates the neural regulation of the anterior pituitary]. These two ideas were threaded together in 1955 in light of a study of the hormones involved in the stress cascade, where it was observed that hypothalamic extracts stimulated the release of the

pituitary stress hormone adrenocorticotropic hormone (ACTH) (Saffran and Schally, 1955). These three revolutionary findings solidified the concept of a neuroendocrine system and led to the beginning of the race to identify the first neural peptide in the late 1950's, which began at the Department of Physiology in the College of Medicine at Baylor University in Houston, Texas. The winners of this race ultimately were awarded the Nobel Prize (Wade, 1981).

Isolation of a bioactive neuropeptide was the sought after proof of principle, and was the intense research focus of many scientists, most notably Roger Guillemin and Andrew Schally (there were others, including Sam M. McCann and Geoffrey Harris, who will be briefly noted). The paths of Guillemin and Schally were intertwined; both of European descent they each received PhDs in Montreal, Canada; however Guillemin left for Baylor Medical College one year before Schally's arrival in 1954. Their training in Montreal, Guillemin's in physiology and Schally's in biochemistry, led them to strive to achieve the same goal: to be the one to prove the neuroendocrine principle. The two first became aware of each other while independently seeking to isolate the hypothalamic component that they each had shown to stimulate ACTH release in pituitary cultures [referred to as corticotrophin releasing factor (CRF)]. Guillimen reported his findings in 1955 (Guillemin, 1955; Guillemin and Rosenberg, 1955), however he was overshadowed by Schally's publication of similar results (Saffran and Schally, 1955), which were far more detailed and received greater attention (Wade, 1981). The two met at a scientific conference and decided to join forces, and so it was that Andrew Schally moved to Baylor Medical College in 1957 to become part of the Guillemin team.

The idea was simple: isolate any hypothalamic releasing factor; any one would do, although at the time luteinizing hormone releasing factor (LRF), the hypothalamic substance thought to regulate the reproductive system, was considered the gold mine due to the potential medical applications in the field of reproductive physiology. Despite this interest in LRF, they initially chose CRF as they each had experience in the area, and the assay for ACTH was the most developed pituitary response test available. Right from the start Schally and Guillemin despised each other, Guillemin saw no one as his equal and Schally expected to be just that. In 1960 Guillemin was appointed to a position in Paris, and ran both laboratories from France, and had the advantage of being included on all projects developed by his team. On the other hand, Schally was exposed to the possibility of being left behind if another person on Guillemin's team identified CRF. After years of floundering, clashing and controversy over the validity and possible contamination of their hypothalamic extracts the two split. Schally was offered a position at the VA hospital in New Orleans in 1962, and he jumped at the opportunity to improve his station. In 1963 Guillemin returned to Houston due to political fall out in Paris. Schally and Guillemin, now 350 miles apart, as competitors, rivals and bitter enemies, developed similar strategies: build a large interdisciplinary team and to temporarily leave CRF behind and begin the hunt for thyrotropin-releasing factor (TRF), the supposed hypothalamic component that drives the pituitaries release of thyroid-stimulating hormone-which regulates thyroid function (Wade, 1981).

Over the next seven years each team spent millions of dollars, and came under intense pressure from the National Institutes of Health (NIH), their funding source, to produce or be denied funding. Each had come to the conclusion that TRF was a

tripeptide, possibly linked to some other non-amino acid component (Guillemin et al., 1966; Schally et al., 1966), however they were having immense difficulty with purification and sequence analyses. By 1969 they each knew the amino acid composition to be glutamate (Glu), histadine (His) and proline (Pro), although they didn't know in which order. Each tested six synthetic peptides representing the different possible arraignments of the three amino acids, none of which showed TRF function except for a weak response in the Glu-His-Pro sequence (Schally et al., 1969). The key revelation came to each team practically simultaneously: the peptides ends were blocked. Both teams scrambled to synthesize the tripeptide with a pyro-glutimate on the N-terminal end and an amidated C-terminal end (Pyr-Glu-His-Pro-NH_2), perform their bioassays and rush to publication. Andrew Schally's report of the isolation of the neurohormone TRF was published in *Biochemical and Biophysical Research Communications* on November 6th, 1969 (Boler et al., 1969), 6 days before Guillemin's report was published in *Comptes Rendus* (Burgus et al., 1969).

With the structure of TRF solved, the attention was immediately turned to the structure of LRF, which was widely considered the true endgame. With Schally and Guillemin's teams so focused on TRF, the competition, McCann and Harris, were drawing close to determining the structure of LRF. Additionally, Schally's team dissolved due to internal strife and Guillemin lost valuable time during his move to the Scripps Institute in La Jolla, California in 1970. Having published the structure of TRF their NIH funding was secured and they created even larger teams to help them not only be the first to describe the structure of LRF, but of most importance to beat the other. Both Schally and Guillemin obtained millions of hypothalami (multiple tons) Guillemin

used sheep brains and Schally used pig brain, from which they had isolated micro-gram quantities of LRF. Subsequently both groups had incorrectly concluded that it was a nine amino acid peptide in their initial studies (Wade, 1981). Hisayuki Matsuo, the lead chemist on Schally's team, realized the error that was leading to their false results: the particular assay they were doing was incompatible with tryptophan, which needed to be assayed independently (Wade, 1981). With these results, Schally's group identified the amino acid sequence of LRF, a ten amino acid peptide, which was synthesized and shown to be functional via bioassay. At the Endocrine Society's meeting in San Francisco in 1971, Andrew Schally presented his results, publicly and unquestionably seized victory over Guillemin (Wade, 1981). The chemical structure presented by Schally led Guillemin's group to obtain the sequence of LRF from the sheep, and each group reported their findings, however this time Schally's publication was first by more then six months ahead of Guillemin's publication (Burgus et al., 1972; Matsuo et al., 1971).

In 1977 Andrew Schally and Roger Guillemin split one half of the Nobel Prize in Medicine "for their discoveries concerning the peptide hormone production of the brain". The contribution of neither man was particularly intellectual, because the conceptual groundwork had been previously established by Geoffrey Harris (who notably was very close to discovering the structure of LRF and was thought to potentially have been awarded the Nobel Prize for his contributions to the field, however he died in November of 1971 (Wade, 1981). Schally and Guillemin placed themselves in a strategic position to investigate a topic of extreme importance in a field of excellent researchers. However, they differed from their competitors due to their strong commitment; they operated at an industrial level in an academic setting, gambling everything they had: their reputation,

millions of dollars and even their careers. Their genius, in the end, was in the forward thinking teambuilding strategy they used, which in combination with their 100% commitment to their relentless pursuit won them the ultimate prize in science.

APPENDIX C

CELL CULTURE (COS7 CELLS)

The overview of this protocol is as follows: General considerations, supplies, plating cells, changing media, and finally splitting cultures.

General Considerations

-The key to successful cell culture is good technique. This means all movements while performing cell culture should be deliberate, and much care and patients is needed to perform proper aseptic technique.

- While working in the hood never pass your hand over an open container or piece of equipment that you wish to remain sterile. The air in the hood flows downward, which potentially can carry contaminants from your hand to anything below it.
- Never touch or let anything come in contact with tubes or bottle near the cap.
- When working in a bottle or tube, remove the cap and hold onto it right side up until you place it back on, never put it down or invert it.

-Have all of the tubes/bottles that will be used unscrewed, with the caps resting on the tube/bottle.

- When opening a sleeve of culture dishes, cut open with a razor blade on the bottom of the bag, so the plates come out the right side up, this way there is no chance that dirt will fall from your gloves and get into the side of the dish.
- Never work in the front 1/4 of the hood, as it is the least protected.
- When using a pipet, open the pipet just prior to use, and peel back each side of the wrapper and don't touch it. And when mixing by pipetting up and down, never go all the way to either extreme, thereby avoiding bubbles. When pipeting don't hold your hand directly above the tube/bottle, always keep it at an angle to avoid contamination.
- If there is ever question as to whether a pipet or other container has been contaminated discard said item and use a fresh one.
- Always wear gloves, and spray your hands often with 70% EtOH.
- Put all biohaz in biohaz waste, all other trash, like pipet wrappers just through on the floor, and pick up later on.

-At least 30 minutes prior to starting the flow hood needs to be sterilized.

- First liberally spray the entire inside of the hood with 70% EtOH and wipe down all surfaces with a paper towel. Next, turn on the blower and re-spray the entire inside of the hood with 70% EtOH, close the sash of the hood and turn on the germicidal (UV) light. After a minimum of 30 minutes the hood will be sterile. From this point on, everything that enters the hood should be liberally sprayed down with 70% EtOH.
- **Also during this time get the media pre-warmed to 37°C.**

-There are two viewpoints on using antibiotics/antimycotics in the culture. On one hand they are good to use to keep contamination down, although probably not completely out. On the other hand, not using any antibiotics/antimycotics you will know if you have contamination right away, and a fresh culture can be prepared. Generally it is suggested

that you start your cultures without any antibiotics/antimycotics to make sure your technique is sound, and then use them after a few passages.
-Cells grow optimally at 37°C in 5% CO_2 in a humid environment, which is why we used the humidified CO_2 incubator at 37°C.

Supplies

Dulbecco's modified Eagles medium (DMEM)
Fetal bovine serum (FBS)
1X trypsin-EDTA
Antibiotic/antimycotic (Optional)
Phosphate buffered saline, pH 7.4 (PBS)
(137 mM NaCl, 2.7 mM KCl, 10 mM Na2HPO4, 2 mM KH2PO4)
Dissolve: 8 g NaCl, 0.2 g KCl, 1.44 g Na2HPO4 and 0.24 g of KH2PO4 in 800 mL MQ H2O and adjust pH to 7.4. Fill to 1 L, and I split into 2 500 mL bottles. Sterilize by autoclaving for 20 minutes at 15 psi.
5% CO_2
100X20 mm culture dishes
10 mL pipets
1 mL pipets
9" sterile Pasteur pipets
50 mL falcon tubes
Metal tray
Rubber stopper

Plating Cells (THIS IS A GENERAL DESCRIPTION)

*This will be described based on a culture began 1 mL culture stocks. If the starting material is different in any way protocol modifications may be necessary.

Start 1 culture in a T75 culture plate. Note, using one stock to start more then 1 culture can result in very low cell densities, which can take a long time to grow. Therefore, starting one culture is recommended, which can soon after be split into multiple cultures.

Set up a 50 mL falcon tubes in a tube stand. This tube is used to make DMEM with ~10% fetal bovine serum (FBS). Pipet 18 mL of DMEM from the DMEM bottle into the falcon tube. The FBS, which will separate during the thaw, should be resuspended by pipeting up and down gently. Add 2 mL of FBS to tube 1, and mix by pipet.

When dealing with cells it is common practice to freeze them slowly (generally you can freeze the cells in a Styrofoam container in a –80°C freezer, then from there put them in liquid nitrogen) and to thaw them quickly (to avoid sheering from ice formation). Remove a tube of cells from the liquid nitrogen and place it in a rack at room temperature for just a few minutes (this can be dangerous as tubes can explode during this step, which can cause eyes to fall out). When all the nitrogen is gone the thaw the cells in 37°C water bath until almost thawed. Centrifuge the tube to pellet the cells, remove media (which contains 10% DMSO), and resuspend cells in 1 mL of media. Transfer cells to the T75, add 14 mL of medium and put culture in the 5% CO_2 incubator, which should be set at 37°C.

At this time put the media away. Each bottle should be closed, and sealed with 70% EtOH sprayed aluminum foil, which protects the cap/bottle junction.

To clean up the hood, organize everything, turn off the blower and spray the entire hood down with 70% EtOH. Close the sash, and turn off the light.

Changing Media
Liberally spray down the Pasteur pipet container and bring into the hood. Unfold the foil carefully, without touching the inside, so it stays sterile.

Turn on the vacuum from the hood, which has a line running into the sterile hood. Using your pinky, remove the lid of the Pasteur pipets, keeping in mind to always keep the cap open side down and never moving your hand over an opening that you want to keep sterile. Holding the Pasteur pipet container parallel to the bench top, gently shake and twist the bottle such that one single pipet will slide out of the bottle, which you should grab with your thumb and pointer finger. When the Pasteur pipet is pulled out, the tip should be held high...as in your hand should be kept lower then the tip of the Pasteur pipet. Place the cap back on the container of Pasteur pipets. Fix the Pasteur pipet onto the vacuum. With your left hand uncover the culture dish, holding it to the side (correct side up), bring the Pasteur pipet to the lowest part of the culture dish to aspirate NOTE keep the Pasteur pipet at an angle such that your hand is not directly over the open culture dish.

The best way to switch the media is to make up a fresh stock of 10% FBS in DMEM in a clean, sterile, 50 mL falcon tube, which would then be used to apply the 15 mL to each culture ahead of time.

Put cells in incubator

Trypsinizing Cells
Prep the hood and pre-warm DMEM, FBS and PBS to 37°C. Thaw and warm trypsin at the last possible moment.

Prepare appropriate volume of 10% FBS in DMEM ahead of time. You will need ~40 mL for each culture line.

Aspirate cultures described above.

Add 10 mL PBS to wash. Aspirate.

Add 2 mL of 1X Trypsin-EDTA to each culture, and swirl to coat. Incubate at 37°C. Incubate plates for 10 minutes- 15 max, but never longer. After 10 minutes you can see the cells floating around in the culture, and it becomes slightly cloudy.

Back in the hood, add ~5 mL of 10% FBS in DMEM to each plate and pipet up and down a few times to mix. Count cells using a hemocytomoter with trypan blue (dilute to achieve appropriate concentration for counting). Add appropriate volume of culture (based on cell density and how many cells you wish to seed) to new T75s. Bring to 15 mL with 10% FBS in DMEM.

Put cells in incubator. Monitor daily.

APPENDIX D

CELL CULTURE MATERIALS LIST

General
COS7 Cells (ATCC Cat# CRL-1651)
Dulbecco's modified Eagle's medium (Invitrogen Cat# 11965-092)
Fetal bovine serum-Certified American Origin (Invitrogen Cat# 16000-044)
Trypan Blue (Sigma Cat# T8154-100mL)
PBS, pH 7.4
1x Trypsin/EDTA (Invitrogen Cat# 25300-054)
pcDNA3.1 HisTopo Cloning Kit (Invitrogen Cat# K4900-01)
Lipofectamine (Invitrogen Cat# 18324-012)
Opti-MEM-I (Invitrogen Cat# 31985-062)
T75 flasks (Fisher Cat# 10-126-37)
T25 flasks (Fisher Cat# 10-126-28)
100 mM culture plates (VWR Cat# 25382-166)
60 mM culture plates (VWR Cat# 25382-100)
10 mL pipets (VWR Cat# 53300-523)
50 mL falcon tubes (VWR Cat# 21008-178)
15 mL falcon tubes (VWR Cat# 21008-103)
1.5 mL eppendorf tubes (USA Scientific Cat# 1615-5500)
9" Pasture pipets
10 mL sterile pipets
50 mL sterile pipets
CO_2 (NorthEast AirGas Cat# CD50)

IP Assay
M199 (Invitrogen Cat# 12350-039)
Dialyzed FBS (Sigma Cat# f0392-100mL)
Myo-[^{3}H]-Inositol (GE Healthcare Cat# TRK317)
HBSS (Invitrogen Cat# 14175-095)
HEPES (Invitrogen Cat# 15630-080)
Phenol Red (Sigma Cat# P3532)
LiCl (Sigma Cat# L9650)
12 well culture plates (VWR Cat# 62406-165)
AG1 Resin (BioRad Cat# 1432445)
PolyPrep chromatography columns (BioRad Cat# 731-1550)
Perchloric Acid (Sigma Cat# 311421)
KOH (Fisher Cat# P250-500)
Formic Acid (Sigma Cat# 39938-8)
Ammonium Formate (Sigma Cat# 516961)
Sodium Tetraborate (AKA Borax) (Sigma Cat# 221732)
RIA SolveII (RPI Cat# 111180)
20 mL Scintillation vials (Fisher Cat# 03-337-7)
7 mL Scintillation vials (VWR Cat# 66022-300)

cAMP Assay

BioTrak EIA cAMP assay kit (GE Healthcare Cat# RPN225)
96 well culture plates (VWR Cat# 62406-081)
3-Isobutyl-1-methylxanthine (Sigma Cat# I5879)
Forskolin (Sigma Cat# F3917)

Intact, Whole Cell Binding Assay

HEPEs modified DMEM (Invitrogen 12320-032)
BSA (Sigma Cat# A-2153)
NaOH (Fisher Cat# S318-500)
SDS (Sigma Cat# L4390)
Acetic Acid (Fisher Cat# 95150-03)
NaCl (Sigma Cat# S-3014)
^{125}INa (PE Life Sciences Cat# NEX-1630)
Glass culture tubes (VWR Cat# 47729-570)
γ-tubes (USA Scientific Cat# 1505-7000)

APPENDIX E

RECEPTOR ASSAY PROTOCOLS

Transfection with Lipofectamine for IP assays

Transfection and Assay Procedural Overview

Day 1-Seed 60 mm plates

Day 2-Transfect

Day 3-Transfer ~$1.5x10^5$ cells to 12 well plate

Day 4-Switch media to 2% dialyzed FBS, 2 μCi/mL Myo-[^{3}H]-inositol in Medium 199

Day 5-Stimulate cells, extract IPs, isolate IPs, count in liquid scintillation counter (can be split into a 6th day)

Specifics

Day 1

-Trypsinize cells in T75's with 2.5 mL of 1X trypsin for 10 minutes at 37°C. Wash cells off the growing surface with 5 mL of 10% FBS in DMEM, and combine cells from each plate in a 50 mL tube (15 mL total volume) and mix well.

-Count cells with hemocytometer.

-Seed experiment specific number of 60 mm culture plates with $5x10^5$ cells in 5 mL 10% FBS in DMEM.

-Incubate overnight in humidified CO_2 (5%) 37oC incubator.

Day 2

-Set up 2 tubes (sterile eppendorf 1.5mL, 2.0 mL or any other), labeled I and II

-Tube I: 5 μg of vector in 300 μL Opti-MEM-I

-Tube II: 15 μL Lipofectamine and 285 μL Opti-MEM-I

-Mix contents of tube I and II, and incubate at room temperature for 30 minutes.

-Aspirate medium off of cultures and wash one time with 3 mL of Opti-MEM-I (and aspirate).

-Add 1.8 mL of Opti-MEM-I to I/II mixture, and pipet 2.4 mL of transfection mixture onto cells. Alternatively, a third tube can be set up, which contains the 1.8 mL of Opti-MEM-I, to which you can add the I/II mixture.

-Incubate for 5 hours in humidified CO_2 (5%) 37°C incubator.

-Add 2.4 mL of 20% FBS in DMEM to each culture (brining them to 10% FBS in an Opti-MEM-I and DMEM mix), gently mix by swirling, and incubate over night in humidified CO_2 (5%) 37°C incubator.

Day 3

-cut cells with 1 mL of 1X trypsin for 10 minutes.

-Transfer cells to a sterile 1.5 mL tube, and centrifuge at 1500 rpm for 5 minutes (setting 1.5 on the eppendorf mini centrifuge in Rm 322).

-Remove Supe carefully. Resuspend pellet in appropriate volume of 10% FBS in DMEM and combine when necessary (i.e. if you were to seed 9 wells with ~$1x10^5$ cells/well, resuspending in 10 mL of medium would be wise as you would seed 1 mL per well (assuming you set up 2-60 mm cultures for transfection and recovered 100% of the cells after trypsinizing them). Count cells to be sure.

-Plate $1x10^5$ to $2x10^5$ cells/well (or more) in a total of 1mL of 10% FBS in DMEM. If set up right you would just seed 1mL of resuspended cells.
-Incubate overnight.

Day 4

-Aspirate media off of cells
-Add 1 mL of 2% dialyzed FBS, 2 μCi/mL Myo-[2-^{3}H]-inositol in Medium 199
-Incubate over night
-Prepare Columns for IP isolation:
    -Use 1g resin per column (which will pour a 1mL column)
    -let resin soak in MQ H_2O for 30 minutes at room temp(10 mL/gram)
    -pour off most of the water
    -while PolyPrep column is plugged, pipet resin and let settle until bed volume is approximately 1mL.
    -remove column plug and run 20 mL MQ H_2O through each column to clean.
    -NOTE: resin is in hydroxyl form, and needs to be in Formate form.

-Resin Conversion:
    -run 2 mL of 1N Formic Acid threw column. This is a harsh treatment; some bubbles will form in the bed and the resin will appear lighter. Let the acid completely run out of the column (i.e. no more drops)
    -Add 1 mL 1N Formic Acid to the resin bed and pipet up and down two or three time to remove bubbles and to make sure all of the resin is converted. Test effluent pH with litmus paper, should be ~2.0
    -Wash column with ~40 mL MQ H_2O, 10 mL at a time. Column is ready when effluent ~pH = ~MQ H_2O pH
    -Add 5 mL MQ H_2O, cap and plug column. Column can sit overnight at room temp.

Day 5

<u>Stimulation and IP Extraction</u>

-In hood:
    -Carefully remove medium from wells (into a 50 mL labeled Falcon)
    -Wash wells 2x with 1mL IP buffer (20mM HEPES, 20mM LiCl in HBSS) (save buffer for proper disposal)
-Pre-Incubation: add 1 mL IP buffer and incubate cells at 37 °C with gentle shaking for 15 minutes
    Note-NOT in CO_2 incubator, HBSS does not have sodium bicarbonate and cultures will quickly become extremely acidic
-Carefully remove medium and save in 50 mL falcon
-Add 1mL of stimulant (control and some range of concentrations of stimulant), generally using IP buffer, and incubate 1 hour at 37 °C with gentle shaking.
-Immediately put plates on ice and add 200 μL of pre-chilled (on ice) 20% Perchloric Acid. Incubate on ice for 30 min. OPTIONAL this incubation is supposed to be at ~4 °C, so ice bucked with plate(s) could be moved into the cold room during this time, but it isn't necessary.

-During this time set up 1.5 mL tubes (1 for each well), to which add 5 μL of EDTA.
-After the 30 minute incubation transfer medium from wells (first mix by pipet 1 time (just be consistent) to the 1.5 mL tube from the previous step.

-Neutralize. This is where it gets tedious...All sample need to be approximately pH 7.4, which is a light pink color (IP has phenol red). All samples need to be as close in color as possible, but it doesn't need to be exact.

-To the first tube, add ~155 μL of 5N KOH, close and mix. Check color, if it's light pink (probably not), test with litmus to verify. Otherwise, add 5N KOH in 5 μL increments until you get there (again, verified by litmus).

-Use this first tube to 1) determine how much KOH to add to each tube (very few of which will come out perfect) and 2) as a color reference.

-Incubate at 4 °C (cold room) for 1 hour
-Centrifuge samples at 5000 rpm (40%) at 4 °C
-Carefully transfer 1.2 mL (2x600 μL) of the Supe to a clean 1.5 mL tube.
AT THIS POINT SAMPLES CAN BE STORED AT -20 °C OVERNIGHT

IP Isolation

Reagents:

-Wash Buffer
60 mM Ammonium Formate
5 mM Sodium Tetraborate (A.K.A. Borax)

-Elution Buffer
100 mM Formic Acid
1 M Ammonium Formate

-Regeneration Buffer
100 mM Formic Acid
3 M Ammonium Formate

-MQ H_2O
55.5 M MQ H_2O

Column Running

-load 1 mL sample to column (in formate form-see above)
-Wash with 10 mL Wash Buffer
-Elute with 3 mL Elution Buffer. Collect sample in a 20 mL Scintillation vial.
-Regenerate column with 5 mL Regeneration Buffer
-Rinse with 10 mL MQ H_2O
-Repeat with next sample.
-NOTE-Resin bed is very fragile and will be disturbed by essentially every step

-Resin has a very inconveniently high affinity for phenol red, which will accumulate as you load samples. As a result each column can be used 6-10 times, and then should be discarded. The PolyPrep column itself can be reused numerous times.

-For example, if 36 samples are to be isolated, 6 columns could be used, which would each be loaded 6 times, after which the resin is discarded.

-10 mL pipetman is very useful-set up four big beakers for each solution and four small beakers to hold the 10 mL pipetman tips.
-Total run time for 1 cycle, from loading sample to the end o f the water rinse, is about 28 minutes.

-Add 500 μL sample to 5 mL of RIA SolveII (in a 7 mL scint vial), mix and let sit in the dark for 2 hours. Count 10 min/sample.
-Analyze data using Excel and/or Prism (GraphPad).

Transfection with Lipofectamine for cAMP assays

Transfection and cAMP Assay Procedural Overview
Day 1-Seed 60 mm plates
Day 2-Transfect
Day 3-Transfer cells to 96 well plate
Day 4-Switch media to 0.5% FBS
Day 5-Stimulate cells and run cAMP assay
Specifics
Day 1

-Cut cells in T75s with 2.5 mL of 1X trypsin for 10 minutes at 37°C. Wash cells off the growing surface with 5 mL of 10% FBS in DMEM, and combine cells from each plate in a 50 mL tube (15 mL total volume) and mix well.
-Count cells with hemocytometer.
-Seed experiment specific number of 60 mm culture plates with $5x10^5$ cells in 5 mL 10% FBS in DMEM.
-Incubate overnight in humidified CO_2 (5%) 37oC incubator.

Day 2

-Set up 2 tubes, labeled I and II
-Tube I: 5 μg of vector in 300 μL Opti-MEM-I
-Tube II: 15 μL Lipofectamine and 285 μL Opti-MEM-I
-Mix contents of tube I and II, and incubate at room temperature for 30 minutes.
-Aspirate medium off of cultures and wash one time with 3 mL of Opti-MEM-I (and aspirate).
-Add 1.8 mL of Opti-MEM-I to I/II mixture, and pipet 2.4 mL of transfection mixture onto cells. Alternatively, a third tube can be set up, which contains the 1.8 mL of Opti-MEM-I, to which you can add the I/II mixture.
-Incubate for 5 hours in humidified CO_2 (5%) 37°C incubator.
-Add 2.4 mL of 20% FBS in DMEM to each culture (brining them to 10% FBS in an Opti-MEM-I and DMEM mix), gently mix by swirling, and incubate over night in humidified CO_2 (5%) 37°C incubator.

Day 3

-cut cells with 1 mL of 1X trypsin for 10 minutes.

-Transfer cells to a sterile 1.5 mL tube, and centrifuge at 1500 rpm for 5 minutes (setting 1.5 on the Eppendorf mini centrifuge in Rm 322).
-Remove Supe carefully. Resuspend pellet in appropriate volume of 10% FBS in DMEM (i.e. if you were to seed 9 wells with ~$5x10^4$ cells/well, resuspending in 1 mL would be the way to go-as you would have 1 mL of $5x10^5$ cells/mL which would set you up for the next steps). Count cells to be sure.
-Plate ~$5x10^4$ cells/well in a total of 100 µL of 10% FBS in DMEM. If set up right you would just seed 100 µL of resuspended cells. NOTE-the exact number of cells seed doesn't matter, what matters is that the seeding is consistent between wells, as ultimately the analysis will be relative to control, and therefore fluctuations in seed number between experiments will not matter.
-Incubate overnight.

Day 4

-Aspirate media off of cells
-Add 100 µL of 0.5% FBS in DMEM
-Incubate over night

Day 5

-Aspirate media
-Add 100 µL of stimulant (control and some range of concentrations of stimulant), generally using ID buffer (1mM 3-isobutyl-1-methylxanthine in DMEM), and a 1 hour incubation at 37 °C.
-At this point you are into the cAMP assay kit (BioTrak system from GE Health Division- Cat # RPN 225). Aspirate medium and lyse cells and continue their protocol.
Notes
-Data Analysis is performed using Excel and Prism GraphPad
-Making all the reagents should be done on this morning, generally it' good to do it prior to the stimulation.
-Running the standard curve in duplicate is unnecessary.
-The GE protocol should be followed exactly (hence I'm not going to re-write it), however there is one exception: The last step of the cAMP EIA is a colorization reaction, which they say to do for 60 minutes and that you should terminate the reaction with 1M sulfuric acid. If this protocol is followed the wells will be out of range in the spec analysis. So, add the 1M sulfuric acid at **20** minutes into the colorization reaction, then immediately read plate.

Transfection with Lipofectamine for Whole [Intact] Cell Receptor Binding Assays

Transfection and Assay Procedural Overview
Day 1-Seed $5x10^5$ cells in 60 mm culture dishes
Day 2-Transfect using Lipofectamine
Day 3-Transfer cells to 24 well plate
Day 4-Check cells (make sure they look ok)
Day 5-Affinity binding assay, competitive binding assay or internalization assay)
Solutions
Assay Buffer

0.1% BSA in DMEM with 25 mM HEPES

PBS

1X PBS at pH 7.4

Solubilizing Reagent

0.5M NaOH / 1% SDS

Acid Wash Solution

150 mM NaCl, 50 mM acetic acid, pH 2.8-3.0

Labeled Peptide

^{125}I-lamprey GnRH-I

Specifics

Day 1

-Cut cells in T75's with 2.5 mL of 1X trypsin for 10 minutes at 37°C. Wash cells off the growing surface with 5 mL of 10% FBS in DMEM, and combine cells from each plate in a 50 mL tube (15 mL total volume) and mix well.
-Count cells with hemocytometer.
-Seed experiment specific number of 60 mm culture plates with $5x10^5$ cells in 5 mL 10% FBS in DMEM.
-Incubate overnight in humidified CO_2 (5%) 37oC incubator.

Day 2

-Set up 2 tubes (sterile eppendorf 1.5mL, 2.0 mL or any other), labeled I and II
  -Tube I: 5 μg of vector in 300 μL Opti-MEM-I
  -Tube II: 15 μL Lipofectamine and 285 μL Opti-MEM-I
-Mix contents of tube I and II, and incubate at room temperature for 30 minutes.
-Aspirate medium off of cultures and wash one time with 3 mL of Opti-MEM-I (and aspirate).
-Add 1.8 mL of Opti-MEM-I to I/II mixture, and pipet 2.4 mL of transfection mixture onto cells. Alternatively, a third tube can be set up, which contains the 1.8 mL of Opti-MEM-I, to which you can add the I/II mixture.
-Incubate for 5 hours in humidified CO_2 (5%) 37°C incubator.
-Add 2.4 mL of 20% FBS in DMEM to each culture (brining them to 10% FBS in an Opti-MEM-I and DMEM mix), gently mix by swirling, and incubate over night in humidified CO_2 (5%) 37°C incubator.

Day 3

-Cut cells with 1 mL of 1X trypsin for 10 minutes.
-Transfer cells to a sterile tube (i.e. 1.5 mL), and centrifuge at 1500 rpm for 5 minutes (setting 1.5 on the eppendorf mini centrifuge in Rm 322 or 4 clicks using the countertop mushroom centrifuge).
-Remove Supe carefully. Resuspend pellet in appropriate volume of 10% FBS in DMEM and combine when necessary (i.e. if you were to seed 24 wells with ~$1x10^5$ cells/well, resuspending in 13 mL of medium would be wise as you would seed 1 mL per well (assuming you set up 4-60 mm cultures for transfection and recovered 100% of the cells after trypsinizing them). Count cells to be sure.
-Plate $1x10^5$ to $1.5x10^5$ cells/well (or more) in a total of 0.5 mL of 10% FBS in DMEM. If set up right you would just seed 0.5 mL of resuspended cells.

-Plate setup should include 4 wells for each treatment, 2 for specific binding and 2 for NSB. So if 6 different hot ligand concentrations will be used, the entire 24 well plate will need to be used.
-Incubate overnight.

Day 4

-Look at cells under scope to make sure they look ok
-The assay could be done this day, but waiting keeps the time line consistent with the IP and cAMP assays

Day 5

Saturation Binding Assay

-First thing, make fresh Assay Buffer and get all reagents chilling on ice in the cold room except for the Solubilizing Solution
-Make dilutions of 2x hot ligand solutions (i.e. if assay will be done in 1 well with 200 µL of 10^{-8}M analog, make 100 µL of $2x10^{-8}$M analog, which will be combined with 100 µL of assay buffer in the well to give the appropriate volume and concentration - this is done so cold ligand can be added to the NSB wells), and pre-chill on ice. Enough ligand should be made so that there is enough for each well and one extra for determination of "total added", and a little extra for pipetting error.
-In the hood, wash cells 1x with 500 µL of Assay Buffer, and aspirate.
-In the cold room, add 100 µL of Assay Buffer or cold ligand ($2x10^{-4}$M) to appropriate wells, then carefully add 100 µL hot ligand to each appropriate well. Incubate one ice at 4°C for 3.5 hours in order to reach equilibrium (or at least steady state).
-Remove medium from each well. Wash each well 2x with 500 µL ice cold PBS.
-Add 300 µL of Solubilizing Solution and incubate at room temperature for 2-5 minutes to disrupt cells.
-Transfer cell lysate to γ-tube and count (program 16-60 seconds/tube).
-Analyze using Excel and GraphPad to average results and perform non-linear regression/Rosenthal plot, respectively.

Competitive Binding Assay

-Starts as described above, from day one through washing cells in the hood with 500 µL of Assay Buffer.
-Competition is set up between the ^{125}I-lamprey GnRH-I and cold (non-labeled) peptides. In this case, plate is set up in a series of triplicates, one set for total binding (only assay buffer) and the rest of the triplicates receive increasing concentrations of cold competitor (generally ranging form 10^{-14} M to 10^{-6} M, depending on the affinity of the ligand). Wells receive 100 µL of 20 nM hot ligand (to deliver a final concentration of 10 nM) along with 100 µL of either Assay Buffer of competitor.
-Incubate on ice in the 4°C cold room for 3.5 hours, wash, solubilize and count as described above.

Internalization Assay

-Starts as described above, from day one through washing cells in the hood with 500 µL of Assay Buffer.

-All treatments set up in triplicate and each time point is set up in a separate 24 well plate. This is can be wasteful, but if 4 receptors are being assays and blank cells are used as control, 15 wells are used for each time point. Plates should be set up for a series of times, to include 0 min through 90 min, with the most time points during the first 30 minutes of the assay (i.e. 0 min, 5 min, 15 min, 30 min, 60 min and 90 min).

-Each well receives 200 µL of 10 nM 125I lamprey GnRH-I in Assay Buffer. Incubate on ice in the 4°C cold room for 3.5 hours.

-Cultures are transferred to 37oC incubator for appropriate period of time.

-Remove medium from cells, wash 2x with PBS. Add 300 µL of Acid Wash and incubate for 12 min on ice in the 4oC cold room.

-At the end of the 12 min collect the acid wash into γ-tubes, which represents the cell surface associated ligand.

-Add 300 µL of Solubilizing Solution and incubate cells at room temperature for 2-5 minutes to disrupt cells, and transfer to γ-tubes, which represents the internalized ligand.

-Repeat for every time point. The timing can be tricky during the first several time points, so plan well and be prepared.

-Analysis is performed based on percent internalization, which is calculated as:

% internalized=(internalized -NSB)/[(internalized-NSB)+(surface bound –NSB)] X 100

-Curve is fit using a single component exponential equation:

$$Y=Y_{max}(1-e^{Kt})$$

Where Y is % bound, K is the rate constant (%/min) and t is time in min.

NOTES:

The two most important aspects of these protocols are time and care. That is, everything needs to be done quickly yet gently, which can be difficult to balance, however a compromise is essential. Why?

-Cells need to be washed rapidly so bound ligand remains bound. Cells need to be washed in the first place to remove free ligand, and two washes does the trick; in fact, the dpm of the second wash is generally very low (however you don't usually count the washes).

-Cells need to be washed gently to avoid cell loss. If, for example, you do not wash gently large patches of cells will be lost, leading to massive amounts of error. To avoid this, never pipet directly onto cells, rather hold the culture plate at an angle, and slowly and carefully pipet onto the sidewall. It is a good idea to look at the cells using the inverted microscope to verify no cells were lost.

^{125}I is not harmless and at the same time it is not terribly dangerous. The most perilous time is during the iodination procedure, while at the time of the binding assays the amounts used are quite low. Regardless, basic safety precautions should be followed, such as using a lab coat, wearing double gloves, wearing dosimeter badges and using good sense.

During large assays, and even small, standing in the cold room can become, well, cold. Put on warm cloths under your lab coat, for more then health reasons. After a while dexterity can become an issue and fast yet gentle washing can become difficult. Further, if you get a little shaky it can be difficult pipet accurately and can lead to contamination issues. And finally, after a while it can become easy to lose track of which sample is which and how many times a certain culture has been washed.

Be careful not to contaminate anything. At greatest risk will be the ends of the P1000 and P200, which should be cleaned with MeOH (for both radioactivity and peptides). Swipes must be done after each use, and it should be noted that when dealing with ^{125}I, greater then 20 dpm over background is considered contaminated. Items to be swiped should include such things that common sense would dictate, such as pipetmen, tube racks, door handles (if inter-room travel is required, which it is), bench top, cold room...etc. All washes should be collected into 50 mL falcon tubes (or some other disposable tube), and samples need to be counted in order to determine the activity of the generated liquid waste.