

**Characterisation of the Tissue-Specific
Expression, Pharmacology and Signalling
Cascades Activated by Chicken GnRH Receptor
Subtypes Suggested Evolutionary Specialisation
of Type III cGnRH Receptor Function**

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Declaration

The experiments described within this thesis were the sole work of the author, where this is not so, credit has been duly given. No part of the work has been previously submitted in support of any other degree.

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Dedication

This thesis is dedicated to my mother, **'Elizabeth Theresa Joseph'**, whom I know would have been proud of my every achievement. With love and thanks for all the time I had to share with her.

Publications and Presentations

Refereed Papers

N. T. Joseph, K. Morgan, R. Sellar, D. McBride, R. P. Millar and I. C. Dunn. (2009). The chicken type III GnRH receptor homologue is predominantly expressed in the pituitary, and exhibits similar ligand selectivity to the type I receptor. *Journal of Endocrinology* **202**:179-190

Reviews

I.C. Dunn, N. A. Ciccone and **N. T. Joseph**. (2009). Endocrinology and genetics of the HPG axis. In *Biology of Breeding Poultry*. Proceedings of the 29th Poultry Science Symposium Meeting. Vol. 29, Ed. P Hocking, ISBN: 9781845933753.

Papers in Preparation

N.T. Joseph, M. Shimizu, R. Millar and G. Bedecarrats. Splice Variants of the Chicken Gonadotrophin Releasing Hormone Receptor III can inhibit cell surface expression and signalling of the Full-Length Receptor. (*In preparation*)

H. McFarlane, **N. T. Joseph**, S.R. Maddineni, R.Ramachandran and G. Bedecarrats. Development, validation and utilisation of a novel antibody specific to the type III chicken gonadotrophin-releasing hormone receptor. (*In preparation*)

Abstracts Selected for Oral Presentation

N. T. Joseph, K. Morgan, R. Millar and I. Dunn. (2009). Does the chicken Type III GnRH Receptor differentially interact with Src Homology 3 (SH3) domain containing proteins through SH3 binding motifs? 16th International Congress of Comparative Endocrinology, 20th-26th June, Hong Kong SAR, China.

N. T. Joseph, A. Stewart, I. C. Dunn, R. P. Millar and K. Morgan. (2008). Do the SH3 binding domains on the Type III GnRH Receptor facilitate additional ERK activation? 24th Conference of European Comparative Endocrinologists, 2nd-6th September, Genoa, Italy.

N. T. Joseph, W. Carre, I. C. Dunn, R. P. Millar and K. Morgan. (2008). Comparison of the amphibian and avian GnRH systems. 24th Conference of European Comparative Endocrinologists 2nd-6th September, Genoa, Italy.

N. T. Joseph, K. Morgan; I. C. Dunn and R. P. Millar. (2008). A Pharmacological approach to dissect the physiological functions of the two chicken GnRH receptor isoforms. 9th International Symposium on Avian Endocrinology 11th-15th July, Leuven, Belgium.

N.T. Joseph, R. Sellar, K. Morgan, R. Millar and I.C. Dunn. (2006). A Novel Chicken Gonadotrophin Releasing Hormone Receptor: expression, binding affinity and functional activity. 23rd Conference of European Comparative Endocrinologists, Manchester 29th August-2nd September, UK.

Abstracts Selected for Poster Presentation

M. Shimizu, **N. T. Joseph**, R. Millar and G. Bedecarrats. (2008). Chicken gonadotropin releasing hormone receptor III splice variants can interfere with normal signalling of the full-length receptor. 9th International Symposium on Avian Endocrinology 11th-15th July, Leuven, Belgium.

N. T. Joseph, I. C. Dunn, R. P. Millar and K. Morgan (2008). Signalling pathways recruited by avian GnRH Receptors. Society for Reproduction and Fertility Annual Meeting, 29th June- 1st July, Edinburgh, UK.

N. T. Joseph, K. Morgan; R. Millar and I. C. Dunn. (2007). Does the novel cGnRH-R-III mediate reproductive function in the Chicken? 29th Poultry Science Symposium Meeting 23rd-25th July, Edinburgh, UK.

N.T. Joseph, K. Morgan, R. Millar and I.C. Dunn. (2007). Distribution and Pharmacological Comparison of the type I GnRH receptor with the newly discovered type III receptor in Chicken. 89th Meeting of the Endocrine Society 2nd-5th June, Toronto, Canada.

Abstract

Variant GnRH ligand and receptor subtypes have been identified in a number of non-mammalian vertebrate species, however research into avian species GnRH systems is lacking. Two isoforms of GnRH are present in the domestic chicken, the evolutionarily conserved GnRH-II and diverged cGnRH-I. The expression of two GnRH ligands parallels the expression of two chicken GnRH receptor subtypes; cGnRH-R-I and the novel cGnRH-R-III. The occurrence of two isoforms of the receptor in the chicken raises questions about their specific biological functions and interactions with the two ligands. Differential roles for these molecules in regulating gonadotrophin secretion or other functions are currently unclear. To investigate this, cGnRH-R-III cDNA was cloned from a broiler chicken anterior pituitary gland and its structure and expression was compared with cGnRH-R-I. Expression profiling of cGnRH-R-III cDNA showed that it is predominantly expressed in the anterior pituitary, approximately 1400 times more abundantly than cGnRH-R-I suggesting that cGnRH-R-III is the predominant regulator of chicken gonadotrophin synthesis and secretion. Additionally, pronounced sex and age differences existed, with higher pituitary cGnRH-R-III mRNA levels in sexually mature females versus juvenile females. In contrast, higher mRNA expression levels occurred in juvenile males compared to sexually mature males. Determination of ligand-binding selectivity and the level of cGnRH-R-III activation in response to the endogenous ligands, cGnRH-I and GnRH-II, was anticipated as facilitating the elucidation of the physiological roles of the receptor subtypes. Additionally, the development of analogs that differentially promote or inhibit activation of the receptor subtypes may be valuable tools for determining the role of receptor types in the regulation of gonadotrophin production. To investigate this, pharmacological profiling of cGnRH-R-III in terms of ligand-binding selectivity and inositol phosphate production in response to GnRH analogs was determined in comparison with the pharmacological profile of cGnRH-R-I. Functional studies in COS-7 cells indicated that cGnRH-R-III has a higher

binding affinity for GnRH-II than cGnRH-I (IC_{50} : 0.57 v 19.8 nM) and more potent stimulation of inositol phosphate production (EC_{50} : 0.8 v 4.38 nM). Similar results were found for cGnRH-R-I, (IC_{50} : 0.51 v 10.8 nM) and (EC_{50} : 0.7 v 2.8 nM). Mammalian receptor antagonist 27 distinguished between cGnRH-R-I and cGnRH-R-III (IC_{50} : 2.3 v 351 nM), and application of this synthetic peptide may facilitate delineation of receptor subtype function either *in-vitro* or *in-vivo*. The length of the C-terminal tail of cGnRH-R-III is 8 residues longer than that of cGnRH-R-I and this observation stimulated investigation of differences in ligand-induced internalisation between the two receptor subtypes. The initial rate of receptor internalisation was faster for cGnRH-R-III than for cGnRH-R-I ($26\% \cdot \text{min}^{-1}$ v $15.8\% \cdot \text{min}^{-1}$). Although proteins encoded by cGnRH-R-III splice variants do not bind GnRH ligands independently and mRNAs were not detectable by Northern blot analysis, cGnRH-R-III_SV2 significantly reduced maximum ligand-binding of cGnRH-R-III, suggesting that it may impair the function of the full-length type III cGnRH receptor. It was anticipated that the two cGnRH-R subtypes may have differential roles in the regulation of luteinising hormone (LH) and follicle stimulating hormone (FSH) gene transcription through the activation of differential second messenger pathways. Three putative Src homology domain 3 (SH3) binding motifs were identified in the type III cGnRH receptor cytoplasmic C-terminal tail domain which are not present in the type I cGnRH-R and suggested the potential for differential coupling to the Mitogen Activated Protein Kinase (MAPK) cascade. To investigate this possibility, activation of the MAPK cascade via cGnRH-R-III and cGnRH-R-I was determined by quantifying elevation of phosphorylated ERK (pERK 1/2) in response to GnRH. Studies performed in COS-7 cells showed a 4-6 fold increase in ERK 1/2 phosphorylation via the type I and type III receptors within 10 minutes of GnRH-I or GnRH-II stimulation, indicating that both receptors signal through the ERK 1/2 pathway in response to cGnRH-I or GnRH-II. The responses were dose-dependent at cGnRH-R-I and cGnRH-R-III. Effects of pre-treatment with PLC and c-Src inhibitors showed that both cGnRH-Rs may activate pERK 1/2 independently of PLC but dependently upon

c-Src. However, it must be noted that 100% of the PLC activity was not inhibited by PLC inhibitor as measured by inositol phosphate production at 60 minutes, and the PLC inhibitor has not been shown to inhibit PLC in the same time frame used for the pERK experiments. Mutagenesis of the individual SH3 binding motifs of cGnRH-R-III were performed and the effects on pERK 1/2 levels quantified. The results indicated that the SH3 binding motifs of cGnRH-R-III do not contribute to additional MAPK activation when compared to the native cGnRH-R-III. Both cGnRH-R-I and cGnRH-R-III were HA epitope-tagged (HA-cGnRH-R-I and HA-cGnRH-R-III) and the methodology was optimised for HA-cGnRH-R-III immuno-precipitation. Several size forms of HA-cGnRH-R-III were detectable by immuno-precipitation, facilitating characterisation of the composition of the receptor protein-protein complexes formed using a western blot approach. In summary, the abundance of cGnRH-R-III expression compared to cGnRH-R-I suggests it is probably the major mediator of pituitary gonadotroph function, and that tissue-specific recruitment of cGnRH-R-isoforms has occurred in the avian pituitary during evolution. Pharmacological profiling demonstrated that cGnRH-R-III, like cGnRH-R-I, has a higher ligand-binding selectivity and induction of inositol phosphate production in response to GnRH-II than with cGnRH-I, although cGnRH-I is established as the physiological regulator of gonadotroph function. These results suggest that evolutionary recruitment of ligand-receptor pairing for particular physiological processes does not correlate with *in-vitro* properties such as highest ligand-binding affinity or efficacy of inositol phosphate production. Therefore evolutionary plasticity has occurred in the tissue-specific adoption of GnRH ligand and receptor subtypes for regulation of particular physiological functions in birds.

Chapter 1.

Literature Review

1.1. Introduction

The main scope of this literature review is to introduce the Gonadotrophin-Releasing Hormone (GnRH) isoforms and GnRH receptor (GnRH-R) subtypes across various orders of species. It also includes a brief description of the second messenger cascades stimulated on activation of the GnRH receptor and motifs pertaining to pharmacological properties of GnRH receptors. More detailed information (e.g. signal transduction pathways) is provided in the introduction sections of each specific chapter. The GnRH system is fundamental to the understanding of reproductive biology and has been researched for several decades. This review aims to highlight the plethora of GnRH system research in several classes of animals and the topical relevance of research in avian species. Studies of GnRH systems in avian species promise to provide a unique perspective of the interplay between GnRH isoforms and their receptors in the control of reproduction at a particular branch in the vertebrate evolutionary tree. These studies may facilitate comprehension of the function of the avian hypothalamic-pituitary-gonadal axis (HPG-axis) which impinges upon the success of the poultry industry and on captive breeding programs to preserve certain endangered species. In this chapter the current understanding of the GnRH system is described in avian species and other animals, and the research objectives of this thesis using the domesticated chicken as a commercially important species are outlined.

1.2. Overview of reproductive biology in egg laying animals

Many vertebrates are seasonal breeders which are driven by environmental cues e.g. availability of food, weather and changes in day-length. With the exceptions of some rare viviparous species, most fish, amphibians and reptiles are oviparous. All birds are oviparous. However, the method of fertilisation in oviparous animals does not follow a universal process. For example, although a few oviparous fish achieve internal fertilisation through an intromittent organ (e.g. horn shark or skates) most fish achieve fertilisation of their eggs externally. In amphibians fertilisation can be achieved in a variety of ways; the order *Anuran* (e.g. frogs) mostly uses external fertilisation, whereas the *Caudata* (e.g. salamander) fertilisation is mainly internal (Beck, 1998). In contrast, caecilians and tailed frogs use internal fertilisation. Large amount of eggs are produced in fish and amphibians (e.g. 5 million in cod and 1000 in common frogs) over the spawning periods, which vary over a number of days. The number of eggs produced is roughly proportional to the body size and length of time required for *in-ovo* development. It is also possible that the number of eggs produced is a function of the probability of ensuring fertilisation and long-term survival due to the inefficiency of external fertilisation. In fish and amphibians, multiple ovarian follicles develop simultaneously and in synchrony to enable mass spawning, therefore ovarian function and its regulation may be expected to reflect these required needs. An integrated GnRH system has been postulated to be functional in teleost fish and amphibians with GnRH released from the hypothalamus stimulating the pituitary to synthesise and release gonadotrophins. There is some speculation that an intra-gonadal GnRH system may be functional in teleosts (Foran and Bass, 1999). In tunicates, there are several GnRH forms which directly activate the gonads (Adams et al., 2003; Powell et al., 1996), suggesting that GnRH regulation of the gonads was an early-evolved function prior to the neuroendocrine role facilitated by the development of the pituitary gland (Millar, 2005). The spawning events of fish are probably controlled by luteinising hormone (LH) and follicle stimulating hormone (FSH), but research into the relevance of both gonadotrophins in fish is not completely understood (Swanson et al., 2003), although

it is established that the final maturation and ovulation of the oocyte is controlled by LH (Swanson, 1991). Teleost fish lack a hypophyseal portal system and the GnRH in the preoptic area of the brain may directly supply and stimulate the pituitary gland (Oka and Ichikawa, 1990; Peter et al., 1990). This differentiates teleost fish from other vertebrates and changes in fish GnRH neurons, may be more rapidly reflected in blood gonadotrophin levels (Foran and Bass, 1999).

The divergence of fish and amphibians is generally thought to have occurred 400-360 million years ago during the early Devonian Period and the divergence of amphibians and amniotes (reptiles, birds and mammals) is thought to have occurred approximately 350 million years ago, during the late Paleozoic Era, however, the common ancestor remains unidentified (Elinson and Beckham, 2002). The defining attribute of amniotes is the cleidoic egg (Reisz, 1997), which, encased in a hard egg shell and comprising extraembryonic membranes, removed amniotes' reliance on aquatic development (Elinson and Beckham, 2002). Unlike most fish and amphibians, fertilisation occurs within the oviduct in amniotes. In reptiles and birds, fewer eggs are produced per season (e.g. 12 in viper and 16 in chickens) and this is a reflection of body size and length of time required for *in-ovo* development and also reflects the efficiency of internal fertilisation. Our understanding of how the GnRH system integrates with reproductive physiology in reptiles and avian species is deficient in both groups of animals (Morgan and Millar, 2004).

Birds are a diverse group of species that evolved from an ancestral reptilian lineage during the Mesozoic Era about 150 million years ago, with the crocodilian lineage being the most closely related extant reptile lineage to that of birds. The organisation of the avian GnRH system is distinct from that of reptiles (Ensembl database), (Morgan and Millar, 2004), suggesting the GnRH systems in these orders of animals may have evolved differently to accommodate the different reproductive systems. The reproductive physiology of reptiles and birds is similar in that they both possess a hierarchical order of pre-ovulatory ovarian follicles (Ikemoto and Park, 2007;

Johnson and Leone, 1985). The domestic chicken (like most birds) is unusual in that it ovulates daily (Johnson and Leone, 1985) with one egg produced per ovulation due to the synchronised hierarchy of follicles. Some differences in reproductive general endocrinology between fish, amphibians, reptiles and birds are outlined below (**Table 1.1**).

Reproductive tissues develop partially during embryogenesis (George and Wilson, 1994), and GnRH may play a role during this time period (Livne et al., 1993; Salisbury et al., 1982; Simonian and Herbison, 2001). These tissues become quiescent in the sexually immature animal. Later, GnRH plays a role at the onset of puberty when reproductive tissues develop further to become fully functional (Millar et al., 2004). The onset of puberty is co-ordinated by the hypothalamus in response to metabolic cues, and various hormones are involved in this process. Reproductive behaviour involves hypothalamic integration of visual, auditory, olfactory, tactile and nutritional inputs (see **Table 1.1. for references**).

Table 1. 1

Reproductive peptide hormones and endocrine glands of the HPG-axis in various fish, amphibian, reptilian, avian and mammalian species

	Fish	Amphibians	Reptiles	Birds	Mammals
Species studied include	Zebrafish, salmon, goldfish	Ranid frogs, bullfrog, African clawed frog	Leopard gecko, Green anole, garter snake	Chicken, sparrow, pigeon, zebra finch, starling, ring dove	Cow, sheep, rat, pig, primates, human
GnRH	GnRH-I GnRH-II GnRH-III	GnRH-I GnRH-II -----	GnRH-I GnRH-II -----	GnRH-I GnRH-II -----	GnRH-I GnRH-II (some) -----
GnIH	GnIH-RP-1 GnIH-RP-2 GnIH-RP-3	GnIH GnIH-RP-1 GnIH-RP-2 GnIH-RP-3	GnIH*	GnIH GnIH-RP-1 GnIH-RP-2	GnIH-RP-1 GnIH-RP-3
Kisspeptin	Kiss 1 Kiss 2	Kiss 1a and b Kiss 2	Kiss 2	NONE (?)	Kiss 1

Note: Fish-GnIH-RP 1,2,3 = LPXRFa , Amphibian GnIH, RP1,2,3 = fGRP, * = unpublished

Table 1.1 Continued

Endocrine glands of the HPG-axis		Fish	Amphibians	Reptiles	Birds	Mammals
	Species studied include	Zebrafish, salmon, goldfish	Ranid frogs, bullfrog, African clawed frog	Leopard gecko, Green anole, garter snake	Chicken, sparrow, pigeon, zebra finch, starling, ring dove	Cow, sheep, rat, pig, primates, human
	Hypothalamus	-Seasonal, visual and social cues (Parhar et al., 2005) (Hofmann, 2006)	-Seasonal and auditory cues (Burmeister and Wilczynski, 2005)	-Seasonal, auditory and pheromonal cues -Temperature regulates reproduction (Crews, 1983)	-Seasonal and auditory cues regulates GnRH production (Foster et al., 1987) (Cheng et al., 1998)	Seasonal, enviromental and social cues and oestrous cycles (Bronson, 1985)
	Pituitary	<u>No</u> hypophyseal portal vasculature -separate population of LH and FSH gonadotrophs	-co-expression of LH and FSH in some gonadotrophs	- co-expression of LH and FSH in some gonadotrophs	- separate population of LH and FSH gonadotrophs	- co-expression of LH and FSH in some gonadotrophs (except bovine)
	Ovary	-1000's synchronized follicles/oocytes -local GnRH systems	-100-1000's synchronized follicles/oocytes	-Hierarchical follicles -6-30 synchronized follicles - Suggested local GnRH system	-Hierarchical follicles -2-10 synchronized follicles -no studies into local GnRH system to date	1-6 synchronised follicles

1.2.1. Reproductive biology of birds

Most birds are seasonal breeders and reproduce under the influence of day-length (photoperiods). Daylight perceived by retinal and hypothalamic photoreceptors impinges upon the brain and influences internal circadian rhythms (Sharp, 2005). This information is integrated within the hypothalamic region of the brain, where multiple hormonal and metabolic signals are processed.

When sexual maturity is attained at around 24 weeks after hatching in chickens, mating can proceed. The male ejaculates semen onto the everted cloaca of a hen and fertilisation involves entry of several spermatozoa into the egg in the oviduct. The large yolky egg of birds does not cleave after each successive cell division and this anatomical feature makes union of male and female pronuclei and the formation of the blastocyst unique among vertebrates. The embryonic development commences in the oviduct of the hen, where the embryo grows up to 4mm in diameter. Once the egg is laid (22 hours after ovulation) and cooled to ambient temperature the embryo then enters embryonic diapause. The embryonic development is reactivated on incubation, and is dependent on environmental conditions. Jungle fowl lay 12-20 eggs over 17-25 days and once maternal behaviour is expressed, egg production is curtailed. The hen incubates the eggs and rears the young for several weeks before returning to another phase of egg production. A chicken can endure a series of reproductive cycles and live to approximately 4-6 years. The roles of GnRH in the brain during these physiological events are poorly characterised.

Chickens were first domesticated from the Red Junglefowl and Grey Junglefowl in south-east Asia before the 6th millennium BC (Eriksson et al., 2008). Identification of the Leghorn strain during the Middle Ages in Livorno, Italy was a revolutionary milestone in poultry breeding (Etches, 1996). This strain maintains egg production for long periods and displays maternal behaviour infrequently. During the years of

domestication, the appearance and reproductive physiology of chickens has been transformed. By the 1950s two distinct lines of commercial chicken emerged; Layers (egg production) and Broilers (meat production) (Etches, 1996). Layer chickens have been genetically selected for high egg productivity whereas broiler chickens are selected for fast growth rate and increased body size. Commercial laying hens are capable of producing up to 310 eggs per year (<http://www.lohmanngb.co.uk/brown-char.html>), whereas broilers selected for rapid growth rate have a poor reproductive efficiency, mainly due to dysfunction in the orderly hierarchical ovarian follicular development (Robinson et al., 1993). The domestic chicken is of great economic importance as it provides a nutritious source of protein in the form of meat and eggs. The highest consumption rates are in industrialised western countries with the US ranking first (54kg/capita/year) and the European Union ranking fifth (22kg/capita/year) in 2006 (Magdelaine et al., 2008). Poultry comprises one quarter of the world's meat consumption and table egg production is around 280 billion per year (Sharp, 1998). The success of the poultry industry and the power of genetic selection for desirable reproductive traits rely on birds with correct functioning of the HPG-axis to control ovulation rate, ovulation, semen production, incubation behaviour, the development of photorefractoriness and features associated with sexual senescence (Dunn I. C. et al., 2009). Further characterisation of the avian GnRH system may therefore provide unique information to enable better comprehension of the interplay between GnRH ligands and their receptors in the control of avian reproduction. Additionally, studying the evolutionary biology of GnRH systems enables a further comprehension of how GnRH systems may have evolved to assume their function in modern taxa. (Okubo and Nagahama, 2008).

The next sections 1.3 and 1.4 will outline the GnRH ligand and receptor subtypes found in vertebrates and non-vertebrates. This will provide information on the complexity of GnRH systems. In section 1.6, information available on GnRH systems in avian species is presented to lead into the experimental chapters in which the GnRH system in the domestic chicken was investigated.

1.3. Gonadotrophin-Releasing Hormone (GnRH)

The co-ordinated functions of the HPG-axis regulate the reproductive system. The 'HPG-axis' refers to three endocrine glands (hypothalamus, pituitary and gonad) as an entity, acting in a co-ordinated fashion. The hypothalamus integrates signals from the environment (external cues/positive and negative), gonads and body (internal cues/positive and negative) to direct synthesis and release of GnRH. This hormone synchronises the development and maturation of the reproductive system and modulates its function in mature animals through appropriate activation of gonadotrophin secretion from the pituitary gland. Gonadotrophins stimulate growth and maintain the functions of the gonads. The cellular and molecular complexity of the HPG-axis continues to be elucidated and is presented in outline in **Fig. 1.1**, using the mammalian (human) HPG-axis as an example where there is a well-developed pituitary gland and reproduction is dependent on oestrous cycles.

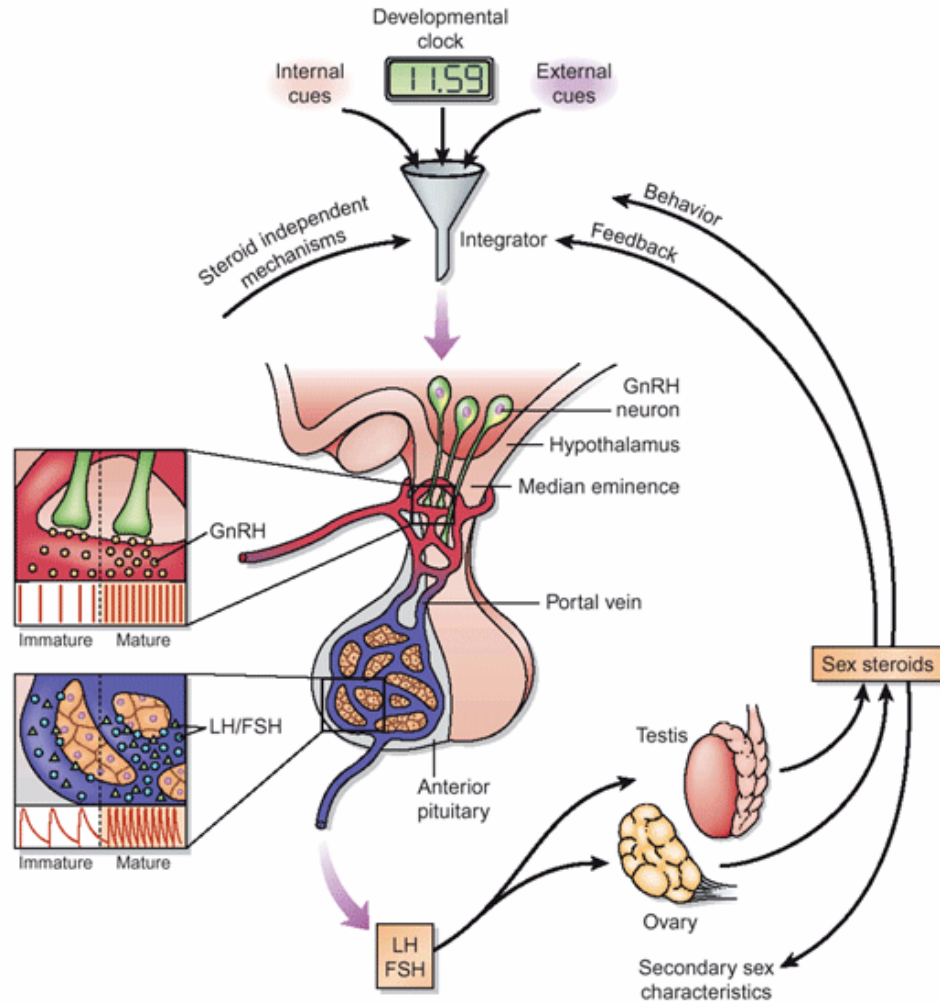
Figure 1. 1

Fig. 1.1 A schematic diagram of the hypothalamo-pituitary-gonadal axis (HPG-axis) from Sisk C and Foster D, *Nature Neuroscience* 2004; 7(10):1040-1047. Internal and external cues and the developmental clock regulate the synthesis of GnRH in neurons which arise in the hypothalamus and project to the median eminence. GnRH enters the hypophyseal portal vasculature where it binds to pituitary gonadotrophs to elicit the synthesis and release of LH and FSH. These regulate development of the gonads and production of sex steroids (e.g. oestrogen and testosterone). A neuroendocrine feedback loop exists, where the sex steroids influence the function of hypothalamic GnRH neurons.

1.3.1. GnRH isoforms

It is well established that GnRH plays a pivotal role in the control of reproductive function and is a central initiator of the reproductive hormonal cascade in pubertal and mature animals (Millar, 2005). The first GnRH isoform to be characterised was isolated from extracts of porcine and ovine hypothalami in 1971 as a decapeptide (pGlu-His-Trp-Ser-Tyr-Gly-Leu-Arg-Pro-Gly.NH₂) (Amoss et al., 1971; Baba et al., 1971; Matsuo et al., 1971; Schally et al., 1971). This finding pioneered the identification of GnRH peptides and/or the genes which encode them in a wide range of species. Multiple forms of GnRH have been identified and their features have been extensively reviewed (Millar, 2005) (Tsai and Zhang, 2008). To date, the GnRH family consists of 28 members found within two animal superphyla: Deuterostoma (vertebrates, echinoderms, tunicates, etc.) and Protostome (nematodes, molluscs, arthropods, etc.). These superphyla differ in embryonic development. In Deuterostoma the blastopore becomes the anus, whereas in Protostomes it develops into the mouth, however, together with other smaller phyla they make up the Bilateria in having bilateral symmetry. Four longer peptide hormones consisting of twelve amino acids have recently been placed within the GnRH family. These GnRH-like dodecapeptides are found within the Protostome superphyla, three within the phylum Mollusca (octopus, aplysia and the owl limpet) and one in the phylum Annelida (annelid) (Iwakoshi et al., 2002; Tsai, 2006; Tsai et al., 2003; Tsai and Zhang, 2008; Zhang et al., 2008). The remaining twenty-four GnRH decapeptide forms have been identified within the Deuterostome superphyla, and are restricted to the phylum Chordata, with nine unique forms found in the protochordate tunicates and fifteen forms of GnRH found in vertebrates (Adams et al., 2003; Millar et al., 2004; Tello and Sherwood, 2009; Tsai, 2006). The Deuterostome GnRH peptides possess a common molecular architecture, with conservation of the NH₂-terminal amino acids (pGlu-His-Trp-Ser) and COOH-terminal domain (Pro-Gly.NH₂), with the exception of the two conservative Tyr substitutions in the NH₂-terminal in the guinea-pig (pGlu-**Tyr**-Trp-Ser) and lamprey I (pGlu-His-**Tyr**-Ser) (Adams et al., 2003; Millar et al., 2004) (**Fig. 1.2**). The most basal chordates (or protochordates) in

which GnRH peptides have been identified are tunicate and amphioxus (common lancelet) (Adams et al., 2003; Chambery et al., 2009). The amphioxus GnRH peptide amino acid sequence is identical to that of mammalian GnRH (Chambery et al., 2009) whereas, the nine tunicate GnRH isoforms all vary from mammalian GnRH (Adams et al., 2003; Tello et al., 2005) (**Fig. 1.2**). GnRH sequence variants have also been identified in lamprey, fish, frog, chicken and guinea pig (**Fig. 1.2**). The GnRHs found within Deuterostoma phylum Chordata include representatives from cartilaginous fish (e.g. dogfish and shark), teleost fish, amphibians, reptiles, aves and mammals (Kah et al., 2007). Significantly, the identification of two GnRH peptides in the chicken between 1979 and 1984 (King and Millar, 1979; King and Millar, 1982a; King and Millar, 1982b; Miyamoto et al., 1983; Miyamoto et al., 1982; Miyamoto et al., 1984) precipitated investigations into the identification of multiple forms of GnRH in vertebrates (Okubo and Nagahama, 2008). In most vertebrate species there are usually two to three forms of GnRH (Millar, 2005), with fewer GnRH genes in mammals compared to protochordates, fish and amphibians (Morgan and Millar, 2004). The most ubiquitous and highly conserved form of GnRH is chicken GnRH-II (Millar, 2005), which was first isolated from a chicken brain (Miyamoto et al., 1984).

There is a lack of consensus regarding the nomenclature and forms of GnRH. In vertebrate species GnRH forms have been generally designated names according to the order in which they were identified, sometimes making for complications in terminology where more than one GnRH form is present in the species (Morgan and Millar, 2004; Okubo and Nagahama, 2008). A universal nomenclature based on phylogenetic analysis was suggested for molecular forms of prepro-GnRH in which three GnRH forms are classified (Fernald and White, 1999; White et al., 1998). The classification of three forms of GnRH was further characterised based on genes and cDNA encoding GnRH (White et al., 1994; White et al., 1995) and comparative genomic analysis (Okubo et al., 2002). Phylogenetic analysis confirmed the clustering of three forms of GnRH (Millar et al., 2004). Thus, in the vertebrate

lineage there are three distinct paralogous forms of GnRH but all three forms to date are known to co-exist only in species of the teleost lineage (Okubo and Nagahama, 2008). It is unclear which phylogenetic group lamprey GnRH-I and GnRH-III and dogfish GnRH belong to (Okubo and Nagahama, 2008). It has been suggested that lamprey and tunicate GnRH are unique to their species (Chambery et al., 2009). A recent phylogenetic study comparing GnRH genes from representatives of both the Protostome and Deterostome superphyla classified the GnRHs into five major clusters (**Fig. 1.3**), GnRH-I, GnRH-II, GnRH-III, GnRH-IV (the lamprey forms) and GnRH-V (the Protostome forms) (Tsai and Zhang, 2008). Additionally, the tunicate GnRH isoforms are shown to be distantly related to GnRH-III and GnRH-I (Tsai and Zhang, 2008) (**Fig. 1.3**).

Figure 1. 2

GnRH	1	2	3	4	5	6	7	8	9	10		
Sturg/Mam	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Arg	Pro	Gly-NH ₂		
Whitefish	pGlu	His	Trp	Ser	Tyr	Gly	Met	Asn	Pro	Gly-NH ₂		
Seabream	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Ser	Pro	Gly-NH ₂		
Pejerrey	pGlu	His	Trp	Ser	Phe	Gly	Leu	Ser	Pro	Gly-NH ₂		
Salmon	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly-NH ₂		
Herring	pGlu	His	Trp	Ser	His	Gly	Leu	Ser	Pro	Gly-NH ₂		
Catfish	pGlu	His	Trp	Ser	His	Gly	Leu	Asn	Pro	Gly-NH ₂		
Dogfish	pGlu	His	Trp	Ser	His	Gly	Trp	Leu	Pro	Gly-NH ₂		
Chicken-I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly-NH ₂		
Chicken-II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly-NH ₂		
Lamprey-I	pGlu	His	Tyr	Ser	Leu	Glu	Trp	Lys	Pro	Gly-NH ₂		
Lamprey-II	pGlu	His	Trp	Ser	His	Gly	Trp	Phe	Pro	Gly-NH ₂		
Lamprey-III	pGlu	His	Trp	Ser	His	Asp	Trp	Lys	Pro	Gly-NH ₂		
Guinea pig	pGlu	Tyr	Trp	Ser	Tyr	Gly	Val	Arg	Pro	Gly-NH ₂		
Frog	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Trp	Pro	Gly-NH ₂		
Tunicate-1	pGlu	His	Trp	Ser	Asp	Tyr	Phe	Lys	Pro	Gly-NH ₂		
Tunicate-2	pGlu	His	Trp	Ser	Leu	Cys	His	Ala	Pro	Gly-NH ₂		
Tunicate-3	pGlu	His	Trp	Ser	Tyr	Glu	Phe	Met	Pro	Gly-NH ₂		
Tunicate-4	pGlu	His	Trp	Ser	Asn	Gln	Leu	Thr	Pro	Gly-NH ₂		
Tunicate-5	pGlu	His	Trp	Ser	Tyr	Glu	Tyr	Met	Pro	Gly-NH ₂		
Tunicate-6	pGlu	His	Trp	Ser	Lys	Gly	Tyr	Ser	Pro	Gly-NH ₂		
Tunicate-7	pGlu	His	Trp	Ser	Tyr	Ala	Leu	Ser	Pro	Gly-NH ₂		
Tunicate-8	pGlu	His	Tyr	Ser	Leu	Ala	Leu	Ser	Pro	Gly-NH ₂		
Tunicate-9	pGlu	His	Trp	Ser	Asn	Lys	Leu	Ala	Pro	Gly-NH ₂		
Apl	pGlu	Asn	Tyr	His	Phe	Ser	Asn	Gly	Trp	Tyr	Ala	Gly-NH ₂
Oct	pGlu	Asn	Tyr	His	Phe	Ser	Asn	Gly	Trp	His	Pro	Gly-NH ₂
Lim	pGlu	His	Tyr	His	Phe	Ser	Asn	Gly	Trp	Lys	Ser	Gly-NH ₂
Ann	pGlu	Ala	Tyr	His	Phe	Ser	His	Gly	Trp	Phe	Pro	Gly-NH ₂

Fig 1.2 Primary amino acid sequence of naturally occurring GnRHs. This figure was adapted from the original kindly donated by Dr. J. Tello. The GnRH amino acids in the Protostome superphyla are dodecapeptides whereas those in the Deuterostoma superphyla are decapeptides. The boxed regions show the conserved NH₂ and COOH terminal residues in the decapeptides with the exception of the two Tyr residues in the Guinea pig and Lamprey I. Note that the GnRH isoforms are named according to the species in which they were discovered and identical GnRHs may be present in more than one species.

Figure 1. 3

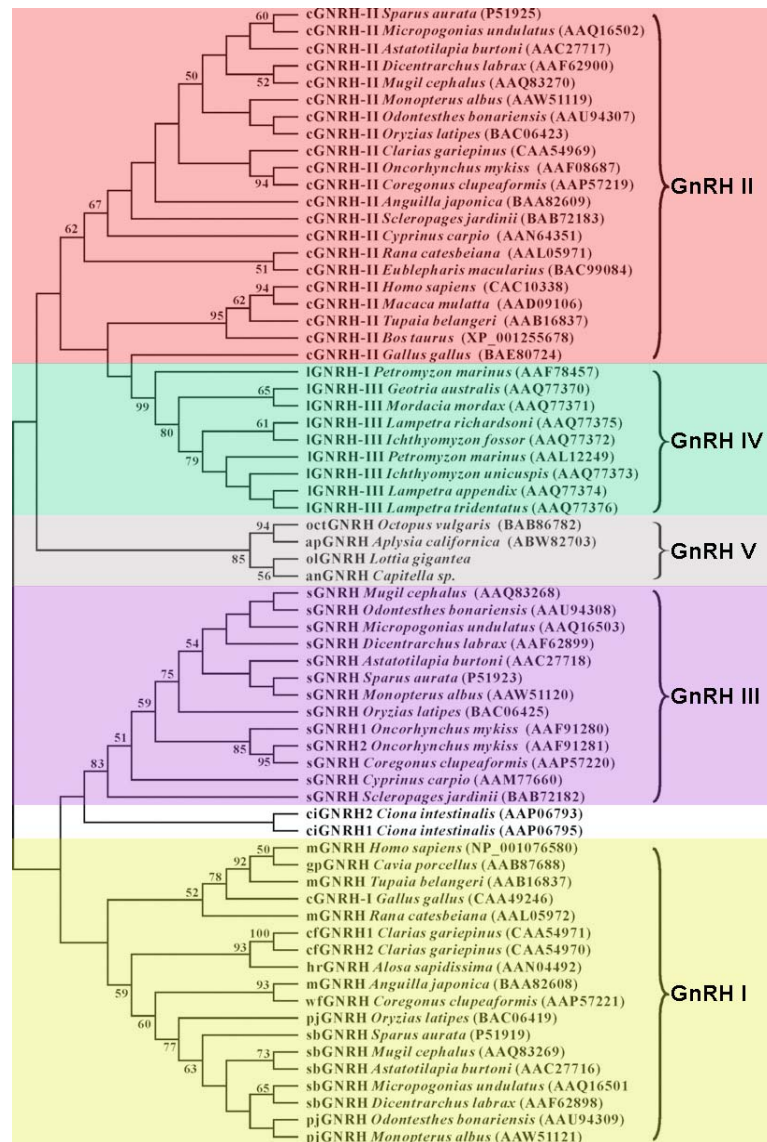


Fig. 1.3. Phylogenetic tree of prepro-GnRHs in chordates and protostomes generated by the minimum evolution method adapted from Tsai P and Zhang L Biol. Reprod. 2008;79:798-805. The bootstrap values (in %) over 50% are given at each branch point. Prepro-GnRHs are clustered into five groups (GnRH I to GnRH V). Each sequence is denoted by the name of its GnRH peptide form, the species from which it was isolated (in italics), and a GenBank accession number. Arabic numbers at the end of vertebrate sequences are used to indicate different precursors that encode the same GnRH peptide. Arabic numbers at the end of ciGnRH (tunicate) indicate different molecular forms of GnRH. The following abbreviations are used to denote the GnRH peptide forms: cGnRH-I, chicken GnRH-I; cGnRH-II, chicken GnRH-II; cfGnRH, catfish GnRH; ciGnRH, *Ciona* GnRH; gpGnRH, guinea pig GnRH; hrGnRH, herring GnRH; lGnRH-I, lamprey GnRH-I; lGnRH-III, lamprey GnRH-III; mGnRH, mammalian GnRH; pjGnRH, pejerro GnRH; sGnRH, salmon GnRH; sbGnRH, sea bream GnRH; wfGnRH, whitefish GnRH; octGnRH, octopus GnRH; apGnRH, *Aplysia* GnRH; anGnRH, annelid GnRH; olGnRH, owl limpet GnRH (Tsai and Zhang, 2008).

A seminal study on African cichlid fish showed that three distinct GnRH genes are expressed in three separate cell populations and differential selective pressure on different parts of each gene suggested different functions for the three GnRH genes (White et al., 1995). Studies investigating the anatomical localisation of GnRH-producing neurons show that there is a heterogeneous distribution of the GnRH forms in the brain, which is likely to reflect different physiological roles (Fernald and White, 1999; Okubo and Nagahama, 2008).

The structure of the mature chicken GnRH-II decapeptide hormone is conserved in species from bony fish to humans, and was suggested as the most primitive evolved form (Millar and King, 1987) and has subsequently been designated GnRH-II (Millar et al., 2004). GnRH-II is produced in the midbrain tegmentum (Fernald and White, 1999; Okubo and Nagahama, 2008). In contrast, GnRH-I was designated due to its hypothalamic localisation (Sealfon et al., 1997; Troskie et al., 1998) and is released exclusively into the pituitary through projections to the median eminence (Fernald and White, 1999; Okubo and Nagahama, 2008). In non-mammalian vertebrate members within the GnRH-I cluster, GnRH-I vary diversely in peptide sequence, although mammalian GnRH has been suggested to represent the ancestral form (Okubo and Nagahama, 2008), as it has been found in ancient fish (King et al., 1995; Lepretre et al., 1993; Sherwood et al., 1991) and more recently the peptide has been purified from amphioxus (Chambery et al., 2009). Interestingly, in several mammals the GnRH-II gene has been inactivated or deleted and therefore they only possess the mammalian GnRH form (Morgan and Millar, 2004; Stewart et al., 2009). A recent review on the GnRH-II genes in mammals shows that the GnRH-II is functionally compromised in 12 out of 22 mammalian GnRH-II genes studied, although large sections of the GnRH-II gene are retained in most mammalian species, suggesting that mammalian ancestors had a functional GnRH-II system (Stewart et al., 2009).

The third form of GnRH is localised to the terminal nerve ganglion in the forebrain in teleost fish and is designated GnRH-III (Fernald and White, 1999; Okubo and

Nagahama, 2008; White et al., 1998). Like GnRH-II, the peptide sequence for GnRH-III is completely conserved but is known to be present only in the teleost lineage (Millar et al., 2004; Okubo and Nagahama, 2008). It was suggested that the gene encoding this peptide was generated after the divergence of the teleosts from the vertebrate (tetrapod) lineage (Millar et al., 2004). However, more recent analysis of GnRH gene loci suggests that the third form of GnRH did not arise from a teleost-specific genome duplication, and the gene may have been deleted during the early evolution of tetrapods (Okubo and Nagahama, 2008). The GnRH forms may have either been adopted for specialised physiological function, or their roles may have become redundant, resulting in conversion to a pseudogene or may have been completely lost in certain species during the evolution of vertebrates. It is also possible that the GnRH gene which retains function may have been able to compensate for the role of the non-functional or deleted genes (Okubo and Nagahama, 2008).

1.3.2. GnRH functions

The anatomical localisation of the neurons producing GnRH-I in the hypothalamus and their projections to the median eminence allows for its release into the pituitary vasculature (Millar, 2005; Sealfon et al., 1997; Troskie et al., 1998). Therefore, it is the GnRH isoform primarily responsible for regulating production and secretion of pituitary gonadotrophins. In mammals it is well established that GnRH-I plays a fundamental role in the control of reproduction by regulating gonadotrophins and promoting steroidogenesis and gametogenesis (Millar et al., 2004). Pulsatile release of GnRH-I into the hypophyseal portal system stimulates the biosynthesis and secretion of LH and FSH (Fink, 1988). Interestingly, studies have shown GnRH to induce production of other endocrine hormones, including growth hormone (Marchant et al., 1989), prolactin (Weber et al., 1997) and somatolactin (Kakizawa et al., 1997) in primary pituitary cell cultures from teleost species. It is therefore feasible that GnRH may have a role in the control of various pituitary hormones in teleosts (Okubo and Nagahama, 2008).

The conservation of GnRH-II decapeptide sequence over 500 million years of evolution suggests it has an important physiological role although its precise functions remain unclear (Millar et al., 2004; Okubo and Nagahama, 2008). Localisation of GnRH-II in the midbrain tegmentum (Okubo and Nagahama, 2008), and in the central and peripheral nervous systems (Millar et al., 2004) suggests that it acts as a neuromodulator (Millar et al., 2004; Okubo and Nagahama, 2008). In support of this neuromodulatory role, GnRH-II expression occurs in amphibian sympathetic ganglia (Troskie et al., 1997) and GnRH-II treatment results in regulation of ion channels in bullfrog sympathetic ganglion cells (Bosma et al., 1990; Jones, 1987). Functions for GnRH-II in modulation of reproductive behaviour and as a sensor of metabolic states have been proposed and intensively reviewed by Millar et al., 2004 and Okubo and Nagahama, 2008 and include functions in sexual arousal, reproductive behaviour, sexual behaviour, food intake and energy balance (Millar et al., 2004; Okubo and Nagahama, 2008).

GnRH-III has been localised to the terminal nerve ganglion in the forebrain in teleost fish (Okubo and Nagahama, 2008; White et al., 1995). Terminal nerve GnRH neurons project to the olfactory placode, optic nerves and are transported to a wide range of regions in the brain (Gonzalez-Martinez et al., 2001; Gonzalez-Martinez et al., 2002; Okubo and Nagahama, 2008). The putative functions of GnRH-III have been reviewed by Okubo and Nagahama, 2008 and include functions in reproductive behaviour (e.g. nest-building), olfactory and visual sensory effects and possible hypophysiotropic functions through their overlap with GnRH-I neurons in the forebrain (Mohamed and Khan, 2006; Mohamed et al., 2005; Okubo and Nagahama, 2008; Pandolfi et al., 2005; Vickers et al., 2004; Wong et al., 2004).

1.4. Gonadotrophin-releasing hormone receptor (GnRH-R)

It has been assumed that GnRH isoforms elicit physiological effects by binding to cognate receptors, termed the Gonadotrophin-Releasing Hormone Receptors (GnRH-R). As outlined above (**section 1.3**) GnRH isoforms are heterogeneously distributed in the nervous system and the peripheral nervous system and this may reflect their physiological roles (Okubo and Nagahama, 2008). However, the principle established function of GnRH is the control of reproduction through activity within the HPG-axis, and this is reflected in the expression of vertebrate GnRH receptors in pituitary gonadotrophs which constitute 8-15% of the anterior pituitary cells (Ruf et al., 2003; Stojilkovic et al., 1994). The activation of the GnRH receptor through binding GnRH maintains the homeostatic activity within the reproductive hormonal cascade, by stimulating transcription of gonadotrophin genes and hormone secretion which in turn result in the functional maturation of the gonads (**Fig. 1.4**).

1.4.1. GnRH receptor isoforms

The earliest report of cloning a GnRH receptor cDNA occurred 21 years after the first GnRH peptide hormone was identified. The first GnRH receptor cDNA was cloned from the mouse pituitary α T3 gonadotrope cell line and the amino acid sequence deduced and shown to be characteristic of GPCRs (Tsutsumi et al., 1992). The functionality of the receptor was confirmed by injecting sense RNA transcripts into *Xenopus* oocytes and determining receptor ligand-binding affinity (Tsutsumi et al., 1992). GnRH-Rs are guanine nucleotide binding protein (G Protein) coupled receptors (GPCRs) (Limor et al., 1989; Perrin et al., 1989) specific for GnRH. GnRH receptors possess a common molecular architecture typical of rhodopsin family GPCRs, consisting of 7 trans-membrane domains connected by three extracellular loops (ECL), and three intracellular loops (ICL), an amino terminal extracellular domain and an intracellular carboxyl terminus, although mammalian GnRH receptors lack the intracellular cytoplasmic tail domain (Millar et al., 2004; Tsutsumi et al., 1992) (**Fig.1.4**). Like GnRH isoforms, GnRH receptors have been identified within two animal superphyla, Deuterostomes and Protostomes. The GnRH receptors found within the Protostomes lie within the phylum Mollusca (octopus) (Kanda et al., 2006). The GnRH receptors found within the Deuterostomes are restricted to the phylum Chordata and include representatives from the protochordates (e.g. tunicates and amphioxus) (Tello and Sherwood, 2009) and vertebrates, inclusive of cartilaginous fish (e.g. dogfish), teleost fish, amphibians, reptiles, aves and mammals (Kah et al., 2007; Millar et al., 2004).

Retention of several functional forms of GnRH in teleost species and two in most non-mammalian vertebrates suggested that two or three types of functionally different cognate GnRH receptors may exist, with each GnRH isoform functioning through its cognate GnRH receptor (Millar et al., 2004; Morgan and Millar, 2004). The high conservation of ECL 3 facilitated the PCR amplification of this region from genomic DNA from various vertebrates (Troskie et al., 1998). Subsequently,

numerous full-length GnRH receptor sequences have been identified, with more than one receptor isoform identified within a single species. In most vertebrates there are usually two to three forms of GnRH receptor present (Millar, 2005), although there are fewer GnRH ligand genes in mammals compared to protochordates, fish and amphibians (Morgan and Millar, 2004).

Several distinct GnRH receptor genes have been identified in certain species of fish, with five identified in the masu salmon (Jodo et al., 2003; Jodo et al., 2005), four identified in zebrafish (Tello et al., 2008), and three in medaka (Okubo et al., 2003). This may reflect whole-genome duplication in ancestral fish followed by slow sequence divergence resulting in retention of functionally identical genes (Hordvik, 1998; Morgan and Millar, 2004). In amphibians, up to three separate GnRH receptor genes have been identified in the bullfrog (Wang et al., 2001a) and two genes have been identified in African clawed frog (Troskie et al., 2000). In reptiles and avian species, studies of GnRH systems have lagged behind and warrant further attention (Morgan and Millar, 2004). Only one GnRH receptor was identified in the leopard gecko prior to 2005 (Ikemoto et al., 2004), although a recent report shows that there are now three separate genes (Ikemoto and Park, 2007). In avian species, one GnRH receptor was identified prior to 2006 (Sun et al., 2001a; Sun et al., 2001b) and in this thesis the cloning and characterisation of a novel GnRH receptor from an avian species (chicken) is described (**See Chapter 3**). In most mammalian species there is only one functional GnRH receptor gene, although in some primates (e.g. macaque and green monkey) a second functional GnRH receptor gene exists (Millar, 2003; Morgan and Millar, 2004; Neill et al., 2001). In a recent study, 22 sequences of the mammalian GnRH receptor gene encoding the type II GnRH receptor were analysed and eight were found to be genetically intact (orangutan, African green monkey, macaque, marmoset, tree shrew, kangaroo, rat, pig and elephant), whereas the remaining 14 exhibited gene disruption or deletion (Stewart et al., 2009).

There is a lot of confusion regarding the nomenclature of GnRH receptor subtypes, with no standard nomenclature being adopted. GnRH receptor genes have been designated names on an '*ad hoc*' basis according to the chronology of their sequence derivation, or their pharmacological characteristics or tissue-specific expression, which makes interspecies comparisons particularly confusing (Millar et al., 2004). The re-naming of GnRH receptors according to phylogenetic classification is being adopted, in which three distinct classes of GnRH receptors from the vertebrate lineage can be grouped into separate clusters (Millar et al., 2004).

A recent phylogenetic study examining GnRH receptor genes, with representatives from both the Protostome (octopus) and Deterostome superphyla inclusive of protochordates (amphioxus and tunicates) and chordates (fish, amphibian, avian and mammals), classify the GnRH receptors into 6 major clusters (Tello and Sherwood, 2009) (**Fig. 1.5**). There are three vertebrate clusters (as described above); type I GnRH receptors (mammalian and non-mammalian), type II GnRH receptors, type III GnRH receptors plus protochordate-specific GnRH receptors (urochordate and amphioxus) and a more distantly related cluster present in invertebrates (octopus and adipokinetic hormone receptor (AKHR) -like) (**Fig. 1.5**). Interestingly, unlike the lamprey-specific GnRH genes (Tsai and Zhang, 2008) (**Fig. 1.3**), the lamprey GnRH receptor is classified with the type II GnRH receptors (**Fig. 1.5**). Similarly, the tunicate GnRH genes are distantly related to GnRH-III and GnRH-I (Tsai and Zhang, 2008) (**Fig. 1.3**) and the receptors lie in a separate protochordate lineage which does not coincide with the protochordate amphioxus receptors (**Fig. 1.5**). This suggests that there was a rapid lineage-specific divergence in urochordate GnRH receptor sequences after the split from an amphioxus-like ancestor (Tello and Sherwood, 2009). This relationship appears to confirm the classification of GnRH variants (**Fig. 1.2**) found in tunicates (**Fig. 1.3**). The Protosomian GnRHs were shown to represent a monophyletic lineage and were designated the name GnRH-V (Tsai and Zhang, 2008), this coincides with the separate cluster in which octopus GnRH receptor resides along with AKHR-like receptors (**Fig. 1.5**).

Surprisingly however, two of the four GnRH receptors identified in amphioxus (protochordate) coincide with the cluster in which the Protostome octopus GnRH receptor resides, along with AKHR receptors. Adipokinetic hormone (AKH) (an invertebrate neuropeptide metabolic hormone found in insects) which is similar to the gene encoding vertebrate GnRH and also possesses a NH₂-terminal pGlu and a COOH-terminal amide, is the cognate ligand for a GnRH receptor ortholog identified in the common fruit fly (*Drosophila melanogaster*) (Hauser et al., 1998; Staubli et al., 2002). The nematode roundworm (*C. elegans*) AKH-GnRH-like peptide was also identified, and its precursor was similar to AKH found in insects and the GnRH precursor genes from protochordates and vertebrates (Lindemans et al., 2009). The identification of a GnRH receptor homolog in the nematode roundworm enabled studies that showed both the nematode AKH-GnRH-like peptide and fruit fly AKH activate the nematode ‘GnRH receptor’ (Lindemans et al., 2009; Vadakkadath Meethal et al., 2006). Additionally, gene silencing of the nematode roundworm AKH-GnRH-like peptide or the ‘GnRH receptor’ resulted in a delay in the egg laying process (Lindemans et al., 2009). Therefore a functional role for AKH-‘GnRH receptor’ in reproduction probably arose prior to the divergence of the Protosomian and Deuterostomian superphyla (Lindemans et al., 2009). The presence of fruit fly GnRH receptor ortholog and nematode roundworm, amphioxus and octopus GnRH receptor homologs coinciding in a separate cluster and/or the response to AKH-GnRH like peptides suggest that this hormonal system evolved early during life on earth but was lost during vertebrate evolution.

Figure 1. 5

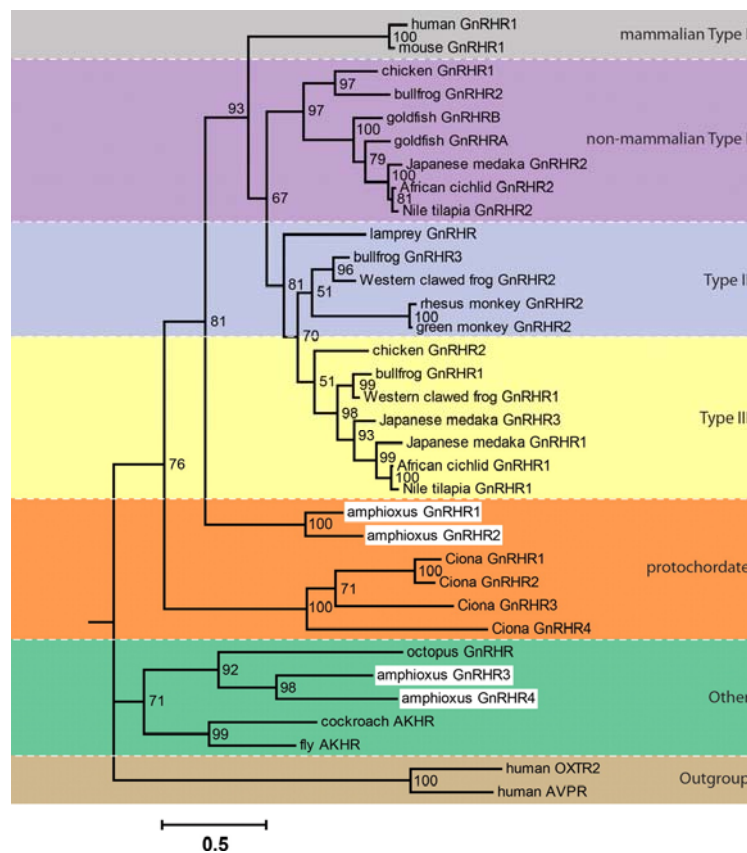


Fig. 1.5. Consensus maximum likelihood phylogenetic tree of vertebrate GnRH-Rs and invertebrate GnRHR-like sequences (1000 bootstrap replicates) from Tello, J. A. et al. *Endocrinology* 2009;150:2847-2856. Consensus maximum likelihood phylogenetic tree of vertebrate GnRHRs and invertebrate GnRHR-like sequences (1000 bootstrap replicates). Bootstrap proportions (shown as a percentage) for each hypothesized receptor group are indicated next to the branch leading to the group. The receptors sequences used to create the phylogram are: human (*Homo sapiens*) GnRHR1 (NP_000397); green monkey (*Cercopithecus aethiops*) GnRHR2 (AAK52746); rhesus monkey (*Macaca mulatta*) GnRHR2 (NP_001028014); house mouse (*Mus musculus*) GnRHR1 (AAA37716); two chicken (*Gallus gallus*) GnRHRs (GnRHR1: NP_989984, GnRHR2: NP_001012627); three bullfrog (*Rana catesbeiana*) GnRHRs (GnRHR1: AAG42575, GnRHR2: AAG42949, GnRHR3: AAG42574); two pipid frog (*Xenopus tropicalis*) GnRHRs [GnRHR1, compiled from the Ensembl *X. tropicalis* genome project (version JGI 4.1) using peptide ID ENSXETP00000038462 and scaffold 22, GnRHR2: compiled from Ensembl peptide ID ENSXETP00000036235 and scaffold 3972]; two Nile tilapia (*Oreochromis niloticus*) GnRHRs (type 1: BAC77240, type 2: BAC77241); three Japanese medaka (*Oryzias latipes*) GnRHRs (GnRHR1: BAB70506, GnRHR2: BAB70505, GnRHR3: BAC97833); two goldfish (*Carassius auratus*) GnRHRs (type A: AAD20001, type B: AAD20002); two African cichlid (*Astatotilapia burtoni*) GnRHRs (GnRHR1: AAU89433, GnRHR2: AAK29745); lamprey (*Petromyzon marinus*) GnRHR (AAQ04564), four tunicate (*Ciona intestinalis*) GnRHRs (GnRHR1: NP_001028997, GnRHR2: NP_001028996, GnRHR3: NP_001028995, GnRHR4: NP_001028994); octopus (*Octopus vulgaris*) GnRHR (BAE66647); fruitfly (*Drosophila melanogaster*) AKH receptor (AKHR; AAC61523); and the cockroach (*Periplaneta Americana*) AKHR (AAQ17230). The human arginine vasopressin type 1A (AVPR1A; NP_000697) and oxytocin (OXTR; NP_000907) receptors were added as outgroups to root the tree.

1.4.2. GnRH receptor isoform functions

According to phylogenetic classification, the vertebrate type I GnRH receptors are represented in teleost fish (numerous), amphibians (bullfrog), reptiles (leopard gecko) and avian (chicken) species and in mammals (human, mouse, rat). The type II receptors include receptors from primitive fish (lamprey), amphibians (bullfrog, Western clawed frog), reptiles (leopard gecko) and mammals (rhesus monkey, African green monkey). The type II GnRH receptors are more closely related to type III GnRH receptors than to type I receptors and it has been suggested that these two GnRH receptor subtypes may have arisen from genome duplication in an ancestral gene in lower vertebrates (Millar et al., 2004). Unlike the type II GnRH receptors, the type III GnRH receptors include sequences from teleost fish (numerous), amphibians (bullfrog, pipid frog) and reptiles (leopard gecko) but do not occur in mammals (**Fig.1.5**).

Comparisons of the expression with the three distinct classes of GnRH receptors from the vertebrate lineage [type I (mammalian and non-mammalian), type II and type III GnRH receptors] (Millar et al., 2004), will further aid attribution of function to specific receptor types. Sometimes however, more than one GnRH receptor identified in a single species resides in the same phylogenetic clade as another GnRH receptor identified in the same species. For example, the three medaka GnRH receptors classify into two distinct lineages (Okubo et al., 2003). Two of the medaka GnRH receptors are type III and the other one is a type I (Tello et al., 2008) (**Fig.1.5**). In the zebrafish, where four GnRH receptors have been identified, two distinct GnRH receptor types reside in the type III clade and two in the type I clade (Tello et al., 2008). In the bullfrog and the leopard gecko, the three GnRH receptors that were identified (Ikemoto and Park, 2007; Wang et al., 2001a) segregate into three separate GnRH receptor groups (type I, type II and type III). The first chicken GnRH receptor that was identified is designated a type I non-mammalian GnRH receptor type (Millar et al., 2004; Sun et al., 2001b) and the novel chicken GnRH receptor described in this thesis is a type III receptor (**Chapter 3**). In most

mammalian species there is only one functional GnRH receptor gene which is classified as a mammalian type I GnRH receptor. Eight GnRH receptor genes encoding a second form of GnRH receptor were found to be functionally intact are classified as type II GnRH receptors (Millar, 2003; Morgan and Millar, 2004; Stewart et al., 2009)

The attribution of physiological significance to each GnRH receptor type by investigating the spatial expression of GnRH receptors is further complicated as studies have shown that more than one receptor type can be expressed in the same tissue. For example, in the zebrafish, the anatomical distribution of the four GnRH receptors (type III and type I) is widespread in the brain, eye and gonads and additionally, all four of the GnRH receptors are expressed in the pituitary (Tello et al., 2008). The sea bass possesses five isoforms of GnRH receptor (type III and type I) and all but one are expressed in the pituitary (Moncaut et al., 2005). In goldfish, two subtypes of the GnRH receptor have been identified (both type I) (Illing et al., 1999) and they are both expressed in the pituitary. Therefore, GnRH systems exhibit species-specific recruitment of receptor subtypes and ligands within different tissues and there is also plasticity in the spatiotemporal expression patterns of GnRH receptor subtypes (Ikemoto and Park, 2007; Illing et al., 1999; Moncaut et al., 2005; Troskie et al., 2000; Wang et al., 2001a). Additionally, in the catfish, both identified GnRH receptors (both type I) are expressed in a number of tissues (testes, brain and pituitary), there is a clear difference in levels of expression between the two in the brain and pituitary, and one receptor subtype is exclusively expressed in the ovary and heart (Bogerd et al., 2002). In the bullfrog, three subtypes of GnRH receptor are expressed but only one (type III) predominates in the pituitary (Wang et al., 2001a). Two subtypes of GnRH receptor occur in the African clawed frog (*Xenopus laevis*) (Troskie et al., 2000) and one (type I) predominates in the pituitary. The leopard gecko expresses three receptor subtypes in the pituitary, with one receptor subtype (type III) predominating in the anterior pituitary (Ikemoto and Park, 2007). Therefore, these observations also suggest that there is plasticity in evolutionary

recruitment of GnRH receptor subtypes for regulation of pituitary gonadotrophin production. The type I GnRH receptor identified in the chicken is widely expressed in a range of tissues at low transcript levels. Comparisons of this receptor and the novel chicken GnRH receptor expression levels were investigated in this thesis (**Chapter 3**).

Studies of GnRH receptor activation by the naturally occurring endogenous ligands relevant to each species should help in the attribution of physiological function. Understanding ligand-binding interactions is important for understanding receptor function, as it is indicative of receptor activation (Millar et al., 2004). Most non-mammalian GnRH peptides are at least an order of magnitude less active than mammalian GnRH at mammalian type I GnRH receptors as they lack a charged residue in position eight of the decapeptide (**Fig. 1.2**) (see **section 1.3.1**) (Millar et al., 1989). Non-mammalian GnRH receptors are less selective for mammalian GnRH compared to non-mammalian GnRHs (Barran et al., 2005; Bogerd et al., 2002; Illing et al., 1999). It is now firmly established that GnRH-II (relative to GnRH-I) has a higher binding affinity for all non-mammalian vertebrate receptors irrespective of their classification based on structural similarities (Pfleger et al., 2002). The promiscuity of receptor subtype activation *in-vitro* by different endogenous GnRH ligand isoforms as seen in teleost fish (Illing et al., 1999; Moncaut et al., 2005), amphibians (Troskie et al., 2000; Wang et al., 2001a) and reptilian (Ikemoto and Park, 2007) species, suggests that a complex interplay between ligands and receptors may be possible *in-vivo*. For example, in the goldfish, the two GnRH receptors (both type I) have a higher ligand selectivity for GnRH-II although they exhibited an 84 fold difference between them in affinity for GnRH-III (Illing et al., 1999). In the catfish, one of the two identified GnRH receptors (both type I) had a higher ligand-binding selectivity for the endogenous GnRH-II than for GnRH-I (Bogerd et al., 2002). Both receptors also had higher ligand-binding selectivity for GnRH-II compared to other analogs (Bogerd et al., 2002). In the leopard gecko two of the three identified GnRH receptors (type I and III), were more

potently activated by GnRH-II than GnRH-I, although there was a marked difference in the potency of GnRH-II at these two GnRH receptors (Ikemoto and Park, 2007). Interestingly, however, there were no statistically significant differences in GnRH-II and GnRH-I stimulation at the third GnRH receptor (type II) (Ikemoto and Park, 2007). In the bullfrog, the three GnRH receptors (type I, II and III) were more potently stimulated with GnRH-II and GnRH-III compared to GnRH-I (Wang et al., 2001a). The greatest potency was observed with GnRH-II (Wang et al., 2001a). However, the receptor activation results in the leopard gecko and the bullfrog are not solely indicative of ligand-binding since receptor coupling efficiency may contribute to the observed differences. The type I chicken GnRH receptor was shown to exhibit a higher ligand-binding selectivity with GnRH-II and GnRH-II exhibits greater potency at the receptor compared to the endogenous cGnRH-I and mammalian GnRH-I (Sun et al., 2001b). In this thesis the comparison of the ligand-binding affinities and potency of endogenous ligands and GnRH analogs at the type I and the novel type III chicken GnRH receptor is compared (**Chapter 4**). Interestingly, one of the protochordate GnRH receptors identified in amphioxus, has a higher ligand selectivity for GnRH-I than GnRH-II, and this preferential selectivity has never been observed in any other non-mammalian GnRH receptor (Tello and Sherwood, 2009).

1.4.3. GnRH receptor isoform structure

As mentioned previously, non-mammalian GnRH receptors are typical GPCRs which consist of seven alpha-helical transmembrane domains (TMD), connected by three extracellular loops (ECL) and three intracellular loops (ICL) plus a cytoplasmic C-terminal domain (Millar et al., 2004; Sealfon et al., 1997). However, unlike all most GPCRs, mammalian GnRH receptors lack the intracellular cytoplasmic tail domain (Millar and Pawson, 2004; Tsutsumi et al., 1992). The ECLs and the extracellular ends of the TMDs are mostly involved in binding GnRH analogs (Millar et al., 2004). Conformational changes in the TMDs elicited by ligand-

binding are involved in receptor activation and the ICLs participate in interactions with G Proteins and signal transduction apparatus (Millar et al., 2004).

The arginine residue in position 8 of mammalian GnRH-I is a crucial determinant for high-affinity binding and ligand-binding selectivity for mammalian GnRH receptors (Flanagan et al., 1994; Illing et al., 1999; Millar et al., 1989; Millar and King, 1983). It is believed to interact with an acidic residue of the GnRH receptor (**Fig. 1.4**) to induce a β II conformation of the ligand (Fromme et al., 2001; Millar et al., 2004). The glutamate residue in ECL3 at position 301 in the mouse receptor was shown to have a role in receptor recognition of Arg⁸ of mammalian GnRH (Flanagan et al., 1994) possibly through an ionic interaction with the positively charged arginine side chain (Flanagan et al., 1994; Millar et al., 2004). Interestingly, ECL3 of each receptor type possesses a distinctive sequence motif and this has a key influence on ligand-receptor selectivity (Millar et al., 2004). Mammalian type I receptors possess a S-E/D-P motif whereas non-mammalian type I receptors have a P-X-S/Y motif (Millar et al., 2004; Wang et al., 2004). A site-directed mutation study has shown that the residues surrounding the glutamic acid residue (E) are important for differential ligand-binding selectivity (mGnRH-I vs GnRH-II) between mammalian and non-mammalian GnRH receptors (Wang et al., 2004). Ligand contact sites have been identified in type I GnRH receptors and have been shown to be conserved in all vertebrate type I GnRH receptors (Millar et al., 2004). These include: **(1)** A highly conserved lysine residue in TMD3 of GnRH receptors which preserves agonist binding to GnRH receptors (Zhou et al., 1995) and interacts with His² or pGlu of GnRH ligands. These residues are conserved in all ligands except in the guinea pig and where His² is substituted with a Tyr residue (**Fig. 1.2**). This interaction is facilitated by a hydrogen bond formation (Hoffmann et al., 2000; Millar et al., 2004; Sealfon et al., 1997). **(2)** An asparagine residue located near the extracellular surface of TMD2 is a critical determinant of ligand potency, particularly for ligands with glycineamide(10) (Davidson et al., 1996), which is conserved in all native GnRH ligands (**Fig 1.1**). This Asn residue serves as a docking site for the C-terminal

Gly¹⁰NH₂ of the ligands through hydrogen-bond formation (Millar et al., 2004). (3) The aspartic acid (Flanagan et al., 2000) located at the extracellular boundary of TMD2 may form an inter-helical interaction with a lysine residue in TMD3 of GnRH receptors (Zhou et al., 1995) to contribute to the formation of the agonist binding pocket (Flanagan et al., 2000). This residue may also interact with His² of GnRH ligands (Flanagan et al., 2000).

The C-terminal tail of GnRH receptors influences receptor ligand-specificity, receptor expression at the cell surface, receptor phosphorylation, internalisation and desensitisation (Blomenrohr et al., 1999; Heding et al., 1998; Willars et al., 1999). Desensitisation of receptors is caused by sustained stimulation of the receptor (McArdle et al., 2002) and is a consequence of receptor phosphorylation. Phosphorylation stabilises β -arrestin association with the receptor, resulting in the inhibition of G protein binding, dissociation from signalling complexes and subsequent inactivation of effector proteins (McArdle et al., 1999; McArdle et al., 2002). The C-terminal cytoplasmic domain is important in receptor desensitisation as phosphorylation of amino acids located in this region facilitates β -arrestin association with the receptor (McArdle et al., 2002), although residues in the intracellular loops may also facilitate β -arrestin binding (Ferguson, 2001).

Aspartic acid residues in both loci of the functional helix2/helix7 micro-domain are conserved in non-mammalian GnRH receptors and serve to regulate G protein coupling and expression (Flanagan et al., 1999; Zhou et al., 1994). Functionally verified residues in mammalian GnRH receptors known to be important for receptor G protein coupling and signal transduction include alanine in ICL3 (Myburgh et al., 1998), and the Arginine cage motif (DRXXX(I/V)) which resides at the cytosolic end of TMD3 (Ballesteros et al., 1998).

1.5. GnRH Receptor Signalling

GnRH receptors are GPCRs (Limor et al., 1989; Perrin et al., 1989) specific for GnRH. The heterotrimeric G proteins consist of an α subunit which binds guanine nucleotides and a complex consisting of tightly bound β and γ subunits (Spiegel et al., 1992). This structure has been determined at the atomic level by X-ray crystallography (**Fig. 1.6**). Stimulation of the receptor by the ligand (GnRH) results in a conformational change in $G\alpha$ resulting in the release of GDP and the association of GTP with the α subunit (Spiegel et al., 1992). This causes a further conformational change of the α subunit leading to G protein activation and a dissociation of the $\beta\gamma$ -complex (Bourne et al., 1991; Spiegel et al., 1992) (**Fig. 1.6**). Thus GPCRs act as guanine nucleotide exchange factors for the associated G proteins, which on ligand-receptor stimulation result in the $G\alpha$ and $G\beta\gamma$ subunits activating effector systems (Naor, 2009). The activation of the effector proteins initiates diverse downstream second messenger signalling pathways that characterise the nature of the response to receptor activation (Kraus et al., 2001; McArdle et al., 1999). Activation of GnRH receptors by GnRH initiates a cascade of intracellular signal transduction pathways that results in the synthesis and release of gonadotrophins from the pituitary gonadotrophs into the systemic circulation (Conn and Crowley, 1994; McArdle et al., 2002; Sealfon et al., 1997; Stojilkovic and Catt, 1995).

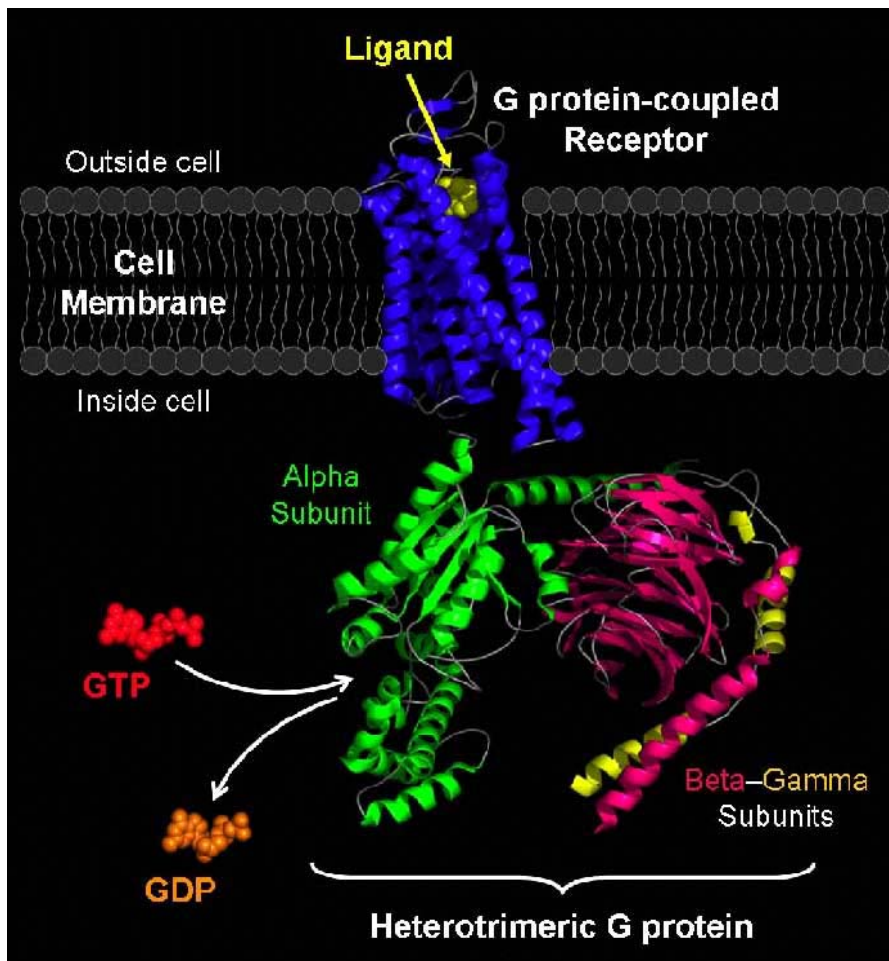
Figure 1. 6

Fig. 1.6. Schematic diagram of a three-dimensional crystal structure of a GPCR embedded in a cell membrane adapted from:

<http://www.vanderbilt.edu/vicb/Articles/LensSummer2005/WhereAreTheNewDrugs.htm>

The GPCR is loosely attached to a heterotrimeric G protein, consisting of alpha, beta and gamma subunits, inside the cell. On ligand activation, the receptor changes shape eliciting the release of guanosine diphosphate (GDP) from the alpha subunit. GDP is replaced by the higher-energy guanosine triphosphate (GTP). Hydrolysis of GTP causes the alpha subunit to dissociate from the beta and gamma subunits. The subunits then interact with other intracellular proteins to transmit signals via two independent pathways. Within a few seconds, GTP is hydrolysed to GDP by $G\alpha$ -GTPase, the G protein subunits reassociate, and the signalling is “turned off”.

1.5.1. G protein dependent signalling

G proteins consist of a family of alpha, beta and gamma subunits. Different alpha subunit isoforms include $G\alpha_{q/11}^*$, $G\alpha_s$, $G\alpha_i$, and $G\alpha_{12/13}$ (Kraus et al., 2001). GnRH receptors primarily couple to $G\alpha_q$ in pituitary gonadotrophs (Grosse et al., 2000; Hsieh and Martin, 1992; Naor et al., 1986; Naor et al., 2000), but evidence shows that GnRH receptors may also couple to $G\alpha_i$ and $G\alpha_s$ (Hawes et al., 1993; Liu et al., 2002; Stanislaus et al., 1998). GnRH receptors interact with multiple G proteins in a 'cell-context' dependent manner and may affect different signal transduction mechanisms according to cell type (Dobkin-Bekman et al., 2006). GnRH receptors couple to $G\alpha_q$ proteins (Anderson et al., 1992; Hsieh and Martin, 1992) which exert signal transduction principally by activating membrane associated phospholipase C (PLC) (Hsieh and Martin, 1992). GnRH receptors additionally couple to $G\alpha_i$ and $G\alpha_s$ proteins (Ruf et al., 2003), which results in the inhibition or stimulation of adenylyl cyclase respectively (Birnbaumer, 1992; Stojilkovic et al., 1994). $G\alpha_i$ can also transmit signals via transactivation of receptor tyrosine kinases (RTKs) (Luttrell et al., 1999). $G\alpha_{12/13}$ signals through protein tyrosine kinases (PTKs) (Jiang et al., 1998). In this review, only $G\alpha_q$ will be described in great detail as this is most relevant to the results.

1.5.2. $G\alpha_q$ coupling

GnRH-Rs couple to $G\alpha_q$ proteins which on GnRH stimulation cause an increase of PLC beta (PLC β) activity (Naor et al., 1986; Naor et al., 2000). There are six distinct isoforms of PLC (Stewart et al., 2007), although the full details remain unclear regarding which isoforms couple to $G\alpha_q$ proteins on GnRH receptor activation. GnRH induced activation of GnRH receptor and subsequent activation of $G\alpha_q$ results in a rapid Ca^{2+} independent stimulation of PLC β (Naor et al., 1986). This increases intracellular levels of Ca^{2+} which acts by positively stimulating more activation of PLC β (Kunkel et al., 2007).

** $G\alpha_{q/11}$ comprise two genes of similar sequence identity and are usually presented 34 together. In this thesis, $G\alpha_q$ will be used for simplification although coupling may also include $G\alpha_{11}$ protein.*

Phospholipase A₂ (PLA₂) and phospholipase D (PLD) are both activated by GnRH after a time delay of about 5-15 minutes after PLC activation (Naor, 2009; Poulin et al., 1996; Zheng et al., 1994). Activation of PLD results in the formation of phosphatidic acid (PA) by acting on a membrane phospholipid (phosphatidylcholine) which is then converted to diacyl-glycerol (DAG) by a PA-phosphohydrolase, which facilitates a prolonged activation of protein kinase C (PKC) (Naor, 2009). The PLA₂ generates long chain unsaturated fatty acids, for example arachidonic (AA), linoleic and linolenic acids which have been implicated in the activation of PKC (Naor, 1991; Naor, 2009). Activation of PLC β results in the hydrolysis of phosphatidylinositol 4,5-bisphosphate (PIP₂) and formation of inositol(1,4,5)-triphosphate (IP3) and DAG. IP3 and DAG second messengers result in Ca²⁺ mobilisation from the endoplasmic reticulum by activating calcium channels present and PKC activation respectively (Naor, 2009; Naor et al., 2000; Naor et al., 1998; Ruf et al., 2003; Stojilkovic et al., 1994). Ca²⁺ is a ubiquitous second messenger with a wide range of actions (Berridge et al., 2000).

1.5.3. Mitogen-Activated Protein Kinase

The organisation of the mitogen-activate protein kinase (MAPK) cascade is complex. Activation of the MAPK cascade is initiated through a GTP-binding protein (smGP or RAS family of proteins) or alternatively, by certain adaptor proteins. These then transduce their signal directly or through a mediator kinase to the MAPK kinase kinase (MAP3K) point of the MAPK cascade. A series of phosphorylation events facilitated by the enzymes e.g. MAPK kinase (MAPKK), MAPK and MAPK-activated protein kinase (MAP-KAPKs) can occur (Naor et al., 2000) (**Fig. 1.7**). There are four separate modules of the MAPK cascade that are named according to their MAPK component and include extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK), p38MAPK (p38) and big MAPK (BMK) (Naor et al., 2000) (**Fig. 1.7**).

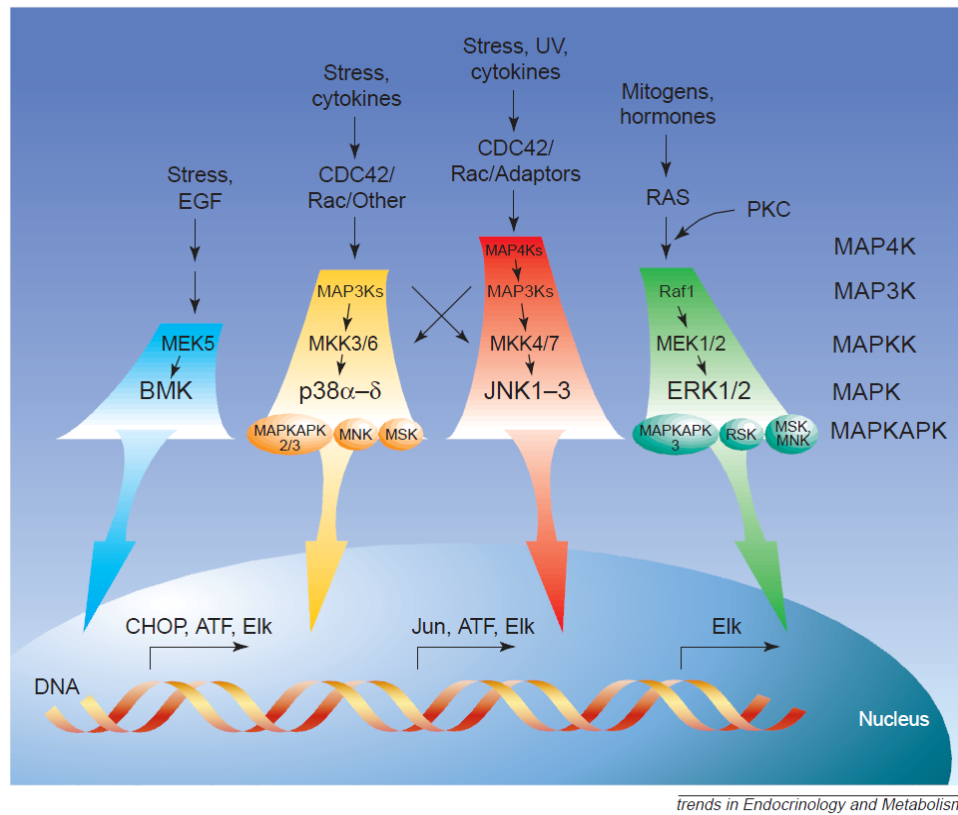
Figure 1. 7

Fig. 1.7. Schematic representation of the mitogen-activated protein kinase (MAPK) cascades from Naor Z et. al., Trends in Endocrinology and Metabolism 2000; 1(3):91-99. Abbreviations: Extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK), p38MAPK and big MAPK (BMK) (Naor et al., 2000).

1.5.4. GPCR signalling to the MAPK cascade via G proteins

As mentioned, following ligand stimulation, activated GPCRs affect $G\alpha$ and $G\beta\gamma$ subunit functions (Naor, 2009). Activated $G\alpha_q$ primarily PLC β which produces IP $_3$ (which induces Ca^{2+}) and DAG (Naor et al., 2000). Ca^{2+} is a ubiquitous second messenger signalling molecule with a wide range of actions (Berridge et al., 2000). The signal transduction continues with the free Ca^{2+} activating CaM kinase II which phosphorylates and inhibits RAS-GTPase-activating protein (RAS-GAP), inducing RAS and subsequently MAPK activation (Chen et al., 1998). Alternatively Ca^{2+} and DAG activate PKC or through Ca^{2+} /RAS-guanine nucleotide exchange factors

(GEFs) and RAS activation lead to MAPK activation (Farnsworth et al., 1995; Naor et al., 2000; Nishizuka, 1992). Activated PKC activates c-RAF which is partially dependent on RAS, resulting in stimulation of the ERK cascade (Kolch et al., 1993). Additionally, Ca^{2+} and PKC may induce activation of receptor PTKs (Zhang et al., 1996) which, influenced by interaction with cell adhesion integrin complexes, lead to MAPK activation (Naor et al., 2000). Thus a variety of signalling pathways (which have not been fully elucidated) result in PTK transactivation via the adaptor protein Shc and activate the Grb-SOS-RAS protein complex, resulting in MAPK activation (Naor et al., 2000). $G\alpha_s$ interacts with adenylyl cyclase to stimulate production of cAMP (Birnbaumer, 1992), which activates protein kinase A (PKA) (Beebe, 1994), or RAS-GEF (de Rooij et al., 1998). Both induce activation of RAP1 and through RAF activation result in activation of the MAPK cascade (Naor et al., 2000). $G\alpha_i$ transduces signal transduction through interaction with RAP-GAP (Jordan et al., 1999; Mochizuki et al., 1999) which in turn inactivates RAP1 activity, resulting in ERK activation (Naor et al., 2000). $G\alpha_{12}$ and $G\beta_\gamma$ may also signal through the MAPK cascade, although the signalling mechanisms have not been elucidated in detail (Naor et al., 2000). $G\alpha_{12}$ primarily signals through Bruton's tyrosine kinase (BTK) stimulation (Jiang et al., 1998), whereas $G\beta_\gamma$ complexes can signal through phosphoinositide 3-kinase (PI3K) to activate the MAPK cascade (Naor et al., 2000). The pathways from $G\alpha_q$ and $G\beta_\gamma$ to RAS-RAF-MEK-ERK is complex, and may involve some cell type specific components (Dobkin-Bekman et al., 2006; Karnoub and Weinberg, 2008). Signalling to the MAPK cascade by $G\alpha_q$, $G\alpha_s$, $G\alpha_i$, $G\alpha_{12}$ and $G\beta_\gamma$ as outlined above is summarised below (**Fig. 1.8**). There is evidence for feedback regulation involving particular protein phosphatases resulting in transient effects on the MAPK pathway (Karnoub and Weinberg, 2008; Omerovic et al., 2007). The feedback regulation may also involve some cell-type specific components (Karnoub and Weinberg, 2008; Omerovic et al., 2007). The complexity of RAS-MAPK signalling complexes continues to be elucidated such that cell context-specific signalling may be better understood in the future (Karnoub and Weinberg, 2008).

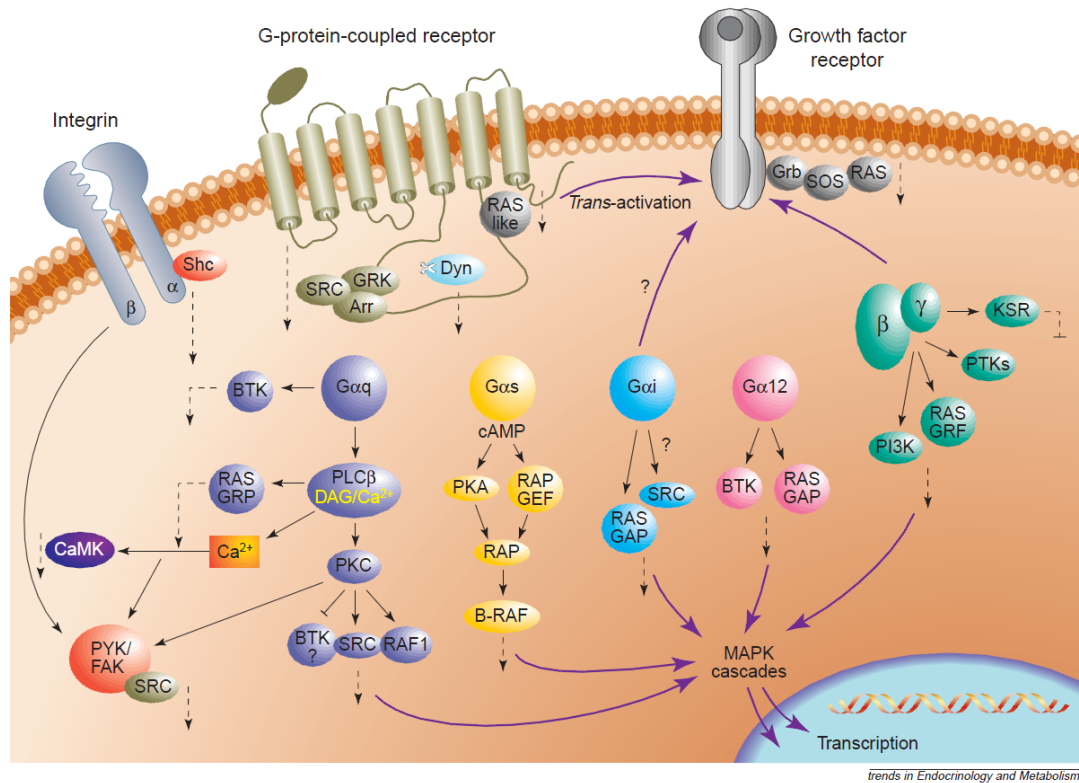
Figure 1. 8

Fig. 1.8. Schematic representation of G protein-coupled receptor (GPCR) and G protein signalling towards mitogen-activated protein kinase (MAPK) cascades from Naor Z et. al., Trends in Endocrinology and Metabolism 2000; 1(3):91-99. Dashed arrows represent indirect activation/inactivation of the MAPK cascades. Abbreviations: BTK, Bruton tyrosine kinase; Dyn, dynamin; FAK, focal adhesion kinase; GAP, GTPase-activating protein; GEF, guanine nucleotide exchange factor; GRF, guanine-specific nucleotide-releasing factor; GRK, GPCR kinase; PI3K, phosphoinositide 3-kinase; PKA, protein kinase A; PKC, protein kinase C; PLC, phospholipase C (Naor et al., 2000).

1.5.5. Signalling cascades and their involvement in GnRH regulation of gonadotrophin gene expression

MAPK regulate gene transcription by activating nuclear transcription factors or through phosphorylation of protein kinases (Kraus et al., 2001). A role for PKC, ERK and JNK but not p38 were implicated in GnRH regulation of the common alpha gonadotrophin subunit (Fowkes et al., 2002; Roberson et al., 1999; Weck et al., 1998; Xie et al., 2005). LH beta subunit gene transcription was shown to be

mediated by Ca^{2+} (Weck et al., 1998), although the role of Ca^{2+} , ERK, and c-Src, but not JNK have been implicated in the transcriptional regulation of the common alpha gonadotrophin subunit in $\alpha\text{T3-1}$ cells (Harris et al., 2003). In contrast studies have shown that Ca^{2+} is involved in common alpha gonadotrophin subunit activation, although the effect of GnRH on LH beta is mediated by PKA and ERK (Call and Wolfe, 1999; Saunders et al., 1998). JNK but not PKC and ERK has been implicated in LH beta regulation (Yokoi et al., 2000), although in L β -T2 cells PKC, ERK and JNK but not Ca^{2+} have been implicated in LH beta gene expression (Harris et al., 2003). FSH beta subunit gene regulation appears to be mediated by PKC, ERK, JNK, p38 and c-Src but not Ca^{2+} (Bonfil et al., 2004). These conflicting results may reflect the different cell types, gene promoters from different species and cell culture conditions used experimentally (Naor, 2009).

Activation of the MAPK cascade results in transcriptional regulation, as certain components are able to translocate to the nucleus (Dobkin-Bekman et al., 2006; Naor et al., 2000). A number of studies have shown that ERK can translocate to the nucleus (Bonfil et al., 2004; Caunt et al., 2006; Harris et al., 2003; Liu et al., 2002). ERK is able to phosphorylate the ternary complex factor Elk 1, and Elk 1 can bind to GnRH-response element located within the common alpha subunit gene promoter region (Marais et al., 1993; Roberson et al., 1995). c-Jun and c-Fos may be activated by JNK and ERK respectively, which then act on AP-1 sites present on the FSH beta promoter (Bonfil et al., 2004; Coss et al., 2004; Huang et al., 2001). C-Jun substrate, has been implicated in GnRH activation of the LH beta subunit promoter (Yokoi et al., 2000). Additionally, c-Jun and ATF-2 (both substrates of JNK) can bind to the CRE domain of the human common gonadotrophin alpha subunit promoter (Heckert et al., 1996)

1.6. Avian GnRH system

1.6.1. Endogenous chicken GnRH ligands

Two GnRH isoforms are present in the chicken (cGnRH-I and GnRH-II) (Sharp and Ciccone, 2005). Chicken GnRH-I (cGnRH-I) was the first non-mammalian GnRH identified (King and Millar, 1982a; King and Millar, 1982b; Miyamoto et al., 1982). cGnRH-I differs from mammalian GnRH (mGnRH) by the substitution of a glutamine in place of an arginine residue in position 8 of the mammalian decapeptide (Miyamoto et al., 1983). The second form of GnRH, GnRH-II was first identified in the chicken, and later found to be present in all vertebrate classes (Miyamoto et al., 1984). Recently a third variant of an avian GnRH has been suggested in the hypothalamus of songbirds, and has been deduced to be structurally similar to lamprey GnRH-III (l-GnRH-III) (Bentley et al., 2004). Contrary to this however, there is no genomic DNA evidence for a third GnRH ligand in the chicken to date (Morgan and Millar, 2004), suggesting that if it does exist in songbirds, it is not present in the chicken. Despite their early identification the function of the two GnRH ligands in the chicken still presents a conundrum. Pulsatile release of GnRH controls differential LH and FSH secretion in mammals, with higher GnRH pulse frequencies leading to increased LH release, whereas lower frequencies result in increased FSH secretion (Wildt et al., 1981). Although there have been no direct evidence of GnRH pulsatility in birds, GnRH release is probably pulsatile in birds. In support of this view, chicken plasma LH is episodically released which is likely to be a reflection of GnRH pulsatility (Senthilkumaran et al., 2006; Vizcarra et al., 2004). In quail there is evidence from experiments performed on hypothalamic slices to show that GnRH release is pulsatile (Li et al., 1994), further supporting the probably pulsatile release of GnRH in chickens.

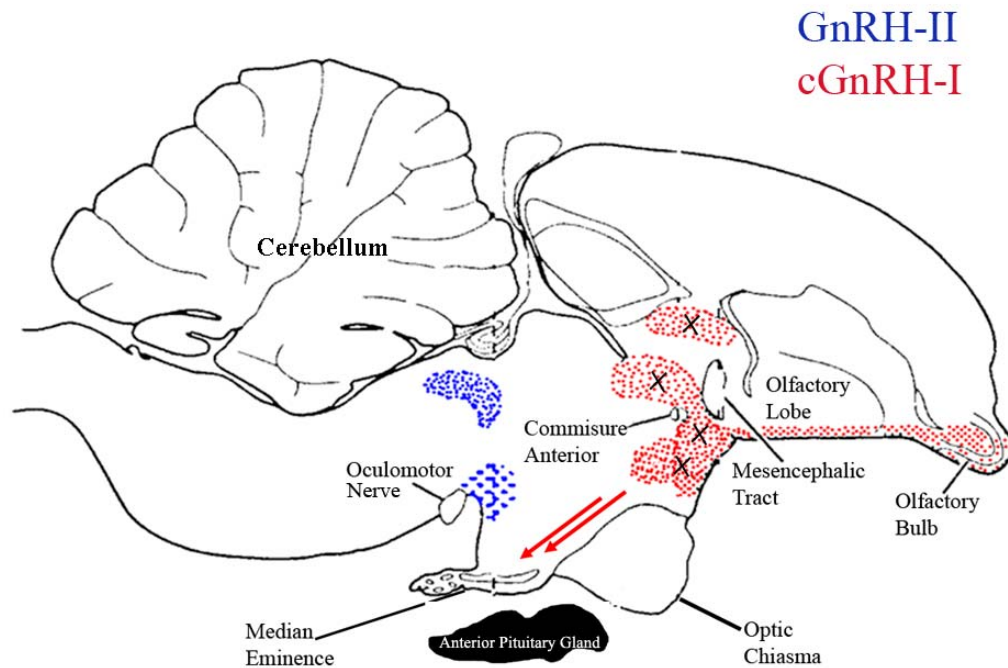
Conflicting studies have been reported on the ability of the cGnRH ligands to stimulate gonadotrophin production, but age, sex and reproductive status of the experimental birds needs to be considered (Bédécarrats et al., 2006). Both cGnRH-I

and GnRH-II stimulate LH release from chicken pituitary *in-vitro* (Hattori et al., 1986a; Millar et al., 1986) and *in-vivo* (Chou et al., 1985b; Hattori et al., 1986b; Proudman et al., 2006; Sharp et al., 1986). More recent studies have shown exogenous lGnRH-III is capable of inducing release of LH in song sparrows (Bentley et al., 2004). Pituitary responsiveness to cGnRH-I and GnRH-II is sexually differentiated in the domestic hen, with LH release being greater in the cockerel than in the hen. The relative potencies of cGnRH-I and II ligands are identical in mature male chickens (cockerel) and juvenile chicken of either sex, while GnRH-II is more potent in releasing LH in the mature laying hen (Sharp et al., 1987). The study performed by Sharp *et al.* in 1987 confirmed previous *in-vivo* observations in cockerels (Chou et al., 1985a) and hens (Sharp et al., 1986), and studies *in-vitro* (Hattori et al., 1986a; Millar et al., 1986) showing that GnRH-II is more potent in releasing LH when compared with cGnRH-I in the hen, but not in the cockerel. cGnRH-I and GnRH-II have been reported to stimulate FSH release *in-vitro*, (Hattori et al., 1986a; Millar et al., 1986). Using a newer specific antibody, there is no evidence *in-vivo* of cGnRH-I, GnRH-II and lGnRH-III having a significant effect on FSH release from chicken pituitary gonadotrophs (Bruggeman et al., 1998; Dunn et al., 2003; Krishnan et al., 1993; Proudman et al., 2006). Probably, cGnRH-I does not directly stimulate FSH release, but may stimulate FSH biosynthesis, while a role for GnRH-II in FSH production has not been established (Dunn et al., 2003; Sharp and Ciccone, 2005). It must be noted that evidence on release of LH and FSH in the experiments outlined above does not necessarily implicate a physiological role of cGnRH-I and GnRH-II as hypothalamic portal delivery of the cGnRH ligands to the pituitary needs to be considered.

Immunisation of laying hens against cGnRH-I, but not against GnRH-II, results in a complete regression of the reproductive system and a decrease in plasma LH (Sharp et al., 1990), suggesting that gonadotrophin secretion is controlled by cGnRH-I rather than GnRH-II. This is consistent with the presence of cGnRH-I in the median eminence (Mikami et al., 1988a; Sharp et al., 1990) (**Fig. 1.9.**) and the correlation

between hypothalamic cGnRH-I peptide content and reproductive status (Sharp et al., 1990). In contrast, immuno-histochemical studies have not found GnRH-II in the chicken median eminence using antibodies specific for GnRH-II (**Fig. 1.9.**) (Mikami et al., 1988a; Sharp et al., 1990), and no change in hypothalamic GnRH-II peptide content is seen in cockerels around the onset of puberty (Sharp et al., 1990). Moreover, in the purification and structural identification of GnRH from 249,000 chicken median eminence samples, only one form of GnRH (cGnRH-I) was found (King and Millar, 1982a; King and Millar, 1982b). Controversially however, three different studies suggest GnRH-II is present in the median eminence of the chicken (Vangils et al., 1993) and quail (Clerens et al., 2003; D'Hondt et al., 2001; Vangils et al., 1993). These studies used the same antibody and its specificity remains questionable; given that these claims are so controversial no attempt to negate the staining with cGnRH-I was made. Another study has suggested the presence of a lGnRH-III-like peptide in the chicken hypothalamus (Berghman et al., 2000), although appropriate controls for antibody specificity were not included in the experiment, leaving the finding open to criticism.

Therefore, cGnRH-I is thought to be the hypothalamic peptide responsible for regulating the pituitary gonadotroph (Mikami et al., 1988a; Sharp et al., 1990). However hypothalamic GnRH-II levels do decrease when the pre-ovulatory release of LH is maximal or declining in the hen. Thus GnRH-II may be involved in neuroendocrine events preceding ovulation (Wilson et al., 1990) or serve other reproduction related functions, such as behaviour. In other avian species GnRH-II affects reproductive behaviour in ring doves and soliciting behaviour in female song sparrows (King and Millar, 1995; Maney et al., 1997). The cGnRH peptides achieve their physiological roles through chicken GnRH receptors (cGnRH-R); however the effects of pharmacological doses of the peptides may be misleading because they may activate either or both of the cGnRH receptor isoforms. Therefore investigations in to the pharmacological responses of endogenous ligands at the cGnRH receptor isoforms will provide better understanding.

Figure 1. 9

PARASAGITTAL SECTION

Fig. 1.9. Schematic representation of a parasagittal section of an adult chicken brain indicating the location of GnRH immunoreactive perikarya, adapted from Dunn, I. and Millam, J. *Poultry and Avian Biology Reviews* 9(2):61-85. The blue regions represent the sites of GnRH-II and the red regions represent the sites of cGnRH-I immunostaining. Majority are in regions marked with an X. Other regions of the brain are indicated on the diagram. The anterior pituitary gland is anatomically positioned relative to the brain. Note the size of the pituitary gland is not to scale. Arrows represent axon projections to the median eminence. (Dunn and Millam, 1998)

1.6.2. The HPG-axis in the chicken

cGnRH-I neurons integrate the signals from the external and internal environment to produce pulsatile output of cGnRH-I which subsequently plays a pivotal role in the control of reproduction through the HPG-axis (Dunn I. C. et al., 2009). cGnRH-I neurons emerge from the olfactory placode or the presumptive ectoderm of the nasal cavity (Elamraoui and Dubois, 1993) and migrate to the olfactory epithelium along the olfactory nerve to the hypothalamus (Norgren, 1996) (**Fig. 1.9.**). In adult

chickens, 12% of cGnRH-I cell bodies are located in the pre-optic hypothalamus and the majority of cGnRH-I neurons are extra-hypothalamic in the septal region of the subpallium, which distinguishes the axis from several mammalian species (Kuenzel and Golden, 2006). The hypothalamic control of reproduction is accomplished at two levels. (1) cGnRH-I synthesis in the cell bodies in the pre-optic hypothalamus (2) cGnRH-I release from nerve terminals at the median eminence (Dunn I. C. et al., 2009).

Sexual maturation in cockerels coincides with increases in hypothalamic cGnRH-I peptide content and there is also an increased capacity to release cGnRH-I in the median eminence in sexually maturing cockerels (Knight, 1983; Knight et al., 1985). The changes in day-length (photoperiod) are perceived in the hypothalamus, which results in increased hypothalamic cGnRH-I mRNA content (Dunn and Sharp, 1999). However, there is an inhibitory effect of sex steroid hormones on hypothalamic neurons, which reduces cGnRH-I peptide content and expression of cGnRH-I mRNA (Dunn I. C. et al., 2009) (**Fig. 1.10**). This has been demonstrated by an increase in hypothalamic peptide content after castration (Knight et al., 1983; Sharp et al., 1990; Sun et al., 2001a) and release of cGnRH-I from the median eminence (Lal et al., 1990). The effects of surgical castration on cGnRH-I peptide content and mRNA were reversible on administration of oestrogen (Dunn et al., 2003; Sharp et al., 1994; Sun et al., 2001a). There is a pre-ovulatory release of cGnRH-I which is induced by circulating progesterone derived from the dominant pre-ovulatory ovarian follicle (Wilson and Sharp, 1973). The onset of incubation behaviour coincides with a dramatic decrease in cGnRH-I mRNA (Dunn et al., 1996). Interestingly in ageing reproductively active hens, there is no decrease in hypothalamic cGnRH-I content (Sharp et al., 1992) although cGnRH-I levels in the hypothalamus are more susceptible to negative-stimuli (Contijoch et al., 1992). Studies on ageing hens show there is a decrease in cGnRH-I in the median eminence (Contijoch et al., 1992). There is a lower cGnRH-I mRNA in food-restricted birds versus those fed ad-libitum and food-restriction reduces the release of cGnRH-I from the median eminence

(Cicccone et al., 2007; Lal et al., 1990). Studies performed by immunoneutralisation of inhibin in cockrels showed that there was accelerated puberty and hindered age-related sexual senescence in these birds (Satterlee et al., 2006). Therefore, it is feasible that inhibin has an inhibitory effect on gonadotrophin biosynthesis (Dunn I. C. et al., 2009).

However, in 2000, the discovery of a novel inhibitory hypothalamic peptide from the Japanese quail hypothalamus that belongs to the RF-amide neuropeptide family characterised by a C-terminal Arg-Phe-NH₂ motif that inhibits gonadotrophin secretion from the pituitary gland was identified, and termed Gonadotrophin Inhibitory Hormone (GnIH) (Tsutsui et al., 2000). GnIH nerve terminals are present in the external layer of the median eminence, and are therefore appropriately located to be released into the hypophyseal portal vasculature (Tsutsui et al., 2000). There is also a direct contact between GnIH fibres and GnRH neurons in the hypothalamus, which suggests that GnIH may directly influence the synthesis and release of GnRH-I (Bentley et al., 2003). In primary chicken pituitary cultures, GnIH treatment reduced LH and FSH release as well as mRNAs encoding the common alpha subunit and the FSH beta subunit, but not the LH beta subunit (Cicccone et al., 2004). Chronic treatment with GnIH may affect LH beta subunit expression in quail (Ubuka et al., 2006). GnIH mRNA is increased in incubating hens although it is not increased in out-of-lay hens (Cicccone et al., 2005). GnIH therefore may play a role in regulation of gonadotrophin secretion although more research is required to establish its role in the HPG-axis (Dunn I. C. et al., 2009).

Therefore, there is both a stimulatory and inhibitory arm in the hypothalamic control of reproduction (**Fig. 1.10**). This inhibitory component would be controlled by the activity of GnIH, whereas the stimulatory control is accomplished by both the synthesis and release of cGnRH-I. cGnRH-I synthesis and release which is dependent on external (e.g. day-length, food availability) and internal (sexual maturation, steroids, proteins, incubation, aging, GnIH) cues. However, the release

of cGnRH-I elicits its effects through cGnRH receptor isoforms. It is the activation of the cGnRH receptor isoforms that results in the relay of information from the release of cGnRH-I into the pituitary. Therefore studies of the cGnRH receptor isoforms will be beneficial in understanding the hypothalamic control of reproduction.

Figure 1. 10

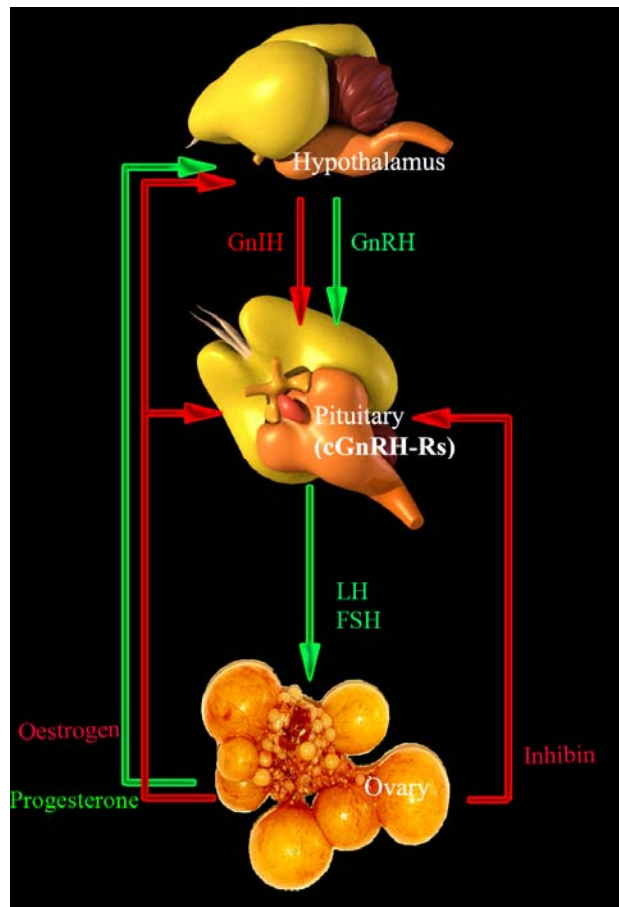


Fig. 1.10. Schematic representation of the hypothalamic-pituitary-gonadal axis (HPG axis) in the chicken showing the key components discussed in this thesis. Factors with stimulatory effects are indicated in green text and green arrows, whereas factors with inhibitory effects are indicated in red text and red arrows. There is both stimulatory (GnRH) and inhibitory (GnIH) hypothalamic control of reproduction influencing the synthesis and release of luteinising hormone (LH) and follicle stimulating hormone (FSH). The release of cGnRH-I elicits its effects through cGnRH-R isoforms which are expressed in the pituitary. It is the activation of the cGnRH receptor isoforms that results in the relay of information from the release of cGnRH-I into the pituitary and the subsequent synthesis and release of LH and FSH. Components of this figure were taken from the following website: http://www.exchange3d.com/cubecart/animals-and-plants/chicken-brain-3d-model/prod_5740.html

1.6.3. Chicken GnRH Receptor Isoforms

cGnRH-I is released from the median eminence into the hypophyseal portal vasculature to the pituitary. Pituitary gonadotrophs are the target cells for GnRH-I released from the median eminence as they express the cognate GnRH receptors (Stojilkovic et al., 1994). Although the expression of cGnRH receptors in gonadotrophs has not been confirmed by *in-situ* hybridisation, chicken pituitary glands are capable of releasing LH in response to GnRH *in-vitro* suggesting the gonadotrophs express cGnRH receptors. Interestingly, in avian species, the two gonadotrophins, FSH and LH, are not expressed in the same cell type (Proudman et al., 1999; Puebla-Osorio et al., 2002). The specific receptor types through which the cGnRH ligands elicit biological responses in the chicken are not known. Identifying the expression of cGnRH-R subtypes on LH and FSH cells would inform of their potential differential function on gonadotrophin biosynthesis. The type I chicken GnRH receptor (cGnRH-R-I) was previously identified in 2001 and found to be expressed in the chicken in a wide range of central nervous and peripheral nervous system tissues at the transcriptional level (Sun et al., 2001a; Sun et al., 2001b). The cloned cGnRH-R-I receptor encodes a 375 amino acid peptide with a signature molecular architecture characteristic of GPCRs (Sun et al., 2001b). Pharmacological studies proved cGnRH-R-I had a higher binding affinity and is more potent at stimulating inositol phosphate accumulation with GnRH-II than cGnRH-I (Sun et al., 2001b), although cGnRH-I is thought to be the hypothalamic peptide responsible for regulating the pituitary gonadotroph (Mikami et al., 1988a; Sharp et al., 1990). This suggested the existence of an alternate receptor which would have a higher binding affinity and inositol phosphate accumulation with cGnRH-I, the ligand primarily responsible for gonadotrophin synthesis. The advent of the chicken genome sequence (Ensembl 2004) made it feasible to perform sequence homology searches to identify novel cGnRH receptor isoforms. A novel cGnRH-R cDNA sequence was identified using this approach, and is the focus of this thesis.

1.7. Aims of this Thesis

The overall aim of this thesis is to gain further understanding of the control of reproduction in the chicken with particular reference to the physiological roles of the two cGnRH receptor subtypes. The studies presented in this thesis focus on comparisons of the expression, pharmacology and signalling cascades activated by the two cGnRH receptor subtypes. Specific hypotheses and aims are presented in subsequent chapters as follows:

CHAPTER 3

Hypothesis: It is hypothesised that the chicken GnRH system utilises receptor subtypes and ligands in a tissue-specific fashion.

Aims: To clone the cDNA encoding the novel isoform of the cGnRH-R from a broiler chicken (commercial meat-type chicken) and to compare it with the type I cGnRH receptor. Comparisons of functionally important amino acid residues and sequence micro-domains can be made using amino acid sequence alignment of the two cGnRH receptors and phylogenetic classification can be performed. The use of real-time RT-PCR will enable the quantification of cGnRH receptor mRNA expression in various tissues; in addition to the changes in expression of the two receptors in birds of varying reproductive stages can be determined. This will be supported by determining the number of different transcripts arising from the novel cGnRH receptor isoform by Northern blot analysis and determining the cellular localisation of the cGnRH receptor isoform expression in the pituitary gland by *in-situ* hybridisation

CHAPTER 4

Hypothesis: It is hypothesised that since cGnRH-R-III mRNA is predominantly expressed in the pituitary (**Chapter 3**) it may be more functionally “selective” for the GnRH ligand that primarily regulates the pituitary gonadotroph function.

Aims: The aims of this study were to compare and further characterise the pharmacological properties of the novel isoform of cGnRH receptor with the type I

cGnRH receptor. This includes analysis of ligand-binding and inositol phosphate production in response to the endogenous ligands, cGnRH-I and GnRH-II, which may elucidate the physiological roles of the receptor subtypes. To identify a GnRH analog that differentially binds and activates one of the chicken GnRH receptor homologs which would facilitate *in-vivo* and *in-vitro* studies to allow delineation of the role of each cGnRH-R subtype in terms of gonadotrophin synthesis and release. To evaluate the ability of the splice variants of the novel cGnRH receptor to bind to cGnRH ligands and determine their effects on full-length receptor function. Additionally, to perform a comparison of the ligand-induced internalisation of both cGnRH receptors

Chapter 5

Hypothesis: It is hypothesised that since three Src homology 3 (SH3) binding motifs were identified in the novel cGnRH receptor (and not the type I cGnRH-R) (**Chapter 3**) it may differentially target signalling pathways through subtype-specific interactions with SH3 domain-containing proteins to differentially activate the MAPK cascade when compared to the type I cGnRH-R subtype.

Aims: In an attempt to elucidate the physiological roles of the receptor subtypes and attribute a differential functional capacity to the two receptor subtypes, the ability of the two receptors to differentially activate the MAPK signalling cascade was investigated. This included comparative dose and time-dependent analysis of pERK 1/2 activation via the two chicken GnRH receptor subtypes. To identify whether the SH3 binding motifs identified exclusively in the novel type III cGnRH receptor attribute a differential capacity in terms of pERK 1/2 activation. To examine the protein complexes directly interacting with the two chicken GnRH receptor subtypes using immuno-precipitation and western blotting.

Chapter 2.

Materials and Methods

2.1. Introduction

This chapter details the methods and laboratory techniques used throughout this thesis, and the procedures used in the experiments. In addition explanations are given of the technicalities of procedures where necessary. All materials used and suppliers information is provided. All work not performed by myself has been acknowledged. Where RNase free H₂O is used, it applies to H₂O treated with 0.1% Diethylpyrocarbonate (DEPC) overnight then autoclaved.

2.2. Animal husbandry and welfare

Birds were sacrificed by cervical dislocation or barbiturate overdose in accordance with the UK Home Office guidelines for animal welfare. Tissue samples were collected according to the experimental purpose.

2.3. Phylogenetic Tree Construction

Phylogenetic tree construction of avian, reptilian and amphibian GnRH receptors amino acid sequences was performed using the MEGA4 program using the default settings (Tamura et al., 2007). The evolutionary history was inferred using the neighbour-joining method (Saitou and Nei, 1987). The neighbour-joining method of phylogenetic tree construction uses distance measures to correct for multiple hits at the same sites, and chooses a topology showing the smallest value of the sum of all branches as an estimate of the correct tree (Saitou and Nei, 1987). Simplistically, it uses an iterative algorithm, which is based on a topology that gives the least total branch length at each step of the algorithm (Gascuel and Steel, 2006). The reliability of the inferred tree was estimated by applying a bootstrap test, which is evaluated using a bootstrap resampling technique (Felsenstein, 1985). Bootstrapping analysis gives a way to judge the strength of support for nodes on phylogenetic trees (Felsenstein, 1985). A number is presented by each node, which reflects the

percentage of bootstrap trees which also resolve that clade. If a bootstrap value for a give interior branch is 95% or higher, then the topology at that branch is considered reliably correct (Efron et al., 1996).

2.4. Ribonucleic Acid (RNA) extraction from neuroendocrine tissues and non-neuroendocrine tissues

2.4.1. Collection of neuroendocrine and non-neuroendocrine samples for RNA extraction

The tissues were rapidly dissected with sterile dissection instruments, weighed and placed in RNAase-free eppendorfs which were snap frozen in liquid nitrogen and stored at -80°C.

2.4.2. Trizol Extraction method

The tissue was placed in matrix D tubes (Q-biogene-Alexis, Nottingham, UK) containing 600µl of Trizol (Invitrogen, Paisley, UK) which is a mono-phasic solution of phenol and guanidine isothiocyanate. Homogenisation was performed to disrupt the tissue architecture and release the RNA with a FastPrep FP120 homogeniser (Q-biogene-Alexis, Nottingham, UK) using two 20-second pulses at speed 4.5. 100µl of chloroform was added, the solution was then vortexed, incubated on ice for 15 minutes and then vortexed again to ensure lysis of cells. The solution was centrifuged at 18630xg (g centrifugal force) for 15 minutes to separate the RNA from tissue debris, DNA and proteins. The upper aqueous layer which contains the RNA was transferred into an eppendorf containing 2µl (40µg) of molecular biology grade glycogen (Sigma-Aldrich, Dorset, UK). An equal volume of isopropanol was added to the retrieved supernatant to precipitate the RNA. The solution was vortexed vigorously, incubated on ice for 15 minutes and vortexed once more to ensure mixing and precipitation of all RNA molecules. Precipitated RNA was isolated by centrifugation of the tube at 18630xg for 15 minutes and the supernatant was

removed immediately and the RNA pellet was washed twice with 1000µl of 70% EtOH. After each wash the solution was centrifuged at 9505xg for 8 minutes. All residual EtOH was removed from the tube, and the RNA pellet was air dried in a desiccator for approximately 45 minutes. The transparent pellet was dissolved in 100µl of RNase free H₂O and stored at -80°C.

2.4.3. Ultraspec II extraction method on small and large tissue samples

The tissue was placed in matrix D tubes containing 1000µl of Ultraspec II (Biotecx, Abington, UK). Homogenisation was performed to disrupt the tissue architecture and release the RNA with a FastPrep FP120 homogeniser using two 20-second pulses at speed 4.5. After a 5 minute incubation on ice to allow the complete dissociation of nucleoprotein complexes, 200µl of chloroform was added to separate the aqueous and organic phases. The solution was then vortexed, incubated on ice for 15 minutes and then vortexed again to ensure isolation of RNA molecules. The solution was centrifuged at 18630xg for 15 minutes to separate the RNA from tissue debris, DNA and proteins. 400µl (small tissue/neuroendocrine) or 500 µl (large tissue/non-neuroendocrine) of the clear upper aqueous layer supernatant which contains the RNA was transferred into an eppendorf prepared with 200µl (small tissue) or 250µl (large tissue) of isopropanol and 20µl (small tissue) or 25µl (large tissue) of RNA TackTM Resin (Biotecx, Abington, UK) and vortexed thoroughly. The precipitated total RNA attached to the RNA Tack Resin was then separated from the solution by centrifugation of the tube at 18630xg for 5 minutes. The supernatant was removed immediately and the RNA pellet was washed twice with 1000µl of 70% EtOH. After each wash the solution was centrifuged at 16060xg for 8 minutes. All residual EtOH was removed from the tube, and the total RNA attached to the RNA TackTM Resin was air dried in a desiccator for approximately 45 minutes. The RNA TackTM Resin was re-suspended in 30µl of RNase free H₂O and stored at -80°C.

2.4.4. RNA quantification and analysis of integrity

The quality and the quantity of the RNA extracted can be analysed by measuring the optical density (OD) at 260nm and 280nm of a 1:50 dilution of the sample. The ratio of the OD₂₆₀/OD₂₈₀ is an indication of the purity of the RNA and can be compared with pure RNA (ratio =2.1).

$$\text{Ratio} = (\text{OD}_{260} / \text{OD}_{280})$$

$$\text{Quantity} = (\text{OD}_{260} \times 40 \times 50) / 1000 = \text{total RNA } \mu\text{g} \cdot \mu\text{l}^{-1}$$

An aliquot of the dissolved RNA from each sample was analysed by agarose gel electrophoresis (non-denaturing agarose gel) on an agarose gel to check the RNA integrity. Two bands corresponding to the two ribosomal subunits (28s and 18s) within each total RNA sample should be visible. Prior to running the gel, all equipment was cleaned with RNase-Zap® (Ambion, Warrington, UK) and thoroughly rinsed with RNAase free H₂O. The samples were prepared by heating the samples to 65°C for ten minutes to alleviate the RNA secondary structure and run immediately.

2.5. Reverse transcription of total RNA to single stranded cDNA

Reverse transcription of total RNA was done using a First Strand synthesis kit (GE Healthcare, Buckinghamshire, UK) to synthesise single stranded complementary deoxyribonucleic acid (cDNA). 5µg of total RNA made up to 8µl by adding the appropriate amount of RNase free H₂O. The total RNA was then heated to 65°C for 10 minutes to alleviate RNA secondary structure allowing more efficient priming and cDNA synthesis. The RNA was chilled on ice, as rapid cooling prevents the formation of secondary structure. The remaining components listed below were added for first strand cDNA synthesis to give a 15µl total volume reaction mix.

Bulk reaction mix.....	5µl
200mM Dithiothreitol (DTT).....	1µl
0.2 µg/µl Not I-d(T) ₁₈ bifunctional primer	1µl

The reaction mix was then incubated at 37°C for 1hour. A final incubation at 90°C for 5 minutes was performed to denature the reverse transcriptase enzyme. Samples of cDNA were stored at -20°C for long term storage.

2.6. Chicken GnRH-R-III cDNA amplification using PCR in samples from male and female, juvenile and sexually mature birds

2.6.1. Tissue distribution of cGnRH-R-III mRNA expression

Forward (f) and reverse (r) primers which are complimentary to the 3' ends of each of the sense and anti-sense strand of the target DNA were designed to span exon boundaries. cGnRH-R-III-713-735f (exon 2-3) (GCT CGC TGT GCC GCA GCT GT) and cGnRH-R-III-933-952r (exon 3-4) (ACG TCC CTG GAG GAG AAG AG) were used to amplify cGnRH-R-III cDNA from anterior pituitary, hind brain, cerebrum, cerebellum, median eminence, anterior hypothalamus, posterior hypothalamus, olfactory bulb, optic lobe, adrenal gland, kidney, small intestine, spleen, testes, ovary and liver cDNA samples of the pre-pubertal males and females (initial denaturation step, 95°C for 4minutes; 40cycles of 95°C/30s, 62°C/30s, 72°C/45s and a final extension 72°C/7minutes). The PCR products were visualised using agarose gel electrophoresis.

2.7. mRNA Quantification using Real-time PCR

2.7.1. cGnRH-R-I and cGnRH-R-III mRNA Expression using Real-time PCR

Total RNA was prepared as described in methods section (2.4). cDNA synthesis was performed using a standardised quantity of total RNA (5µg), using a First Strand synthesis kit as described in methods section (2.5). Real-time PCR was used to quantify the mRNA expression of cGnRH-R-I and cGnRH-R-III in the pituitary

gland, median eminence, small intestine and gonads using the method based on the fluorescence of SYBR[®] Green I dye (Invitrogen, Paisley, UK) with the recommended amplification conditions. Primers that spanned the exon boundaries; cGnRH-R-III-713-735f (GCT CGC TGT GCC GCA GCT GT) and cGnRH-R-III-933-952r (ACG TCC CTG GAG GAG AAG AG) for cGnRH-R-III mRNA amplification and primers cGnRH-R-I-701-722f (AGC TAA AGA TCA ACA AAA GTC T) and cGnRH-R-I-1100-1082r (TTG GAG CCA CCT GAT GTC A) for cGnRH-R-I amplification were used. Standard curves for amplification of each GnRH receptor homolog cDNA were made with the cGnRH-R-III cDNA in Bluescript II SK⁽⁺⁾ vector (described in 2.10.2), and a cGnRH-R-I cDNA in Bluescript II SK⁽⁺⁾ vector (Agilent Technologies, Cheshire, UK) (containing 488 bp inserted into the *EcoRV/NotI* site of the vector, that corresponds to bases 1-421 of AJ304414 and 1-68 of AJ506779). A linear regression analysis of serially diluted quantities of cDNA template versus cycle threshold values was plotted, allowing the calculation of the quantity of target nucleic acid from the test-sample cycle threshold values. PCR reaction with either template reached plateau phase following the same number of amplification cycles, indicating similar amplification efficiencies.

2.8. Northern Blot Probe Synthesis

2.8.1. Oligonucleotide Probes

5' end labelling using [γ -³²P]-dATP and T4 polynucleotide kinase (PNK) was performed with 20pmol 73-120-cGnRH-R-III_sense (TGG TGC CGA TGT GGG AGC GCT GGG AGC AGT GAG TGG GCG GTG TTT GA) and 73-120-cGnRH-R-III_anti-sense (TCA AAC ACC GCC CAC TCA CTG CTC CCA GCG CTC CCA CAT CGG CAC CA) oligonucleotides using the mirVana Probe and Marker Kit (Ambion, Warrington, UK). The probes were subsequently purified with the components included in the kit. These oligonucleotides, designed in the 5' region

upstream of the translation start site in exon 1 are specific for cGnRH-R-III and are not homologous to cGnRH-R-I.

2.8.2. DNA Probes

A chicken actin alpha 1 skeletal muscle (ACTA1) (Accession Number 001031063) DNA probe was synthesised from 0.25µg of purified insert DNA by random primer labelling with ³²P-dCTP using the Rediprime Kit (Random primer labelling) (GE Healthcare, Buckinghamshire, UK) and purified using Sephadex G50 Nick columns (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's recommendations. The probe was to check for equal loading of sample RNAs in Northern blotting.

2.8.3. RNA probes

A RNA probe was synthesized by *in-vitro* transcription of 1µg of cGnRH-R-III_1210f-3217r-pBSK⁽⁺⁾ using the T7 promoter linearized with *EcoRI*. The fragment was gel purified as above. Transcription was performed using a T7 transcription kit (Promega, Hampshire, UK) according to the manufacturer's instructions using [³²P] UTP (800 Ci/mmol), (GE Healthcare, Buckinghamshire, UK) Free nucleotides were removed using Sephadex G50 Nick columns according to the manufacturer's recommendations.

2.9. Northern blotting

2.9.1. Northern blotting using [γ -³²P] labelled oligonucleotide probes to detect cGnRH-R-III expression

Total RNA was isolated using Ultraspec II according to the manufacturer's instructions as described above (2.4). Poly (A)⁺ RNA was purified from anterior pituitary gland, small intestine and testes total RNA pools using Oligotex mRNA Spin-Columns (Qiagen, Crawley, UK). 3µg of Poly (A)⁺ RNA was loaded on a

denaturing 1% agarose-formaldehyde gel and electrophoresed for 2.5 hours at 100 volts. Transfer of the RNA to a Hybond-N nylon membrane, (GE Healthcare, Buckinghamshire, UK) was performed by capillary action overnight in 10 x saline-sodium citrate (SSC) buffer. The RNA was fixed by baking the membrane for 2 hours at 80°C. Pre-hybridisation and hybridisation of the membrane was carried out using ULTRAHyb-Oligo hybridisation buffer (Ambion, Warrington, UK) according to manufacturer's instructions overnight at 42°C using radiolabelled oligonucleotide probes (1×10^6 cpm probe/ml hybridisation buffer). Post-hybridisation stringency washes were 2x15 minute in 0.1% SSC, 0.1% sodium dodecyl sulphate (SDS) at 70°C. The membrane was exposed to Kodak BioMax MA autoradiographic film (Sigma-Aldrich, Dorset, UK) for 2 days at -80°C prior to development.

2.9.2. Northern analysis using [α - 32 P] labelled RNA probe to detect cGnRH-R-III expression

Alternatively, total RNA was prepared from muscle, brain cortex and pituitary gland tissues and 15 μ g was subsequently prepared for Northern blotting according to the method of Pelle and Murphy (1993). The gel was washed in 50mM NaOH, 10mM NaCl for 45 minutes and the RNA was transferred to Hybond-XL membrane, (GE Healthcare, Buckinghamshire, UK) using capillary transfer overnight with 20 x SSC. After washing in 2 x SSC the RNA was cross-linked to the filter using Ultraviolet light in a StratalinkerTM (Agilent Technologies, Cheshire, UK) set at 120 millijoules. Hybridization and pre-hybridisation was carried out using ULTRAHyb solution (Ambion, Warrington, UK) according to the manufacturer's instructions at 68°C. The cGnRH-R-III_1210f-3217r-pBSK⁽⁺⁾ anti-sense RNA probe was added at a concentration of 1×10^6 cpm/ml and hybridization was performed overnight at 68 °C. The membrane was then rinsed in 2 x SSC, 0.1% SDS twice, each for 5 minutes, followed by a series of more stringent washes to a final wash of 0.01 x SSC, 0.1% SDS (2 x 30 min) at 90°C. The membrane was exposed overnight to a phosphor-imaging screen and digital images were captured using a Molecular Imager FX (Bio-Rad, Hertfordshire, UK).

2.10. Cloning of chicken GnRH receptor isoform cDNA sequences

2.10.1. Cloning the cGnRH-R-I cDNA into pcDNA I/Amp vector

The expression construct containing cGnRH-R-I gene cloned into pcDNA1/Amp (Invitrogen, Paisley, UK) mammalian expression vector was used as previously reported (Sun et al., 2001b). The construct was recovered from a glycerol stock (Bob Millar Lab / MRC, HRSU Edinburgh), by growing the colony for plasmid DNA purification using the QIAprep Miniprep Kit (Qiagen, Crawley, UK) according to the manufacturer's protocols. Once the integrity of the insert DNA was verified, the selected clone was grown for plasmid DNA purification using the HiSpeed Plasmid Maxi Kit (Qiagen, Crawley, UK) according to the manufacturer's instructions.

2.10.2. Cloning the cGnRH-R-III cDNA into pBluescript II SK(+) vector

Trizol RNA extraction method as described in methods (2.4.2) was applied to isolate total RNA from pooled mature male and female pituitary glands. cDNA synthesis was performed on 5µg of total RNA using the First Strand synthesis kit as described in methods section (2.5). NCBI Genbank sequence AY895154 was used as a reference sequence for design of cGnRH-R-III cDNA primers. Primers cGnRH-R-III-1210f (TGA GTG GGC GGT GTT TGA) and cGnRH-R-III-3217r (TAG AGG CAT TGT GGA GCA GA) flanking the entire coding region of the receptor were used to amplify pituitary cDNA using standard PCR conditions (initial denaturation step of 95°C/5minutes, 35 cycles of denaturation 95°C/30s, annealing 61.9°C/30s and elongation 72°C/90s and a final extension 72°C/7minutes). Once amplification of the desired 1386bp PCR product was achieved, blunt ending (filling in recessed 3'ends of DNA fragments) of the PCR product was performed with the addition of 10% by volume of DNA polymerase I (Klenow fragment) (Promega, Hampshire, UK) and subsequent incubation at 37°C for 15 minutes prior to deactivating the enzyme at 75°C for 5 minutes. The residual dNTPs from the PCR provided the necessary nucleotides for the blunt ending. The blunt-ended PCR product band was then electrophoresed on a 2% agarose gel and excised. The DNA was extracted from

the gel using the GeneClean® Spin Kit (Q-Biogene, Nottingham, UK), according to the manufacturer's recommendations. 200ng of a pBluescript II SK⁽⁺⁾ vector (PBSK⁺) was linearised at the *EcoRV* site of the multiple cloning site (MCS) in a final volume of 10µl. A final dilution of the reaction mix by the addition of 30µl H₂O was performed to give a final plasmid concentration of 5ng.µl⁻¹. An additional dephosphorylation step on the linearised plasmid prior to ligation was performed, which reduces the chances of the plasmid re-circularising, by removal of the 5'-terminal phosphates. The procedure makes use of shrimp alkaline phosphatase (Roche Applied Science, West Sussex, UK) which is easily denatured after use by heat treatment at 65°C. A rapid DNA ligation kit (Roche Applied Science, West Sussex, UK) was used to insert the DNA into the vector. The components listed below were added to give a 20µl total volume reaction mix.

~0.4 µg.µl ⁻¹ purified PCR product.....	7 µl
Linearised 5ng.µl ⁻¹ pBSK ⁺ vector.....	1 µl
5x DNA dilution buffer.....	1 µl
T4 DNA ligase.....	1 µl
2x T4 DNA ligation buffer.....	10 µl

The reaction mix was allowed to incubate at room temperature for 5 minutes. An additional *EcoRV* digest was performed after ligation to eliminate any plasmid that had re-circularised without insert. The tubes were then chilled on ice prior to transformation. The ligation reaction was transformed into XL1 Blue *E. coli* competent cells (Agilent Technologies, Cheshire, UK). The ligation reaction was added to a 50µl aliquot of the competent cells and incubated on ice for 30 minutes. The cells were heat shocked for 45 seconds at 45°C and immediately cooled on ice for 60 seconds. 700µl of room temperature SOC was added to each sample and the cells were shaken at 250 rpm at 37°C for 45 minutes. The cells were then plated onto pre-warmed agar plates and incubated at 37°C for 16 hours. Colonies were picked after 16 hours and grown for plasmid DNA purification using the QIAprep Miniprep Kit according to the manufacturer's instructions. Once the integrity of the insert DNA was verified, the selected clone was grown for plasmid DNA purification using the HiSpeed Plasmid Maxi Kit according to the manufacturer's instructions.

2.10.3. Cloning the cGnRH-R-III cDNA into pcDNA 3.1/myc-His-A vector

The cGnRH-R-III fragment between the *Bam*HI and *Hinc*II restriction sites of pBSK⁽⁺⁾ vector cloned above was removed by restriction digest and subcloned into a mammalian expression vector pCDNA3.1/myc-His-A (Invitrogen, Paisley, UK) between the *Bam*HI and *Eco*RV sites of the MCS. The rapid DNA ligation kit was used as described in 2.10.2, to clone the DNA sequence. The ligation reaction was transformed into XL1 Blue *E. coli* competent cells as in 2.10.2. Colonies were picked after 16 hours and grown for plasmid DNA purification using the QIAprep Miniprep Kit according to the manufacturer's instructions. Once the integrity of the insert DNA was verified, the selected clone was grown for plasmid DNA purification using the HiSpeed Plasmid Maxi Kit according to the manufacturer's instructions.

2.11. Verification of the sequence of cloned chicken GnRH Receptor cDNAs

2.11.1. cGnRH-R-I cDNA in pcDNA I/Amp vector

The sequence of the cGnRH-R-I cDNA inserted into pcDNA I/Amp vector was verified by *Hinc*II digest.

2.11.2. cGnRH-R-III cDNA in pBluescript II SK(+) vector

The insert was amplified with primers BSKPF (CGA TTA AGT TGG GTA ACG C) and BSKPR (CAA TTT CAC ACA GGA AAC AG) using the plasmid DNA from a single bacterial colony as a template, with the following conditions (inactivation step, 95°C for 5minutes; 30cycles of 95°C/15s, 56°C/20s, 72°C/45s and a final extension 72°C/2minutes). The entire amplicon containing the insert was analysed by sequencing with M13-forward and M13-reverse infrared dye (IRD)-labelled primers. Additionally, the sequence of the cGnRH-R-III cDNA inserted into pBSK⁽⁺⁾ vector was verified by *Bam*HI, *Hind*III, *Hinc*II, *Ava*I and *Pst*I restriction digest.

2.11.3. cGnRH-R-III cDNA in pcDNA 3.1/myc-His-A vector

The clone was amplified with primers pcDNA3.1/myc-His_826f (TAA CTA GAG AAC CCA CTG CTT ACT), and pcDNA3.1/myc-His_1060r (AAT GGT GAT GGT GAT GAT GA), and subsequently sequenced with IRD-labelled primer T7-prom-IRD-700 (TAA TAC GAC TCA CTA TAG GG), to further validate the GnRH receptor expression construct. Additionally, the sequence of the cGnRH-R inserted into pcDNA3.1/myc-His was verified by *BamHI*, *BsmI*, *PstI* and *HincII* restriction digest.

2.12. *In-situ* Hybridisation Probe synthesis

2.12.1. Synthesis of Dioxygenin (DIG)-labelled RNA probes using T7 and T3 RNA polymerase

The cGnRH-R-III cDNA in pBSK⁽⁺⁾ vector was linearised with either *BamHI* (antisense / T7 promoter) or *HindIII* (sense / T3 promoter) restriction endonucleases. 1µg of the plasmid was used to synthesis RNA run-off transcripts from the T3 or T7 promoter using RNase polymerase, by adding the following solutions and adjusting the total reaction volume to 20µl with RNase free H₂O, prior to incubating the reaction for four hours at 37°C.

5 x Transcription buffer	4 µl
DTT	2 µl
RNA labelling mix –DIG	2 µl
RNase inhibitor	0.5 µl
RNA polymerase.....	1 µl

A probe derived from pro-opio-melanocortin (POMC) cDNA was used as a positive control for experimentation. The POMC plasmid was designed to reference sequence NM001031098. Hypothalamic cDNA was amplified with POM3-f (ATG CTG GGA GAA CAG CAA GTG CC) and POM5-r (GTT CCA ACC CTT CTC CAT CTT C) and cloned into pBSK⁽⁺⁾ vector. The POMC cDNA in pBSK⁽⁺⁾ vector

was individually linearised with either *EcoRI* (antisense / T7 promoter) or *HindIII* (sense / T3 promoter). 1µg of the plasmid was used to synthesis RNA run-off transcripts from the T3 or T7 promoter using RNase polymerase as above. The probes were then purified using Probe Quant G-50 Micro Columns (GE Healthcare, Buckinghamshire, UK) according to the manufacturer's instructions. The quality and the quantity of the RNA synthesised was analysed by measuring the optical density (OD) at 260nm and 280nm and by electrophoresis on a 2% agarose gel.

2.12.2. Synthesis of [³⁵S] dATP(alphaS) labelled oligonucleotide probes using 3' End labelling with Terminal Transferase

3' end labelling using [³⁵S]dATP(alphaS) (GE Healthcare, Buckinghamshire, UK) and Terminal Transferase, was performed with 10pmol cGnRH-R-III_sense-cocktail (73-120-cGnRH-R-III_sense (TGG TGC CGA TGT GGG AGC GCT GGG AGC AGT GAG TGG GCG GTG TTT GA) and 1261-1290-cGnRH-R-III_sense (CGG GTT CCT TCC ACT GTT CGG CCT CAT CCC)) and, cGnRH-R-III_antisense-cocktail (73-120-cGnRH-R-III_anti-sense (TCA AAC ACC GCC CAC TCA CTG CTC CCA GCG CTC CCA CAT CGG CAC CA) and 1261-1290-cGnRH-R-III_anti-sense (GGG ATG AGG CCG AAC AGT GGA AGG AAC CCG)) oligonucleotides using the Terminal Transferase recombinant DNA labelling kit (Roche-Applied Sciences, West Sussex, UK) according to the manufacturers instructions, with the exception of the incubation time being prolonged by 30 minutes. These oligonucleotides, designed in the 5' region upstream of the translation start site in exon 1 are specific for cGnRH-R-III and are not homologous to cGnRH-R-I. The labelled oligoprobes were then purified using the QIAquick nucleotide removal kit (Qiagen, Crawley, UK) according to the manufacturer's instructions. The POMC probes were used as positive controls for each experiment and were labelled according to the method described for cGnRH-R-III oligoprobes. The quantification of the incorporation of the radioactivity in 2µl of the oligoprobe was measured in 4ml of scintillation fluid on a beta-counter. A count greater than 50000dpm per 2µl of labelled oligoprobe was

acceptable for subsequent hybridisations. Additionally, polyacrylamide gel electrophoresis was performed to check the incorporation of the radioactivity by running the oligonucleotides on a 50cm 6% acrylamide gel at a constant current of 80W. The gel was then dried and exposed to a phosphoscreen and analysed with a phosphoimager (Biorad, Hertfordshire, UK). The sample was stored at -20°C or used immediately.

2.13. In-situ Hybridisation to detect cGnRH-R-III mRNA expression

2.13.1. Whole-mount in-situ hybridisation using Digoxigenin-labelled RNA probes

Collection of neuroendocrine tissue samples for *in-situ* hybridisation

The tissues were rapidly dissected, with sterile RNase free instruments. The tissues were immediately immersed in sterile 1x phosphate buffered saline (PBS) (pH 7.4) and either used immediately or frozen on dry ice or alternatively in -20°C 100% hexane placed on dry ice and chilled to -65°C. The tissue samples were stored wrapped in a container with hexane solution and stored at -80°C till required.

Fixation and dehydration of neuroendocrine tissue samples

The tissue samples were placed in a 4% paraformaldehyde (PFA)/PBS (pH 7.4) and maintained at 4°C overnight. The samples were then subjected to the following dehydration stages at room temperature, series of 10 minute dehydration washes twice in 1 x PBT buffer (PBS and 0.1% Tween 20), 25% MeOH/PBT, 50% MeOH/PBT, 75% MeOH/PBT and finally twice in 100% MeOH. The samples were then stored at -20°C or immediately subjected to tissue permeabilisation.

Tissue Permeabilisation

The tissues were transferred to 4°C MeOH and DMSO (dimethyl sulphoxide) (1:1) solution, and closely monitored. Once the tissue drops, immediately add 25% by

volume of 10 x Triton-X-100 (Sigma-Aldrich, Dorset, UK) for the appropriate times (10 minutes for pituitary samples, 30 minutes for whole brain samples). The samples were then subjected to 3 x 10 minute washes in PBT.

Pre-hybridisation and Hybridisation

The samples were placed in pre-hybridisation solution (50% formamide, 5 x SSC, 2% Blocking Reagent (<2% maleic acid) (Roche Applied Science, West Sussex, UK), 0.1% Triton-X-100, 0.1% CHAPS (VWR, Leicestershire, UK), 20µg/ml tRNA (Boehringer, Berkshire, UK), 5mM EDTA, 50µg/ml Heparin (Sigma-Aldrich, Dorset, UK) overnight at 65°C, and then hybridised for 2-3 days in hybridisation solution (50% formamide, 5 x SSC, 2% Blocking Reagent (Roche Applied Science, West Sussex, UK), 0.1% Triton X, 0.1% CHAPS, 20µg/ml tRNA, 5mM EDTA, 50µg/ml Heparin with a minimum of 25% of RNA in 2ml of hybridisation buffer.

Immuno-detection with Anti-DIG antibody

The samples were washed following hybridisation. The following series of washes were performed, two washes in 2 x SSC each for 10 minutes at 70°C, three washes in 2 x SSC/0.1% CHAPS each for 20 minutes at 70°C, three washes in 0.2 x SSC/0.1% CHAPS each for 20 minutes at 70°C and two final 10 minute washes in KTBT (50mM Tris-HCL pH7.5, 150mM NaCl, 10mM KCl, 1% TritonX-100) at room temperature. The sample was blocked in 20% heat inactivated FCS/KTBT for 3 hours at 4°C and then incubated in a 1:1000 dilution of mouse anti-DIG-AP Fab fragment (Boehringer, Berkshire, UK) in 20% heat inactivated FCS/KTBT overnight at 4°C. The sample was then subjected to five or more 1 hour washes in KTBT at room temperature and left at 4°C overnight in KTBT.

Colour detection

The sample was washed twice each for 15 minutes in freshly made NTMT (100mM Tris-HCl pH9.5, 50mM MgCl, 100mM NaCl, 0.1% TritonX-100) at room temperature. In the dark, add 3.5µl/ml nitro-blue tetrazolium chloride (NBT) (Sigma-Aldrich, Dorset, UK) and 3.5µl/ml 5-bromo-4-chloro-3-indolyl-phosphate

(BCIP) (Sigma-Aldrich, Dorset, UK) in NTMT. The colour development was terminated in 4% formal saline (10% formalin neutral buffered in PBS) once sufficient staining was observed. If a deeper blue staining was required, the sample was incubated NTET (1ml, 5M NaCl, 1ml, 0.5 EDTA pH8, 5ml 1M Tris-HCl pH8, 0.5ml Tween20, 42.5ml dH₂O) at 4°C for several days if necessary. Once the desired staining was achieved, the sample was fixed in 4% formaldehyde/PBS.

Gelatin Embedding and Cutting Sections

The sample was transferred to 5% sucrose, PBS until the specimen sank. The specimen was then transferred to 20% sucrose, PBS at 4°C and stored overnight. The tissue samples were pre-warmed to 38°C in 20% sucrose, PBS and then delicately placed in 38°C molten gelatine sucrose mixture (7.5% gelatin in 15% sucrose, PBS) and maintained at 38°C to infiltrate, until they sunk. The sample was then mounted in the correct orientation for sectioning and allowed to cool at room temperature in a mould. The blocks were either stored for up to 5 days at 4°C or used immediately. The trimmed gelatin block was then mounted on a chuck and frozen in a small volume of -20°C isopentane which was placed on solid CO₂ (dry ice) and chilled to -80°C. The tissue was sectioned with a cryostat at 30µm thickness and mounted onto Superfrost plus slides (Menzel-Glaser, Braunschweig, Germany).

2.13.2. *In-situ* hybridisation using DIG-labelled RNA probes on tissue sections

Collection and sectioning of neuroendocrine samples for *in-situ* hybridisation

The tissues were rapidly dissected, with RNase free instruments. The tissues were immediately immersed in sterile PBS and either used immediately or frozen on dry ice or alternatively in -20°C hexane placed on dry ice and chilled to -65°C. The tissue samples were stored wrapped in paper tissue in a container with hexane and stored at -80°C till required. The tissue was sectioned with a cryostat at 25µm

thickness and mounted onto Superfrost slides (Menzel-Glaser, Braunschweig, Germany) and stored at -80°C.

Fixation and Hybridisation

The sections mounted on the slides were fixed with 4% PFA/PBS for 10 minutes prior to hybridisation. The slides were then subjected to 2 x 5 minute washes in sterile PBS. The probe was diluted in hybridisation buffer to 1µg/ml. 100µl of the probe mix was added to each slide, and then covered with a coverslip and hybridised overnight at 65°C in a sealed perspex box humidified with Whatman paper (VWR, Leicestershire, UK) saturated with 1x NaCl/50% formamide.

Post-hybridisation washes

The slides were transferred into slide racks and subjected to 1 x 15 minute and 2 x 30 minute washes in washing solution (1 x SSC, 50% formamide, 0.1% Tween-20) pre-warmed to 65°C. The slides were then subjected to 2 x 30 minute washes in MABT (100mM Malic acid, 150mM NaCl, 0.1% Tween 20) at room temperature.

Blocking and antibody staining

The sections were blocked with blocking buffer (MABT + 2% blocking reagent + 20% heat inactivated sheep serum) for 60 minutes at room temperature. 100µl per slide of a 1/2000 dilution of the anti-DIG antibody (Boehringer, Berkshire, UK) in blocking buffer was added to the slides and incubated at room temperature overnight in a humidified chamber.

Post antibody washes and staining reaction

The excess antibody was washed off at room temperature by subjecting the slides to 5 washes in MABT, each for 20 minutes. The sections were then washed twice in staining buffer (without NBT and BCIP) each for 10 minutes. The sections were then stained in staining buffer in the dark until adequately stained. Once the required staining was achieved, the reaction was stopped by subjecting the sections to 2 x PTw (PBS, 0.1% Tween-20) + EDTA washes. The samples were then subjected to

dehydration washes; (twice in 1 x PBT buffer (PBS and 0.1% Tween 20), 25% EtOH/PBT, 50% EtOH/PBT, 75% EtOH/PBT and finally twice in 100% EtOH. The slides were immersed briefly into HistoClear (VWR, Leicestershire, UK) twice and mounted using DPX (VWR, Leicestershire, UK) for visualisation.

2.13.3. *In-situ* hybridisation protocol using [³⁵S] dATP(alphaS) labelled oligonucleotide probes on tissue sections

Collection and sectioning of neuroendocrine samples for *in-situ* hybridisation

The tissues were rapidly dissected, with RNAase-free instruments. The tissues were immediately frozen on dry ice or alternatively frozen in -20°C hexane placed on dry ice and chilled to -65°C. The tissue samples were stored wrapped in paper tissue in a container with hexane and stored at -80°C till sectioning. The tissue was sectioned with a cryostat at 15µm thickness and mounted on superfrost slides which were immediately placed on dry ice and transferred to -80°C for storage or used immediately. The slides were removed from the freezer and dried in a stream of air from a fan to remove the frost and H₂O prior to fixation.

Fixation and dehydration of neuroendocrine samples

The slides were then subjected to the following fixation and dehydration stages at room temperature; 4% PFA for 5 minutes, twice in 1 x PBS buffer for 5min, RNAse free H₂O for 10 seconds, 0.1M triethanolamine (TEA) + Acetic anhydride (AA) (25mls AA to 100mls TEA) for 10 minutes, 2 x SSC for 3 minutes, 70% EtOH for 3 minutes, 95% EtOH for 3 minutes, 100% EtOH for 3 minutes, chloroform for 3 minutes, 100% EtOH for 3 minutes and 95% EtOH for 3 minutes. The slides were then blown dry in a stream of air from a fan or air dried. The slides were either stored in appropriate slide storage boxes at room temperature at this stage, as they remain stable for a number of months or used immediately for hybridisation with the oligoprobes.

Hybridisation of radiolabelled oligonucleotide probes

The following hybridisation reaction mix per slide was made immediately prior to hybridisation.

Hybridisation cocktail	90 μ l
Yeast tRNA10mg/ml	1.0 μ l
Denhardt's x100 (Sigma-Aldrich)	1.25 μ l
Oligonucleotide Probe.....	3.75 μ l
5M DTT	4.0 μ l

The hybridisation reaction mix was then placed onto the sections, and covered with parafilm. The hybridisation was performed at 42°C and 52°C overnight, in a humidified chamber (50% formamide + 50% 1 x SSC). The control hybridisation was performed using a 10 x higher concentration of unlabelled probe in hybridisation buffer. After the overnight hybridisation, the slides were washed with 1 x SSC at 56°C four times, each for 45 minutes. The slides were air dried or step alcohol dehydrated (dip in RNase free H₂O, 70%, 95% and 100% EtOH, 3min/each). The slides were then subjected to emulsion autoradiography.

Emulsion autoradiography

Emulsion autoradiography was performed in the dark with a safe light. K2 emulsion (Ilford, Staffordshire, UK) (diluted to 50% with RNase-free H₂O) was heated to 42-45°C for 20 minutes prior to use in a slide dipping chamber placed within a water-bath to maintain the temperature. The slides were dipped and placed vertically in a light-proof box to dry for a minimum of two hours. The slides were then stored in a light-proof slide box and stored at 4°C for the appropriate time prior to development. All development of slides was performed in the dark room. The slides were transferred into slide racks and exposed to Phenisol Developer (Ilford, Staffordshire, UK) for 2.5 minutes, RNase-free H₂O for 30 seconds and Hypam Rapid Fixer (Ilford, Staffordshire, UK) (diluted to 25% with RNase free H₂O) for 3 minutes. At this stage, the samples can be exposed to light. The slides were run under tap H₂O for approximately 20 minutes. The slides were then subjected to a haemotoxylin and

eosin counterstaining, by immersing the slide in haematoxylin (Sigma-aldrich, Dorset, UK) for 1 minute, followed by washing the slides for 1 hour in H₂O. The slides were then dipped in eosin (Sigma-aldrich, Dorset, UK) 3 times, 60% EtOH five times, 95% EtOH eight times, 100% EtOH twice each for 2 minutes, and HistoClear twice, each for two minutes, and immediately mounted with DPX mountant.

2.14. Immuno-histochemistry (IHC) to detect LH in Gonadotrophs

2.14.1. Rapid IHC to detect LH specific gonadotrophs on sectioned pituitary glands

Collection and sectioning of neuroendocrine samples for IHC

One method that was applied prior to collection of the sample tissue was to perfuse the birds through the jugular vein. Prior to perfusion, the birds were terminally anaesthetised with 0.8ml 60mg.µl of Expiral anaesthetic (Sanofi Animal Healthcare, Watford, UK). The birds were perfused through the jugular vein with 400ml of heparinised saline (1L 0.9% saline + 10 mls heparin @1000U/ml) at a rate of 20ml/minute. Fixation of the tissue is performed with Zamboni fixative (1L-350ml H₂O, 40g PFA, 150ml saturated picric acid and 500ml of 0.2M Buffer PB). The birds are then perfused with 500ml of 5% sucrose solution through the jugular vein. The pituitary glands were dissected, and placed in a 30% sucrose solution overnight, alternatively, until the tissue sunk to the bottom of the solution. The tissue was frozen in -20°C isopentane closely monitored to -65°C. The tissue was sectioned with a cryostat at 15µm thickness and mounted on Superfrost plus slides which were immediately placed on dry ice and transferred to -80°C for storage or used immediately.

Alternatively, fresh tissues were rapidly dissected, with RNase-free instruments cleaned with RNase-Zap® and thoroughly rinsed with RNAase free H₂O

(alternatively sterile autoclaved instruments). The tissues were immediately immersed in sterile PBS and frozen on dry ice immediately. The tissue was sectioned with a cryostat at 8 μ m thickness and mounted onto Superfrost plus slides and stored at -80°C.

Fixing the tissue

The non-perfused frozen sections were thawed at room temperature for 30–60 seconds taking care not to dry the tissue section as this would have resulted in increased tissue adhesion to the slide. The slides were immersed in cold acetone for 5 minutes and then rinsed in 1 x PBS (pH 7.4) for 1 minute.

Immunohistochemistry

Non-specific binding to tissue sections using 10% goat serum for 3 minutes, and then immediately incubated with the primary anti-turkey LH- β antibody (produced in a rabbit immunized with turkey LH beta prepared by Prof. W.H. Burke and provide as a gift to Prof. P. Sharp, Roslin Institute, Edinburgh, UK), of varying concentration to optimise the staining technique, for 5-10 minutes. The antibody has previously been shown to cross reacted with chicken LH, but not with chicken FSH, GH nor prolactin (Burke et al., 1979). The slides were then subjected to 3 x 15 second washes in PBS prior to incubating with the secondary antibody (donkey anti-rabbit immunoglobulin) for 5-10 minutes. The slides were then washed 3 times in PBS, each for 15 seconds and incubated in Vectastain ABC (Vector labs, Peterborough, UK) reagent for two minutes prior to incubating in 200-400u/ml 3,3'-Diaminobenzidine (DAB) for 5 minutes. The sections were then subjected to dehydration by immersing in 75% EtOH, and 80% EtOH each for 45 seconds and finally in 100% EtOH for 1 minute. The sections were then immersed twice in 100% Xylene solution each for 5 minutes and left to dry at room temperature for 10 minutes, and then stored overnight in an air-tight container with dry silica crystals.

Laser Capture Micro-dissection

Laser capture micro-dissection using the Acturus PIX Cell III Laser Capture Instrument (Genetic Research Instrumentation, Essex, UK) was performed according to manufacturer's instructions. Once the cells of interest were located, a thermostatic film coated CapSure macro LCM cap (Arcturus, California, USA) was mounted over the target area. The laser was then pulsed (set at various combinations of pulse power, laser pulse duration, and laser spot diameter) through the cap resulting in the thermostatic film to form a thin protrusion that bridges the gap between the cap and tissue and adheres to the target cell by the activated laser over the target cell. Lifting the cap, results in the removal of the target cell(s) which have adhered to the cap. The cap containing the cell was then placed onto an eppendorf tube containing RNA extraction buffer PicoPure RNA extraction kit, (Arcturus, California, USA).

2.15. Transient gene expression following transfection by electroporation in COS-7 cells

COS-7 cells were cultured in Dulbecco's modified Eagles medium (DMEM) (Sigma-Aldrich, Dorset, UK) containing a final concentration of 10% FCS, 4mM L-glutamine and 1 x penicillin/streptomycin for at least 48 hours prior to transfection. COS-7 cells were transiently transfected by electroporation with 10µg of expression construct per 4mm cuvette (Biorad, Hertfordshire, UK). A single 150cm² dish of 80% confluent COS-7 cells (approximately 1x10⁷ cells) was sufficient for a single electroporation (960µF capacitance and 0.23kV) in a cuvette diluted in 700µL Opti-Mem reduced serum buffer (Invitrogen, Paisley, UK). The transfected cells from a single cuvette were seeded into two 12-well culture plates (Corning, Avon, France) in DMEM containing a final concentration of 10% FCS, 4mM L-glutamine and 1 x penicillin/streptomycin. The cells were incubated in a humidified atmosphere of air and 5% CO₂ at 37°C for two days prior to performing required assays.

2.16. Transient gene expression following co-transfection by electroporation in COS-7 cells

COS-7 cells were transiently transfected by electroporation with cGnRH-R-III expression construct variants. The ability of the cGnRH-R-III splice variants (SVs) to bind endogenous chicken GnRH ligands was assessed in cells transfected with 10µg of cGnRH-R-III_SV1 and cGnRH-R-III_SV2 expression constructs. In addition, cells co-transfected with 3.3µg of full-length construct and 6.6µg of empty pcDNA3.1 (10µg of vector in total) were used for comparative purposes. Effects of the splice variants on full-length receptor binding were determined by co-transfecting 3.3µg of the full length cGnRH-R-III construct and 6.6µg of each cGnRH-R-III splice variant. All assays were performed in triplicate, and each experiment repeated three times. The transfected cells from a single cuvette were seeded into two 12-well culture plates in DMEM containing a final concentration of 10% FCS, 4mM L-glutamine and 1 x penicillin/streptomycin. The cells were incubated in a humidified atmosphere of air and 5% CO₂ at 37°C for two days prior to performing required assays.

2.17. Iodination of [His⁵-D-Tyr⁶] GnRH-I

[His⁵-D-Tyr⁶]-GnRH-I peptide was radioactively labelled with Iodine¹²⁵ using Chloramine-T method (Sigma-Aldrich, Dorset, UK) and purified using Sephadex G25 chromatography (Sigma-Aldrich, Dorset, UK), kindly provided by Robin Sellar, MRC, HRSU, Edinburgh. The specific activity of the various batches produced were between 100-125Ci/mmol.

2.18. ¹²⁵I-[His⁵-D-Tyr⁶]-GnRH-I whole cell binding assays

Radioligand binding assays were performed with ¹²⁵I-[His⁵-D-Tyr⁶]-GnRH-I on intact cells 48 hours after transfection. Cells were placed on ice and washed twice

with 4°C Dulbecco's phosphate buffered saline with calcium chloride and magnesium chloride (PBS+Ca²⁺+Mg²⁺), (Sigma-Aldrich, Dorset, UK). The cells were then incubated for 4 hours at 4°C with 10⁵cpm/500ul ¹²⁵I-[His⁵-D-Tyr⁶]-GnRH and various concentrations of unlabelled cGnRH-I and GnRH-II in HEPES, DMEM, 0.1%BSA in triplicate. The cells were then washed twice with PBS+Ca²⁺+Mg²⁺ and subsequently lysed with 0.5ml of 0.1M NaOH. Radioactivity was measured and non-specific binding was determined in the presence of 1μM unlabelled cGnRH-I or GnRH-II using a gamma counter. Each assay was repeated at least three times on separate occasions. Competition curves were fitted using GraphPad Prism (GraphPad) by applying a 'One Site Competition' non-linear regression and were fitted to a sigmoidal dose-response curve with a assumed HILLSLOPE equal to -1 with no constraints applied to the curve fitting. This analysis describes the competition of the ligand for the receptor binding. The IC₅₀ value derived is the concentration of the competing ligand required to compete half of the specific binding. The IC₅₀ values were calculated for individual experiment performed in triplicate, followed by calculating the mean IC₅₀ value for the number of times the experiment was performed. In all cases, the IC₅₀ is used as a measure of the relative affinity. As the affinity of the radiolabelled ligand to cGnRH-R-I and cGnRH-R-III was not determined, the Cheng-Prusoff equation was not used to determine the "affinity" (K_i) or "dissociation constant" of each ligand.

2.19. Determining agonistic and antagonistic effects of GnRH analogs on Inositol Phosphate (IP) accumulation

24 hours after transfection cells were labelled with 1μCi/ml [³H]-myo-inositol, (Amersham Pharmacia Biotech) in 1ml of inositol-free "special DMEM" (Invitrogen, Paisley, UK) and incubated for a further 24 hours in a humidified atmosphere of air and 5% CO₂ at 37°C. Media was removed and cells were incubated at 37°C with 0.5ml HEPES, DMEM (Sigma-Aldrich, Dorset, UK) containing 10mM LiCl for 5 minutes and repeated for further 30 minute incubation. LiCl inhibits inositol

phosphatases resulting in the accumulation of inositol phosphate metabolites (Berridge et al., 1982). The cells were then incubated for 60 minutes with a concentration gradient of GnRH analogs in 0.5ml HEPES, DMEM, 10mM LiCl to determine agonistic effects. The antagonistic effects were determined by incubating for 60 minutes with a concentration gradient of GnRH analogs in the presence of 1nM GnRH-II in 0.5ml HEPES, DMEM, 10mM LiCl. In both cases, the incubation was promptly terminated by the removal of the media and the subsequent addition of 1ml of 10mM formic acid for 60 minutes at 4°C. The solution was then applied to tubes containing 500µl AG 1-X8 resin (Dowex) (Bio-rad Hertfordshire, UK). The resin was allowed to settle and the supernatant aspirated off. The resin was washed with 1ml of H₂O, 1ml of 60mM ammonium formate and 1ml of 5mM sodium tetraborate. Each wash was followed by aspirating off the supernatant prior to the addition of the next wash solution. Finally, inositol phosphate product was eluted in 1ml of 1M ammonium formate, 0.1M formic acid. 800µL of the supernatant was removed from the resin and the β-emission was measured by the radioactivity counted in 2.5mls of scintillation fluid (Optiphase HiSafe 3). Inositol phosphate experiments were performed in triplicate and repeated at least three times. Functional data were analysed using GraphPad Prism (GraphPad) by applying non-linear regression and were fitted to a sigmoidal dose-response curves with an assumed slope of 1. No further constraints were applied to the curve fitting. Curves generated were used to calculate EC₅₀ (excitatory concentration) or IC₅₀ (inhibitory concentration) values for receptor stimulation or inhibition respectively. The EC₅₀ or IC₅₀ values were calculated for each individual experiment performed in triplicate, followed by calculating the mean EC₅₀ or IC₅₀ value for the number of times the experiment was performed.

2.20. Receptor mediated internalisation of ¹²⁵I-[His⁵-D-Tyr⁶]-GnRH-I

Internalisation of the radioligand ¹²⁵I-[His⁵-D-Tyr⁶]-GnRH-I was measured across time points and expressed as a percent of total radioligand interacting with the cell.

Receptor transfected COS-7 cells were seeded into 12-well culture plates and incubated at 37°C/5% CO₂ for 48 hours prior to performing the internalisation assays. The plates were placed on ice and the media removed and replaced with ice cold 0.5ml HEPES, DMEM, 0.1%BSA containing 10⁵cpm/500ul ¹²⁵I-[His⁵-D-Tyr⁶]-GnRH-I. The plates were incubated at 4°C for 5 hours. Initiation of internalisation of the radioligand in the transfected cells was performed at 37°C at 5, 15, 45 and 90 minutes with the control remaining at 4°C. The internalisation was halted at each time point by removing the cells from 37°C and placing them immediately on ice and then removing the medium containing the radioligand. The plates were then immediately washed with 0.5 ml ice cold PBS+Ca²⁺+Mg²⁺ twice. The cells were subsequently incubated on ice for 10 minutes in the presence of 0.5 ml ice cold acid wash solution (150mM NaCl, 50mM acetic acid, pH2.8) to determine all surface bound radioligand i.e. ligand that is bound to the receptor but not internalised. The acid wash solution was removed and the cells were solubilised with 0.5ml 1M NaOH to quantify the content of internalised radioligand. The internalisation percent calculated from time point 0 was subtracted from all other time point per replicate, as a means of removing the background radiolabelled ligand associated with the cell. Curves were fitted using GraphPad Prism (GraphPad) by applying the “One phase exponential association” which describes the pseudo-first association kinetics of the interaction between a ligand and its receptor.

$$\% \text{ internalisation} = \frac{\text{NaOH}}{\text{Total (acid and NaOH)}} \times 100.$$

2.21. Generation of cGnRH-R-III SH3 Binding Motif Mutants in pcDNA 3.1/myc-His-A vector

Mutagenesis of three identified putative SH3 binding motifs located at amino acid positions 369-373, 370-375 and 398-402 of cGnRH-R-III was carried out using the QuikChange site-directed mutagenesis kit (Agilent technologies, Chesire, UK)

according to the manufacturers instructions. The proline codon was mutated to an alanine codon. In brief, the insert of the cGnRH-R-III cDNA cloned into the pCDNA3.1/myc-His-A expression vector was PCR amplified with each individual SH3 motif mutant primer pair, each complementary to each opposite strands of the vector; SH3P317A-F (TGT GGG CAC AGC TCT GAG GCT GAG CCA CCC TCC CCA GCC ACG) and SH3P317A-R (CGT GGC TGG GGA GGG TGG CTC AGC CTC AGA GCT GTG CCC ACA); SH3P319A-F (TGT GGG CAC AGC TCT GAG CCT GAG GCA CCC TCC CCA GCC ACG) and SH3P319A-R (CGT GGC TGG GGA GGG TGC CTC AGG CTC AGA GCT GTG CCC ACA); and SH3P346A-F (GTA TGG GGG GCA CAG AGG GGG CAC ACC CAC CTA TCG AGC TGG) and SH3P346A-R (CCA GCT CGA TAG GTG GGT GTG CCC CCT CTG TGC CCC CCA TAC). A *Pfu* Turbo DNA Polymerase (Agilent technologies, Cheshire, UK) was used to amplify cDNA according to manufacturers instructions using the following amplification conditions (initial denaturation step of 95°C/30s, 16 cycles of denaturation 95°C/30s, annealing 55°C/60s and elongation 68°C/90s) to incorporate the oligonucleotide primers generating a mutated plasmid containing staggered nicks. The reaction mixes are then cooled on ice for 2 minutes to ~37°C prior to the addition of 1µl of *DpnI* restriction enzyme (target sequence: 5'-Gm6ATC-3') (10U/µl). The non-mutated supercoil dsDNA was then digested for one hour at 37°C. The *DpnI* treated DNA (i.e. the nicked vector DNA) was then used to transform XL1 Blue *E. coli* super competent cells provided with the QuikChange site-directed mutagenesis kit according to manufacturers provided instructions. The cells were then plated onto pre-warmed agar plates and incubated at 37°C for 16 hours. Colonies were picked after 16 hours and grown for plasmid DNA purification using the QIAprep Miniprep Kit according to the manufacturer's instructions. Once the integrity of the insert DNA was verified, the selected clone was grown for plasmid DNA purification using the HiSpeed Plasmid Maxi Kit according to the manufacturer's instructions.

2.22. Cloning of HA epitope-tagged cGnRH-R-I and cGnRH-R-III into pcDNA 3.3 vector

The insert of the cGnRH-R-I cDNA cloned into the pcDNA/Amp expression vector was PCR amplified with primers Type I-cGnRH-R-HA-tag-F (GTT ATG TAT CCT TAT GATGTTCCCAACTATGCTATG TGC GTA CCA GCT GCT TTA ATC) and Type I-cGnRH-R-HA-tag-R (TCA GCA CAC CGT GTT AAC GGT T). The insert of the cGnRH-R-III cDNA cloned into the pcDNA 3.1/myc-His-A expression vector was PCR amplified with primers Type III-cGnRH-R-HA-tag-F (GTT ATG TAT CCT TAT GAT GTT CCC AAC TAT GCT ATG GCC CGG CTC GGC) and Type III-cGnRH-R-HA-tag-R (TCA CAG CGC ACT GCT CTG G). The forward primers for both receptors were designed to encode an HA epitope-tag to the N-terminal of either cGnRH receptor included the Kozak translation initiation consensus sequence (GTT ATG) and an HA epitope-tag (TAT CCT TAT GAT GTT CCC AAC TAT GCT). A PfuUltra® Hotstart Polymerase (Agilent technologies, Chesire, UK) was used to amplify cDNA according manufacturers instructions using the following amplification conditions (initial inactivation step of 95°C/1minute, 30cycles of denaturation 95°C/60s, annealing 55°C/60s and elongation 72°C/90s and a final extension 72°C/10minutes). The PCR products were run on a 2% agarose gel to ensure the PCR product amplified was of the desired length (1161bp for cGnRH-R-I and 1293bp for cGnRH-R-III). The PCR products were excised and purified with Ultrafree-MC Filter Units (Millipore, Watford, UK) according to the manufacturer's instructions. 3' A-overhangs necessary for TOPO® cloning were then added post amplification by adding the following solutions to give a total reaction volume of 50 µl with RNase free H₂O:

Purified PCR product	*µl
Taq Polymerase	1µl
dNTPs.....	1 µl
10X Buffer	5 µl.

The reaction mix was incubated at 72°C for 10 minutes. The DNA was extracted from the reaction mixture by applying a phenol-chloroform extraction to remove the

polymerase. The DNA was then ethanol-precipitated and re-suspended in an appropriate amount of TE buffer. The HA epitope-tagged cGnRH-R-I and cGnRH-R-III inserts were then ligated into the pcDNA 3.3-vector using the pcDNA 3.3-TOPO® TA Cloning Kit (Agilent technologies, Chesire, UK) according to the manufacturers instructions. The constructs were then used to transform competent *E. coli*. One Shot® TOP10 Chemically Competent *E.Coli* provided with the pcDNA 3.3-TOPO® TA Cloning Kit were used according to the manufacturers instructions. The cells were then plated onto pre-warmed agar plates and incubated at 37°C for 16 hours. Colonies were picked after 16 hours and grown for plasmid DNA purification using the QIAprep Miniprep Kit according to the manufacturer's instructions. Once the integrity of the insert DNA was verified, the selected clone was grown for plasmid DNA purification using the HiSpeed Plasmid Maxi Kit according to the manufacturer's instructions.

2.23. Verification of the DNA sequence of the cloned chicken GnRH Receptor cDNAs

2.23.1. cGnRH-R-III SH3 binding motif mutants in pcDNA 3.1/myc-His-A vector

The insert of both the cGnRH-R-III SH3 binding motif mutants insert was sequenced with a CMV-F primer (CGC AAA TGG GCG GTA GGC GTG) whose complimentary sequence is present in the MCS of the pcDNA 3.1/myc-His-A vector.

2.23.2. HA Epitope-Tagged cGnRH-R-I and cGnRH-R-III in pcDNA 3.3 vector

The insert of both the HA epitope-tagged cGnRH-R-I and cGnRH-R-III insert was sequenced with a CMV-F primer (CGC AAA TGG GCG GTA GGC GTG) whose complimentary sequence is present in the MCS of the pcDNA 3.3 vector. The

sequence of the HA epitope-tagged cGnRH-R-I and cGnRH-R-III inserted into pCDNA3.3 vector was also verified by *PvuII* restriction enzyme digest.

2.24. GnRH ligand stimulations and inhibition of second messenger signalling cascades and preparation of NP40 cell lysates for immunoblotting

cGnRH Receptor subtype transiently transfected COS-7 cells were seeded in 6-well culture plates (1 cuvette = 2x6-well culture plates). After 24 hour incubation period, the media was removed and replaced with a serum-free media (Hepes, DMEM) for 16 hours prior to stimulations. The cells were then stimulated with varying concentrations of GnRH analogs and/or inhibitors, for specific time points. The stimulations were terminated by placing the cells on ice, aspirating off the media and washing the cells once with 4°C PBS+Ca²⁺+Mg²⁺. Cell lysis was performed with 150µl per well of 4°C NP40-based solubilisation buffer (250mM NaCl, 5mM HEPES, 10% glycerol, 0.5% Nonidet P-40, 2mM EDTA (pH8) supplemented with 1mM sodium orthovanadate, 1mM phenylmethsulphonyl fluoride (PMSF) and 1mg/ml Leupeptin). Cell lysates were scraped with a cell scraper and placed in chilled eppendorf tubes for centrifugation at 14000rpm for 10 minutes at 4°C. 100µl of the supernatant was recovered to a fresh eppendorf. An equal volume of 2 x Laemmli Sample Buffer (LSB) was added to the clarified lysate. Samples were stored at -20°C until required.

2.25. GnRH ligand stimulations, preparation of NP40 cell lysates and immuno-precipitation

cGnRH Receptor subtype transiently transfected COS-7 cells were seeded in 10cm culture dishes. (1 cuvette = 1x10cm culture dish). After 24 hour incubation period, the media was removed and replaced with a serum-free media for 16 hours prior to stimulations. The cells were then stimulated with varying concentrations of GnRH

analogs and/or inhibitors, for specific time periods. The stimulations were terminated by placing the cells on ice, aspirating off the media and washing the cells once with 4°C PBS+Ca²⁺+Mg²⁺. Cell lysis was performed with 1000µl per well of 4°C NP40-based solubilisation buffer (as above). Cell lysates were scraped with a cell scraper and placed in chilled eppendorf tubes for centrifugation at 14000rpm for 10 minutes at 4°C. The cells lysates were then sonicated and subjected to another centrifugation at 14000rpm for 10 minutes at 4°C. 500µl of the supernatants was removed and added to a fresh eppendorf. 50µl of Anti-HA Affinity Matrix (Roche Applied Science, West Sussex, UK) was added to 500µl (or a fixed amount of protein) of the cell lysate supernatant, and incubated while shaking overnight at 4°C. The column was centrifuged at 14000rpm for 5 minutes, and then washed with 1000ul of 4°C NP40-based solubilisation buffer twice, followed by centrifugation. 50µl of 2x LSB was added to the beads and vortex thoroughly. Samples were stored at -20°C indefinitely until required for immuno-blotting.

2.26. SDS Polyacrylamide Gel Electrophoresis (SDS-PAGE)

4-20% Tris-Glycine gels (Invitrogen, Paisley, UK) were loaded with protein samples denatured by heating to 100°C for 3 minutes. 15-20µl of sample per well was separated under electrophoresis at 90mA for 60 minutes in 1x SDS-PAGE Buffer (29g Tris Base, 144g Glycine, 10g SDS). Pre-stained broad range SDS-PAGE standards (Biorad, Hertfordshire, UK) were electrophoresised alongside the samples to determine the protein sizes. Polyvinylidene difluoride (PVDF) membranes (Perkin Elmer, Waltham, USA) were prepared by washing in 100% EtOH for 5 minutes and then 3 x 10 minute washes with distilled H₂O. A wash in Semi-Dry Transfer Buffer (20mM Tris, 192mM glycine, 20% MeOH, 0.1% SDS) was performed before protein transfer. The gel was placed above the prepared PVDF membrane, and sandwiched with blotting pads (VWR, Leicestershire, UK) pre-soaked in Semi-Dry Transfer Buffer. Proteins were transferred from the gel to the membrane

in a Trans-Blot[®] SD Semi-Dry Electrophoretic Transfer Cell (Biorad, Hertfordshire, UK) at 200mA, 25V, 300W for 60 minutes.

2.27. Immuno-blotting

The protein samples transferred onto the membrane was blocked with 10ml Blocking Buffer (4% BSA in 1 x TBS-T (100mM Tris-HCl (pH7) 150mM NaCl, 0.05% Tween 20, 0.05% NP40)) with shaking for 60 minutes. The appropriate primary antibody (1:1000 dilutions in Blocking Buffer, 0.1% Sodium Azide) was added onto the membrane and incubated for 60 minutes while shaking. After removal of the primary antibody, the membrane was subjected to 3x 10 minute washes in 1 x TBS-T (100mM Tris-HCl, 150mM sodium chloride, 0.05% Tween 20, 0.05% NP40). 10ml of the appropriate alkaline phosphatase-conjugated polyclonal IgG secondary antibody (Sigma-Aldrich, Dorset, UK) diluted at 1:10000 in Blocking Buffer was added onto the membrane and incubated for 60 minutes while shaking. Prior to protein visualisation, the membrane was subjected to 3 x 10 minute washes in 1 x TBS-T. Enhanced chemifluorescence (ECF) substrate (Amersham Pharmacia Biotech, Buckinghamshire, UK) was added according to the manufactures instructions and scanned using the Typhoon 9400 Phospho-Imager and subsequently quantified using Image-Quant TL software (Molecular Dynamics, Amersham Biosciences, Buckinghamshire, UK). The membrane was prepared for subsequent probes by removing the ECF substrate from the membrane by incubation in 40% MeOH for 30 minutes. Antibodies were then removed from the membrane by incubation at 80°C for 30 minutes in 10ml of Stripping Buffer (25mM Tris-HCl (pH7) 8% SDS, 0.72M β -mercaptoethanol). The membrane was then washed 3x 10 minute washes in 1 x TBS-T. The membranes were either stored dry or used immediately for alternate probes after blocking the membranes with 10ml Blocking Buffer.

Table 2. 1**Primers used in experiments**

Primer ID	5' to 3' sequences
cGnRH-R-III-713-735f (exon 2-3)	GCT CGC TGT GCC GCA GCT GT
cGnRH-R-III-933-952r (exon 3-4)	ACG TCC CTG GAG GAG AAG AG
cGnRH-R-I-701-722f	AGC TAA AGA TCA ACA AAA GTC T
cGnRH-R-I-1100-1082r	TTG GAG CCA CCT GAT GTC A
73-120-cGnRH-R-III_sense	TGG TGC CGA TGT GGG AGC GCT GGG AGC AGT GAG TGG GCG GTG TTT GA
73-120-cGnRH-R-II_anti-sense	TCA AAC ACC GCC CAC TCA CTG CTC CCA GCG CTC CCA CAT CGG CAC CA
cGnRH-R-III-1210f	TGA GTG GGC GGT GTT TGA
cGnRH-R-III-3217r	TAG AGG CAT TGT GGA GCA GA
M13 F	GTT GTA AAA CGA CGG CCA GT
M13 R	CAG GAA ACA GCT ATG ACC
pcDNA3.1/myc-His_826f	TAA CTA GAG AAC CCA CTG CTT ACT
pcDNA3.1/myc-His_1060r	AAT GGT GAT GGT GAT GAT GA
T7-prom-IRD-700	TAA TAC GAC TCA CTA TAG GG
CMV-F primer	CGC AAA TGG GCG GTA GGC GTG
SH3P317A-F	TGT GGG CAC AGC TCT GAG GCT GAG CCA CCC TCC CCA GCC ACG
SH3P317A-R	CGT GGC TGG GGA GGG TGG CTC AGC CTC AGA GCT GTG CCC ACA
SH3P319A-F	TGT GGG CAC AGC TCT GAG CCT GAG GCA CCC TCC CCA GCC ACG
SH3P319A-R	CGT GGC TGG GGA GGG TGC CTC AGG CTC AGA GCT GTG CCC ACA
SH3P346A-F	GTA TGG GGG GCA CAG AGG GGG CAC ACC CAC CTA TCG AGC TGG
SH3P346A-R	CCA GCT CGA TAG GTG GGT GTG CCC CCT CTG TGC CCC CCA TAC
Type I-cGnRH-R-HA-tag-F	GTT ATG TAT CCT TAT GAT GTT CCC AAC TAT GCT ATG TGC GTA CCA GCT GCT TTA ATC
Type I-cGnRH-R-HA-tag-R	TCA GCA CAC CGT GTT AAC GGT T
Type III-cGnRH-R-HA-tag-F	GTT ATG TAT CCT TAT GAT GTT CCC AAC TAT GCT ATG GCC CGG CTC GGC
Type III-cGnRH-R-HA-tag-R	TCA CAG CGC ACT GCT CTG G

Chapter 3.

Cloning, Characterisation and Tissue-Specific Expression of Type III cGnRH Receptor

3.1. Abstract

Variant GnRH ligand and receptor subtypes have been identified in a number of non-mammalian vertebrate species, however research into avian species GnRH systems is lacking. Two isoforms of GnRH are present in the domestic chicken, the evolutionary conserved GnRH-II and cGnRH-I. The expression of two GnRH ligands coincides with the two identified chicken GnRH receptor subtypes; cGnRH-R-I and a novel form I have designated as cGnRH-R-III. The occurrence of two isoforms of the receptor in the chicken raises questions about their specific biological functions and interactions with the two ligands. Differential roles for these molecules in regulating gonadotrophin secretion or other functions are currently unclear. To investigate this I cloned cGnRH-R-III cDNA from a broiler chicken anterior pituitary gland and compared its structure and expression with cGnRH-R-I. The broiler cGnRH-R-III cDNA was 100% identical to the sequence recently reported in the red jungle fowl and white leghorn breed and had a 53% sequence identity with cGnRH-R-I in the protein coding region. Expression profiling of cGnRH-R-III cDNA showed that it is predominantly expressed in the anterior pituitary, ~1400 times more abundant than cGnRH-R-I cDNA. A pronounced sex and age difference existed, with higher pituitary transcript levels in sexually mature females versus juvenile females. In contrast, higher expression levels occurred in juvenile males compared to sexually mature males. Northern blot analysis indicated a single cGnRH-R-III transcript. The research presented in this chapter, demonstrates the mRNA expression of a novel pituitary-predominant type III GnRH receptor homolog in the chicken. The higher abundance of expression of cGnRH-R-III mRNA in the anterior pituitary when compared to cGnRH-R-I suggests it is probably the major mediator of pituitary gonadotroph function, and that tissue specific recruitment of cGnRH-R isoforms has occurred during evolution.

3.2. Introduction

Two GnRH isoforms are present in the chicken (cGnRH-I and GnRH-II) (Sharp and Ciccone, 2005). There is no genomic DNA evidence for a third GnRH ligand in the chicken to date (Morgan and Millar, 2004). Both cGnRH-I and GnRH-II stimulate luteinising hormone (LH) release from chicken pituitary *in-vitro* (Hattori et al., 1986a; Millar et al., 1986) and *in-vivo* (Chou et al., 1985b; Hattori et al., 1986b; Proudman et al., 2006; Sharp et al., 1986). LH secretion in response to GnRH-II is markedly greater than to cGnRH-I in mature laying hens. However, this is not the case in juvenile chickens of either sex, nor the mature male (Sharp et al., 1987). There is conflicting evidence for an effect of cGnRH-I or GnRH-II on follicle stimulating hormone release (FSH) *in-vitro*, (Hattori et al., 1986a; Millar et al., 1986) and *in-vivo* (Bruggeman et al., 1998; Dunn et al., 2003; Krishnan et al., 1993; Proudman et al., 2006). Probably, cGnRH-I does not directly stimulate FSH release, but may stimulate FSH biosynthesis, while a role for GnRH-II in FSH production has not been established (Dunn et al., 2003; Sharp and Ciccone, 2005). Immunisation of laying hens against cGnRH-I, but not against GnRH-II, results in a complete regression of the reproductive system and a decrease in plasma LH (Sharp et al., 1990), suggesting that gonadotrophin secretion is controlled by cGnRH-I rather than GnRH-II. This is consistent with the presence of cGnRH-I in the median eminence (Mikami et al., 1988a; Sharp et al., 1990) and the correlation between hypothalamic cGnRH-I peptide content and reproductive status (Sharp et al., 1990). In contrast, abundant levels of GnRH-II (Mikami et al., 1988; Sharp et al., 1990; Vangils et al., 1993) are not observed in the median eminence and no change in hypothalamic GnRH-II peptide content is seen in cockerels around the onset of puberty (Sharp et al., 1990). However hypothalamic GnRH-II levels do decrease when the pre-ovulatory release of LH is maximal or declining in the hen (Wilson et al., 1990). Thus GnRH-II may be involved in neuroendocrine events preceding ovulation or other non-neuroendocrine elements of reproduction. In other avian species, GnRH-II affects reproductive behaviour in ring doves and soliciting behaviour in female song sparrows (King and Millar, 1995; Maney et al., 1997).

The specific receptor types through which the GnRH ligands elicit biological responses in the chicken are not known. Localisation of receptor gene expression using *in-situ* hybridisation would help to formulate hypothesis but such information is not available for the avian species. Other clues to GnRH system organisation may be available from comparative anatomy, endocrinology, evolutionary biology and genomics.

The divergence of amphibians and amniotes (reptiles, birds and mammals) is generally thought to have occurred approximately 350 million years ago (Elinson and Beckham, 2002), during the late Paleozoic Era (Elinson and Beckham, 2002). Birds are a diverse group that evolved from reptiles during the Mesozoic Era about 150 million years ago, with the crocodylian lineage being most closely related to birds (Rogers, 1999). 22,000 species of ray-finned fish share a common ancestor which experienced a large-scale gene or genome duplication event (Taylor et al., 2003) leading to tetraploidy in most fish species. Most amphibians are also tetraploid as seen in *Xenopus laevis* (Hughes and Hughes, 1993), whereas some amphibians are diploid, with *Xenopus tropicalis* being the only diploid species in the *Xenopus* genus, (Amaya et al., 1998; Fierro et al., 2007). Reptilian genomes range in diploid chromosome number from $2n=20$ in most lizards to $2n=68$ in some turtles (Schmid et al., 1994). Most avian karyotypes are composed of a diploid number of 78-82 chromosomes (Federico et al., 2005). Fewer GnRH ligand and GnRH receptor genes are retained in the genomes of mammals and birds compared to protochordates, fish and amphibians (Morgan and Millar, 2004).

There is some evidence that GnRH systems exhibit species-specific recruitment of receptor subtypes and ligands within different tissues. For example, the sea bass possesses five isoforms of GnRH receptor and all but one are expressed in the pituitary (Moncaut et al., 2005). In goldfish, two subtypes of the GnRH receptor have been identified (Illing et al., 1999) and they are both expressed in the pituitary. In the bullfrog, three subtypes of GnRH receptor are expressed but only one

predominates in the pituitary (Wang et al., 2001a). Two subtypes of GnRH receptor occur in the African clawed frog (*Xenopus laevis*) (Troskie et al., 2000), whereas the leopard gecko expresses three receptor subtypes in the pituitary, with one receptor subtype predominating in the anterior pituitary (Ikemoto and Park, 2007). The pituitary-specific GnRH receptor subtypes expressed in the bullfrog (Wang et al., 2001a), African clawed frog (Troskie et al., 2000) and leopard gecko (Ikemoto and Park, 2007) differ structurally, according to the nomenclature proposed by Millar et al., and by phylogenetic analysis (Flanagan et al., 2007; Millar et al., 2004; Tello et al., 2008). The predominant pituitary GnRH receptor subtype expressed in the bullfrog is type III, in the African clawed frog is type I and in the leopard gecko is type III. This suggests plasticity in evolutionary recruitment of GnRH receptor subtypes for regulation of pituitary gonadotrophin production. There is also plasticity in the spatiotemporal expression patterns of GnRH receptor subtypes (Ikemoto and Park, 2007; Illing et al., 1999; Moncaut et al., 2005; Troskie et al., 2000; Wang et al., 2001a).

The promiscuity of receptor subtype activation by different endogenous GnRH ligands (Ikemoto and Park, 2007; Illing et al., 1999; Moncaut et al., 2005; Troskie et al., 2000; Wang et al., 2001a) suggests that a complex interplay between ligands and receptors may occur. In this respect, the organisation of the avian GnRH system is distinct from those of teleosts, amphibians, reptiles or mammals, having two receptor subtypes for two GnRH ligands (Morgan and Millar, 2004). Further characterisation of the avian GnRH system may therefore provide unique information to enable further comprehension of the interplay between GnRH ligands and their receptors in the control of reproduction.

The first chicken GnRH-R (cGnRH-R) isoform to be cloned, cGnRH-R-I is widely expressed (Sun et al., 2001a; Sun et al., 2001b) and GnRH-II has a higher binding affinity and is more potent in stimulating inositol phosphate accumulation than cGnRH-I at this receptor (Sun et al., 2001b). This suggested that the chicken pituitary might contain an alternative GnRH receptor more selective for cGnRH-I, since this appears to be the GnRH isoform most likely to be biologically important

for regulation of LH production. Therefore, the aims of this study were to clone the cDNA encoding the novel isoform of the cGnRH-R from a broiler chicken (commercial meat-type chicken) and undertake further characterisation of this second GnRH receptor isoform, including comparisons with the type I receptor. The novel isoform of cGnRH-R was subsequently cloned by ourselves and others using RT-PCR (Joseph et al., 2006; Shimizu and Bedecarrats, 2006) (Genbank AY895154). Using bioinformatics analysis, I confirmed that the novel cGnRH-R is a type III GnRH receptor homolog. Critically, I have established the preponderance of cGnRH-R-III mRNA relative to cGnRH-R-I mRNA in the pituitary.

3.3. Methods

The detailed methodologies performed to generate the results presented in this chapter are described in chapter 2 (2.1-2.14.)

3.4. Results

The identification and cloning of the novel cGnRH receptor isoform is described in this chapter and was performed first in order to identify that the sequence was identical to the predicted GnRH receptor sequence identified by bioinformatic analysis in the Chicken Genome (Ensembl 2004) (results section 3.4.5 and 3.4.6). It is presented after the RT-PCR amplification and tissue distribution (3.4.1), the Northern blot analysis to detect mRNA transcripts (3.4.2) and the real-time RT-PCR quantification in various tissues from birds in different physiological stages. The results are presented in this manner for purposes of simplification. In the results section and chapters that follow, the amino acid residues are referred to as the actual amino acid number as illustrated in Fig.3.8 and the Ballesteros and Weinstein 'Numbering Convention' was not adopted. However, all residues have been converted to the Ballesteros and Weinstein Number and are available in the Appendix A.

3.4.1. cGnRH-R-III cDNA is predominantly expressed in the pituitary

The amplification of GnRH receptor cDNA was ensured by designing primers based on predicted cDNA sequence determined from the *Gallus gallus gallus* Genome (Ensembl 2004) subsequent to performing basic local alignment search tool 'BLAST' analysis with other identified GnRH-R subtype cDNAs. The retrieved cDNA sequence was latterly found to be identical to a published cGnRH-R subtype cDNA sequence (Accession Number AY895154). The PCR primers were designed to extend over exon boundaries, therefore eliminating genomic DNA amplification (**Fig. 3.1A**). Total RNA from dissected tissues was reverse transcribed as described in the methods chapter (2.5) to synthesize cDNA. A single 238bp cDNA amplification product derived from exon 3 was detected in the anterior pituitary (**Fig. 3.1A**). The amplification of cGnRH-R-III cDNA was also detected in all the tissues tested (anterior pituitary, optic lobe, hind brain, cerebrum, cerebellum, median eminence, anterior hypothalamus, posterior hypothalamus, adrenal gland, olfactory bulb, kidney, small intestine, spleen, testes, liver) from pre-pubertal males and females although the intensity of the amplicons in the anterior pituitary was distinctly greater than any other tissue (**Fig. 3.1B and 3.1C**).

3.4.2. Single cGnRH-R-III transcript identified by Northern blot analysis

Hybridisation of [γ - 32 P]-dATP labelled 73-120-cGnRH-R-III_anti-sense oligonucleotide probe designed to the 5' upstream region of the translation start site in exon 1 of cGnRH-R-III was detected in samples from the anterior pituitary, but not from the small intestine or the testes of pooled Poly (A)⁺ RNA samples purified from ten 14-week old male birds of an advanced intercross of broiler and layer strains (AIL) (**Fig. 3.2A**) indicating a single cGnRH-R-III mRNA transcript. [γ - 32 P]-dATP 73-120-cGnRH-R-III_sense oligonucleotide used as a hybridisation negative control did not hybridise to the Poly (A)⁺ RNA samples as expected (**data not shown**). Skeletal muscle actin-alpha-1 (ACTA1) 32 P-dCTP labelled DNA probe hybridised to the pituitary, small intestine and testes samples (**Fig. 3.2B**), demonstrating equal RNA loading of samples and estimation cGnRH-R-III mRNA transcript size. This ACTA1 gene whose full-length transcript size is 1736bp was used to estimate the size of the cGnRH-R-III mRNA transcript size, by overlapping the two Northern blots. In another study, cGnRH-R-III mRNA of approximately 1600bp bound the cGnRH-R-III_1210f-3217r-pBSK⁽⁺⁾ riboprobe in sexually mature male and female white Leghorn layer type bird anterior pituitary samples, but not to cerebral cortex or to muscle RNA (**Fig. 3.2C**). This confirmed the estimated size of the cGnRH-R-III transcript as estimated in (**Fig. 3.2A**). There was no evidence for expression of cGnRH-R-III mRNA transcripts (splice variants) in any tissues, in either study.

3.4.3. Comparative real-time RT-PCR quantification of cGnRH-R-I and cGnRH-R-III mRNA.

The comparative quantification of cGnRH-R-I and cGnRH-R-III mRNA expression by real-time RT-PCR, in 3 male and 3 female, 6 week old (pre-pubertal) Ross broiler type birds raised on 21 hours light period and 3 hours dark period (21L/3D) lighting and 3 male and 3 female, 24 week old (sexually mature) Ross broiler breeder birds raised on 14L/10D lighting, demonstrated that the overall expression of

cGnRH-R-III was greater than cGnRH-R-I in the median eminence, gonads and small intestine, but particularly so in the anterior pituitary where cGnRH-R-III expression was ~1373 times that of cGnRH-R-I (**Fig. 3.3C**). The data analysis was performed by applying an ANOVA analysis, as this data is composed of various explanatory variables (e.g. age, sex and tissue), and ANOVA analysis partitions the observed variance into components due to the different explanatory variables. ANOVA analysis demonstrated a significant relationship between tissue type on cGnRH-R-III concentrations ($p < 0.01$). This is primarily due to higher cGnRH-R-III mRNA expression in the anterior pituitary when compared to the median eminence, gonads and intestine (**Fig. 3.3A**). Anterior pituitary expression of cGnRH-R-III was ~92.5 times greater than in the testes or ovary. No significant relation to tissue type was detected with cGnRH-R-I expression, where expression in the testes and ovary was ~1.4 times greater than in the anterior pituitary (**Fig. 3.3B**). ANOVA analysis demonstrated a significant relationship between age and sex on pituitary cGnRH-R-III mRNA expression (**Fig. 3.3D**). Anterior pituitary cGnRH-R-III expression was ~8.4 fold lower in sexually mature males compared to juvenile males whereas the reciprocal relationship was observed in females, with ~2.8 fold more anterior pituitary cGnRH-R-III expression in sexually mature females compared to juveniles (**Fig. 3.3D**).

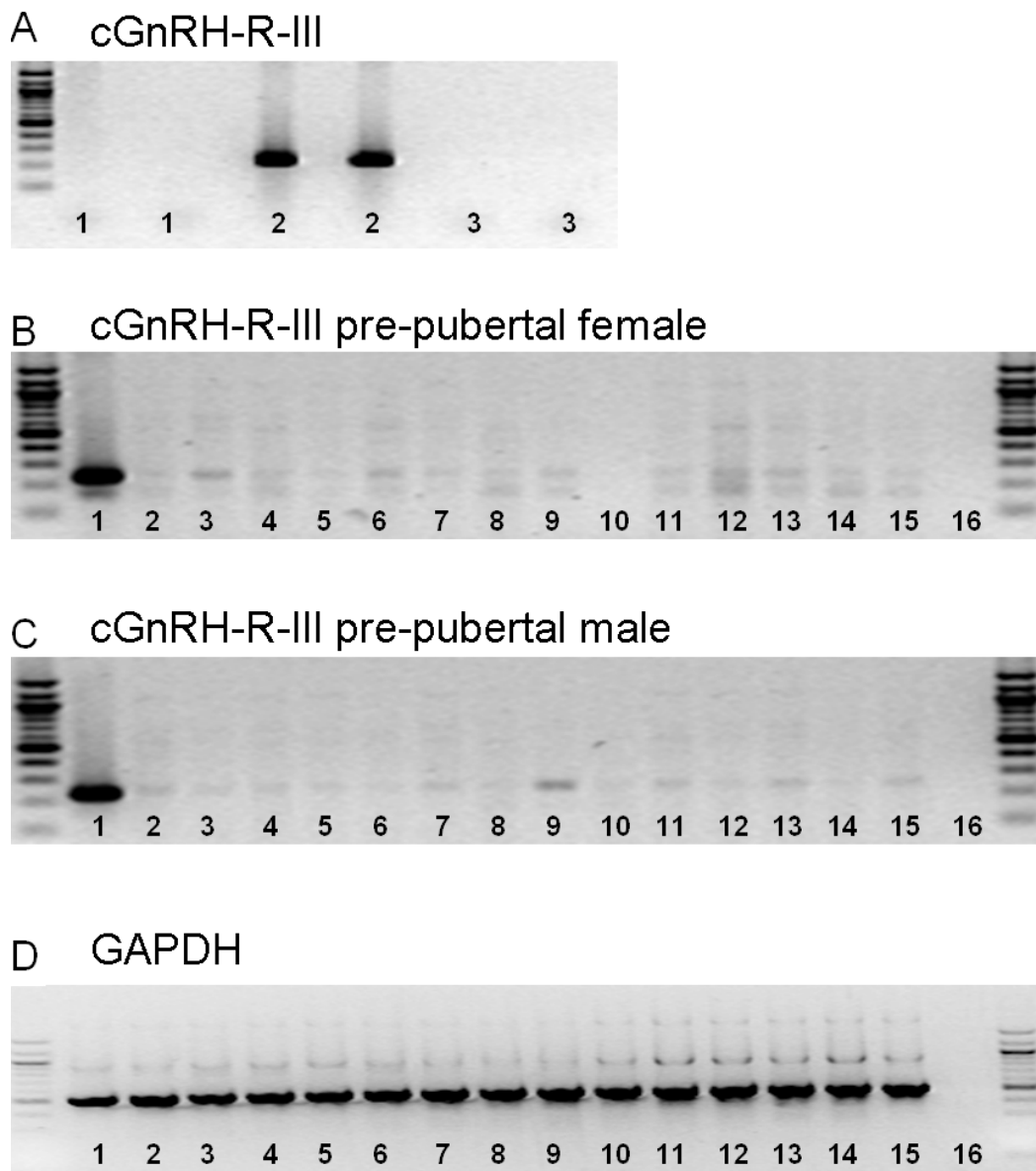
Figure 3. 1

Fig. 3.1. Ethidium bromide stained agarose gels of cGnRH-R-III cDNA PCR amplification with primers cGnRH-R-III-713-735f (exon 2-3) and cGnRH-R-III-933-952r generating a 238bp product which corresponds to exon 3 of cGnRH-R-III. PCR cycle number = 40. (A) cGnRH-R-III amplification; 1 = genomic DNA control, 2 = anterior pituitary complementary DNA, 3 = non-template control. (B) cGnRH-R-III amplification on pre-pubertal female samples, (C) cGnRH-R-III amplification on pre-pubertal male samples, (D) GAPDH amplification. (B, C and D) 1 = anterior pituitary, 2 = hind brain, 3 = cerebrum, 4 = cerebellum, 5 = median eminence, 6 = anterior hypothalamus, 7 = posterior hypothalamus, 8 = olfactory bulb, 9 = optic lobe, 10 = adrenal gland, 11 = kidney, 12 = small intestine, 13 = spleen, 14 = testes or ovary, 15 = liver, 16 = non-template control.

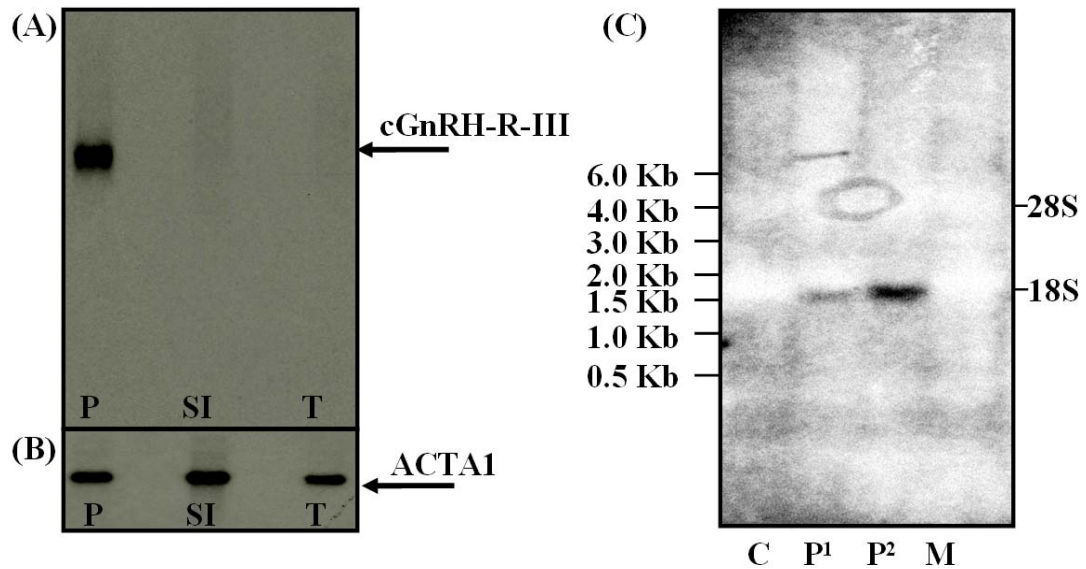
Figure 3. 2

Fig. 3.2. Northern blots of cGnRH-R-III mRNA indicating a single transcript detectable with both oligoprobes and riboprobes. (A, B) Northern blot with pituitary (P), small intestine (SI) and testes (T) Poly (A)⁺ RNA samples (3 μ g) of 14 week old AIL male birds, hybridised with (A) cGnRH-R-III [γ -³²P]-dATP 5' end labelled oligoprobe and (B) Skeletal muscle alpha-actin-1 (ACTA1) ³²P-dCTP random primer labelled DNA probe. (C) Northern blot with pooled male and female cerebral cortex (C), pooled male and female muscle (M) and anterior pituitary glands (P¹=female, P²=male) total RNA samples (15 μ g) of white Leghorn layer type birds. Hybridised with GnRH-R-III antisense riboprobe spanning all 4 exons. The blot shows a single detectable band in the pituitaries and no detectable bands in the muscle or brain cortex tissues. Millennium RNA size markers were added to the original gel and sizes were transferred to the image by interpolation. The position of the 28S and 18S ribosomal DNA is indicated and can be seen as areas of reduced background on the image.

Figure 3. 3

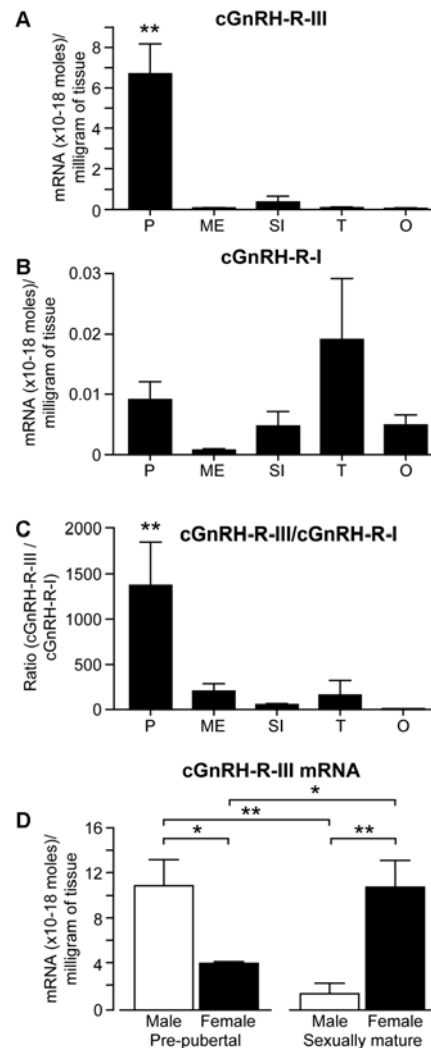


Fig. 3.3. Comparative quantitative real-time PCR analysis of cGnRH-R-I and cGnRH-R-III mRNA expression in tissues of pre-pubertal and sexually mature, male and female broiler chickens. (A) cGnRH-R-III mRNA expression observed per milligram of tissue. (B) cGnRH-R-I mRNA expression observed per milligram of tissue. (C) Ratio of absolute expression of cGnRH-R-III over cGnRH-R-I mRNA. The statistical significances in each experiment were calculated by applying an ANOVA to determine the effects of tissue type on cGnRH receptor expression. In (A) and (C) values of $p < 0.01$ are represented by two asterisk (**) and represents statistical significance compared to all other tissues. Values are mean (attomoles/milligram of tissue), represented as 1×10^{-18} moles/milligram of tissue \pm SEM. P = pituitary, ME = median eminence, SI = small intestine, T = testes and O = ovary. (No significant differences were detected between male and female gonads). (D) Relationship between sex and age on pituitary cGnRH-R-III mRNA expression in pre-pubertal ($n=6$) and sexually mature ($n=6$), male ($n=6$) (open columns) and female ($n=6$) (solid columns) broiler chickens. Values are mean (attomoles/milligram of tissue) \pm SEM * $p < 0.05$, ** $p < 0.01$.

3.4.4. Cloning of the type I GnRH receptor homolog cDNA into pcDNA1/Amp expression vector

An expression construct containing cGnRH-R-I cDNA cloned into pcDNA1/Amp (Invitrogen, UK) eukaryotic expression vector was used (Sun et al., 2001b) in parallel studies. The sequence of the cloned expression construct was confirmed by restriction enzyme digest (**Fig 3.4A-D**). The expression construct contains the entire coding sequence and included 69bp upstream from the start codon through to the stop codon (Sun et al., 2001b).

3.4.5. Cloning of the type III GnRH receptor homolog cDNA into pBluescript II SK⁽⁺⁾ vector

The sequence of the cGnRH-R-III_1210f-3217r-pBSK⁽⁺⁾ construct, the cloned PCR product from broiler pituitary cDNA pool amplified with primers cGnRH-R_(new)-1210f and cGnRH-R_(new)-3217r, was mapped by restriction digest (**Fig. 3.5A-D**). Automated sequencing of cGnRH-R-III_1210f-3217r-pBSK⁽⁺⁾ construct using vector primers M13-Forward and M13-Reverse, indicated that the product from a meat type broiler strain was identical to the exons in the genomic DNA sequence from *Gallus gallus gallus* (Ensembl 2004) and White Leghorn strains (matching AY895154), following analysis with ClustalW sequence alignment software (Chenna et al., 2003). The expression construct contained the entire coding sequence and included 30bp upstream from the start codon through to 97bp after the stop codon (**Fig. 3.5A-D**). The cGnRH-R-III gene resides on chromosome 10 (UCSC Genome Browser Chr 10: 20593844bp-20595762bp: Version_Feb2004), which is relatively close to the location of the cGnRH-R-I gene (UCSC Genome Browser Chr 10: 18495117bp-18496853bp: Version_Feb2004) (**Fig. 3.6**).

3.4.6. Cloning of the type III GnRH receptor homolog cDNA into pCDNA3.1/myc-His-A expression vector

The sequence of the cGnRH-R-III_1210f-3217r-pCDNA3.1/myc-His-A expression construct was mapped by restriction digestion (**Fig. 3.7A-D**) and determined by sequencing using vector primer T7-Forward. The expression construct contained the entire coding sequence and included 30bp upstream of the start codon through to 97bp after the stop codon. The myc-His epitope encoded in the expression vector was not utilised.

Figure 3. 4

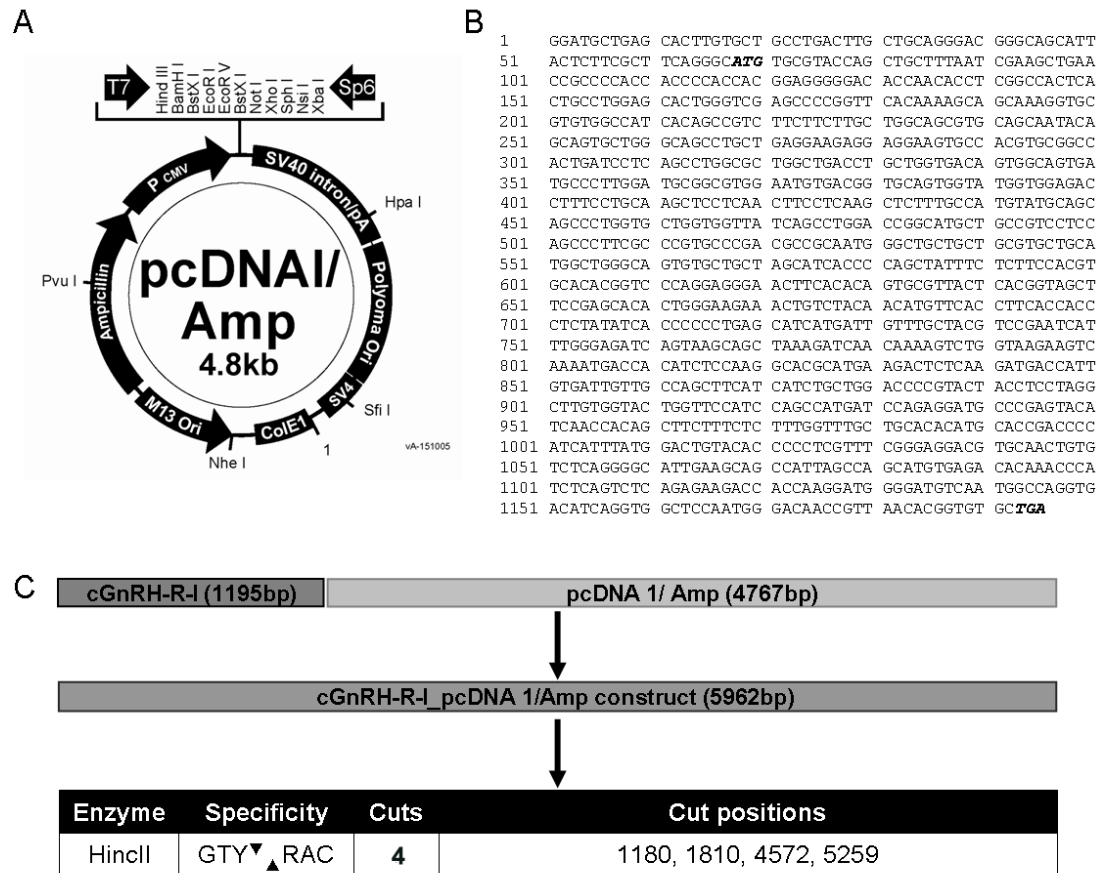


Fig 3.4. Schematic representation of the construction of the cGnRH-R-I_pcDNA 1/Amp construct as described in (Sun et al., 2001a; Sun et al., 2001b). (A) The pcDNA 1/Amp vector MCS. Col E1 origin (1-587bp), M13 origin (588-1180bp), Ampicillin gene (1360-2303bp), CMV promoter (2304-2954bp), T7 primer sequence (2938-2957bp), Polylinker (2956-3074bp), Sp6 primer sequence (3075-3093bp), splice and PolyA (3798-4693bp), SV40 origin (4634-4797). (B) The amplified anterior pituitary cDNA insert is as described (Sun et al., 2001a; Sun et al., 2001b). The translation start site (58-60bp) and the translation termination site (1193-1195) are indicated in bold italics. (C) The restriction enzyme sites used to validate the integrity of the recovered construct. The cut positions are with reference to the 5962bp cGnRH-R-I_pcDNA 1/Amp construct.

Figure 3.6

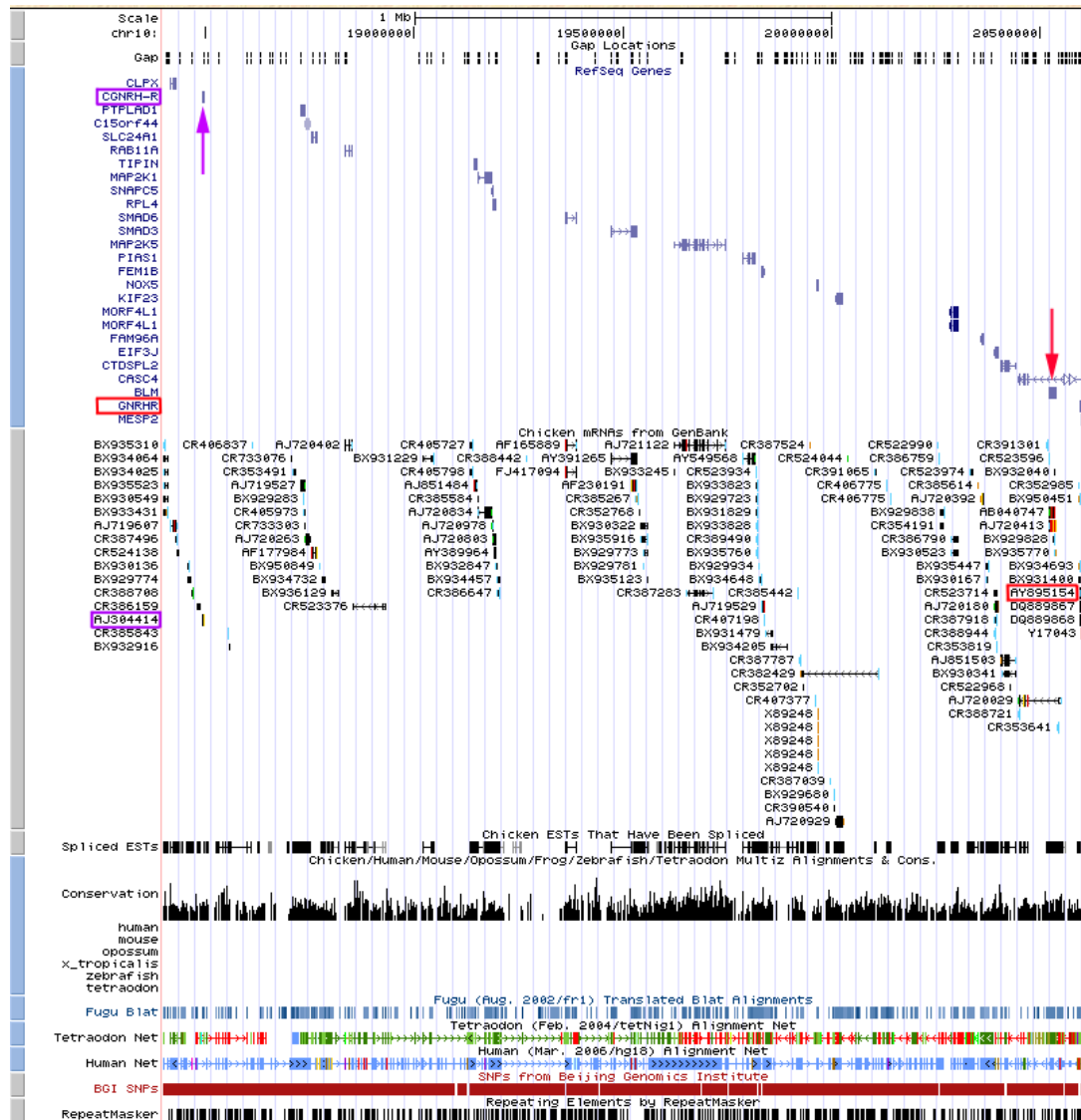


Fig 3.6. UCSC Genome Browser focused on chicken Chromosome 10 position 18,400,000-20,600,000 [Feb. 2004 Assembly]. (<http://genome.ucsc.edu/cgi-bin/hgGateway>). cGnRH-R-III (purple box and indicated with purple arrow) gene resides on chromosome 10:20593844bp-20595762bp, which is close to the location of cGnRH-R-I (red box and indicated with red arrow) gene which resides on chromosome10:18495117bp-18496853bp.

Figure 3. 7

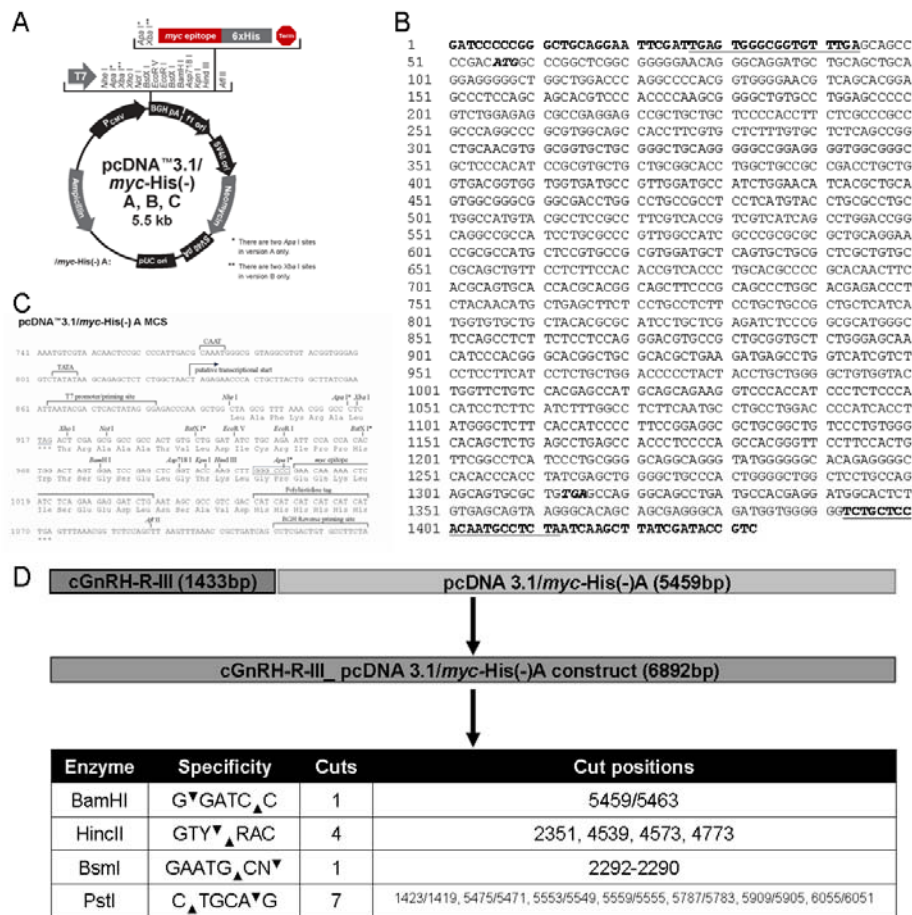


Fig. 3. 7. Schematic representation of the construction of the cGnRH-R-III_pcDNA3.1/myc-His(-)A plasmid. (A) The pcDNA 3.1/myc-His(-)A vector. (C) pcDNA 3.1/myc-His(-)A multiple cloning site region. CMV promoter (209-863bp), T7 promoter/priming site (863-882bp), Multiple cloning site (895-1006bp), myc epitope (1007-1036), Polyhistidine tag 1052-1069bp), BGH reverse priming site (1113-1130bp), BGH polyadenylation signal (1116-1343bp), f1 origin (1389-1817bp), SV40 promoter and origin (1844-2152bp), Neomycin resistance gene (2227-3021bp), SV40 polyadenylation signal (3195-3325bp), pUC origin (3708-4381bp), Ampicillin resistance gene (5426-5386bp:complementary strand). (B) The insert sequence removed from cGnRH-R-III_1210f-3217r-pBSK(+) construct, using restriction enzymes *BamHI* and *HincII*. The regions of the removed pBSK(+) vector are annotated in bold. The primer sequences used to amplify the original cGnRH-R-III_1210f-3217r insert are annotated in bold and underlined characters. The translation start site (56-58bp) and the translation termination site (1315-1318) are indicated in bold italics. (D) The restriction enzyme sites used to validate the integrity of the construct cloned by inserting the cGnRH-R-III-pBSK(+) insert into the pcDNA3.1/myc-His(-) vector between the *BamHI* and *HincII* sites of the MCS. The base pair sites are numbered with reference to the 6892bp cGnRH-R-III_pcDNA3.1/myc-His(-) A construct.

3.4.7. Type III GnRH receptor gene structure and amino acid sequence motif identification

Bioinformatics comparisons of the chicken type III GnRH receptor cDNA and genomic DNA sequences confirmed that the predicted receptor sequence consists of a 419 amino acid protein encoded by 4 exons on chicken chromosome 10 linked to the type I GnRH receptor. The amino acid sequence identity of the two cGnRH-Rs is 53% (**Fig. 3.8A**). The amino acid sequences of both cGnRH-R receptors were compared with human, bullfrog, leopard gecko and African clawed frog GnRH receptor subtypes (**Table 3.1**). The highest homology of cGnRH-R-I was with bullfrog-II GnRH-R. The highest homology of cGnRH-R-III was with bullfrog-I and leopard gecko-I GnRH-Rs. (Note: the existing nomenclature for the bullfrog and leopard gecko subtypes is confusing, as they have not been designated according to phylogenetic classification) (**Table 3.1**). Phylogenetic analysis (**Fig. 3.8B**) clearly classes this receptor as a type III GnRH receptor homolog, with a high bootstrap value (99%) supporting the classification between type II and type III GnRH receptor clusters. The bullfrog-I GnRH-R and the leopard gecko-I cGnRH receptor homologue lie within the type III GnRH receptor homolog cluster.

Using cDNA sequence alignment with genomic DNA sequence, the gene structure of cGnRH-R-III was determined. The putative translation start codon is coded in exon 1 and the stop codon in exon 4. The amino terminal extracellular domain is coded by exon 1 and exon 2. Exon 2 also encodes transmembrane domain (TMD) 1, intracellular loop (ICL) 1, TMD2, extracellular loop (ECL) 1, TMD3, ICL2 and part of TMD4. Exon 3 encodes the rest of TMD4, ECL2, TMD5 and part of ICL3. Exon 4 encodes the rest of ICL3, TMD6, ECL3, TMD7 and the C-terminal cytoplasmic domain (**Fig. 3.8A**). Comparisons of functionally important amino acid residues and sequence micro-domains were performed using amino acid sequence alignment. cGnRH-R-III like cGnRH-R-I has aspartic acid residues (Asp/D) in both loci of the helix2/helix7 micro-domain (amino acid positions; cGnRH-R-I = 87 and 310, cGnRH-R-III = 113 and 345) (**Fig 3.8A**). The arginine cage motif (DRXXX(I/V) and

alanine residue (Ala/A) in ICL3 are maintained in cGnRH-R-III at positions 164 and 286 respectively (**Fig 3.8A**). The two cGnRH-Rs exhibit conservation of the aspartic acid (Asp/D) (amino acid positions; cGnRH-R-I = 98 and cGnRH-R-III = 124) and asparagine (Asn/N) (amino acid positions; cGnRH-R-I = 102 and cGnRH-R-III = 128) residues in TMD2 (**Fig 3.8A**). The lysine (Lys/K) residue in TMD3 at position 121 of cGnRH-R-I is conservatively substituted with an arginine (Arg/R) residue at position 147 of the cGnRH-R-III. The glutamate (Glu/E) residue in ECL3 is not conserved in cGnRH-R-III at position 288 whereas it is present in cGnRH-R-I at position 328. cGnRH-R-I has a PEY motif in ECL3 at position 291 - 293 whereas in cGnRH-R-III this motif is PPS at position (327-329) (**Fig 3.8A**). The cytoplasmic tail domain of cGnRH-R-III is longer by an additional 8 residues (**Fig 3.8A**). Three putative Src homology domain 3 (SH3) binding motifs were identified exclusively in the type III cGnRH receptor cytoplasmic C-terminal tail domain (sequence = EPEPP, EPPSP, GPHPP), which are not present in the type I cGnRH-R. These motifs are located at amino acid positions 369-373, 370-375 and 398-402 encoded by exon four of cGnRH-R-III (**Fig 3.8A**).

Figure 3. 8

A

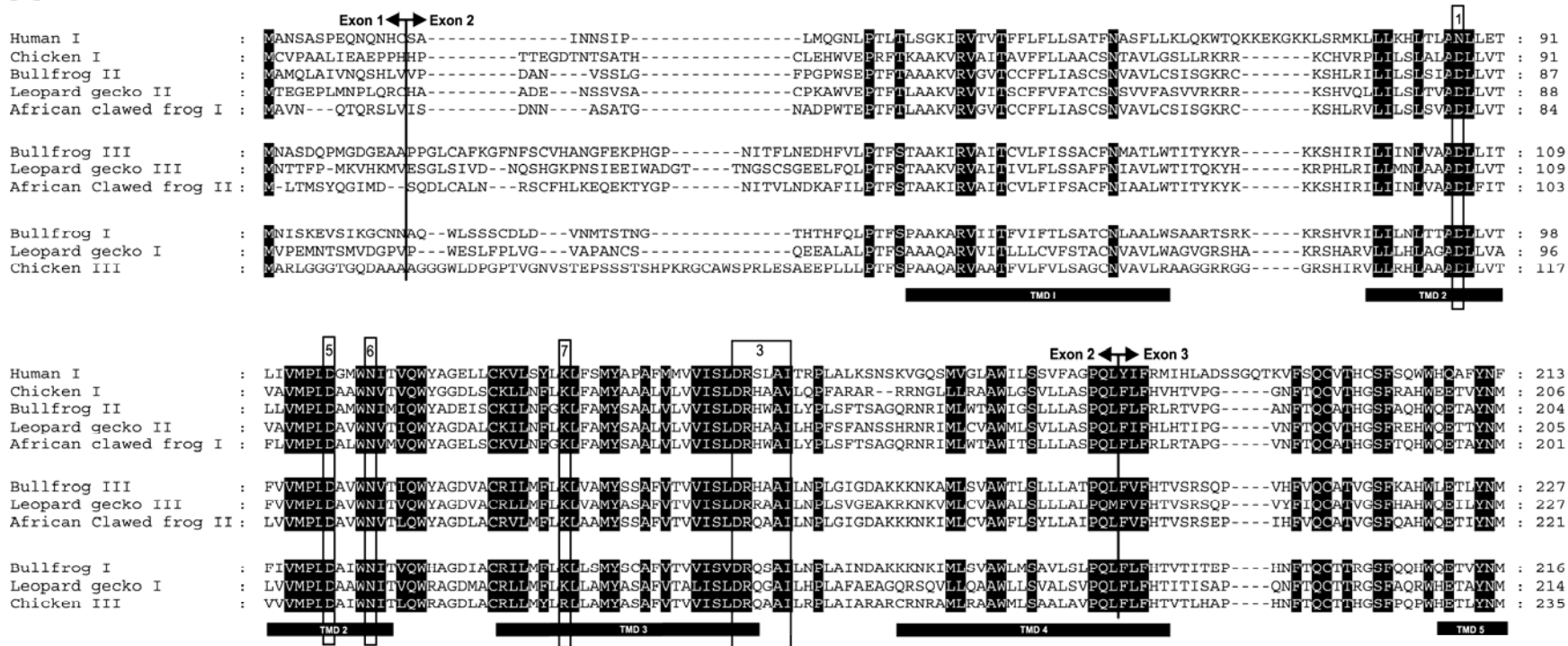


Fig. 3.8A continued on next page

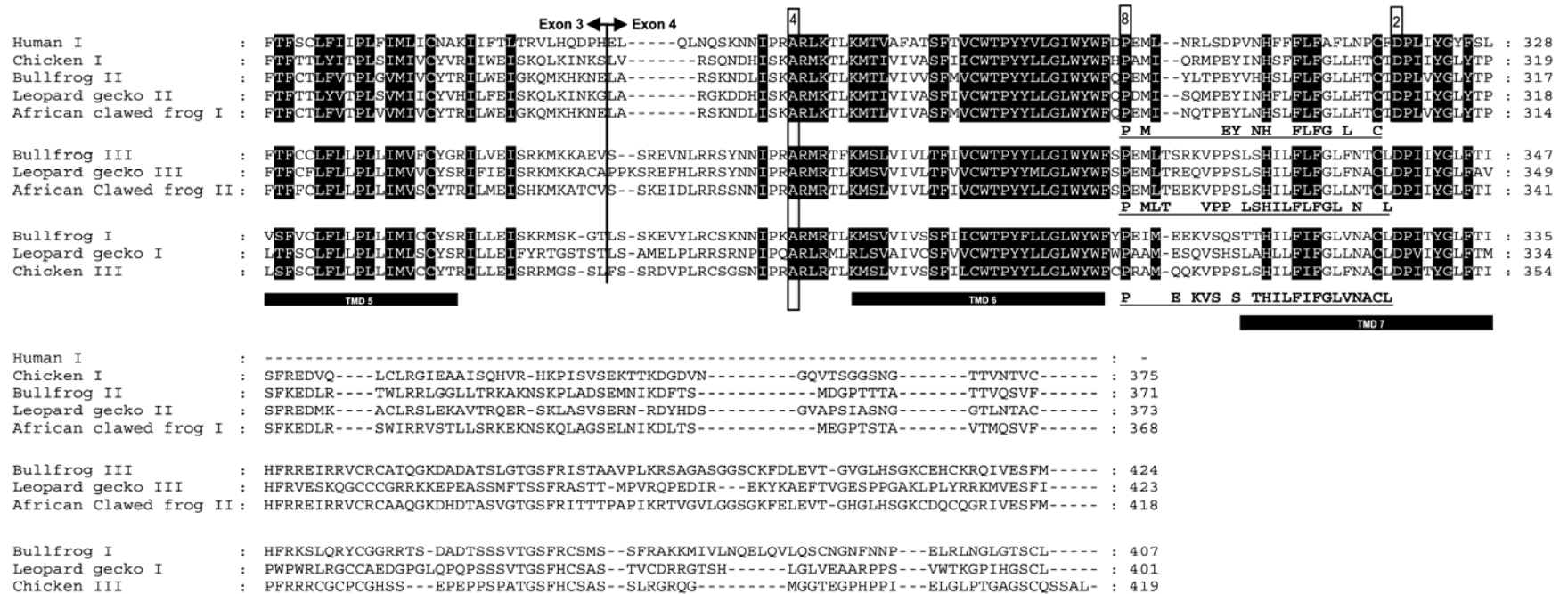


Fig. 3. 8. (A) Alignment of human, bullfrog, leopard gecko, African clawed frog and chicken GnRH Receptor sequences, representative sequences of GnRH receptor types I, II and III. Motifs in ECL3 are depicted in bold and underlined. Ubiquitously conserved amino acid residues are shown with dark background shading. Boxed amino acids are as follows: 1 and 2 = aspartic acid residue in the functional helix2/helix7 microdomain of non-mammalian GnRH receptors, 3 and 4 = conserved coupling arginine cage motif and alanine residue, 5, 6, 7 and 8 = ligand binding residues; aspartic acid residue, asparagine residue, lysine residue and arginine residue respectively. Predicted transmembrane domains (TMD 1-7) are indicated by a bold horizontal line. GnRH receptor subtypes are labelled according to the published terminology and presented in Roman numerals (Ikemoto and Park, 2007; Wang et al., 2001a).

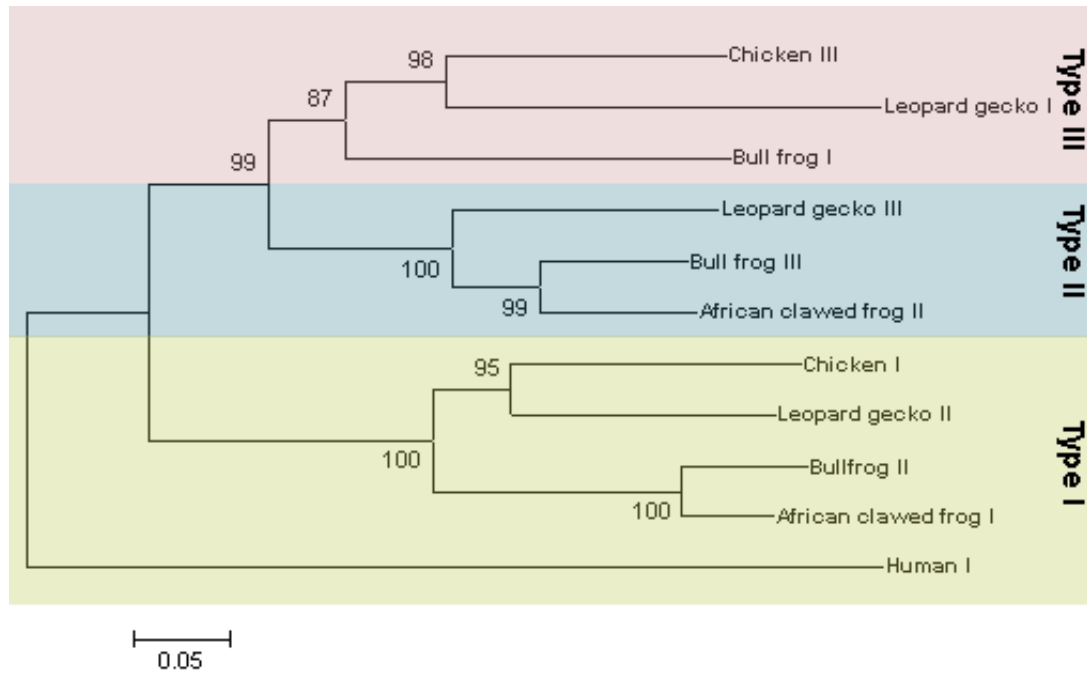
B

Fig. 3.8. (B) A phylogenetic tree inferred using the neighbour joining method based on multiple sequence alignment of representative, reptilian, amphibian and avian GnRH receptor amino acid sequences. Phylogenetic analyses were conducted in MEGA4 (Tamura et al., 2007). The bootstrap consensus tree inferred from 1000 replicates is taken to represent the evolutionary history of the taxa analyzed (Felsenstein, 1985). Branches corresponding to partitions reproduced in less than 50% bootstrap replicates are collapsed. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (Felsenstein, 1985). The tree is drawn to scale, with branch lengths in the same units as those of the evolutionary distances used to infer the phylogenetic tree. The evolutionary distances were computed using the Poisson correction method (Zuckerandl and Pauling, 1965) and are in the units of the number of amino acid substitutions per site. All positions containing gaps and missing data were eliminated from the dataset (Complete deletion option). There were a total of 300 positions in the final dataset.

Table 3. 1

Comparison of the amino acid identities of GnRH-R subtypes. The GnRH receptor names of the bullfrog and the leopard gecko are according to the published terminology of each receptor subtype, presented in Roman numerals (Ikemoto and Park, 2007; Wang et al., 2001a).

GnRH-R subtype	Identity (%) with cGnRH-R-I	Identity (%) with cGnRH-R-III
Chicken I	100	53
Chicken III	53	100
Bullfrog I	51	65
Bullfrog II	63	51
Bullfrog III	51	60
African clawed frog I	65	52
African clawed frog II	51	61
Leopard gecko I	50	65
Leopard gecko II	69	51
Leopard gecko III	51	58
Human	42	43

3.4.8. *In-situ* hybridisation to detect cGnRH-R-III mRNA

***In-situ* hybridisation using Digoxigenin (DIG)-labelled RNA probes on whole tissues**

No DIG-specific staining could be identified in the 18 month old female layer (**Fig. 3.9A, C**) pituitary gland hybridised with DIG-labelled cGnRH-R-III antisense RNA probe. There was clear DIG-specific staining in the pituitary gland of an 18 month old female layer pituitary gland hybridised with DIG-labelled pro-opiomelanocortin

(POMC) antisense RNA probe used as a positive control (**Fig 3.9B, D**). Whole-mount pituitary glands (**Fig** were sectioned to try to identify the cellular localisation of DIG-specific labelling (**Fig. 3.10A-D**). DIG-specific staining localised in the pituitary gland hybridised with DIG-labelled POMC antisense RNA probe (**Fig. 3.10C, D**), demonstrating that the probe permeated only the outer surface of the tissue and the methodology used was optimal for detecting mRNA expression on the outer surface of the pituitary gland. The expression of cGnRH-R-III mRNA was not detectable on the outer surface of the pituitary gland.

***In-situ* hybridisation using DIG-labelled RNA probes on sectioned tissues**

DIG-specific staining was identified in 18 month old male layer bird (**Fig. 3.11A-B**) pituitary gland sections hybridised with DIG-labelled POMC antisense RNA probe. The DIG-labelled cGnRH-R-III sense RNA probe did not hybridise to 18 month old female layer pituitary gland sections (**Fig. 3.11C-D**). There was no evidence of DIG-specific staining of sections hybridised with DIG-labelled cGnRH-R-III antisense RNA probe (**Fig. 3.11E-F**).

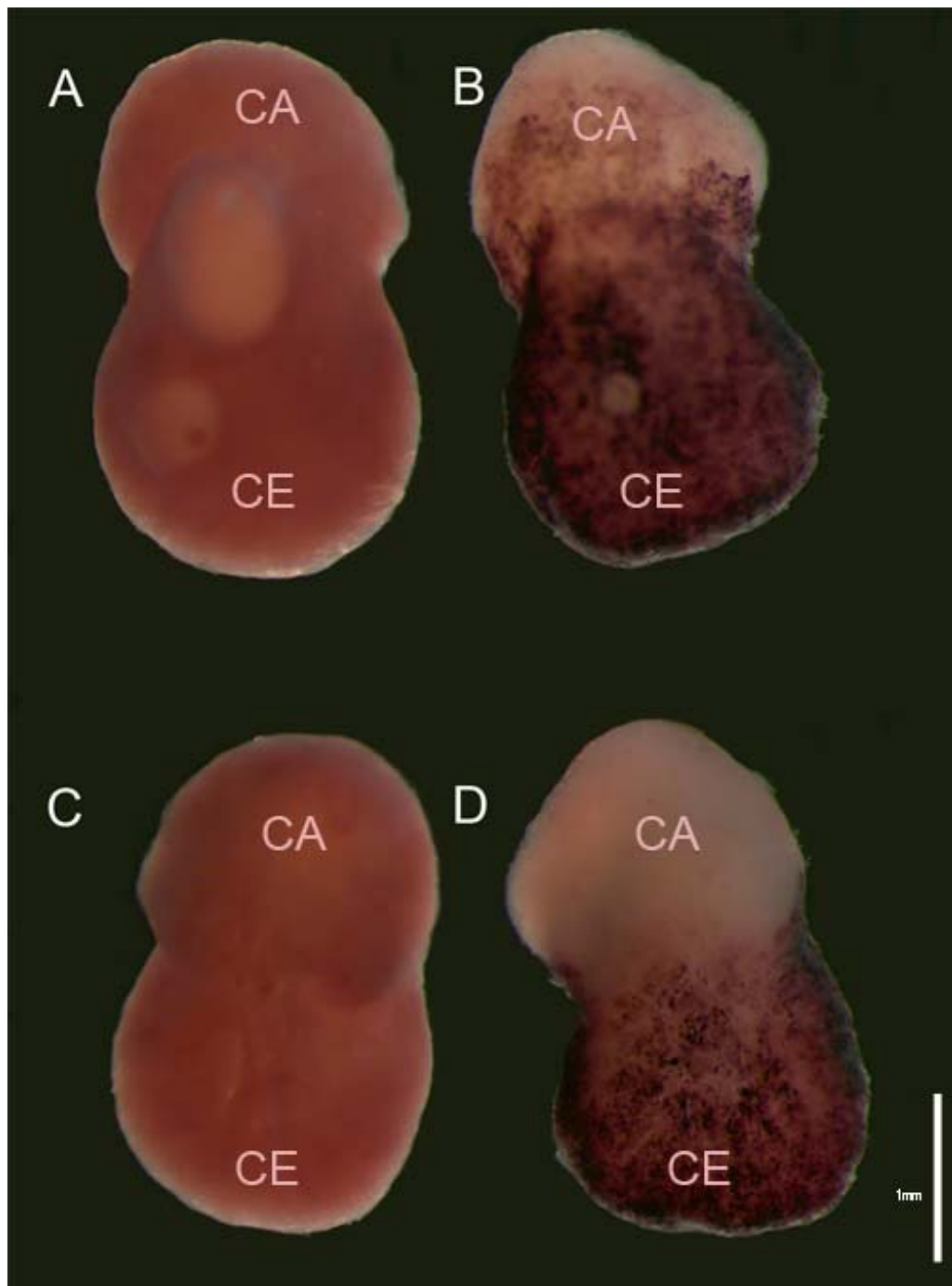
Figure 3. 9

Fig. 3. 9. 18 month old female layer anterior pituitary gland whole-mount *in-situ* hybridisation with DIG-labelled cGnRH-R-III antisense RNA probe (A, C) and DIG-labelled POMC antisense RNA probe (B, D). (A, B) Dorsal view of cephalic (CE) and caudal (CA) lobes of the pituitary gland. (C, D) Ventral view of cephalic (CE) and caudal (CA) lobes of the pituitary gland.

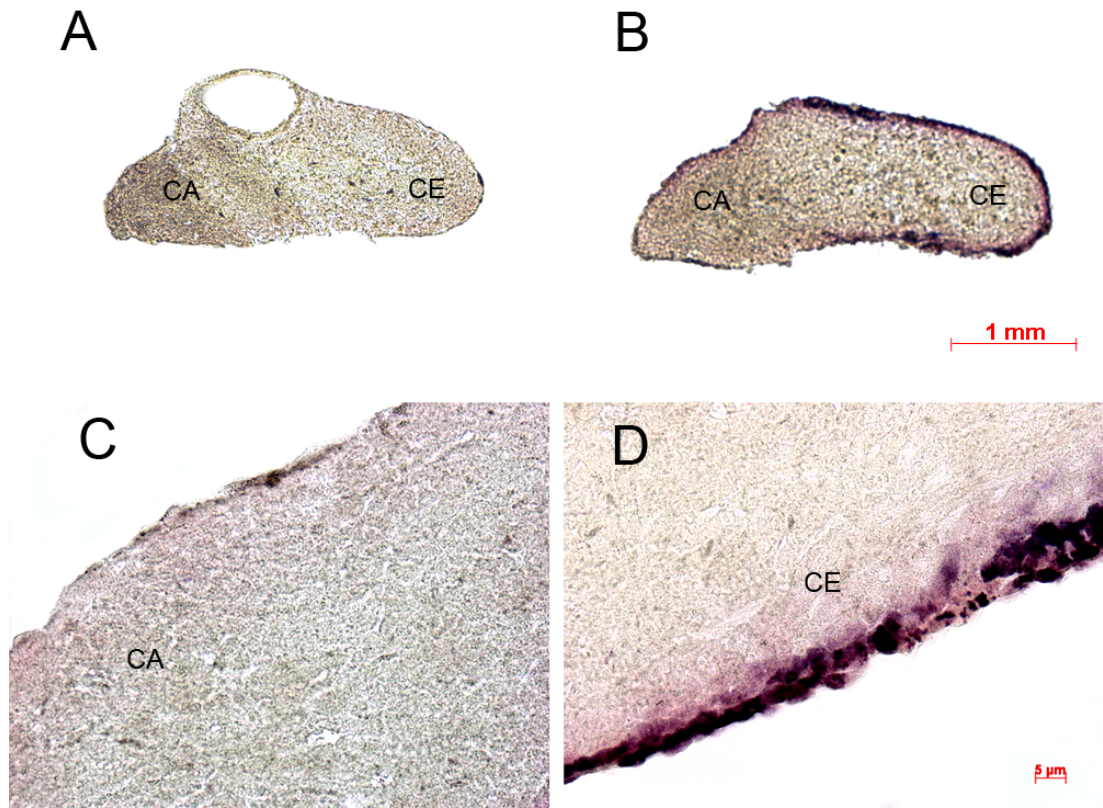
Figure 3. 10

Fig. 3.10. 30µm sagittal sections of sectioned whole-mount chicken anterior pituitary gland *in-situ* hybridisation samples. (Note: the whole-mount samples were sectioned subsequent to performing the *in-situ* hybridisation). CE=cephalic lobe and CA-caudal lobe. (A, C) DIG-labelled cGnRH-R-III antisense RNA probe. (B, D) DIG-labelled POMC antisense RNA probe. (Note: strong surface staining with POMC probe)

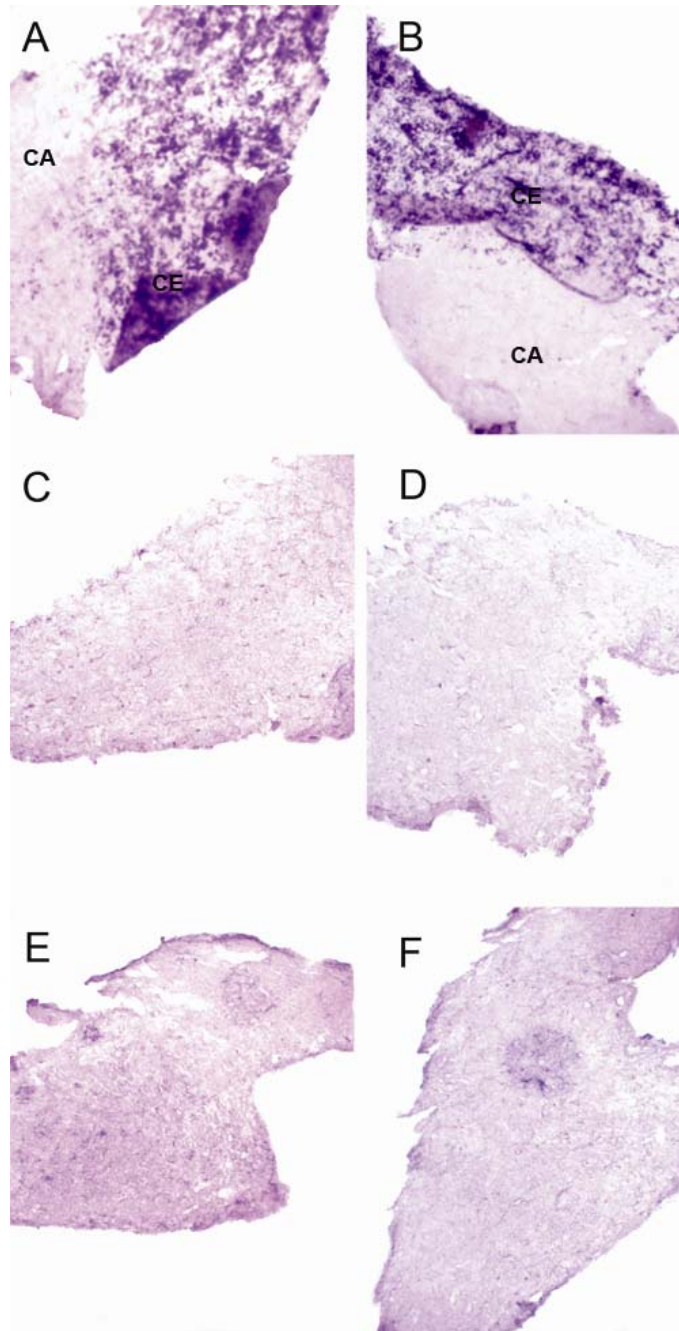
Figure 3. 11

Fig. 3.11. 18 month old male layer pituitary parasagittal sections hybridised with; (A, B) DIG-labelled POMC antisense RNA probe. (C, D) DIG-labelled cGnRH-R-III sense RNA probe. (E, F) DIG-labelled cGnRH-R-III anti-sense RNA probe. The tissues were sectioned at 25 μ m thickness, and images were captured at 10X objective magnification. Cephalic lobe = CE, Caudal lobe = CA.

***In-situ* hybridisation using [³⁵S] dATP(alphas) labelled oligonucleotide probes on sectioned tissues**

No obvious differences in the density of silver grains were detected in 26 week old broiler breeder pituitary sections hybridised with [³⁵S]dATP labelled 73-120-cGnRH-R-III_sense, 73-120-cGnRH-R-III_antisense, 1261-1290-cGnRH-R-III_sense or 1261-1290-cGnRH-R-III_antisense oligonucleotide probes exposed to photographic emulsion for 30 days (**data not shown**). Silver grain distribution did suggest sites of transcript expression for cGnRH-R-III. However, silver grains were present on both the area of the section and the slide, indicating that this represents the level of non-specific background. Therefore in an attempt to amplify the signal, [³⁵S]dATP cGnRH-R-III sense (73-120-cGnRH-R-III_sense + 1261-1290-cGnRH-R-III_sense) negative control and [³⁵S]dATP cGnRH-R-III antisense (73-120-cGnRH-R-III_antisense + 1261-1290-cGnRH-R-III_antisense) oligonucleotide cocktail probes were used. Hybridisation using [³⁵S]dATP POMC oligonucleotide probes was detected abundantly in the cephalic lobe of the anterior pituitary sections of sexually mature advanced intercross layer (AIL) birds, reflected by a high and clustered silver grain density (**Fig. 3.12E**). There was no increase in abundance of silver grains detected in the sections hybridised with [³⁵S]dATP cGnRH-R-III sense cocktail (**Fig. 3.12A, B**) probes or [³⁵S]dATP cGnRH-R-III antisense cocktail probes (**Fig. 3.12C, D**). Although in one particular pituitary section hybridised with [³⁵S]dATP cGnRH-R-III antisense probe (**Fig. 3.12F, G, H**) an abundance of silver grains distributed in a network pattern were evident, which potentially indicated detection of cGnRH-R-III mRNA.

In another study, hybridisation of [³⁵S]dATP POMC antisense oligonucleotide probe was detected abundantly in the cephalic lobe of 15µm thick anterior pituitary gland sections, reflected by a high silver grain density in both male (**data not shown**) and female (**Fig. 3.13A, B**) sexually mature birds of an advanced intercross lay (AIL) pedigree. There was no evidence of the [³⁵S]dATP cGnRH-R-III sense cocktail

probes hybridising to the anterior pituitary sections of both AIL male (**data not shown**) or female (**Fig. 3.13C, D**) sexually mature birds, as no clustering of silver grains was apparent. There was no increase in abundance of silver grains in (**Fig 3.13E, F**) compared to hybridisation of the cGnRH-R-III sense oligonucleotide cocktail probes (**Fig. 3.13C, D**). Therefore, there was no evidence of hybridisation of the [³⁵S]dATP cGnRH-R-III oligonucleotide antisense cocktail probes to the pituitary glands of sexually mature AIL male (**data not shown**) or female (**Fig. 3.13E, F**) birds. No increase in the abundance of silver grains was detected in sexually mature female (**data not shown**) or male AIL samples exposed to photographic emulsion for an additional 40 days of the [³⁵S]dATP POMC antisense (**Fig. 3.14A, B**) oligonucleotide probe or either the [³⁵S]dATP cGnRH-R-III sense (**Fig. 3.14C, D**) or antisense cocktail probes (**Fig 3.14E, F**).

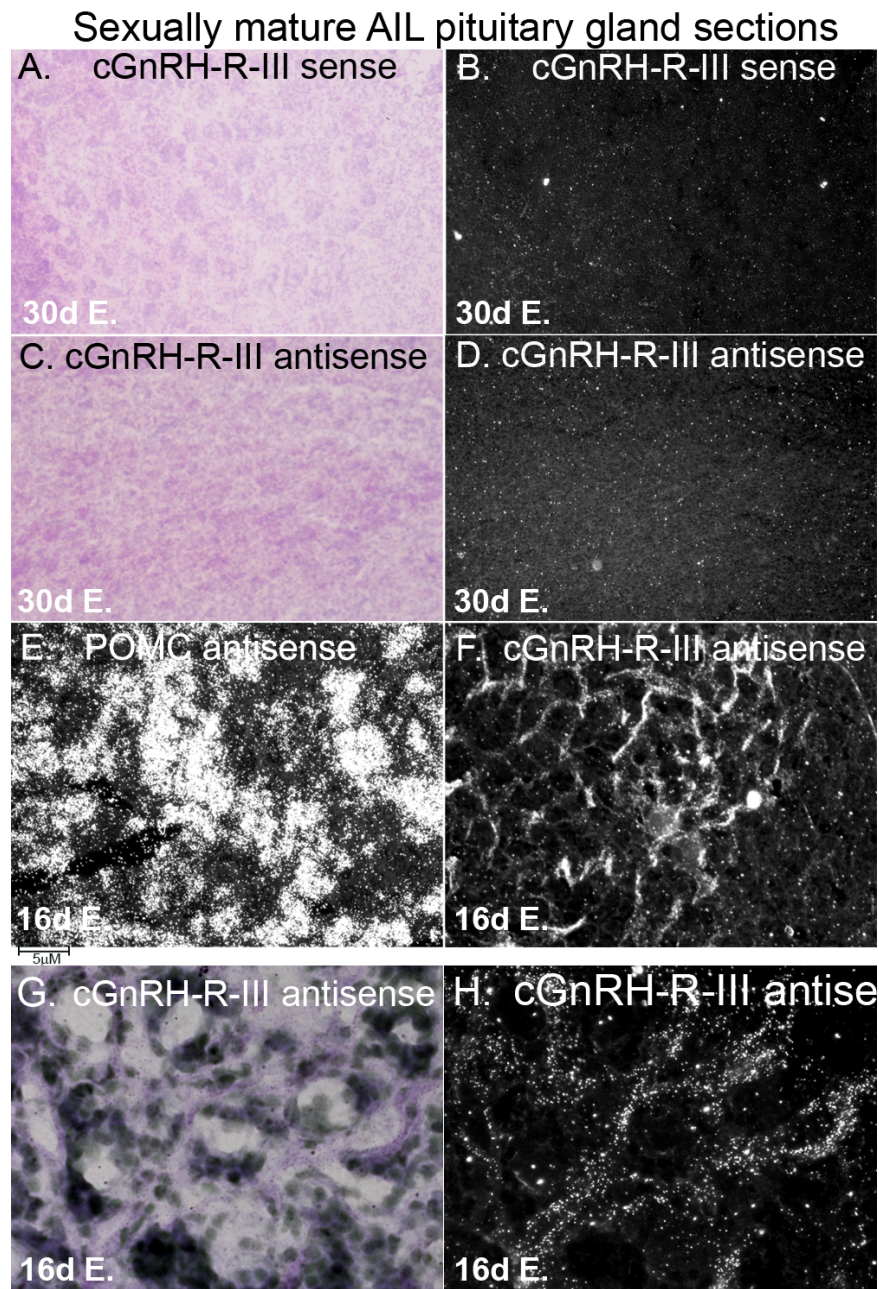
Figure 3. 12

Fig. 3.12. *In-situ* hybridisation of cGnRH-R-III (sense and antisense) and POMC (antisense) [³⁵S]dATP α S oligonucleotide probes to sexually mature AIL pituitary gland sections. (A, B) cGnRH-R-III_sense oligonucleotide cocktail. (C, D, F, G, H) cGnRH-R-III_antisense oligonucleotide cocktail. (E) POMC antisense oligonucleotide. Images are in dark field to visualise silver grains. (B, D, E, F, H) and bright field to visualise Haematoxylin and Eosin stained cells (A, C, G). The slides were exposed to emulsion for 16 days (E, F, G, H) and 30 days (A, B, C, D). (G, H) 2x magnification of section captured in image (F). The tissues were sectioned at 15 μ m thickness.

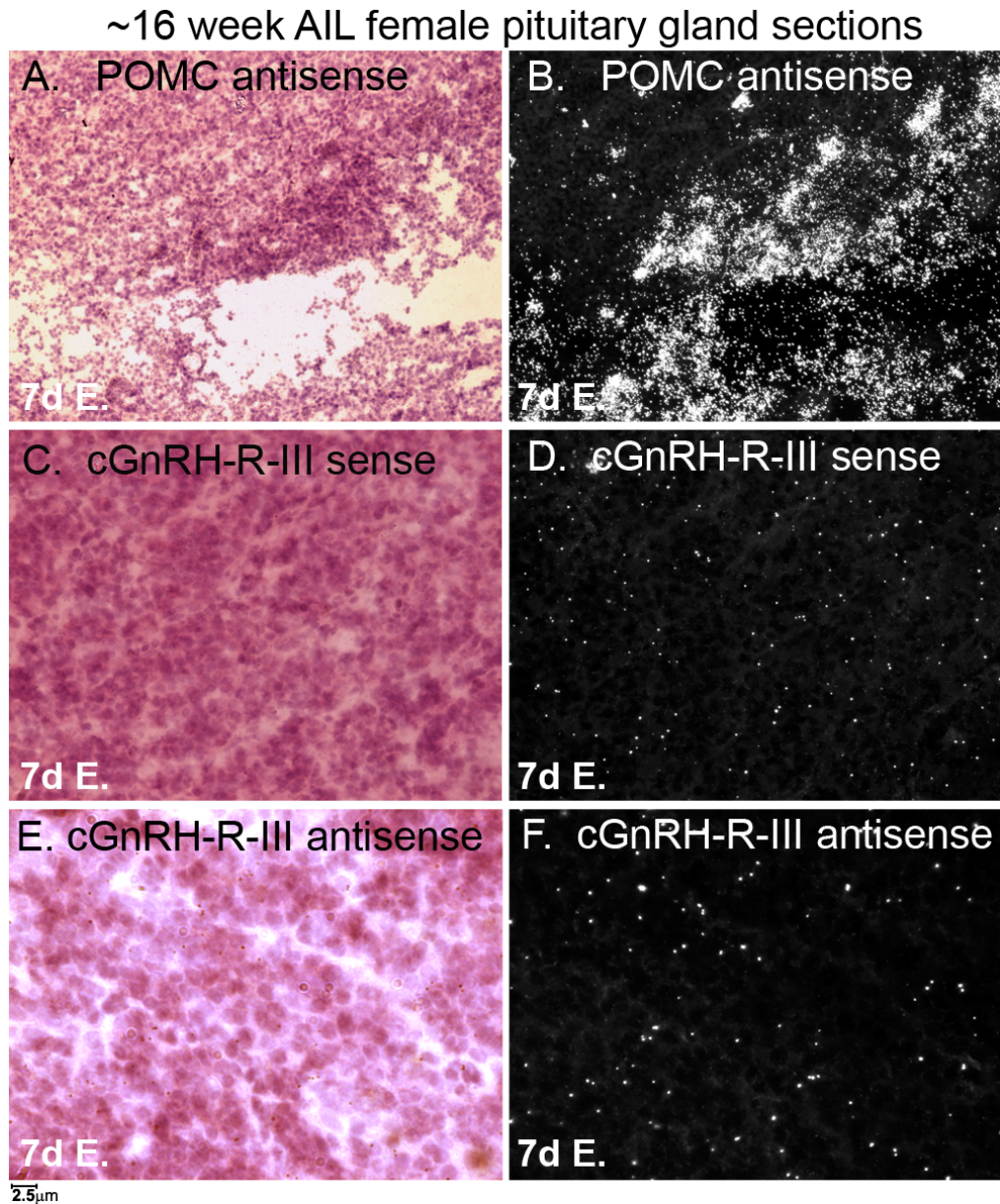
Figure 3. 13

Fig. 3.13 *In-situ* hybridisation of cGnRH-R-III sense and antisense and POMC antisense [³⁵S]dATP α S oligonucleotide probes to sexually mature (approximately 16 weeks old) AIL female pituitary gland sections. (A, B) POMC antisense oligonucleotide. (C, D) cGnRH-R-III_sense oligonucleotide cocktail. (E, F) cGnRH-R-III_antisense oligonucleotide cocktail. Images are in dark field to visualise silver grains. (B, D, F) and bright field to visualise Haematoxylin and Eosin stained cells (A, C, E). The slides were exposed to emulsion for 7 days. The tissues were sectioned at 15µm thickness.

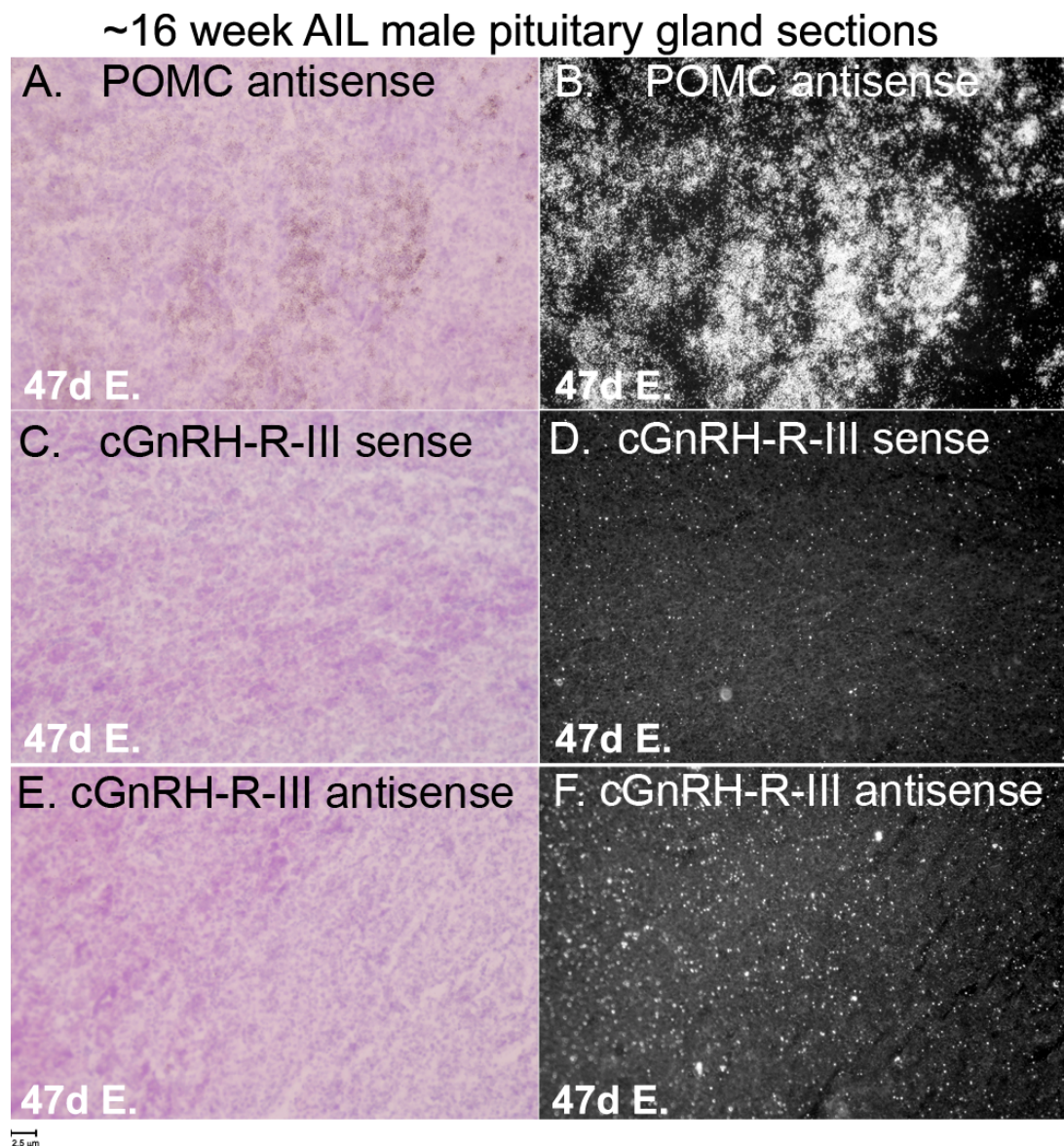
Figure 3. 14

Fig. 3.14. *In-situ* hybridisation of cGnRH-R-III sense and antisense and POMC anti-sense [³⁵S]dATP α S oligonucleotide probes to sexually mature (approximately 16 weeks old) AIL male pituitary sections. (A, B) POMC anti-sense oligonucleotide. (C, D) cGnRH-R-III_sense oligonucleotide cocktail. (E, F) cGnRH-R-III_antisense oligonucleotide cocktail. Images are in dark field to visualise silver grains. (B, D, F) and bright field to visualise Haematoxylin and Eosin stained cells (A, C, E). The slides were exposed to emulsion for 47 days. The tissues were sectioned at 15μm thickness.

3.4.9. Laser capture enrichment of fixed LH gonadotrophs for cGnRH-R subtype RT-PCR quantification

Rapid IHC detection of LH-specific gonadotrophs on sectioned pituitary glands

Female 'out of lay', layer silkie cross birds were perfusion fixed with Zamboni's fixative through the jugular vein or alternatively were dissected and fixed in acetone once sectioned. No effects of the tissue fixation methods were observed on the sections stained with anti-LH- β antibody (**data not shown**). The optimal staining methodology was a 1:800 dilution of the primary anti-turkey LH- β antibody incubated for 3 minutes. Immuno-reactive positive cells were stained as dark brown in colour, and the immuno-negative cells were faint brown (**Fig. 3.15**). Slides were kept in a box containing silica crystals to prevent re-hydration of the cells which inhibits Laser capture micro-dissection (LCM). The smallest laser spot diameter of the Acturus PIX Cell III Laser Capture Instrument is 7 μ m and the average size of a gonadotroph is 10 μ m. Thus it is theoretically possible to dissect out a pure gonadotroph. However, due to the method in which the thermostatic film forms a protrusion that bridges the gap between the cap and the target cell when activated by the laser, the actual size of the site of contact between the melted thermostatic film and the target cell is larger than the set laser spot diameter size. Additionally, lifting the cap results in the removal of the target cell which has adhered to the cap and at times is contaminated by fractions of adjacent cells. Therefore, The LCM proved unsuccessful as the area dissected by LCM was greater than that of a gonadotroph, resulting in contamination from adjacent cell types.

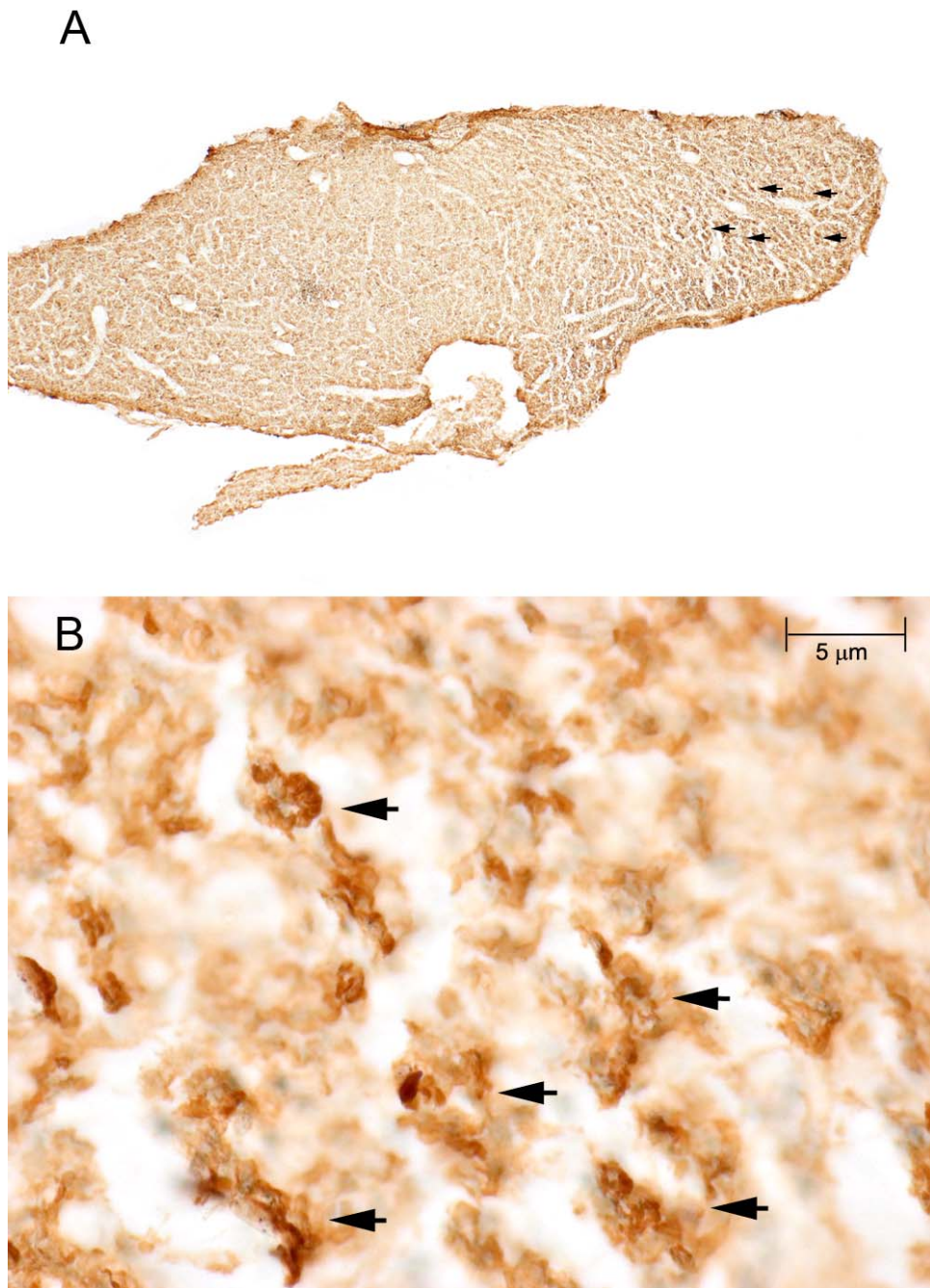
Figure 3. 15

Fig. 3. 15. Immunohistochemistry of pituitary samples from a female ‘out of lay’, layer silkie cross bird. (A) Sagittal 20µm frozen section at 4x objective magnification to detect LH specific-gonadotrophs which are visible as dark brown clusters of cells (indicated with black arrows). **(B)** Sagittal 20µm frozen sections to detect LH gonadotrophs which are visible as dark brown cells (indicated with black arrows).

3.5. Discussion

In this chapter, a novel chicken GnRH receptor cDNA expressed in the pituitary gland was cloned and sequenced (**Fig. 3.5, 3.6, 3.7, and 3.8**). Birds, like certain mammals, possess two GnRH receptor subtypes in contrast to fish, reptiles and amphibians in which three types of receptor are generally found (Ikemoto and Park, 2007; Morgan and Millar, 2004; Stewart et al., 2009). The proximity of the two chicken GnRH receptor subtype genes on chromosome 10 (approximately 2Mb apart) and their high amino acid sequence identity (53%), suggests they may have been derived by gene duplication (**Fig. 3.6**) (Joseph et al., 2009). Phylogenetic analysis indicates that the novel chicken GnRH receptor should be classified as a type III receptor. There has been an inclination by many researchers to designate GnRH receptors that are selective for GnRH-II as type II GnRH receptors. However, all non-mammalian receptors, regardless of their structural similarity appear to preferentially bind GnRH-II (Pfleger et al., 2002). Other investigators have designated GnRH receptors by their tissue-specific expression (type I in pituitary/type II in brain). This classification is also problematic as the predominant GnRH receptor expressed in bullfrog pituitary is structurally a type III receptor, (Wang et al., 2001a) while in African clawed frog it is a type I receptor (Troskie et al., 2000). Thus it is evident that there is considerable plasticity in utilisation of a particular GnRH receptor in a particular tissue. We therefore advocate that GnRH receptors should be designated names according to their structural and phylogenetic relatedness prior to assessing their physiological roles. cGnRH-R-III was first designated as cGNRHR2 (Shimizu and Bedecarrats, 2006), whereas re-designation as cGnRH-R-III is appropriate according to phylogenetic classification (**Fig. 3.8B**) (Joseph et al., 2009).

The first GnRH-R isoform to be cloned in the chicken, cGnRH-R-I is expressed at the transcriptional level in various tissues including the pre-optic anterior hypothalamus, basal hypothalamus, anterior pituitary gland, brain cortex, cerebellum, testes, heart and spleen; but not the liver or kidney (Sun et al., 2001a; Sun et al.,

2001b). The widespread expression of cGnRH-R-I transcripts was confirmed in this study (**Fig. 3.3A**). The novel cGnRH-R-III homolog was also shown to be widely transcribed (**Fig. 3.1B, C**) although levels are predominant in the anterior pituitary gland (**Fig. 3.2A, B and Fig. 3.3B**) (Joseph et al., 2009). Critically, the relative abundance of the two receptors mRNA transcripts in relation to each other has been established in this thesis, with cGnRH-R-III mRNA being expressed at a higher molar concentration than cGnRH-R-I mRNA in all tissues tested (**Fig. 3.3C**) (Joseph et al., 2009). In another study, PCR amplification of cGnRH-R-III cDNA was shown to be pituitary-specific (Shimizu and Bedecarrats, 2006). The comparison of the two cGnRH-R homolog cDNA expression patterns may be useful in determining the physiological importance of each receptor subtype. However, this approach is based on the assumption that cDNA is translated into protein in proportional quantities (**see Appendix B for additional data**). The higher expression (~1373 times) of cGnRH-R-III mRNA compared to cGnRH-R-I mRNA in the anterior pituitary gland (**Fig. 3.3C**), does suggest it is the physiologically important receptor in this organ. Although, more than one GnRH receptor subtype can be expressed within the same tissue, as seen in the sea bass (Moncaut et al., 2005) and goldfish (Illing et al., 1999) and now in the chicken (**Fig. 3.3C**) (Joseph et al., 2009). In the bullfrog and leopard gecko however, as in the chicken (**Fig. 3.3C**) mRNA for one of the three GnRH-R subtypes predominates in the pituitary (Ikemoto and Park, 2007; Joseph et al., 2009; Wang et al., 2001a). **In this study presented in this chapter, sex-specific plasticity in the spatiotemporal expression patterns of GnRH receptor subtypes has been demonstrated.** The predominance of one receptor subtype (type III) over another receptor subtype (type I) in the chicken pituitary is similar to that of the closest evolutionary species within the amphibian and the reptilian orders (Ikemoto and Park, 2007; Illing et al., 1999; Moncaut et al., 2005; Troskie et al., 2000; Wang et al., 2001a). Interestingly, the pituitary-specific GnRH receptor subtypes expressed in the bullfrog (Wang et al., 2001a), African clawed frog (Troskie et al., 2000) and leopard gecko (Ikemoto and Park, 2007) and chicken (**Fig. 3.8B**) (Joseph et al., 2009) differ structurally, according to their phylogenetic classification (Flanagan et al., 2007; Joseph et al., 2009; Millar et al., 2004; Tello et al., 2008). **These observations lend**

further support for the existence of plasticity in evolutionary recruitment of GnRH receptor subtypes for regulation of pituitary gonadotrophin production.

In this study, pituitary cGnRH-R-III mRNA expression levels can be correlated with reproductive status, suggesting that cGnRH-R-III plays a role in the regulation of gonadotroph function (Joseph et al., 2009). In contrast, no sex differences or changes in expression were detected for cGnRH-R-I mRNA (**data not shown**), compared to a pronounced reciprocal sex difference in pituitary cGnRH-R-III mRNA expression (**Fig 3.3.D**) (Joseph et al., 2009). Higher cGnRH-R-III mRNA levels occur in adult females compared to adult males (**Fig 3.3.D**) (Joseph et al., 2009). In contrast, in juvenile chickens, higher cGnRH-R-III mRNA levels occurred in males compared to females (**Fig 3.3.D**) (Joseph et al., 2009). Differences in cGnRH-R-III mRNA expression levels between juvenile and mature males and females as seen in this study (Joseph et al., 2009) were not previously reported in another study (Shimizu and Bedecarrats, 2006). This difference may be due to the precise age of animals used in different studies. The sexual dimorphism in cGnRH-R-III mRNA expression, where levels of mRNA are higher in juvenile males compared to juvenile females, is consistent with the LH response to photo-stimulation seen in juveniles, where there is a robust response in males but not in females (Sreekumar and Sharp, 1998). In adult males and females, cGnRH-R-III mRNA expression levels are inversely related to sex differences in LH responsiveness to GnRH injection (Sharp et al., 1987). When chicken pituitary fragments from adult males or juveniles of either sex are perfused, a large spike followed by a plateau in LH release in response to GnRH stimulation is observed (Liu et al., 1995). Adult laying hens do not exhibit this rapid spike of LH release but do show the plateau phase (Liu et al., 1995). The release of LH in adult hens is also much reduced compared to juvenile hens (Liu et al., 1995). In general, our observations suggest that the level of GnRH-R-III mRNA may be inversely related to the capacity to release LH by gonadotrophs in adult females (Joseph et al., 2009). It can be hypothesised that elevated levels of cGnRH-R-III mRNA in the adult female may be reflective of expression of membrane

cGnRH-R-III receptor number and that increased levels of ovarian steroid or peptide hormones may act to diminish the LH response to GnRH at the gonadotroph, through down regulation of intracellular signalling pathways, including Ca^{2+} mechanism in these cells (Liu et al., 1995) (**see Appendix B for additional data**).

In avian species, the two gonadotrophins, FSH and LH, are not expressed in the same cell type (Proudman et al., 1999; Puebla-Osorio et al., 2002). Both cGnRH-I and GnRH-II stimulate LH release from chicken pituitary *in-vitro* (Hattori et al., 1986a; Millar et al., 1986) and *in-vivo* (Chou et al., 1985b; Hattori et al., 1986b; Proudman et al., 2006; Sharp et al., 1986) however, there is conflicting evidence for an effect of cGnRH-I or GnRH-II on FSH *in-vitro*, (Hattori et al., 1986a; Millar et al., 1986) and *in-vivo* (Bruggeman et al., 1998; Dunn et al., 2003; Krishnan et al., 1993; Proudman et al., 2006). The quantification of the expression of cGnRH-R-I and cGnRH-R-III subtypes in the separate populations of gonadotrophs would allow inference about the role of cGnRH-R subtypes in the control of production of each gonadotrophin and how the secretion of LH and FSH may be differentially controlled by GnRH. However, in this thesis concerted attempts to confirm the cellular localisation of cGnRH-R-III expression within the pituitary gonadotroph cells using *in-situ* hybridisation or laser capture enrichment of gonadotrophs proved unsuccessful.

It was possible to detect POMC mRNA using *in-situ* hybridisation to chicken pituitary sections, indicating that the three methods used (DIG labelled RNA probes on whole-mount or cryostat sectioned pituitary glands and radiolabelled oligoprobes on sectioned pituitary glands) were effective (**Fig. 3.9.B, D; Fig. 3.10.B, D; Fig. 3.11.A, B; Fig. 3.12.E; Fig. 1.13.A, B and Fig. 3.14.A, B**). POMC is a peptide hormone precursor which contains sequences for melanocortins, lipotrophins and β -endorphin (Takeuchi et al., 1999), and is expressed in the cephalic lobe of the chicken pituitary gland (Gerets et al., 2000). High level expression of POMC in the cephalic lobes of the pituitary was confirmed in this study (**Fig. 3.9.B, D; Fig. 3.10.B, D; Fig. 3.11.A, B**). The DIG labelled cGnRH-R-III RNA probes

encompassed the entire cDNA fragment (~1300bp) (**Fig. 3.9.A, C; Fig. 3.10.A, C; Fig. 3.11.C-F**), and perhaps a smaller RNA probe may prove more successful due to greater tissue penetration and hybridisation efficiency. However, in view of the fact that the oligoprobes, which were previously shown to hybridise to the mRNA (**as shown in the Northern blots Fig. 3.2.A**) did not yield a signal in *in-situ* hybridisation indicates that the cGnRH-R-III mRNA may not be expressed abundantly enough to be readily detected by *in-situ* hybridisation (**Fig 3.12.A-D, F; Fig. 3.13.C-F; Fig. 3.14.C-F**). On two occasions however, the presence of silver grain clusters forming a network pattern (**Fig. 3.12F, G, H**) suggested cGnRH-R-III mRNA in pituitary cells, however, this was very difficult to reproduce (**Fig. 3.14**), and is likely to be an artefact, possibly due to cracking of the emulsion during drying. In addition to gonadotrophs, it is possible that GnRH receptors may be expressed in other pituitary cell types, including for example folliculo stellate (FS) cells. A number of growth factors and peptides are expressed in FS cells and FS cells may have a role in paracrine communication in the anterior pituitary gland (Allaerts and Vankelecom, 2005). It has been shown that activin which is expressed in FS cells may have a role in modulating GnRH-R expression (Kaiser et al., 1992; Rispoli and Nett, 2005). Therefore, it is plausible that cGnRH-R-III homolog mRNA may be expressed in the FS cells. More research into the expression of GnRH receptors in FS cells may prove beneficial in understanding cell-type specific expression of cGnRH-R subtypes.

Semi-quantitative studies on GnRH and GnRH receptor mRNA in the sheep have shown that the receptor is expressed approximately 6 times less abundantly in the pituitary compared to the relative expression of GnRH in the hypothalamus (Dolan et al., 2003). When comparing expression levels of genes, hypothalamic cGnRH-I in laying and incubating hens varies about 5 fold (between 38.7 and 7.7×10^{-17} mols per hypothalamus) (Dunn et al., 1996) and is 10-50 fold higher than that of the pituitary expression of cGnRH-R-III which was quantified in this study to be 6.8×10^{-18} mol per mg of tissue (**Fig. 3.3.A**) (Joseph et al., 2009). In general, mRNAs for G protein

coupled receptors are expressed at relatively low levels (Fraser et al., 1996). There have been previous reports of *in-situ* hybridisation detection of cGnRH-R subtypes mRNA in a variety of tissues in other species (Bahk et al., 2008; Bing et al., 2003; Crumeyrolle et al., 1994; Fraser et al., 1996; Quinones-Jenab et al., 1996; Wei-Quan et al., 2001). However, in this study cGnRH-R-III mRNA was not detectable using the same methodology. As mentioned, it is possible that the cGnRH-R-III mRNA is not expressed exclusively in gonadotrophs. GnRH receptor mRNA has been previously shown to be expressed in lactotrophs of the rat anterior pituitary (Mertani et al., 1996). This may explain why cGnRH-R-III mRNA was detectable in the anterior pituitary by Northern analysis (**Fig. 3.2**) but not by *in-situ* hybridisation (**Fig. 3.9-Fig. 3.14**). Receptor transcripts might not be concentrated in one particular subtype of pituitary cells, but may be spread over a number of different pituitary cell types. However, it may have been more appropriate to use a GPCR as a positive control in order to optimise the *in-situ* hybridisation methods, as POMC is expressed in much more abundant levels than GPCRs. Therefore, the methodology may not have been optimised for *in-situ* hybridisation of a GPCR and the optimised methods may thus not have been optimised for detecting cGnRH-R-III mRNA expression. This approach may be the focus of future investigations.

An alternative method to measure the cellular localisation of cGnRH-R-III and cGnRH-R-I mRNA in pituitary gonadotrophs was attempted by the application of immunohistochemical techniques to detect LH gonadotrophs followed by laser capture enrichment of the isolated population of cells and real-time quantitative RT-PCR (**Fig. 3.14.A, B**). Theoretically this methodology is possible for the selective extraction of cells from heterogeneous tissues (Fend et al., 1999; Mojsilovic-Petrovic et al., 2004), and has previously been performed for pituitary cell type enrichment in cichlid fish in combination with post-capture micromanipulation to remove undesirable tissue around the periphery of the targeted cells (Parhar et al., 2005). However, it proved technically difficult for chicken gonadotroph cells in our hands. Although detection of LH stained gonadotrophs was successful, the laser capture of

pure gonadotrophs was impossible because the size limit for the micro-dissected area was greater than that of a single gonadotroph cell (**Fig. 3.14.B**). It is plausible that this method could be applied to *in-vitro* dispersed pituitary cells in the future. A more involved method of employing double immunohistochemistry to detect the co-localisation of the cGnRH receptor subtypes in the gonadotroph cells may prove more effective at some point in the future.

Using RT-PCR, two cGnRH-R-III splice variant transcripts have been identified (Shimizu and Bedecarrats, 2006), a pituitary-specific transcript (1065bp splice variant) (Shimizu and Bedecarrats, 2006), in addition to another 501bp splice variant that is expressed in different brain regions (Shimizu and Bedecarrats, 2006). In this study, these cGnRH-R-III splice variant transcripts (Shimizu and Bedecarrats, 2006) were not detected by oligonucleotide probe or riboprobe using Northern blotting to pituitary gland RNA, (**Fig. 3.2.A-C**), suggesting that their *in-vivo* expression is relatively low and may be of little functional significance in the pituitary. cGnRH-R subtype splice variants have been detected in the bullfrog, mouse, wallaby and human (Cheung and Hearn, 2003; Grosse et al., 1997; Illing et al., 1993; Wang et al., 2001b; Zhou and Sealton, 1994). Of the identified cGnRH-R splice variants, only that of the bullfrog and sheep (Illing et al., 1993; Wang et al., 2001b) have been confirmed by Northern blotting (Cheung and Hearn, 2003; Grosse et al., 1997; Zhou and Sealton, 1994). Experimentally, GnRH-R splice variants were shown to possess inhibitory effects on the signalling of full-length receptors in the human and bullfrog (Grosse et al., 1997; Pawson et al., 2005; Wang et al., 2001b) and have been suggested to interfere with the function of the full-length receptors (Grosse et al., 1997; Pawson et al., 2005; Wang et al., 2001b) possibly through the formation of homo-dimers and hetero-dimers (Cheung and Hearn, 2005). The effects of the cGnRH-R-III splice variants on the function of the full length type III cGnRH receptor are investigated in **Chapter 4**.

Unlike mammalian GnRH receptors, which are encoded by 3 exons (Kakar, 1997; Zhou and Sealfon, 1994), both avian cGnRH-R-I and cGnRH-R-III are encoded by 4 exons (**Fig. 3.8.A**). The presence of functionally important residues and micro-domains in cGnRH-R-III were assessed. cGnRH-R-III like cGnRH-R-I has aspartic acid residues in both loci of the helix2/helix7 micro-domain (**Fig. 3.8.A**), an arrangement unique to non-mammalian GnRH receptors (Flanagan et al., 1999; Zhou et al., 1994), like other GPCRs. Conservation of important coupling motifs and residues, the arginine cage motif (Ballesteros et al., 1998) and alanine in ICL3 (Myburgh et al., 1998) is maintained in cGnRH-R-III at amino acid positions 164 and 286 respectively (**Fig. 3.8.A**). The two cGnRH-Rs exhibit conservation of the aspartic acid (Flanagan et al., 2000) and asparagine (Davidson et al., 1996) ligand-binding residues in TMD2 (**Fig. 3.8.A**). The lysine in TMD3 of the cGnRH-R-I (Zhou et al., 1995) is substituted with an arginine residue in cGnRH-R-III (**Fig. 3.8.A**). This conservative change is believed to preserve agonist binding to the receptor (Zhou et al., 1995). The glutamate residue in ECL3 at position 301 in the mouse which was shown to possess a role in receptor recognition of Arg⁸ in mammalian GnRH (Flanagan et al., 1994) is not conserved in cGnRH-R-III (**Fig. 3.8.A**) whereas it is present in cGnRH-R-I (Sun et al., 2001b). ECL3 of each receptor type has a distinctive sequence motif which has a key influence on receptor selectivity (Millar et al., 2004). cGnRH-R-I has a PEY motif in ECL3 whereas in cGnRH-R-III this motif is PPS (**Fig. 3.8.A**). The conservation of these motifs suggests that the two cGnRH-R receptor homologs may have similar ligand selectivity to endogenous cGnRH ligands. This is investigated in **Chapter 4**

The C-terminal cytoplasmic domain of GnRH-Rs has been shown to be involved in receptor mediated internalisation, receptor expression, phosphorylation and desensitization (Blomenrohr et al., 1999; Heding et al., 1998; Pawson et al., 1998b; Willars et al., 1999). The C-terminal cytoplasmic tail domain of cGnRH-R-III is longer by an additional 8 residues compared to cGnRH-R-I (**Fig. 3.8.A**). The effect

of the additional 8 residues on the rate of ligand induced receptor internalisation is investigated in **Chapter 4**.

Three putative SH3 binding motifs were identified in the type III cGnRH receptor C-terminal cytoplasmic tail domain which are not present in the type I cGnRH-R. SH3 binding proteins contain proline rich regions, with at least one PXXP motif (Cohen et al., 1995). These motifs are located at amino acid positions 369-373, 370-375 and 398-402 encoded by exon four of the cGnRH-R-III gene (**Fig. 3.8.A**). The occurrence of SH3 binding motifs suggest the potential for differential coupling of cGnRH-R-III to the mitogen activated protein kinase (MAPK) cascade (Millar et al., 2004), which is investigated in **Chapter 5**.

As the information on GnRH systems is incomplete in avian and reptilian species (Morgan and Millar, 2004), this thesis provides a new insight into the interplay between GnRH ligands and their receptors in the control of reproduction at a particular branch in the evolutionary tree. To date, there have been no other studies on GnRH receptors in avian species other than the chicken (Joseph et al., 2009; Shimizu and Bedecarrats, 2006; Sun et al., 2001a; Sun et al., 2001b). With the recent release of the Zebrafinch genome (Ensembl Genome Browser, June 2009) however, it has been possible to search for homologous regions of the genetic loci expected to contain GnRH receptor subtypes. Although the current release of the genome is not complete, a putative type III zebrafinch GnRH receptor gene resides on chromosome 10, at location 463841-465318 and a putative type I Zebrafinch GnRH receptor resides on chromosome 10, at location 18405095-18405990. Comparisons of the sequence identity of chicken and zebrafinch GnRH receptors will be of particular interest for future investigations.

Based on the species-specific variation in GnRH receptor subtype predominantly expressed in the anterior pituitary observed in the bullfrog (Wang et al., 2001a),

African clawed frog (Troskie et al., 2000), leopard gecko (Ikemoto and Park, 2007) and here in the chicken (**Fig. 3.8B**) (Joseph et al., 2009) it is evident that there **is likely to be plasticity in pituitary utilisation of GnRH receptor subtypes for functional regulation of gonadotrophin production and secretion.** There is also plasticity in the recruitment of GnRH isoforms for tissue regulation, as seen in the goldfish, where GnRH-II is a regulator of pituitary function (Yu et al., 1998). This plasticity of ligand and receptor recruitment during evolution provides a new perspective on our understanding the interplay between GnRH ligands and their receptors in the control of reproduction. **Delineation of ‘cognate’ ligand/receptor pairing requires the assessment of receptor protein expression in the target cell and assessment of ligand isoform delivery to that cell.** In the chicken there is good evidence that cGnRH-I targets the gonadotroph (Mikami et al., 1988a; Sharp et al., 1990; Vangils et al., 1993). In view of the ~1400 fold higher levels of cGnRH-R-III mRNA in the pituitary (**Fig. 3.3.A**) and its changes in relation to sexual maturity (**Fig. 3.3.D**) the cognate pituitary receptor for cGnRH-I and the mediator of gonadotroph regulation is likely to be cGnRH-R-III.

3.6. Connective Statement

Expression of cGnRH-R-III mRNA was ~1400 fold higher than cGnRH-R-I mRNA in the anterior pituitary gland suggesting that cGnRH-R-III is the predominant regulator of gonadotrophin synthesis and secretion in chickens. However, the capacity of cGnRH-R-III to bind the different cGnRH ligands is unknown. Measurement of the ligand selectivity of the cGnRH-R-III subtype for the endogenous ligands, cGnRH-I and GnRH-II, may help to elucidate the physiological significance of the receptor subtypes. Furthermore, the development of GnRH analogs that differentially promote or inhibit activation of either receptor subtype may be valuable tools for deciphering the role of receptor subtypes in terms of effects on gonadotrophin production or other neuroendocrine functions. Therefore in Chapter 4, using cGnRH-R-I and cGnRH-R-III cDNA expression constructs transiently transfected into COS-7 cells, the pharmacological profiling of cGnRH-R-III in terms of ligand selectivity and activation of inositol phosphate production is described in comparison with the pharmacological profile of cGnRH-R-I.

Chapter 4.

Pharmacological Characterisation of GnRH Agonist and Antagonist at Type I and Type III cGnRH Receptors and cGnRH-R-III Splice Variants

4.1. Abstract

The expression of two GnRH receptor subtypes, cGnRH-R-I and the more recently discovered cGnRH-R-III, in the chicken coincides with the presence of two chicken GnRH ligands, cGnRH-I and GnRH-II. Expression of cGnRH-R-III mRNA was ~1400 fold higher than cGnRH-R-I mRNA in the anterior pituitary gland, suggesting that cGnRH-R-III is the predominant regulator of chicken gonadotrophin synthesis and secretion (Chapter 3). However, the ability of cGnRH-R-III to bind the endogenous ligands was uncharacterised. Determination of ligand-binding selectivity and the extent of activation of the cGnRH-R-III subtype to the endogenous ligands, cGnRH-I and GnRH-II, may help to elucidate the physiological roles of the receptor subtypes. Additionally, the development of GnRH analogs that differentially promote or inhibit activation of the receptor subtypes will be valuable tools for determining the role of receptor subtypes in the regulation of gonadotrophin production or in other functions in the central nervous system or peripheral tissues. To investigate this, pharmacological profiling of cGnRH-R-III in terms of ligand-binding selectivity and inositol phosphate production in response to ligand-receptor activation was investigated in comparison with the pharmacological profile of cGnRH-R-I. Functional studies in transfected COS-7 cells indicated that cGnRH-R-III has a higher ligand-binding affinity for GnRH-II than cGnRH-R-I (IC_{50} : 0.57 v 19.8 nM) with more potent stimulation of inositol phosphate production (EC_{50} : 0.8 v 4.38 nM). Similar results were found for cGnRH-R-I, (IC_{50} : 0.51 v 10.8 nM) and (EC_{50} : 0.7 v 2.8 nM). Effects of GnRH antagonists were compared at the two receptors. Mammalian antagonist 27 distinguished between cGnRH-R-I and cGnRH-R-III (IC_{50} : 2.3 v 351 nM), and application of this synthetic peptide may allow delineation of receptor subtype function *in-vitro* and *in-vivo*. In addition, the initial rate of receptor internalisation was faster for cGnRH-R-III than cGnRH-R-I (26%.min⁻¹ v 15.8%.min⁻¹). Also, although cGnRH-R-III splice variants do not bind GnRH ligands independently, cGnRH-R-III_SV2 significantly decreased maximal

ligand-binding of cGnRH-R-III by approximately 30%, suggesting that it may impair the expression of the full-length type III cGnRH receptor. The research presented in Chapter 4, demonstrates that cGnRH-R-III, like cGnRH-R-I, has a higher ligand-binding selectivity and induction of inositol phosphate accumulation with GnRH-II than with cGnRH-I, although cGnRH-I is the physiological regulator of gonadotroph function. These results suggest that evolutionary recruitment of ligand-receptor pairing for particular physiological processes does not correlate with properties such as highest ligand-binding affinity or potency of inositol phosphate production.

4.2. Introduction

4.2.1. Ligand selectivity and G protein coupling of cGnRH-Rs to endogenous ligands

Two GnRH isoforms, cGnRH-I and GnRH-II are present in the chicken (Sharp and Ciccone, 2005). cGnRH-I differs from mammalian GnRH-I (mGnRH-I) by the substitution of an arginine (Arg) by a glutamine (Gln) residue in position 8 of the decapeptide, while the structure of GnRH-II (His5-Trp7-Tyr8-GnRH) is conserved in all jawed vertebrate classes (Miyamoto et al., 1984). It is firmly established that GnRH-II (relative to GnRH-I) has the highest ligand-binding affinity for all non-mammalian vertebrate receptors irrespective of receptor classification based on structural similarities (Pfleger et al., 2002). The promiscuity of receptor subtype activation *in-vitro* by different endogenous GnRH ligand isoforms, as seen in teleost fish (Illing et al., 1999; Moncaut et al., 2005), amphibians (Troskie et al., 2000; Wang et al., 2001a) and reptilian (Ikemoto and Park, 2007) species, suggests that a complex interplay between ligands and receptors may occur *in-vivo*. Hence, the occurrence of two GnRH ligands and two GnRH receptors expressed in the chicken, cGnRH-R-I (Sun et al., 2001a; Sun et al., 2001b) and cGnRH-R-III (**Chapter 3-Fig. 3.1, 3.2, 3.3, 3.6.**) (Joseph et al., 2009; Shimizu and Bedecarrats, 2006), poses questions concerning the interactions of the two ligands at either receptor subtype. The presence of functionally important residues and micro-domains in cGnRH-R-III were assessed and compared with those in cGnRH-R-I and other GnRH receptors expressed in vertebrate species (**Chapter 3-3.3.7, Fig. 3.8**). It was established in **Chapter 3** that; (A) cGnRH-R-III like cGnRH-R-I has aspartic acid residues in both loci of the helix2/helix7 micro-domain, which regulates GPCR coupling and expression (Flanagan et al., 1999; Zhou et al., 1994). (B) Conservation of other important coupling motifs and residues such as the arginine cage motif (DRXXX(I/V)) which resides at the cystolic end of TMD3 (Ballesteros et al., 1998) and alanine in ICL3 (Myburgh et al., 1998), are maintained in cGnRH-R-III like cGnRH-R-I. (C) The two cGnRH-Rs exhibit conservation of the aspartic acid

(Flanagan et al., 2000) and asparagine (Davidson et al., 1996) ligand-binding residues in TMD2. (D) The lysine in TMD3 of the cGnRH-R-III (Zhou et al., 1995) is conservatively substituted with an arginine residue and this is believed to preserve agonist binding to the receptor (Zhou et al., 1995) (**Fig. 3.8A**). The conservation of these motifs suggests that the two cGnRH-R receptor isoforms may have similar pharmacological profiles in response to endogenous ligands.

Interestingly, ECL3 of each receptor type has a distinctive motif and this has a key influence on ligand-receptor selectivity (Millar et al., 2004). Mammalian type I receptors possess S-E/D-P motifs whereas non-mammalian type I receptors have P-X-S/Y motifs (Millar et al., 2004; Wang et al., 2004). A mutagenesis study has shown that the residues surrounding the glutamic acid residue (E) are important for differential ligand-binding selectivity (mGnRH-I v GnRH-II) between mammalian and non-mammalian GnRH receptors (Wang et al., 2004). cGnRH-R-I has a PEY motif in ECL3 whereas in cGnRH-R-III this motif is PPS. The presence of these motifs suggests that the two cGnRH-R receptor homologs may function similarly in response to endogenous chicken GnRH ligand stimulation with a preference for GnRH-II (although cGnRH-I differs from mGnRH).

Previous pharmacological studies in COS-7 cells expressing chicken GnRH receptors showed that cGnRH-R-I has a higher ligand-binding affinity for GnRH-II ($K_i = 0.6\text{nM}$) than cGnRH-I ($K_i = 5.3\text{nM}$) and GnRH-II is more potent in stimulating inositol phosphate accumulation ($ED_{50} = 0.04\text{nM}$) than cGnRH-I ($ED_{50} = 2.7\text{ nM}$) (Sun et al., 2001b). Earlier studies on cultured pituitary cells showed that GnRH-II is more potent than cGnRH-I ($ED_{50} = 0.055\text{ v } 0.28\text{ nM}$) in stimulating LH release and in stimulating FSH release from dispersed chicken pituitary cells ($ED_{50} = 0.034\text{ v } 0.37\text{ nM}$) (Millar et al., 1986). This was confirmed in later studies on cultured pituitary cells with GnRH-II exhibiting an ED_{50} of 0.35nM compared to an ED_{50} of 1.8nM for cGnRH-I in LH releasing activity (Millar et al., 1989). *In-vivo* injection studies showed that LH secretion in response to GnRH-II is markedly greater than to cGnRH-I in mature laying hens, however, this is not the case in juvenile chickens of

either sex, nor the mature male (Sharp et al., 1987). There is conflicting evidence for an effect of cGnRH-I or GnRH-II on FSH release *in-vitro*, (Hattori et al., 1986a; Millar et al., 1986) and *in-vivo* (Bruggeman et al., 1998; Dunn et al., 2003; Krishnan et al., 1993; Proudman et al., 2006). Probably as derived from other species, cGnRH may stimulate FSH biosynthesis, although there is no evidence of FSH release on cGnRH-I stimulation, while a role for GnRH-II in FSH production has not been established (Dunn et al., 2003; Sharp and Ciccone, 2005).

Research into second messenger systems involved in the control of LH secretion in the avian pituitary is relatively scant when compared to other species. Early studies suggested a role of cAMP in the release of LH (Bonney and Cunningham, 1977b). Later studies showed the requirement for PKC and calcium mobilisation (Davidson et al., 1987a; Davidson et al., 1987b), which confirmed other studies establishing the requirement for elevated intracellular Ca^{2+} (Bonney and Cunningham, 1977a; Luck and Scanes, 1980). More recent research however has provided clear evidence for cAMP, PKC activation and the requirement for Ca^{2+} in regulation of cGnRH-I induced LH secretion from chicken pituitary gonadotrophs (Johnson and Tilly, 1991) confirming earlier reports. However, this data also demonstrates the complexity of signal transduction mechanisms regulating LH secretion, in that intracellular calcium is sufficient, cAMP is not a prerequisite for and additional signalling molecules mediate the effects of PKC activation on cGnRH-I induced LH secretion. Thus multiple signalling mechanisms exist to control the LH secretion from avian pituitary gonadotrophs (Johnson and Tilly, 1991).

It was anticipated that identifying the ligand selectivity and potency of cGnRH-R-III activation in response to endogenous ligands, cGnRH-I and GnRH-II, may elucidate the physiological roles of the receptor subtypes, given that *in-vitro* studies could be performed with dispersed pituitary cells expressing both cGnRH-R-I and cGnRH-R-III. Interaction between cGnRH-I and GnRH-II and the release of LH is sexually differentiated in the domestic chicken (Sharp et al., 1987). Elucidating the ligand

selectivity and the potency of cGnRH-I and GnRH-II at individual chicken GnRH receptor homologs would increase understanding of the functional significance of cGnRH-R-I and cGnRH-R-III in terms of sex-specific responses to the endogenous ligands. Alternatively it could be hypothesised that since cGnRH-R-III is predominantly expressed in the pituitary (**Chapter 3**) it may be more functionally “selective” for cGnRH-I, since this appears to be the GnRH isoform released from the hypothalamus to the pituitary.

In addition to quantifying cGnRH receptor subtype mRNA abundance and ligand-binding selectivity, determining receptor signalling (e.g. in terms of G protein coupling or receptor protein-protein complex formation) is important for elucidating the functional capacity of each GnRH receptor subtype. GnRH receptors preferentially couple to $G\alpha_q$ protein in pituitary gonadotrophs (Grosse et al., 2000; Hsieh and Martin, 1992; Naor et al., 1986), but evidence shows that GnRH receptors can also couple to $G\alpha_i$ and $G\alpha_s$ proteins (Hawes et al., 1993; Liu et al., 2002; Stanislaus et al., 1998). It is important however to take into account the cell system in which the coupling of the G protein is being studied, as variations in G protein complement suggest that coupling of GnRH receptors is “cell-context dependent” (Dobkin-Bekman et al., 2006; Naor, 2009; Ruf et al., 2003). In this chapter the ligand induced potency of $G\alpha_q$ coupling of cGnRH-R-III was assessed by measuring the production of inositol phosphates in transfected COS-7 cells in comparison with cGnRH-R-I.

4.2.2. Activity of GnRH analogs

Mammalian GnRH receptor antagonists 27, 135-18 and 135-25 have been previously shown to have decreased affinities for cGnRH-R-I compared to the human GnRH receptor (Sun et al., 2001b). The binding affinities of the GnRH analogs, 27, 135-18 and 135-25 at cGnRH-R-I were 61nM, 703nM and 4900nM, respectively (Sun et al., 2001b). In cells expressing cGnRH-R-I, the GnRH analog 135-18 acted as a high

potency full agonist ($ED_{50} = 33\text{nM}$), whereas GnRH analog 135-25 was a weak partial agonist ($ED_{50} = 1300\text{nM}$) and GnRH analog 27 showed antagonistic properties ($IC_{50} = 177\text{nM}$) (Sun et al., 2001b). This confirmed previous studies performed with cultured chicken pituitary cells measuring the ability of GnRH analogs to inhibit the GnRH induced LH release (Jacobs et al., 1995). In these studies GnRH analog 27 acted as a pure antagonist, and had an IC_{50} of 137nM , whereas GnRH analog 135-18 acted as a pure agonist (Jacobs et al., 1995). Considering the 53% amino acid sequence identity of the two chicken GnRH receptor subtypes (**Chapter 3-Fig. 3.8**) and taking into consideration the expression of both chicken GnRH receptors in the pituitary (**Chapter 3-Fig 3.3**), it was hypothesised that identifying a GnRH analog that differentially binds and activates one of the chicken GnRH receptor homologs may be valuable. This would facilitate *in-vivo* and *in-vitro* studies to allow delineation of the role of each cGnRH-R subtype in terms of gonadotrophin synthesis and release.

4.2.3. cGnRH-R-III splice variants

In addition to full-length GnRH receptors, several splice variant (SV) mRNAs of GnRH receptors have been identified in the bullfrog, mouse, sheep, wallaby and human (Cheung and Hearn, 2003; Cheung and Hearn, 2005; Grosse et al., 1997; Illing et al., 1993; Wang et al., 2001b; Zhou and Sealfon, 1994). Although their physiological significance is unclear, it has been suggested that the proteins they encode may interfere with full-length receptor function. In experimental systems, expression of human and bullfrog GnRH-R SVs elicits inhibitory effects on the signalling of full-length receptors (Grosse et al., 1997; Pawson et al., 2005; Wang et al., 2001b). Cellular trafficking of truncated receptors varies, as seen in studies performed with the bullfrog and the human GnRH-R SV constructs (Pawson et al., 2005; Wang et al., 2001b). Carboxyl-terminal GFP-tagged bullfrog GnRH-R SVs are either localised exclusively in the intracellular compartment, or alternatively in both the intracellular compartment and at the cell surface in HeLa cells depending on

the type of SVs (Wang et al., 2001b). A human GnRH-R-SV could not be detected in COS-7 cell membranes (Pawson et al., 2005) although, non-functional natural mutants of the human GnRH-R were shown to promote retention of the native receptor in the endoplasmic reticulum, thus reducing ligand-binding ability and inositol phosphate accumulation (Brothers et al., 2004). The inability of receptors to be properly trafficked to the cell surface is often the result of protein misfolding and misrouting (e.g. to lysosome), as demonstrated by several studies (Brothers et al., 2004; Janovick et al., 2002; Leanos-Miranda et al., 2002). This may be due to hetero-oligomerization between the wild type and mutant proteins (Brothers et al., 2004).

A PCR based study has shown that there is expression of two cGnRH-R-III SVs in the chicken, a pituitary-specific transcript (1065bp SV) (cGnRH-R-III_SV2), in addition to another 501bp SV that is expressed in different brain regions (cGnRH-R-III_SV1) (Shimizu and Bedecarrats, 2006). cGnRH-R-III_SV1 mRNA lacks exon II and III, resulting in a predicted 253 amino acid deletion from part of the N-terminal to part of intracellular loop 3. cGnRH-R-III_SV2 mRNA lacks part of exon II, resulting in a predicted 65-amino acid deletion from part of the extracellular domain to part of transmembrane domain 1. Both SVs are unable to signal independently through $G\alpha_q$ (Shimizu and Bedecarrats, 2006), and their RNA expression is not detectable by Northern blot analysis (**Chapter 3-Fig. 3.2**) (Joseph et al., 2009), suggesting that their expression may be of little functional significance. Only the bullfrog and sheep GnRH receptor SVs (Illing et al., 1993; Wang et al., 2001b) have been detected by Northern blot analysis, whereas other GnRH-R SVs have been detected by PCR amplification and subsequent sequencing (Cheung and Hearn, 2003; Grosse et al., 1997; Zhou and Sealson, 1994). However, studies on the bullfrog, mouse and human GnRH-R (Grosse et al., 1997; Pawson et al., 2005; Wang et al., 2001b) show that the SVs (Shimizu and Bedecarrats, 2006) might alter function of the full-length cGnRH-R-III although their endogenous expression has not been confirmed by Northern blot analysis (**Chapter 3-Fig. 3.2**). Thus, in this

chapter, the ability of the cGnRH-R-III SVs to bind to cGnRH ligands and their effects on full-length receptor expression were evaluated. These studies were performed alongside studies in a collaborator's laboratory (Prof. Gregory Bedecarrates, University of Guelph, Canada), where evaluation of the ability of the SVs to be translated into functional proteins and influence second messenger signalling of the full-length receptor were determined.

4.2.4. Ligand induced internalisation of cGnRH-Rs

The C-terminal tail of GnRH-Rs has been shown to be involved in receptor mediated internalisation and desensitisation (Blomenrohr et al., 1999; Heding et al., 1998; Pawson et al., 1998a; Willars et al., 1999). Receptor desensitisation is caused by sustained stimulation of the receptor and can be a consequence of receptor phosphorylation (McArdle et al., 2002). Phosphorylation can stabilise β -arrestin association with GPCRs resulting in the inhibition of G protein binding and subsequent uncoupling from effector proteins (McArdle et al., 1999; McArdle et al., 2002). The phosphorylation of amino acids in the C-terminal-tail facilitates β -arrestin association with the GnRH-Rs (McArdle et al., 2002), although other residues may facilitate β -arrestin binding (Ferguson, 2001). Chicken gonadotroph cells were stimulated with antagonists and no effects on desensitization were observed, whereas cGnRH-I desensitized gonadotroph response measured by LH release at a more rapid rate than determined in mammalian cells. This desensitisation was shown to be dependent on intracellular calcium stores (King et al., 1986). The rate of internalisation of radioligand [His⁵-D-Tyr⁶]-GnRH-I is 11.3%.min⁻¹ at the chicken type I cGnRH receptor in COS-1 cells, which is much more rapid than at the human GnRH-R (0.71%) (Pawson et al., 1998a). This internalisation was demonstrated to be through a caveolae-like, dynamin dependent mechanism and cysteine³²⁸ and the threonine³⁶⁹threonine³⁷⁰ doublet are crucial for rapid internalisation (Pawson et al., 2003). The region crucial for receptor internalisation of cGnRH-R-I is distal to or including Ser³³⁷ (Pawson et al., 1998a).

The highly conserved arginine residue in the cystolic region of TM3 and the highly conserved tyrosine residue of the N/DPXXY motif in TM7 have all been shown to be important for GnRH-R internalisation in the mouse (Arora et al., 1996; Arora et al., 1997). Interestingly mammalian GnRH-Rs lack a cytoplasmic C-terminal domain and internalise more slowly than non-mammalian GnRH-Rs (Millar et al., 2004). The possibility that cGnRH-R-III may differ from cGnRH-R-I in the rate of ligand-induced internalisation was considered because 8 additional residues occur in the cytoplasmic C-terminal tail of cGnRH-R-III (**Chapter 3-Fig. 3.8A**), a domain which predisposes GnRH-Rs to rapid rates of internalisation (Pawson et al., 1998a). Therefore the ligand-induced internalisation of cGnRH-R-III was investigated in this chapter.

4.2.5. Summary

The aims of this study were to compare and further characterise the pharmacological properties of the chicken type III cGnRH receptor with the type I cGnRH receptor. This included analysis of ligand-binding and potency of inositol phosphate production in response to cGnRH-I and GnRH-II and a small panel of GnRH analogs in an attempt to identify a GnRH analog that may be applied to examine the differential effects of receptor subtypes on gonadotrophin synthesis and release. The effect of the cGnRH-R-III splice variants on cGnRH-R-III receptor binding was also examined. Additionally, the ligand induced internalisation of both receptors was compared.

4.3. Methods

The methodologies used to generate the results presented in this chapter are described in chapter 2 (2.15-2.20.). The primary structures of the synthetic peptide GnRH analogs used in all experiments presented in this chapter are described in **Table 4.1.**

Table 4. 1**Primary structure of GnRH analogs.**

Peptide name	Position of Amino Acid										
	1	2	3	4	5	6	7	8	9	10	
cGnRH-II	pGlu	His	Trp	Ser	His	Gly	Trp	Tyr	Pro	Gly	NH ₂
cGnRH-I	pGlu	His	Trp	Ser	Tyr	Gly	Leu	Gln	Pro	Gly	NH ₂
His ⁵ -D-Tyr ⁶ -GnRH	pGlu	His	Trp	Ser	His	D-Tyr	Leu	Arg	Pro	Gly	NH ₂
sGnRH-III	pGlu	His	Trp	Ser	Tyr	Gly	Trp	Leu	Pro	Gly	NH ₂
27	Ac-D-Nal(2) ^a	D- α -Me-4-Cl-Phe	D-Trp	Ser	Ipr-Lys	D-Tyr	Leu	Arg	Pro	D-Ala	NH ₂
135-18	Ac-D-Nal(2)	D-4-Cl-Phe	D-Pal(3)	Ser	Ile	D-Ipr-Lys	Leu	Ipr-Lys	Pro	D-Ala	NH ₂
135-25	Ac-D-Nal(2)	D-4-Cl-Phe	D-Pal(3)	Ser	1-MePal	D-Ipr-Lys	Leu	Ipr-Lys	Pro	D-Ala	NH ₂

^aAbbreviations for synthetic amino acids are: Nal(2), 3-(2-naphthyl)alanine; 4-Cl-Phe, 3-(4-chlorophenyl) alanine; α -Me-4-Cl-Phe, 2-methyl-3-(4-chlorophenyl) alanine; Ipr-Lys, *N*^ε-isopropyllysine.

4.4. Results

The IC_{50} values derived in these experiments are the concentration of the competing ligand required to displace 50% of the specific radioligand binding. In all results presented in this chapter, IC_{50} values derived from competitive binding assays are used as a measure of relative binding affinity of each ligand to make a comparison between the two receptors (cGnRH-R-I and cGnRH-R-III).

4.4.1. cGnRH-R-III, like cGnRH-R-I, has a higher ligand-binding affinity for GnRH-II than for cGnRH-I.

COS-7 cells transiently transfected with cGnRH-R-III and cGnRH-R-I expression constructs, bound cGnRH-I and GnRH-II (**Table 4.2**) and showed displacement of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I with both cGnRH-I and GnRH-II in a concentration dependent fashion. The binding affinities for cGnRH-I and GnRH-II were similar at cGnRH-R-I and cGnRH-R-III (**Fig 4.1**). GnRH-II has a higher binding affinity (35-fold) for cGnRH-R-III than cGnRH-I (IC_{50} : 0.57 vs 19.8 nM) (**Table 4.2**). The ligand selectivity was similar with cGnRH-R-I, where a higher binding affinity for cGnRH-II (35-fold) than cGnRH-I was observed (IC_{50} : 0.51 vs 10.8 nM) (**Table 4.2**). These findings are similar to previous reports (Sun et al., 2001b). cGnRH-R-I showed a 1.8-fold higher ligand-selectivity for cGnRH-I compared to cGnRH-R-III, whereas the ligand-selectivity for GnRH-II at the two receptors was similar.

4.4.2. GnRH-II elicits inositol phosphate accumulation at cGnRH-R-III and cGnRH-R-I more potently than cGnRH-I.

cGnRH-I and GnRH-II stimulated inositol phosphate production in cGnRH-R-I and cGnRH-R-III transfected COS-7 cells (**Fig 4.2**). GnRH-II was more potent in stimulation of inositol phosphate production at cGnRH-R-III than cGnRH-I (EC_{50} : 0.8 vs 4.38 nM). Similar results were found for cGnRH-R-I, where GnRH-II was more potent in stimulating inositol phosphate production than cGnRH-I (EC_{50} 0.7 vs

2.8 nM). The potency of GnRH-II at both receptors was much higher than for cGnRH-I, in accordance with their relative binding affinities. In cGnRH-R-III transfected cells, the potency of GnRH-II stimulation was 6-fold higher than with cGnRH-I (**Table 4.2**). GnRH-II was 4-fold more potent than cGnRH-I for production of inositol phosphate via activation of cGnRH-R-I (**Table 4.2**).

4.4.3. Binding affinity of sGnRH-III and GnRH analogs-135-18, 135-25 and 27 at cGnRH-R-I and cGnRH-R-III.

sGnRH-III bound cGnRH-R-III and cGnRH-R-I with similar affinities (**IC₅₀: 9.31 and 8.40 nM**, respectively) (**Table 4.2**). Because there was no genomic evidence for a GnRH-III isoform in the chicken, the binding affinity of sGnRH-III was tested as an alternative. GnRH-II exhibited a 16-fold higher affinity than sGnRH-III at cGnRH-R-III and cGnRH-R-I, and sGnRH-III exhibited a higher affinity than cGnRH-I at both receptors (**Table 4.2**). Mammalian antagonists 27, 135-18 and 135-25 bound cGnRH-R-III and cGnRH-R-I (**Table 4.2**). Similar binding affinities were observed for all GnRH analogs and mammalian antagonists at either receptor except for mammalian antagonist 27. Mammalian antagonist 135-18 and 135-25 showed respectively a 3.5- and 0.6-fold higher binding affinity at cGnRH-R-I compared to cGnRH-R-III. Remarkably, mammalian antagonist 27 had a much higher affinity for cGnRH-R-I (**IC₅₀: 0.38nM**) than cGnRH-R-III (**IC₅₀: 21.4nM**).

4.4.4. Stimulation of inositol phosphate accumulation in cGnRH-R-I and cGnRH-R-III expressing COS-7 cells by mammalian antagonists

Mammalian antagonist 27 displayed inverse agonist properties at both receptors at 10 μ M concentration, decreasing inositol phosphate production below basal levels (**Table 4.2**), (**Fig 4.2A, C**). However, at concentrations <10 μ M this ligand displayed agonistic activity at both receptors (**Fig 4.2A, C**). Mammalian antagonist 135-25 induced only weak stimulation of inositol phosphate production in transfected COS-7

cells: cGnRH-R-I (EC_{50} : 5970nM) and cGnRH-R-III (EC_{50} : 4860nM) (**Table 4.2**), (**Fig 4.3A, C**). Mammalian antagonist 135-18 also induced a weak stimulation of inositol phosphate production in transfected COS-7 cells; cGnRH-R-III (EC_{50} : 1510nM), but no stimulation of inositol phosphate was detected in cGnRH-R-I transfected cells (**Fig 4.4A, C**).

4.4.5. Inhibition of inositol phosphate accumulation in cGnRH-R-I and cGnRH-R-III expressing COS-7 cells by mammalian antagonists

GnRH-II was used as an agonist to test antagonistic activity of mammalian antagonists, as this would facilitate comparisons with previous publications where cGnRH-R-I was researched (Sun et al., 2001b). Mammalian antagonist 135-18 was able to antagonise stimulation of inositol phosphate production by 1nM GnRH-II. This effect was most potent in cells transfected with cGnRH-R-I (IC_{50} : 2330 nM) than those transfected with cGnRH-R-III (IC_{50} : 3890 nM) (**Fig 4.4B, D**). Mammalian antagonist 135-25 acted as an agonist in cGnRH-R-III transfected cells in the presence of 1nM GnRH-II, with its potency of inositol phosphate stimulation increasing from EC_{50} : 4860nM in the absence of GnRH-II to EC_{50} : 20.1nM in the presence of GnRH-II (**Fig 4.3D**). Antagonistic properties for mammalian antagonist 135-25 were observed at cGnRH-R-I transfected cells as this compound decreased the inositol phosphate stimulation seen with 1nM GnRH-II, but with weak effect (IC_{50} : 245 nM) (**Fig 4.3B**). Surprisingly, mammalian antagonist 27 exhibited large differences in antagonistic potency at cGnRH-R-I (IC_{50} : 2.3 nM) compared to cGnRH-R-III (IC_{50} : 351nM) (**Fig 4.2B, D**).

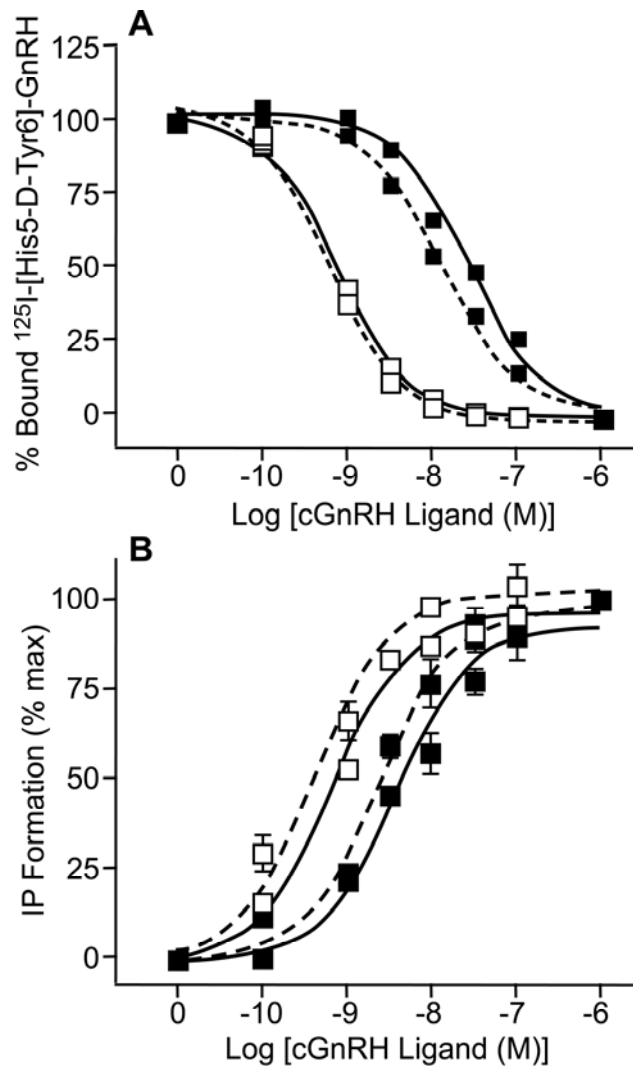
Figure 4. 1

Figure 4.1: (A) Ligand binding of cGnRH-R-I and cGnRH-R-III. Competitive displacement of ^{125}I -[His⁵-D-Tyr⁶]-GnRH with serial dilutions (10^{-11}M to 10^{-6}M) of cGnRH-I (solid squares ■) and (10^{-11}M to 10^{-6}M) GnRH-II (open squares □) in COS-7 cells transiently transfected with cGnRH-R-I (dashed lines) and cGnRH-R-III (solid lines) expression constructs. The data presented is from four independent experiments each performed in triplicate. Non-specific binding, determined in un-transfected cells was subtracted from cpm. **(B) Inositol phosphate production** in response to (10^{-11}M to 10^{-6}M) cGnRH-I (solid squares ■) and GnRH-II (open squares □) in COS-7 cells transiently transfected with cGnRH-R-I (dashed lines) and cGnRH-R-III (solid lines) expression constructs. The data is from four independent experiments performed in triplicate.

Figure 4. 2

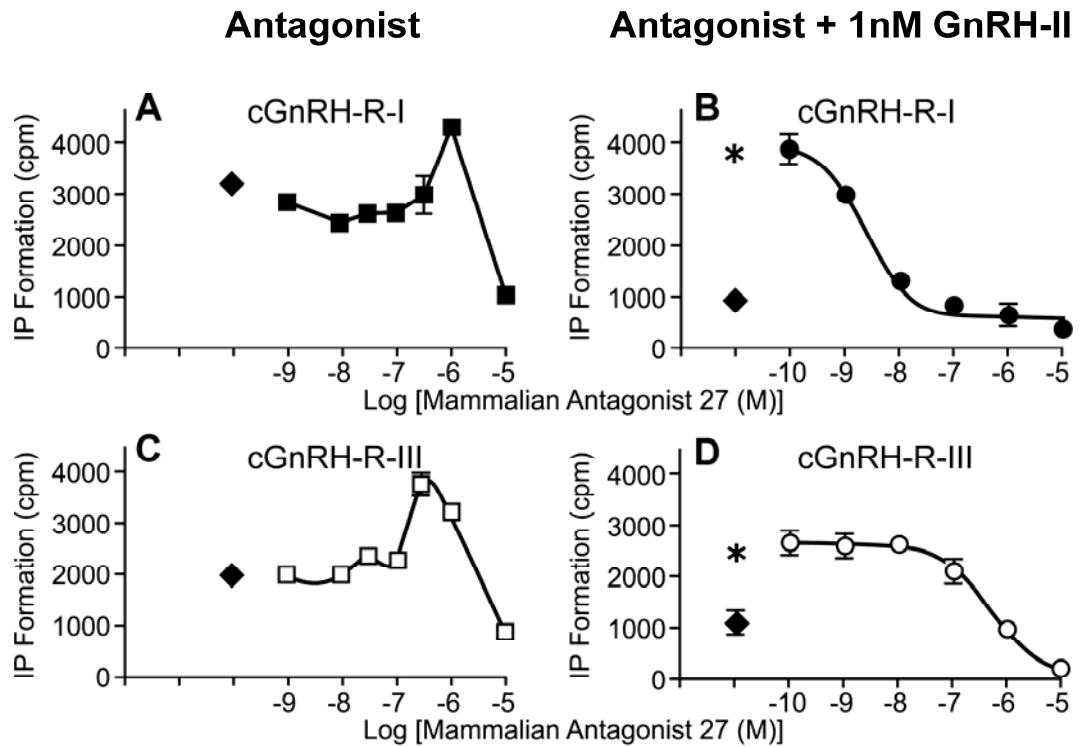


Fig. 4.2. Inositol phosphate production in response to (10^{-10} M to 10^{-5} M) mammalian antagonist 27 (A, C) in COS-7 cells transiently transfected with cGnRH-R-I (filled squares ■ (A)) and cGnRH-R-III (open squares □ (C)). The ability to inhibit inositol phosphate formation by mammalian antagonist 27 (B, D) in the presence of 1nM GnRH-II stimulation was measured in COS-7 transiently transfected with cGnRH-R-I (filled circles ● (B)) and cGnRH-R-III (open circles ○ (D)). Inositol phosphate production in response to 10^{-8} M GnRH-II stimulation (asterisks *). Non-stimulated basal inositol phosphate accumulation (◆). The data are the mean \pm SEM of three independent experiments performed in triplicate.

Figure 4. 3

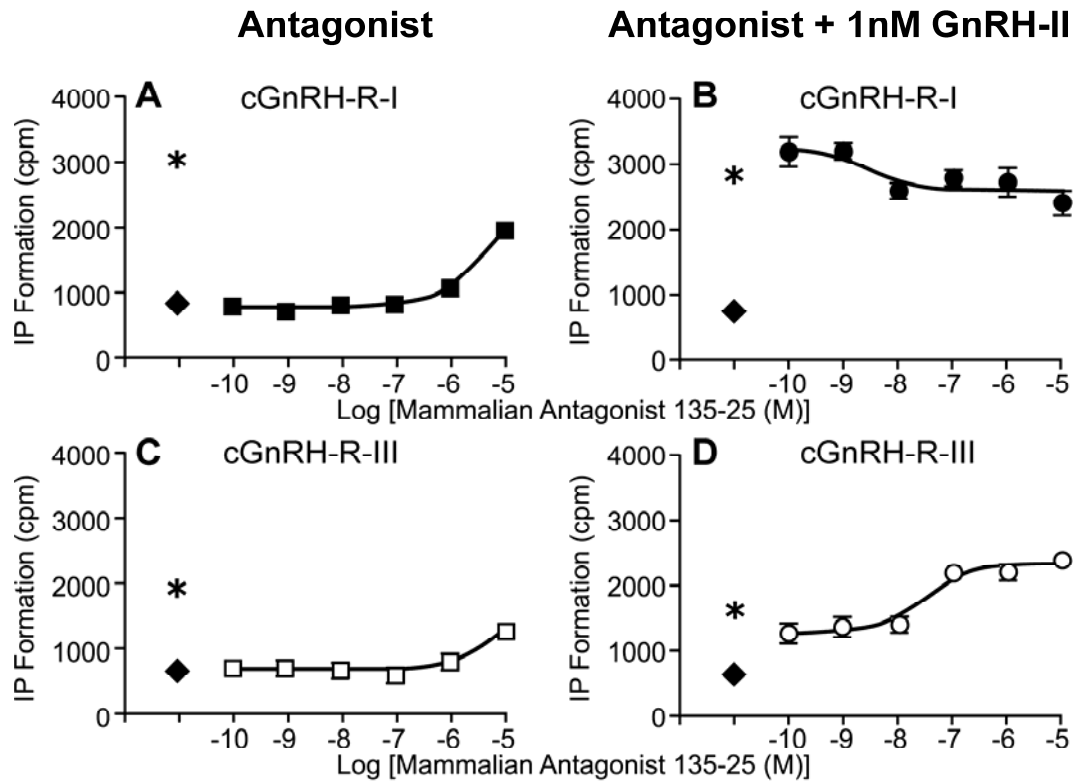


Fig. 4.3. Inositol phosphate production in response to (10^{-10} M to 10^{-5} M) mammalian antagonist 135-25 (A, C) in COS-7 cells transiently transfected with cGnRH-R-I (filled squares ■ (A)) and cGnRH-R-III (open squares □ (C)). The ability to inhibit inositol phosphate formation by mammalian antagonist 135-25 (B, D) in the presence of 1nM GnRH-II stimulation was measured in COS-7 transiently transfected with cGnRH-R-I (filled circles ● (B)) and cGnRH-R-III (open circles ○ (D)). Inositol phosphate production in response to 10^{-8} M GnRH-II stimulation (asterisks *). Non-stimulated basal inositol phosphate accumulation (◆). The data are the mean \pm SEM of three independent experiments performed in triplicate.

Figure 4. 4

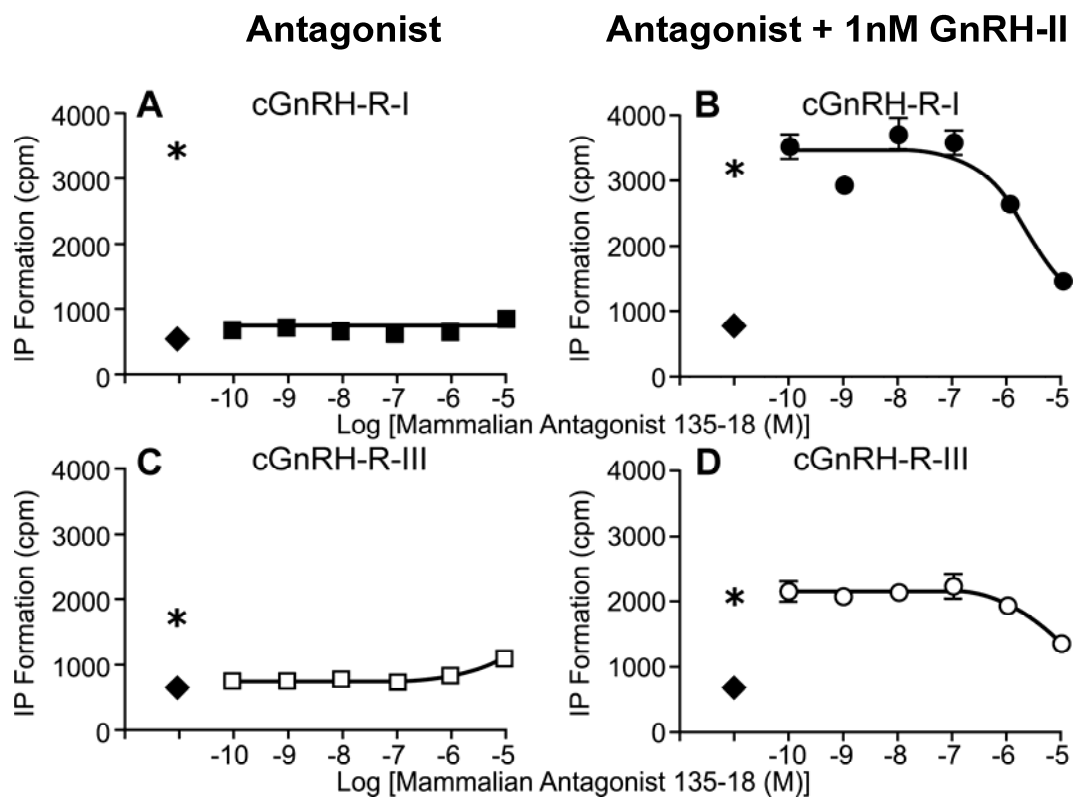


Fig. 4.4. Inositol phosphate production in response to (10^{-10} M to 10^{-5} M) mammalian antagonist 135-18 (A, C) in COS-7 cells transiently transfected with cGnRH-R-I (filled squares ■ (A)) and cGnRH-R-III (open squares □ (C)). The ability to inhibit inositol phosphate formation by mammalian antagonist 135-18 (B, D) in the presence of 1nM GnRH-II stimulation was measured in COS-7 transiently transfected with cGnRH-R-I (filled circles • (B)) and cGnRH-R-III (open circles ○ (D)). Inositol phosphate production in response to 10^{-8} M GnRH-II stimulation (asterisks *). Non-stimulated basal inositol phosphate accumulation (◆). The data are the mean \pm SEM of three independent experiments performed in triplicate.

Table 4. 2

Receptor binding and peptide-stimulated inositol phosphate accumulation in response to GnRH analogs in COS-7 cells expressing cGnRH-I and cGnRH-R-III

Analog	Ligand binding		IP Production			
	cGnRH-R-I	cGnRH-R-III	cGnRH-R-I		cGnRH-R-III	
	IC ₅₀ (nM) ^a		EC ₅₀ (nM) ^b	IC ₅₀ (nM) ^c	EC ₅₀ (nM) ^b	IC ₅₀ (nM) ^c
cGnRH-I	10.8 ± 1.59 ^d	19.8 ± 3.56 ^d	2.8 ± 0.20 ^d		4.38 ± 0.18 ^d	
GnRH-II	0.51 ± 0.07 ^d	0.57 ± 0.15 ^d	0.7 ± 0.14 ^d		0.8 ± 0.14 ^d	
sGnRH-III	8.40 ± 1.21 ^e	9.31 ± 4.31 ^e	10.3 ± 4.5 ^d		21 ± 0.2 ^{d*}	
27	0.38 ± 0.09 ^e	21.4 ± 4.86 ^e	Inverse agonist ^e	2.3 ± 0.3 ^e	Inverse agonist ^e	351 ± 114 ^e
135-18	2090 ± 156 ^e	7260 ± 105 ^e	None ^d	2330 ± 134 ^d	1510 ± 216 ^d	3890 ± 2027 ^d
135-25	14200 ± 1515 ^e	8560 ± 3402 ^e	5970 ± 2510 ^d	245 ± 207 ^d	4860 ± 807 ^d	*None ^d

^a IC₅₀ values (relative binding affinities) for GnRH analogs

^b EC₅₀ values for agonist activity of GnRH analogs

^c IC₅₀ values for antagonism of IP production stimulated by 1nM GnRH-II

^d Data are mean ± SE of three to four experiments

^e Data are mean ± SE of two to three experiments

^{*}20.1 ± 8.9 = agonistic effect of peptide in presence of 1nM GnRH-II

All experiments were performed on up to four separate occasions in triplicate

4.4.6. cGnRH-R-III_SV2 significantly affects cell surface expression of cGnRH-R-III when co-expressed with cGnRH-R-III.

Co-transfections were performed as described in the methods section (2.16) and were controlled for to ensure that co-transfection with splice variant constructs (cGnRH-R-III_SV1 and cGnRH-R-III_SV2) did not result in reduced transcription of the full-length construct (cGnRH-R-III). A titration of varying quantities of full-length cGnRH-R-III cDNA (3µg-10µg) were transfected and the maximal binding of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I was determined. There were no differences in the maximal binding of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I to cGnRH-R-III in cells transfected with 3µg of cDNA versus those transfected with 10µg of cDNA (**data not shown**). In all cases the total amount of transfected cDNA was standardised to 10µg with the addition of empty pcDNA3.1. Independently transfected cGnRH-R-III SVs in COS-7 cells, displayed no binding to ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I (**Fig. 4.5A, B**). A significant reduction ($p < 0.01$) in the maximum binding of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I to the full-length cGnRH-R-III was observed when co-transfected with the cGnRH-R-III_SV2 (**Table 4.3**). A small, but significant increase in the maximum binding of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I was observed when cGnRH-R-III_SV1 was co-transfected with cGnRH-R-III (**Table 4.3**). Cells transfected with cGnRH-R-III alone or in combination with cGnRH-R-III_SV1 or cGnRH-R-III_SV2 bound cGnRH-I and GnRH-II (**Table 4.3**) and showed displacement of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I with both cGnRH-I and GnRH-II in a concentration dependent fashion and with similar IC₅₀ values (**Fig. 4.5A, B**), (**Table 4.3**).

Table 4. 3

Maximum binding and IC₅₀ values calculated from competitive binding assay performed on COS-7 cells transfected with cGnRH-R-III and cGnRH-R-III_SV1 or cGnRH-R-III_SV2.

GnRH Ligand	cGnRH-R-III and SVs	IC₅₀ (nM)	Maximal Binding (%)
cGnRH-I	cGnRH-R-III	7.90 ± 0.02 (12.59nM)	100.0 ± 2.9 ^a
	cGnRH-R-III_SV1	No binding	No binding
	cGnRH-R-III_SV2	No binding	No binding
	cGnRH-R-III + cGnRH-R-III_SV1	7.70 ± 0.03 (19.95nM)	102.9 ± 1.9 ^a
	cGnRH-R-III + cGnRH-R-III_SV2	7.90 ± 0.05 (12.59nM)	70.2 ± 2.2 ^b
GnRH-II	cGnRH-R-III	9.10 ± 0.01 (0.79nM)	100.0 ± 2.3 ^a
	cGnRH-R-III_SV1	No binding	No binding
	cGnRH-R-III_SV2	No binding	No binding
	cGnRH-R-III + cGnRH-R-III_SV1	9.10 ± 0.01 (0.79nM)	109.0 ± 1.6 ^b
	cGnRH-R-III + cGnRH-R-III_SV2	9.30 ± 0.02 (0.50nM)	66.6 ± 0.9 ^c

* Data correspond to mean ± SEM of three independent assays. The statistical significances were calculated by applying a Student's t-test between each maximum binding value determined as a % of cGnRH-R-III transfected cells. ^{a,b} Different superscript letters indicate significant differences within column for each ligand ($p < 0.05$).

Figure 4. 5

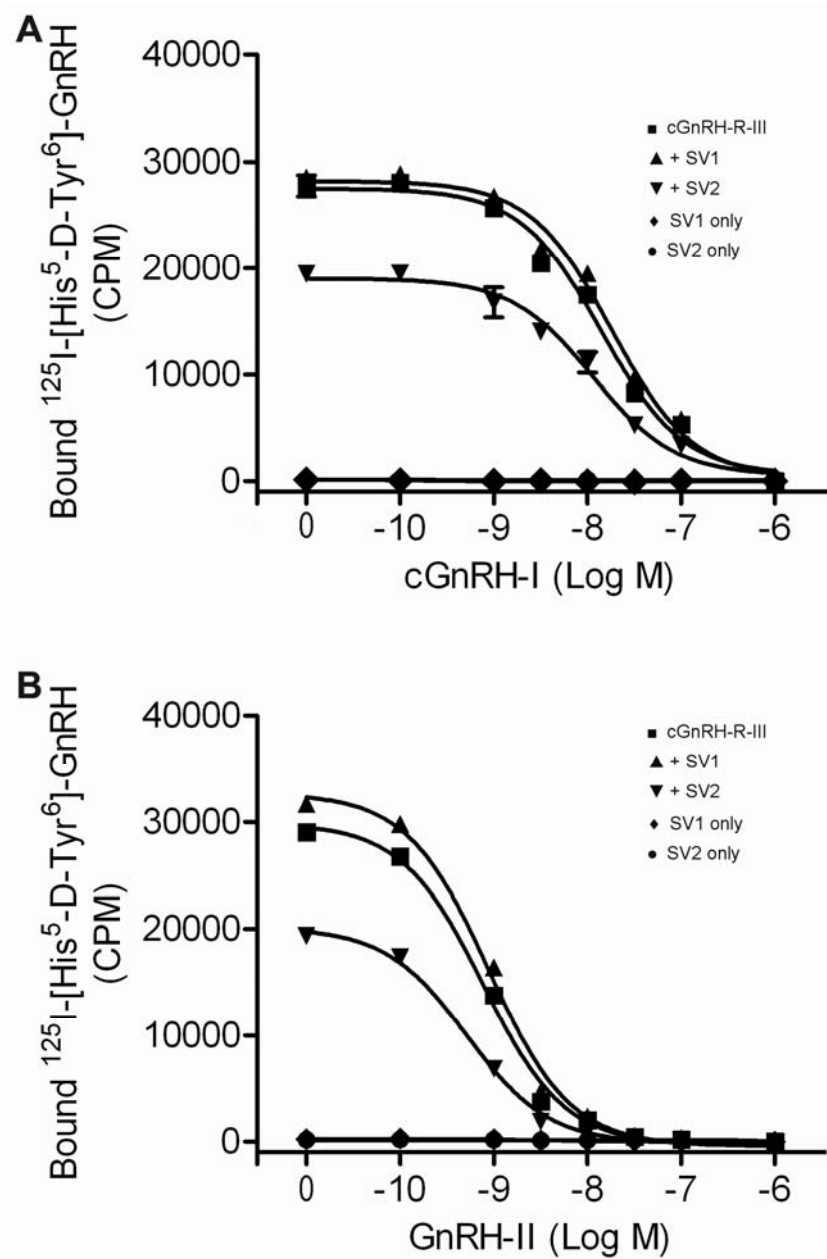


Fig. 4.5. Ligand binding to cGnRH-R-III in cells co-transfected with cGnRH-R-III Splice Variants. Competitive displacement of ^{125}I -[His⁵-D-Tyr⁶]-GnRH with serial dilutions of (A) cGnRH-I and (B) GnRH-II in COS-7 cells transiently transfected with cGnRH-R-III (filled squares ■), cGnRH-R-III & SV1 (filled up-turned triangles ▲), cGnRH-R-III & SV2 (filled down-turned triangles ▼), SV1 (filled diamond ◆) and SV2 (filled circle ●) expression constructs. Data are presented as mean \pm SEM total counts per minute corrected for non-specific binding from three independent experiments performed in triplicate.

4.4.7. Ligand-induced internalisation of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I bound to cGnRH-R-I and cGnRH-R-III.

cGnRH-R-I internalised ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I at an initial rate of $15.8\%.\text{min}^{-1} \pm 1.57$ which was significantly less ($p < 0.005$) than the initial rate of internalisation of $26.0\%.\text{min}^{-1} \pm 2.33$ at cGnRH-R-III. The percent maximal internalisation of cGnRH-R-I was 78.2% versus 68.9% for GnRH-R-III (**Fig 4.6**).

Figure 4. 6

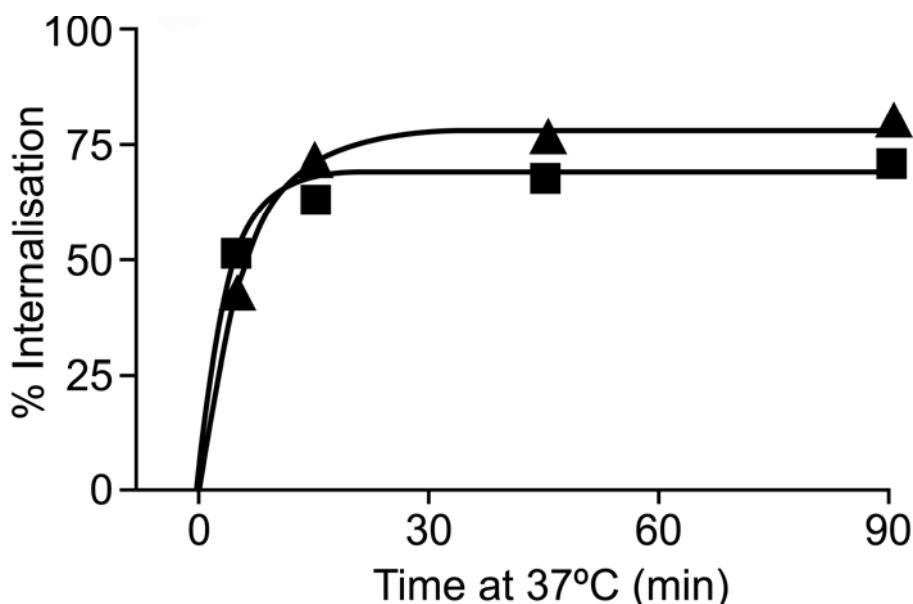


Fig. 4.6. cGnRH-R-I and cGnRH-R-III ligand induced internalisation rate. Percent internalisation of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I ligand mediated by cGnRH-R-I (solid triangle ▲) and cGnRH-R-III (solid square ■). Data points are the mean \pm SEM of four independent experiments each performed with 6 replicates. Background binding, determined in un-transfected cells was accounted for. The statistical significance was calculated by applying a Student's t-test to the initial rates of internalisation for each individual experiment performed.

4.5. Discussion

4.5.1. Ligand binding selectivity and G protein coupling of cGnRH-Rs

The occurrence of two or three forms of GnRH peptide hormone in different vertebrate species suggested the possibility that cognate receptors respond differently to distinguish between the different ligands. However, the analysis performed in this chapter comparing the pharmacological profiles of both cGnRH-R-I and cGnRH-R-III illustrate that the chicken receptors have similar pharmacological profiles in relation to the endogenous GnRH peptides in the chicken (**Fig. 4.1A, B**). cGnRH-R-III, like cGnRH-R-I has a similar GnRH binding selectivity for GnRH-II over cGnRH-I (**Fig 4.1A**). The results confirm a previous observation on ligand selectivity at cGnRH-R-I, where cGnRH-R-I was shown to have a higher binding selectivity for GnRH-II rather than cGnRH-I (Sun et al., 2001b). The ligand selectivity of cGnRH-I for cGnRH-R-I was relatively high (**Fig 4.1A**) despite cGnRH-I lacking a folded β -II' turn conformation (Sun et al., 2001b). The major ligand binding sites identified in mammalian GnRH receptors; Asp(98) (Flanagan et al., 2000), Asn(102) (Davidson et al., 1996) are conserved in cGnRH-R-III and in cGnRH-R-I, whilst the lysine in TMD3 of cGnRH-R-I is conservatively substituted with an arginine residue in cGnRH-R-III, preserving agonist binding to the receptor (**Chapter 3-Fig. 3.8**) (Zhou et al., 1995). The conservation of these residues is consistent with the observed similarity in binding affinities of the two receptors to cGnRH-I and GnRH-II. These receptor residues are believed to interact with amino and COOH terminal residues of the GnRH ligands (Davidson et al., 1996; Flanagan et al., 1994; Zhou et al., 1995). It has been proposed that cGnRH-R-I is configured such that the spatial arrangement of the receptor binding sites accommodates binding of configured and non-configured ligands, supported by the presence of aspartic acid in both loci of the functional helix2/helix7 micro-domain (Sun et al., 2001b). These residues are also conserved in cGnRH-R-III (**Chapter 3-Fig. 3.8**). Preferential selectivity for GnRH-II is emerging as a general phenomenon for non-mammalian GnRH receptors irrespective of their classification based on structural features. This

phenomenon appears to be due to the preconfigured β -II' turn of GnRH-II (Pfleger et al., 2002). The GnRH-II structure is conserved in vertebrates and it has been proposed this results from a co-ordinated evolutionary selection of residues for binding and for ligand configuration resulting in a high affinity binding for all receptors (Pfleger et al., 2002). The current study supports this concept in that both cGnRH-Rs maintain higher ligand selectivity for GnRH-II (**Fig. 4.1**). However, this situation presents an apparent conundrum because cGnRH-I is thought to be the hypothalamic peptide responsible for regulating the pituitary gonadotroph (Mikami et al., 1988a; Sharp et al., 1990).

In accordance with the ligand-binding selectivity of cGnRH-R-III (**Fig. 4.1**), GnRH-II was more potent (4-fold) at stimulating inositol phosphate production at cGnRH-R-III than cGnRH-I (**Fig. 4.1B**) consistent with previous data (Shimizu and Bedecarrats, 2006; Sun et al., 2001b). Due to the conservation of aspartic acid in both loci of the helix2/helix7 micro-domain (Sun et al., 2001b), alanine in IC3 (Myburgh et al., 1998), and the arginine cage motif that resides at the cytosolic end of TMD3 (Ballesteros et al., 1998) (**Chapter 3-Fig. 3.8A**), all of which are known to be important for receptor G protein coupling and signal transduction, it is not surprising that cGnRH-R-III, like GnRH-R-I (Sun et al., 2001b) stimulates inositol phosphate production. Since inositol phosphate and DAG are generated by the recruitment of the $G\alpha_q$ protein and subsequent activation of PLC- β (Ruf et al., 2003; Stojilkovic et al., 1994), cGnRH-R-III can activate the Ca^{2+} and PKC signalling pathways which are the predominant mediators of LH and FSH biosynthesis and secretion (Conn and Crowley, 1994; Sealton et al., 1997). Additionally, recent research using a cAMP responsive element (CRE) luciferase reporter construct suggests that cGnRH-R-I and cGnRH-R-III can couple to $G\alpha_s$ (Shimizu M et al., 2008). However, it should be recognised that CRE-luc can also be activated by the stimulation of more than one second messenger signalling pathway (Naor, 2009). For example, studies show that there is a possible crosstalk between PLC and AC pathways and two isoforms of PKC (PKC Δ and PKC ϵ) may activate AC, resulting in

increased cAMP levels (Lariviere et al., 2007). In COS-7 cells, the human GnRH-R can stimulate cAMP production via Ca^{2+} /calmodulin crosstalk when AC-I is introduced (Grosse et al., 2000). Similarly, it has also been reported that activation of $G\alpha_q$ and the resulting increase in intracellular Ca^{2+} , can lead to the stimulation of CRE-binding protein (CREB) (Matthews et al., 1994). Thus cGnRH-I stimulation of cGnRH-R-I and cGnRH-R-III may result in additional CRE-binding protein activation by mechanisms involving the coupling of several G proteins. An investigation into the precise mechanisms would prove beneficial in future studies. The potency of the endogenous ligands in inducing cAMP in these experiments was in accordance with the determined ligand-binding selectivity shown in (Fig. 4.1A). cGnRH-II had a higher potency in cAMP induction at cGnRH-R-I and cGnRH-R-III (ED_{50} 2.09 and 3.2nM respectively) compared with cGnRH-I (ED_{50} 26.8 and 22.91nM respectively) (Shimizu M et al., 2008).

The results suggest that receptor ligand selectivity measured *in-vitro* does not necessarily reflect the likely *in-vivo* role of GnRH receptor isoforms nor does it necessarily determine the designation of ‘cognate’ receptors. Evolutionary recruitment of ligand-receptor pairing for particular physiological processes is not always determined by selection according to the highest affinity for ligand-binding or potency in inositol phosphate production, but can be determined by tissue-specific expression of ligand and receptor in particular instances. As in the chicken where cGnRH-I is the only ligand that activates the pituitary cGnRH receptors, as GnRH-II is not present in the median eminence (Mikami et al., 1988b; Sharp et al., 1990). However, there may be an adaptive advantage of lower affinity/potency pairing. It can be hypothesised that a ligand (e.g. GnRH-I) having a lower affinity with a receptor (cGnRH-R-I and cGnRH-R-III) will correlate with a greater dissociation of the ligand and the receptor. This would allow a greater proportion of available receptors in a given time-frame of receptor activation. Additionally, lower affinity/potency pairing will enable receptor activation with lower pulses of GnRH and remove the requirement of large pulses to produce an effect.

In contrast, lower affinity/potency pairing may result in less receptor desensitisation on GnRH pulse activation, enabling a more prolonged affect of the ligands.

4.5.2. Activity of GnRH analogs at chicken receptors

Mammalian antagonist 27 has significantly higher binding affinities for cGnRH-R-III and cGnRH-R-I compared with mammalian antagonists 135-18 and 135-25 (**Table 4.2**). Although, the binding affinities of the mammalian antagonists were much lower at both chicken GnRH receptor homologs than previous observations at the human GnRH receptor (Sun et al., 2001b). Mammalian antagonist 27 showed a 56-fold higher binding affinity for GnRH-R-I than for cGnRH-R-III (**Table 4.2**). The D- α -Me-4-Cl-Phe in position 2 of mammalian antagonist 27 may enhance its binding affinity to both receptors compared to the other mammalian antagonists (Sun et al., 2001b). The lysine (Lys/K) in TMD3 at position 121 of the cGnRH-R-I is conservatively substituted with an arginine (Arg/R) residue in cGnRH-R-III at position 147 (**Chapter 3, Fig. 3.8.A**) (Joseph et al., 2009), although this preserves agonist binding to the receptor (Zhou et al., 1995), it may contribute to the altered binding affinity of mammalian antagonist 27 (**Table 4.2**).

The observed low binding affinities of mammalian antagonists 135-25 and 135-18 (**Table 4.2**) are translated into similarly low potencies in their capacity to stimulate or inhibit inositol phosphate accumulation (**Fig. 4.3A-D, Fig. 4.4A-D**). The sizes of these effects are too small to distinguish between the two receptor subtypes. The agonistic and antagonistic effects of 135-25 and 135-18 are extremely small, and this is reflected in the size of standard error values observed (**Table 4.2**). The concentrations of mammalian antagonists 135-25 and 135-18 required to bind both cGnRH-R-I and cGnRH-R-III and to exert an agonistic or antagonistic effect are large, making an accurate estimation of the EC₅₀ and IC₅₀ values difficult (**Table 4.2**). Mammalian antagonist 135-18 behaved as an extremely weak agonist (EC₅₀ = 1510nM) and also antagonised GnRH-II activation of cGnRH-R-III (IC₅₀ = 3890nM)

(**Fig. 4.4C, D**). At cGnRH-R-I, mammalian antagonist 135-18 acted as a full antagonist ($IC_{50} = 2330\text{nM}$) with no agonistic effects (**Fig. 4.4A, B**). This contradicts previous observations, where mammalian antagonist 135-18 was shown to behave as a full agonist at cGnRH-R-I ($EC_{50} = 33\text{nM}$) (Sun et al., 2001b). Mammalian antagonist 135-25 acted as a very weak agonist at cGnRH-R-III ($EC_{50} = 4860\text{nM}$) and cGnRH-R-I ($EC_{50} = 5970\text{nM}$) (**Fig. 4.3C, D**). However, while this ligand was able to weakly antagonise GnRH-II activation of cGnRH-R-I ($IC_{50} = 245\text{nM}$) (**Fig. 4.3A, B**), no antagonism was seen at cGnRH-R-III. Interestingly, the potency of this ligand appeared to be increased in the presence of GnRH-II. Co-operativity may be a possible explanation for the observed effects. It is feasible that in the presence of GnRH-II, mammalian antagonist 135-25 displayed increased potency in inositol phosphate production of cGnRH-R-III due to conformational changes of the receptor. This data confirms previous observations where mammalian antagonist 135-25 was shown to have agonist and antagonist effects at cGnRH-R-I ($EC_{50} = 1300\text{nM}$ and $IC_{50} = 4100\text{nM}$) (Sun et al., 2001b).

Differences in binding affinity were translated into differences in antagonistic effects at the receptors, with antagonist 27 showing a 153-fold higher antagonistic effect at cGnRH-R-I than cGnRH-R-III (**Table 4.2**) (**Fig. 4.2B, C**). The difference in binding affinity of Antagonist 27 was 56-fold higher at cGnRH-R-I than cGnRH-R-III (**Table 4.2**). Although it behaved as an agonist at low concentrations, mammalian antagonist 27 acted as an inverse agonist at both cGnRH-R-I and cGnRH-R-III at $10\mu\text{M}$ concentration (**Fig 4.2A, C**). Mammalian antagonist 27 was also able to strongly antagonise GnRH-II stimulation of cGnRH-R-I ($IC_{50} = 2.3\text{nM}$) and cGnRH-R-III ($IC_{50} = 351\text{nM}$) (**Fig 4.2B, D**). This is an extremely valuable finding in terms of distinguishing between the receptors in terms of their pharmacology. **Therefore, it may be possible to utilise antagonist 27 to preferentially block chicken type I GnRH receptor function *in-vivo* in order to study physiological roles of the chicken type III GnRH receptor.** This data confirms previous observations, where mammalian antagonist 27 acted as a pure antagonist ($IC_{50} = 177\text{nM}$) (Sun et al.,

2001b) at cGnRH-R-I, although previously it was not shown to have inverse agonistic effects, as shown in this study (**Fig. 4.2A, C**). Antagonist 27 displayed a binding affinity of 0.38nM at cGnRH-R-I and 21.nM at cGnRH-R-III (**Table 4.2**), however, inverse agonist effects were only observed at 10 μ M at both receptors, therefore it is plausible that the observed inverse agonism is not receptor-specific mediated effect. It is feasible that the antagonist 27 or the vehicle is effecting the production of inositol phosphate at these concentrations irrespective of the receptor expression.

Analysis of the mammalian antagonist sequences revealed that both 135-25 (which has agonistic activity at cGnRH-R-I and GnRH-R-III) and 135-18 (which has agonistic activity at cGnRH-R-III) have a basic _D-amino acid substitution (_D-Ipr-Lys) in place of Gly⁶ and differ only by the amino acid in position 5 (**Table 4.1**). Previous studies showed that agonism at cGnRH-R-I is conferred by _D-lys or _D-Ipr-Lys in position 6 of the GnRH analog interacting with ECL2 of the receptor by stabilising the receptor active conformation (Sun et al., 2001b). Stabilisation of the active conformation of cGnRH-R-I by 135-18 was not supported by the observation made in this study, where 135-18 was shown to have antagonistic effects at cGnRH-R-I in the concentration range used (**Fig 4.4A, B**). Additionally, the large aromatic side chain (1-MePal) (**Table 4.1**) was suggested to prevent 135-25 acting as a full agonist at cGnRH-R-I (Sun et al., 2001b) by changing the orientation of the adjacent _D-Lys moiety such that its ability to interact with the cognate receptor amino acid is impaired. This observation is maintained in terms of GnRH-R-I; however the data from the present study suggests that the large aromatic side chain of 135-25 does not interfere with the interaction of cGnRH-R-III. However, the smaller aromatic side chain (Ile) (**Table 4.1**) at position 5 of mammalian antagonist 135-18 was suggested to allow this analog to act as a full agonist at cGnRH-R-I (Sun et al., 2001a), however in the current study, this compound acted as a pure antagonist at cGnRH-R-I (**Fig 4.4A, B**), making structure-function interpretations for these analog sequences difficult.

4.5.3. cGnRH-R-III splice variants

In these experiments the maximum binding of the co-transfected full-length and splice variant constructs to ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I was used as a measure of receptor expression at the cell surface. The maximum binding of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I in the absence competing ligand between the two experiments (**Fig 4.5**) should not differ as transfected cells were derived from the same transfection reaction, and they are not dependent on the competing ligand. The differences observed between the two experiments (**Fig 4.5**) are due to interplate variation attributable to the experimental procedure. Although there is a significant increase in the maximum binding observed for cGnRH-R-III when co-transfected with cGnRH-R-III_SV1 (**Table 4.3**), the size of the effect is small (increases of 2.0 and 9%), this may suggest that co-transfection with cGnRH-R-III_SV1 may result in increased trafficking of the full-length receptor to the cell membrane; although the effect is very small. The significant reduction in maximum binding (decreases 29.8 and 33.4%) observed for cGnRH-R-III when co-transfected with cGnRH-R-III_SV2 (**Table 4.3 and Fig. 4.5A, B**) may be a result of cGnRH-R-III_SV2 reducing the trafficking of the full-length receptor to the cell membrane, as seen in a study with the full-length and splice variants of the human GnRH-R (Pawson et al., 2005). Alternatively, cGnRH-R-III_SV2 could potentially dimerize with the full length receptor, as shown in a study using wallaby GnRH receptors, where the GnRH-Rs formed homodimers as well as heterodimers with SVs without agonist stimulation (Cheung and Hearn, 2005). Dimerisation of the full length cGnRH-R-III and cGnRH-R-III_SV2 may induce allosteric conformational changes in the full length cGnRH-R-III, resulting in a disruption of ligand binding sites (N. Joseph et. al., in preparation). This complements observations made in a collaborator's laboratory (Prof Gregory Bedecarrats, University of Guelph), where it has been demonstrated that both SV mRNAs are translated and trafficked in a similar way to the full-length receptor and that a substantial proportion of cGnRH-R-III splice variants (particularly cGnRH-R-III_SV2) co-localize with the full-length receptor in co-transfected COS-7 cells. Expression of cGnRH-R-III_SV2 resulted in a decrease in maximum inositol

phosphate accumulation in COS-7 cells and in CRE-luc activity in GH₃ cells transfected with the full-length receptor, while no effect was observed on EC₅₀ values. Interestingly, the effect of cGnRH-R-III_SV2 was more pronounced on cAMP production than on inositol phosphate accumulation, suggesting that inhibition by this variant is more effective on the G α_s pathway. While the decreased maximal binding induced by co-transfection of cGnRH-R-III_SV2 can explain the decreased ligand stimulation of inositol phosphate and CRE-luc activity, cGnRH-R-III_SV1 had no effect on binding when transfected in COS-7 cells at a ratio of 2:1 to full-length receptor cDNA. However, it did impair inositol phosphate generation in the same cells under these conditions. This implies that cGnRH-R-III_SV1 associates with the full-length receptor to cause uncoupling from G α_q protein (N. Joseph et. al., in preparation). **Therefore, it is plausible that cGnRH-R-III splice variants may modulate cGnRH-R-III activity and may be physiological regulators if expressed at sufficient levels.**

4.5.4. Ligand induced internalisation of cGnRH-Rs

Considering the primary structural differences between the two chicken receptors, the possibility that the rate of ligand-induced internalisation between the two receptors may differ was considered due to the presence of 8 additional residues in the C-terminal tail of cGnRH-R-III (**Chapter 3-Fig. 3.8**). A slightly greater rate of internalisation was measured for cGnRH-R-III compared to cGnRH-R-I (**Fig. 4.6**). The region crucial for receptor internalisation of cGnRH-R-I is distal to residue 336 in cGnRH-R-I (Pawson et al., 1998a). In cGnRH-R-III this region contains all 8 of the additional residues that comprise the elongated tail (**Fig. 4.7**). The cysteine³²⁸ residue of cGnRH-R-I, which was shown to be crucial for internalisation rate is conserved in cGnRH-R-III (Fig. 4.7). The putative threonine³⁶⁹threonine³⁷⁰ phosphorylation doublet of cGnRH-R-I, known to be critical for internalisation (Pawson et al., 2003), is not conserved in cGnRH-R-III (**Fig. 4.7**). However, in cGnRH-R-III, a different phosphorylation doublet (serine doublet) exists near the

carboxyl terminus (**Fig. 4.7**). Therefore, this serine doublet probably maintains the internalisation rate of cGnRH-R-III. The highly conserved arginine residue in the cystolic region of TM3 and the highly conserved tyrosine residue of the N/DPXXY motif in TM7 are important for GnRH receptor internalisation in the mouse (Arora et al., 1996; Arora et al., 1997) and are conserved in both chicken receptors (**Chapter 3-Fig. 3.8**), although these features are also conserved throughout the rhodopsin family of GPCRs. It is possible that the spatial arrangement of amino acid residues in the carboxyl-terminus region of cGnRH-R-III may contribute to the increase in internalisation rate when compared to the internalisation rate of cGnRH-R-I (**Fig. 4.6**).

Figure 4. 7

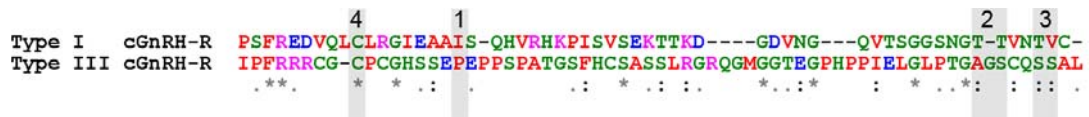


Fig. 4.7. Alignment of the cytoplasmic C-terminal tails of cGnRH-R-I and cGnRH-R-III. The amino acid residues that are important for receptor internalisation are boxed. [1] Region crucial for cGnRH-R-I internalisation is distal to residue 336. [2] Threonine³⁶⁹ and threonine³⁷⁰ doublet of cGnRH-R-I, known to be critical for internalisation. [3] Serine-serine doublet of cGnRH-R-III presenting a phosphorylation doublet near the carboxyl terminus. [4] Conservation of cGnRH-R-I cysteine³²⁸ residue with cGnRH-R-III. The C-tail domain is poorly conserved (~14% sequence identity between the type I and type III cGnRH receptors).

The length of the cytoplasmic tail has also been suggested to contribute to the cell surface expression of the receptor (Pawson et al., 2003), but transient gene expression of both receptors following transfection by electroporation of a standard quantity of cDNA was similar in COS-7 cells (**data not shown**), suggesting that extension of the tail beyond a critical minimum does not further enhance expression at the cell surface. The length of the cytoplasmic tail probably effects cell surface expression by changing the conformation of the receptor. The internalisation of cGnRH-R-I was suggested to be through a caveolae-like, dynamin-dependent manner (Pawson et al., 2003). The internalisation kinetics of the two receptors were

measured in COS-7 cells (**Fig. 4.6**) which have low endogenous levels of β -arrestin (Menard et al., 1997). A further investigation into the mechanisms of internalisation of cGnRH-R-III will prove valuable as internalisation of the receptors may be influenced by cell-type-specific factors. Although unlikely, the receptors may internalise through different mechanisms which would result in differential effects in different tissue cell types. Agonist induced receptor desensitisation has been studied in chicken pituitary cells (King et al., 1986) although no analysis of agonist induced desensitisation of chicken receptor isoforms has been performed. Such studies may give insight into the functional roles of the two receptors and should be a focus of future investigations.

4.5.5. Summary

In summary, I compared the *in-vitro* pharmacological properties of cGnRH-R-I and cGnRH-R-III, and found both receptors to be similar in relation to endogenous chicken GnRH ligands. Pharmacological profiling of the type I and type III GnRH receptors has established that GnRH-II is more selective and more potent in the induction of inositol phosphate accumulation at both receptors than cGnRH-I. cGnRH-R-III has a slightly more rapid internalisation rate than cGnRH-R-I. This may be important in determining the dynamics of cellular responses to receptor activation. Activation and inhibition by GnRH analogs acting on cGnRH-R-I and cGnRH-R-III were characterised using a range of ligands (sGnRH-III, mammalian antagonist 27, 135-18 and 135-25), furthering the pharmacological profile of both receptor subtypes. Major differences between the two receptors were only seen with mammalian antagonist 27. A decrease in expression of cGnRH-R-III by co-transfections with cGnRH-R-III_SV2 was measured and the implications of cGnRH-R-III splice variants on signal transduction was discussed.

The findings presented in this chapter encompassing ligand-binding selectivity, G protein coupling and internalisation of cGnRH-R-III suggest that it could theoretically function similarly to cGnRH-R-I to regulate gonadotrophin synthesis.

However the predominant levels of cGnRH-R-III mRNA in the pituitary gland (~1400 fold more than cGnRH-R-I), and the changes in expression patterns with age and sex suggest a distinct functional capacity of cGnRH-R-III compared to cGnRH-R-I (**Chapter 3**). The application of GnRH analogs such as antagonist 27 which has been shown to differentially affect GnRH receptor subtype function in transfected cells will be valuable for determining the physiological roles of different ligands and receptor subtypes *in-vivo*. **Our studies suggest that evolutionary plasticity in the tissue-specific adoption of GnRH ligand and receptor subtypes for regulation of particular physiological functions may have occurred in birds. Birds appear to have adopted a strategy to regulate gonadotrophin synthesis and release which utilises cGnRH-I and cGnRH-R-III pairing.**

4.6. Connective Statement

Expression of cGnRH-R-III mRNA was ~1400 fold higher than cGnRH-R-I mRNA in the anterior pituitary gland suggesting that cGnRH-R-III is the predominant regulator of gonadotrophin synthesis and secretion (**Chapter 3**). Both cGnRH-R subtypes have similar *in-vitro* pharmacological properties in relation to endogenous ligands, cGnRH-I and GnRH-II (**Chapter 4**) which does not further enhance our understanding of the roles of the cGnRH-R-I and cGnRH-R-III in terms of regulation of gonadotrophin biosynthesis. However a GnRH analog was identified that may be applied to differentially promote or inhibit activation of each cGnRH receptor subtype, providing a valuable tool for deciphering the role of receptor subtypes in terms of gonadotrophin regulation or other putative functions. In an attempt to discover differential functional capacities of the cGnRH-R subtypes, an in-depth study of the GnRH receptor activated MAPK signalling cascade was undertaken (**Chapter 5**). A number of primary structural differences were identified between the two receptors. Of particular interest are the putative Src Homology 3 (SH3) binding motifs identified exclusively in cGnRH-R-III. Identifying if cGnRH-R-I and cGnRH-R-III differentially target signalling pathways may help to elucidate differential physiological roles for the receptor subtypes. Therefore in **Chapter 5** using the cGnRH-R-I and cGnRH-R-III expression constructs, SH3 binding motif mutants and HA epitope-tagged cGnRH-R subtype constructs transiently transfected into COS-7 cells, the activation and interaction of the cGnRH-R subtypes with an array of signalling pathway mediators is described.

Chapter 5.

MAPK Signalling Activated via Type I and Type III cGnRH Receptors

5.1. Abstract

The expression of two GnRH receptor subtypes, cGnRH-R-I and cGnRH-R-III, coincides with the occurrence of two chicken GnRH ligands (cGnRH-I and GnRH-II). Despite the ~1400 fold higher level of mRNA encoding cGnRH-R-III in pituitary compared to cGnRH-R-I mRNA, there was no obvious functional difference of cGnRH-R-III compared to cGnRH-R-I when pharmacological properties were compared using endogenous ligands. cGnRH-R-III, like cGnRH-R-I, had a preferential ligand-binding selectivity and induction of inositol phosphate production in response to GnRH-II compared with cGnRH-I, although cGnRH-I is believed to be the physiological regulator of gonadotroph function. It was anticipated that the two cGnRH-R subtypes may have differential roles in the regulation of LH and FSH transcription through differential activation of second messenger pathways. Characterisation of signalling cascades activated by the two cGnRH-R subtypes may help to elucidate the existence of differential roles for the receptor subtypes. With this in mind, three putative Src homology domain 3 (SH3) binding motifs were identified in the type III cGnRH receptor cytoplasmic C-terminal tail domain (sequence = EPEPP, EPPSP, GPHPP) which are not present in the type I cGnRH receptor. This suggested the potential for differential coupling to the mitogen-activated protein kinase (MAPK) cascade. Therefore to investigate this possibility, activation of the MAPK cascade via cGnRH-R-III and cGnRH-R-I was determined by quantifying phosphorylated ERK (pERK 1/2) produced in response to GnRH. Studies performed in COS-7 cells showed a 4-6 fold increase in ERK1/2 phosphorylation via the type I and type III cGnRH receptors within 10 minutes of cGnRH-I or GnRH-II stimulation, indicating that both receptors signal through the ERK pathway. The effects were dose dependent at cGnRH-R-I and cGnRH-R-III. Effects of pre-treatment with phospholipase C (PLC) inhibitor or c-Src inhibitor showed that both cGnRH-Rs can activate pERK 1/2 possibly independently of PLC but dependently upon c-Src. However, it must be noted that 100% of the PLC activity was not inhibited

by PLC inhibitor as measured by inositol phosphate production at 60 minutes, and the PLC inhibitor has not been shown to inhibit PLC in the same time frame used for the pERK experiments. Mutagenesis of the individual SH3 binding motifs of cGnRH-R-III was performed by substituting an alanine codon for the first proline codon of each SH3 binding motif, and the effects on pERK 1/2 activation were quantified. The results indicated that the individual SH3 binding motifs of cGnRH-R-III do not contribute to MAPK activation when compared to the native cGnRH-R-III. Both cGnRH-R-I and cGnRH-R-III were HA epitope-tagged (HA-cGnRH-R-I and HA-cGnRH-R-III) to facilitate characterisation of receptor protein-protein complexes isolated by immunoprecipitation using western blotting, following transient transfection of COS-7 cells. Both HA-tagged receptors retained ligand-binding and coupling to PLC. Several size forms of HA-cGnRH-R-III were detectable and the composition of the immuno-precipitated protein complexes can be analysed in detail in future studies.

5.2. Introduction

5.2.1. The mitogen-activated protein kinase (MAPK) cascade influences gonadotrophin gene expression

Four groups of MAPK are currently known in mammals; extracellular signal-related kinase (ERK), Jun N-terminal kinase (JNK), p38MAPK (p38) and big MAPK (BMK) (Naor et al., 2000). GnRH activates all four of the MAPK cascades, although the mechanisms involved in GnRH stimulation of the MAPK cascade via GnRH receptors are dependent on “cell-context” (Dobkin-Bekman et al., 2006). Activation of the MAPK cascade results in gene transcription regulation, as certain components are able to translocate to the nucleus (Dobkin-Bekman et al., 2006; Naor et al., 2000). Variation in the frequency of GnRH pulses and in the level of GnRH receptors (which affects signal intensity) have been implicated in the differential regulation of gonadotrophin subunit gene expression (Haisenleder et al., 1991; Kaiser et al., 1995). These variables may lead to differential signalling (Dobkin-Bekman et al., 2006). Mediation of effects through PKC, ERK, JNK, c-Src Ca^{2+} and PKA, PKC, ERK, JNK, Ca^{2+} have been implicated in the transcriptional regulation of the common gonadotrophin alpha subunit and LH beta subunit respectively, although some studies show that p38, JNK and PKC, ERK, Ca^{2+} are not implicated in the transcriptional regulation of the common gonadotrophin alpha subunit and LH beta subunit respectively (Call and Wolfe, 1999; Fowkes et al., 2002; Harris et al., 2003; Roberson et al., 1999; Saunders et al., 1998; Weck et al., 2000; Weck et al., 1998; Xie et al., 2005; Yokoi et al., 2000). FSH beta subunit gene regulation appears to be mediated by PKC, ERK, JNK, p38 and c-Src but not Ca^{2+} (Bonfil et al., 2004). These conflicting results may reflect the different cell types, gene promoters from different species and cell culture conditions used experimentally (Naor, 2009).

5.2.2. MAPK signalling activated by the GnRH receptor through a G protein dependent mechanism

GnRH receptors primarily couple to $G\alpha_q$ in pituitary gonadotrophs (Grosse et al., 2000; Hsieh and Martin, 1992; Naor et al., 1986; Naor et al., 2000), but evidence shows that GnRH receptors can also couple to $G\alpha_i$ and $G\alpha_s$ (Hawes et al., 1993; Liu et al., 2002; Stanislaus et al., 1998). However, GnRH receptors interact with multiple G proteins in a 'cell-context' dependent manner (Dobkin-Bekman et al., 2006). In pituitary gonadotrophs a consensus signalling mechanism has emerged which activates the MAPK cascade, with $G\alpha_q$ activating PLC which generates DAG and IP3 which subsequently activate PKC and Ca^{2+} mobilisation, resulting in activation of ERK, JNK, and p38 (Dobkin-Bekman et al., 2006; Naor et al., 2000). In rat pituitary cultures, ERK activation is PKC dependent and dependent on Ca^{2+} influx, whereas JNK activation is PKC independent and dependent on Ca^{2+} mobilisation (Mulvaney and Roberson, 2000; Mulvaney et al., 1999). In $\alpha T3-1$ (mouse embryo gonadotroph) cells, GnRH stimulated ERK, JNK and p38 activation is dependent on PKC activation [8, 35, 36]. ERK activation has been further studied and shown to be dependent on c-Src, dynamin, Ras and Ca^{2+} influx (Benard et al., 2001), whereas JNK activation is additionally dependent on c-Src and CDC42/Rac1 (Levi et al., 1998). In HEK293 (human embryo kidney) cells, GnRH stimulation of GnRH receptor transfected cells results in ERK activation dependent on focal adhesion kinase (FAK) and c-Src at focal adhesion complexes (sites of integrin-ECM adhesion) mediated by Rac (Davidson et al., 2004). In COS-7 (African Green monkey kidney) cells, $G\alpha_i$ modulates EGFR resulting in c-Src activation and subsequent ERK activation mediated via Ras, and JNK activation mediated by PI3K (Kraus et al., 2003).

5.2.3. MAPK signalling activated by GPCRs through a G protein independent mechanism

It was shown that heptahelical receptors may directly interact with multiple intracellular signalling proteins (Hall et al., 1999) such as β -arrestin, GPCR kinases (GRKs) SH2, SH3 or PDZ (protein-protein interaction motifs) containing proteins, PTKs, small GTPases and other proteins (Naor et al., 2000). The complexes formed act as a scaffold or platform for interaction with additional signalling molecules. These protein-protein interactions mediate signal transduction that activates the MAPK cascade independently of the G proteins (Naor et al., 2000). β -arrestins serve as scaffolds and adaptors for GPCR signalling (Miller and Lefkowitz, 2001). A recent publication demonstrated an interaction of c-Src with β 2-adrenergic receptor through the proline-rich region of β 1-arrestin (Miller et al., 2000). The C-terminal tail of GPCRs serve as phosphorylation sites which facilitate β -arrestin binding (McArdle et al., 2002). The activation of c-Src can subsequently lead to the recruitment of Shc, Grb2, SOS and RAS which may activate the MAPK cascade (Kraus et al., 2001; McArdle et al., 2002; Miller and Lefkowitz, 2001). Presence of SH3 binding motifs in the C-terminal tail domain suggests the potential for differential coupling to the MAPK cascade (Millar et al., 2004). Carboxyl terminal tails of non-mammalian GnRH receptors have been implicated as sites for interactions with regulators of G protein signalling proteins (Castro-Fernandez and Conn, 2002). The possibility of direct interactions between accessory proteins, poly-proline binding proteins, receptor-activity modifying proteins and members of the scaffolding family of proteins and the GnRH receptor has been hypothesised, although the micro-domains or structural motifs within the GnRH receptors required for these interactions have not been identified (Millar et al., 2004).

5.2.4. c-Src interaction with MAPK signalling complexes and GPCRs

Activation of the MAPK cascade is often dependent on c-Src interactions (Benard et al., 2001; Davidson et al., 2004; Gutkind, 1998; Kraus et al., 2003; Levi et al., 1998; Luttrell et al., 1999). PKC activated via $G\alpha_q$ can modulate the activity of non-receptor PTKs e.g. c-Src family kinases (Levi et al., 1998). Additionally $G\alpha_i$ coupling may lead to activation of c-Src family of PTKs which in turn lead to MAPK activation (Gutkind, 1998; Luttrell et al., 1999). In $\alpha T3-1$, HEK293 and COS-7 cells GnRH stimulated ERK activation is c-Src dependent along with other second messengers (Benard et al., 2001; Davidson et al., 2004; Kraus et al., 2003). In $\alpha T3-1$, JNK activation is also shown to be c-Src dependent (Levi et al., 1998).

5.2.5. Signalling cascades involved in regulation of gonadotrophin gene expression in the chicken

Characterisation of second messenger systems involved in the control of LH secretion in the avian pituitary is scant. It has been established that cAMP, PKC activation and elevated intracellular Ca^{2+} are involved in cGnRH-I induced LH secretion from chicken pituitary gonadotrophs (Bonney and Cunningham, 1977a; Bonney and Cunningham, 1977b; Davidson et al., 1987a; Davidson et al., 1987b; Johnson and Tilly, 1991; Luck and Scanes, 1980). However it was also shown that increases in intracellular Ca^{2+} are sufficient for secretion of stored LH, whilst elevated cyclic cAMP is not essential for LH secretion (Johnson and Tilly, 1991). Each second messenger may be required to elicit different effects of gonadotroph function following stimulation with GnRH. Thus multiple signalling mechanisms co-exist to activate and sustain LH secretion from pituitary gonadotrophs (Johnson and Tilly, 1991). Previously, there have been no studies examining the signalling mechanisms involved in the regulation of gonadotrophin gene transcription in the chicken. In this thesis, it has been established that GnRH-II was more potent at stimulating inositol phosphate production at cGnRH-R-III than cGnRH-I (**Chapter 4 - Fig. 4.1B**) (Joseph et al., 2009). Similarly, GnRH-II stimulates inositol phosphate

production more potently at cGnRH-R-I than cGnRH-I (**Chapter 4 - Fig. 4.1B**), consistent with previous data (Joseph et al., 2009; Shimizu and Bedecarrats, 2006; Sun et al., 2001b). Since inositol phosphate and DAG are generated by the recruitment of the $G\alpha_q$ protein and subsequent activation of PLC- β (Ruf et al., 2003; Stojilkovic et al., 1994), cGnRH-R-III and cGnRH-R-I can activate the Ca^{2+} and PKC signalling pathways which are the predominant mediators of LH and FSH biosynthesis and secretion (Conn and Crowley, 1994; Sealfon et al., 1997). Recent research using a cAMP responsive element (CRE) luciferase reporter construct suggests that cGnRH-R-I and cGnRH-R-III couple to $G\alpha_s$ (Shimizu M et al., 2008). However as mentioned in **Chapter 4**, it should be recognised that CRE-luc can also be activated by the stimulation of more than one second messenger signalling pathway (Naor, 2009). The potency of the endogenous ligands in inducing cAMP was in accordance with the GnRH receptor ligand-binding selectivity (**Chapter 4 - Fig. 4.1A**). GnRH-II had a higher potency at cGnRH-R-I and cGnRH-R-III ($ED_{50} = 2.09$ and $3.2nM$ respectively) compared with cGnRH-I ($ED_{50} = 26.8$ and $22.91nM$ respectively) (Shimizu M et al., 2008). Coupling to other G protein subtypes by the cGnRH-R isoforms has not been studied as yet, and to date no G protein independent signalling has been examined using the chicken GnRH receptors.

5.2.6. Research Objectives

Three putative Src homology domain 3 (SH3) binding motifs were identified in the type III cGnRH receptor (sequence = EPEPP, EPPSP, GPHPP) cytoplasmic C-terminal tail domain which are not present in the type I cGnRH-R. SH3 binding motifs contain proline-rich regions with at least one PXXP motif (Cohen et al., 1995). Putative functional motifs are located at amino acid positions 369-373, 370-375 and 398-402 encoded by exon four of cGnRH-R-III (**Chapter 3 - Fig 3.8A**). The occurrence of SH3 binding motifs suggest the potential for differential coupling to the MAPK cascade (Millar et al., 2004). Determining whether cGnRH-R-I and cGnRH-R-III differentially target signalling pathways may help to elucidate

differential physiological roles for the receptor subtypes. Therefore in this chapter, a study of the GnRH receptor-activated MAPK signalling cascade was undertaken. Experiments were performed to test the hypothesis that the type III GnRH receptor may differentially interact with SH3 domain-containing proteins through the SH3 binding motifs.

5.3. Methods

The methodologies used to generate the results in this chapter are described in Chapter 2 (2.21-2.27)

5.4. Results

Note: In all results and diagrams that follow the phosphorylation of ERK 1/2 is denoted as 'pERK' for simplification. The antibody used to immuno-blot for pERK (Cell Signaling, Danvers, USA) detects endogenous levels of ERK1/2 when phosphorylated either individually or dually at Thr202 and 204 of ERK1 (Thr and Tyr of ERK2). Control experiments were carried out to ensure that no pERK increases occurred in non-transfected COS-7 cells treated with vehicle (0.2% propylene glycol) or 1 μ M GnRH-II (data not shown).

5.4.1. cGnRH-I and GnRH-II induce cGnRH-R-I and cGnRH-R-III mediated pERK activation in a dose-dependent and time-dependent manner

Experiments showed that cGnRH-I and GnRH-II induced pERK activation via cGnRH-R-I and cGnRH-R-III in a dose-dependent manner (**Fig. 5.1A-D**). At each increment in concentration of cGnRH-I and GnRH-II, there was an increase in the level of pERK via cGnRH-R-I and cGnRH-R-III. The maximum pERK levels in COS-7 cells transiently transfected with cGnRH-R constructs were as high as 12 fold when compared to basal (**data not shown**). The data presented in (**Fig. 5.1A-D**) were calculated as a fold response over non-stimulated or basal pERK level. cGnRH-I stimulation of cGnRH-R-I elicited a significant increase in pERK level when applied at 10nM ($p < 0.01$), 100nM ($p < 0.01$) and 1 μ M concentrations ($p < 0.05$) (**Fig. 5.1A**). Similarly stimulation of cGnRH-R-I with GnRH-II elicited a significant increase in pERK level when applied at 10nM ($p < 0.01$), 100nM ($p < 0.05$) and 1 μ M concentrations ($p < 0.05$) (**Fig. 5.1B**). cGnRH-I stimulation of cGnRH-R-III elicited a significant increase in pERK level when applied at 10nM ($p < 0.05$), 100nM ($p < 0.01$) and 1 μ M concentrations ($p < 0.05$) (**Fig. 5.1C**). Similarly stimulation of cGnRH-R-III with GnRH-II elicited a significant increase in pERK level when applied at 10nM ($p < 0.01$), 100nM ($p < 0.01$) and 1 μ M concentrations ($p < 0.01$) (**Fig. 5.1D**).

In addition, cGnRH-I and GnRH-II induced ERK activation in a time-dependent manner via cGnRH-R-I or cGnRH-R-III, with a maximum response observed after 15 minutes of stimulation (**Fig. 5.2A-D**). At each time point from 5-15 minutes with either cGnRH-I or GnRH-II, there was an increase in the pERK level whereas after 30 minutes of stimulation there was a decrease in pERK level (**Fig. 5.2A-D**). A significant increase in ERK activation via cGnRH-R-I using cGnRH-I stimulation was observed at 5 ($p<0.01$), 10 ($p<0.001$), 15 ($p<0.001$) and 30 ($p<0.05$) minutes and when using GnRH-II stimulation increases were also observed at 5 ($p<0.001$), 10 ($p<0.001$), 15 ($p<0.001$) and 30 ($p<0.05$) minutes (**Fig. 5.2A-B**). A significant increase in ERK activation via cGnRH-R-III using cGnRH-I stimulation was observed at 5 ($p<0.01$), 10 ($p<0.001$), 15 ($p<0.001$) and 30 ($p<0.05$) minutes and when using GnRH-II stimulation increases were also observed at 5 ($p<0.01$), 10 ($p<0.05$), 15 ($p<0.05$) and 30 ($p<0.01$) minutes (**Fig. 5.2C-D**).

Figure 5. 1

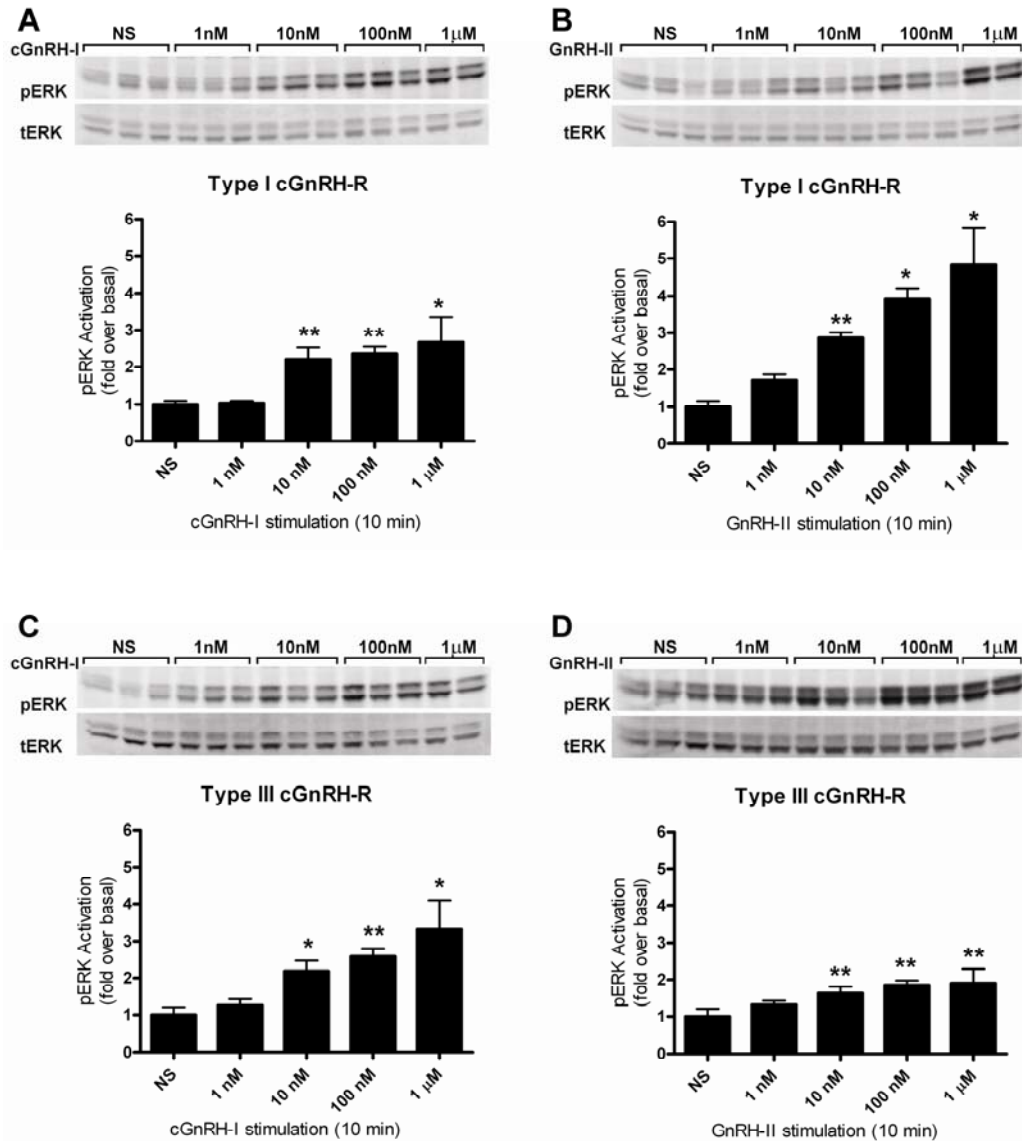


Fig. 5.1. cGnRH-I and GnRH-II induce phosphorylation of ERK via cGnRH-R-I and cGnRH-R-III in a dose-dependent manner. (A-D) COS-7 cells expressing chicken GnRH-R receptors were stimulated for 10 minutes with varying concentrations of cGnRH-I or GnRH-II (1nM-1μM) or left non-stimulated (NS) as indicated. Representative western blots of NP40 cell lysates are shown. Data from at least three independent experiments performed in triplicate or duplicate (as shown above) were quantified (using total ERK (tERK) immunoblot as a loading control) and the mean relative level of increase in pERK +/- S.E. is presented graphically below the corresponding blot. The statistical significance of changes in level was calculated by applying a Student's t-test between the non-stimulated and each individual dose stimulated. Values of $p < 0.05$ are represented by an asterisk * and $p < 0.01$ are represented by two asterisks **. These represent statistically significant differences compared to NS control levels.

Figure 5. 2

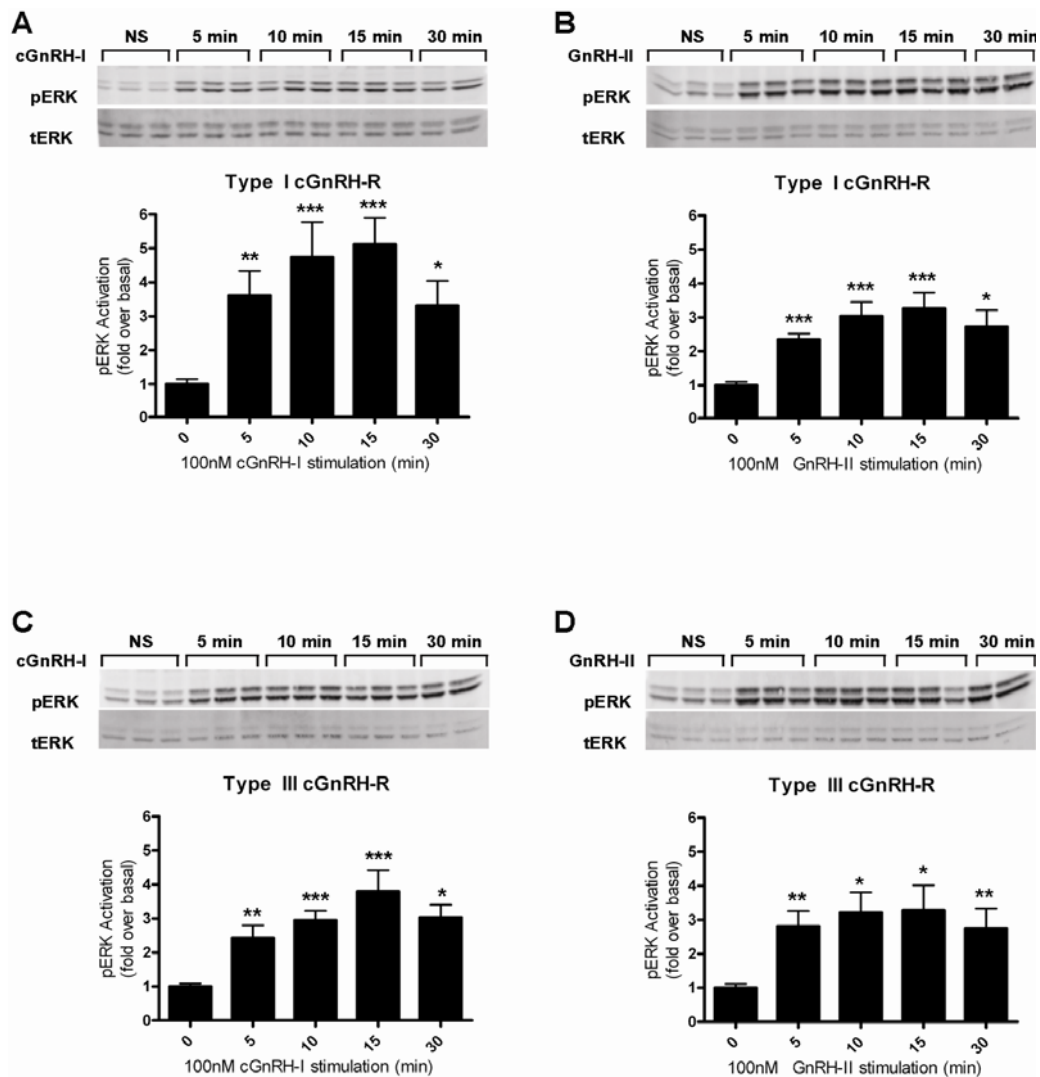


Fig. 5.2. cGnRH-I and GnRH-II induce pERK via cGnRH-R-I and cGnRH-R-III in a time-dependent manner. (A-D) COS-7 cells expressing chicken GnRH-R receptors were stimulated with 100nM cGnRH-I or GnRH-II for different periods of time (5-30mins) or left un-stimulated as indicated. Representative western blots of NP40 cell lysates are shown. Data from at least three independent experiments performed in triplicate or duplicate (as shown above) were quantified (using tERK immunoblot as a loading control) and the mean relative level of increase in pERK +/- S.E. is presented graphically below the corresponding blot. The statistical significance of changes in level was calculated by applying a Student's t-test between the non-stimulated and each individual dose stimulated. Values of $p < 0.05$ are represented by an asterisk * and $p < 0.01$ are represented by two asterisks ** and $p < 0.001$ are represented by three asterisks ***. These represent statistically significant differences compared to NS control levels.

5.4.2. PLC inhibitor (U73122) inhibits cGnRH-I and GnRH-II induced inositol phosphate production (at 60 minutes stimulation) but does not inhibit ERK activation via cGnRH-R-I and cGnRH-R-III (at 10 minutes stimulation)

COS-7 cells transfected with cGnRH-R-III expression constructs were pre-incubated for 45 minutes with 20 μ M U73122, a specific inhibitor of PLC, prior to stimulation with 100nM cGnRH-I for 10 minutes. Following treatment, pERK levels and inositol phosphate production were quantified. The method used to measure inositol phosphate production is only just sensitive enough to detect the response to cGnRH-I after 10 minutes stimulation (**Fig. 5.3A**). However, there was a significant increase ($p<0.01$) in the production of inositol phosphate after 10 minutes of 10nM and 1 μ M cGnRH-I stimulation (**Fig. 5.3A**). The size of the increase in inositol phosphate production was small (3.42% at 10nM and 22.01% at 1 μ M cGnRH-I compared to basal). These data were not sufficient to accurately measure stimulation compared to pre-inhibited samples (**Fig. 5.3A**). Therefore stimulation with GnRH was prolonged to 60 minutes, at which time a clear increase of inositol phosphate production can be observed (up to 284% compared to basal) (**Fig. 5.3B**). Large significant increases in inositol phosphate production were observed in the absence of U73122 after 60 minutes of 10nM and 1 μ M cGnRH-I and GnRH-II stimulation ($p<0.01$) but pre-incubation with U73122 ‘effectively blocked’ accumulation of inositol phosphate production by ~70-80% (**Fig. 5.3B**). A significant increase ($p<0.05$) in inositol phosphate production following 10nM and 1 μ M cGnRH-II stimulation for 60 minutes was observed in cGnRH-R-III transfected COS-7 cells pre-treated with U73122 (**Fig. 5.3B**), however, the size of the effect is small (28.9% at 10nM and 18.8% at 1 μ M cGnRH-II) in comparison to the non-inhibited controls.

The cell surface expression of cGnRH-R-III following DNA transfection was quantified by measuring the maximal specific-binding of His⁵-D-Tyr⁶-GnRH-I (**Fig. 5.4A**) to ensure that the cells expressed a sufficient amount of receptor to be able to measure the stimulation and inhibition. Quantification of pERK was determined (**Fig. 5.4B-C**) and showed that there is no inhibition of ERK activation in cGnRH-R-

III transfected cells pre-incubated with U73122 and stimulated with 100nM cGnRH-I or 100nM GnRH-II when compared to those that were not pre-incubated with U73122. In the presence of U73122, ERK activation via cGnRH-R-III was detectable, on 100nM cGnRH-I ($p < 0.01$) stimulation or 100nM GnRH-II ($p < 0.01$) stimulation (**Fig. 5.4B-C**), suggesting PLC-independent activation of the MAPK cascade.

Figure 5. 3

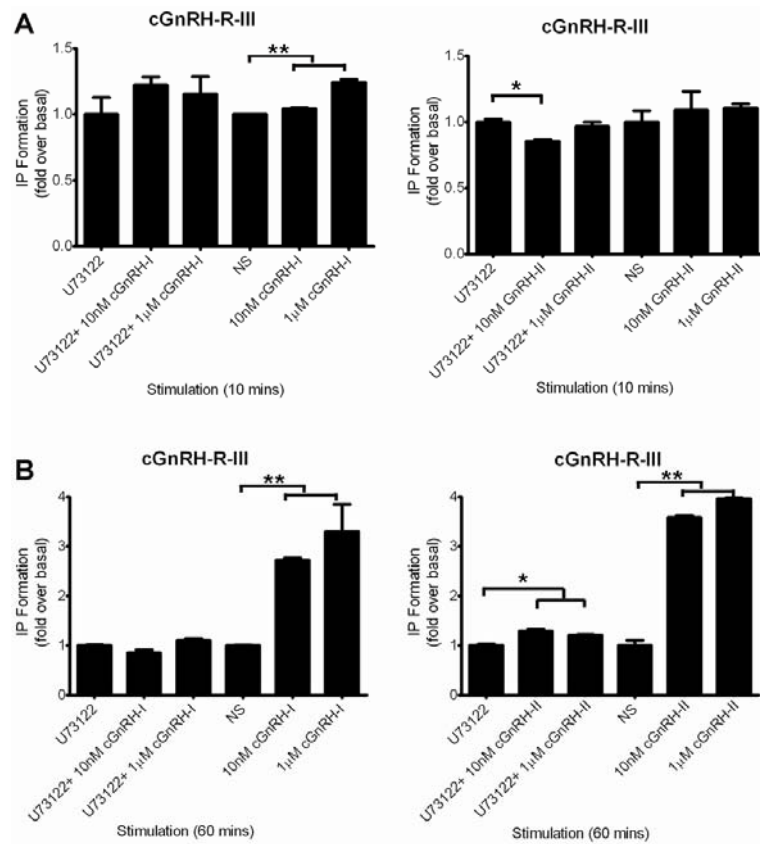


Fig. 5.3. The effects of PLC inhibitor (U73122), on inositol phosphate production in cGnRH-R-III expressing cells. COS-7 cells were transfected with cGnRH-R-III expression constructs and either incubated for 10 minutes with 20μM U73122 prior to stimulation with 100nM cGnRH-I and 100nM cGnRH-II for (A) 10 or (B) 60 minutes or directly stimulated without a pre-incubation with U73122. The amount of inositol phosphate production was then quantified and presented as fold over basal level present in non-stimulated cells. Data is from a single experiment performed in triplicate. The significance was calculated by applying a Student's t-test between the non-stimulated and each individual stimulated time point. Values of $p < 0.05$ are represented by an asterisk * and $p < 0.01$ are represented by two asterisks ** and represent statistical significance compared to basal controls.

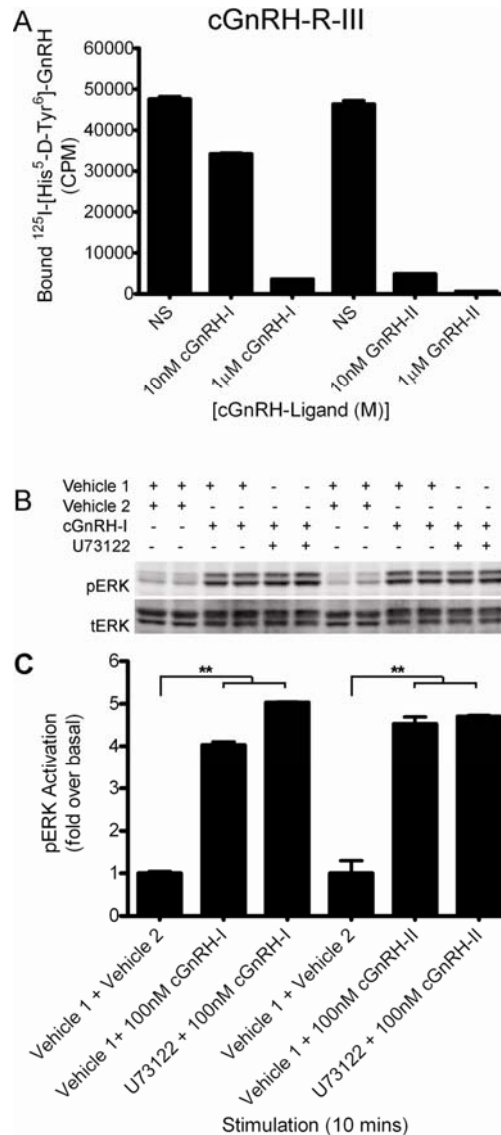
Figure 5. 4

Fig. 5.4. Measure of cell surface cGnRH-R-III expression and the effects of PLC inhibitor (U73122) on pERK activation in cGnRH-R-III expressing cells. (A) The maximal binding and displacement of His⁵-D-Tyr⁶-GnRH-I with 10nM and 1 μ M cGnRH-I or GnRH-II in COS-7 cells expressing cGnRH-R-III. (B, C) COS-7 cells were transfected with cGnRH-R-III expression constructs and either incubated for 10 minutes with 20 μ M U73122 prior to stimulation with 100nM cGnRH-I and 100nM cGnRH-II for 10 minutes or directly stimulated without a pre-incubation with U73122. (B) Representative blots of NP40 cell lysates are shown. (C) Data presented from one experiment performed in duplicate were quantified (using tERK immuno-blot as a loading control) and the mean-fold over control \pm S.E. presented graphically below the corresponding blot. The significance was calculated by applying a Student's t-test between the basal control and each individual stimulated time point. Values of $p < 0.05$ are represented by an asterisk * and $p < 0.01$ are represented by two asterisks ** and represent statistical significance compared to basal control.

5.4.3. cGnRH-I and GnRH-II induced ERK activation via cGnRH-R-I and cGnRH-R-III possibly via a PLC independent and c-Src dependent mechanism

COS-7 cells transfected with cGnRH-R-I and cGnRH-R-III expression constructs were pre-incubated for 45 minutes with 20 μ M U73122, a specific inhibitor of PLC and/or 5 μ M PP2, an inhibitor of the Src family of tyrosine kinases, prior to stimulation with 100nM cGnRH-I for 10 minutes. Following treatment, the ERK activation was quantified in the NP40 cell lysates using western blotting. There was no inhibition in ERK activation in cGnRH-R-I (**Fig. 5.5A**) and cGnRH-R-III (**Fig. 5.5B**) transfected cells pre-treated with U73122 and stimulated with 100nM cGnRH-I when compared to those stimulated with 100nM cGnRH-I alone. Compared to the non-stimulated or basal controls, there was a significant ($p < 0.05$ or $p < 0.01$) increase in pERK level via cGnRH-R-I (**Fig. 5.5A**) and via cGnRH-R-III (**Fig. 5.5B**) in both the U73122 pre-inhibited and non-inhibited treatments. Therefore, despite there being an inhibition in inositol phosphate production in cGnRH-R-I and cGnRH-R-III transfected cells pre-treated with U73122 and subsequently stimulated for 60 minutes (**Fig. 5.3**), the pERK activated on 10 minute stimulation via cGnRH-R-I and cGnRH-R-III transfected cells pre-treated with U73122 was not inhibited. Interestingly, however, compared to the non-stimulated controls, there was no increase in the pERK via cGnRH-R-I (**Fig. 5.5A**) and cGnRH-R-III (**Fig. 5.6B**) in transfected COS-7 cells pre-incubated with PP2. There was a clear inhibition of ERK activation in cGnRH-R-I (**Fig. 5.5A**) and cGnRH-R-III (**Fig. 5.5B**) transfected cells pre-treated with PP2 when compared to those stimulated with 100nM cGnRH-I alone. Therefore, ERK activation via cGnRH-R-I and cGnRH-R-III was inhibited by PP2.

Figure 5. 5

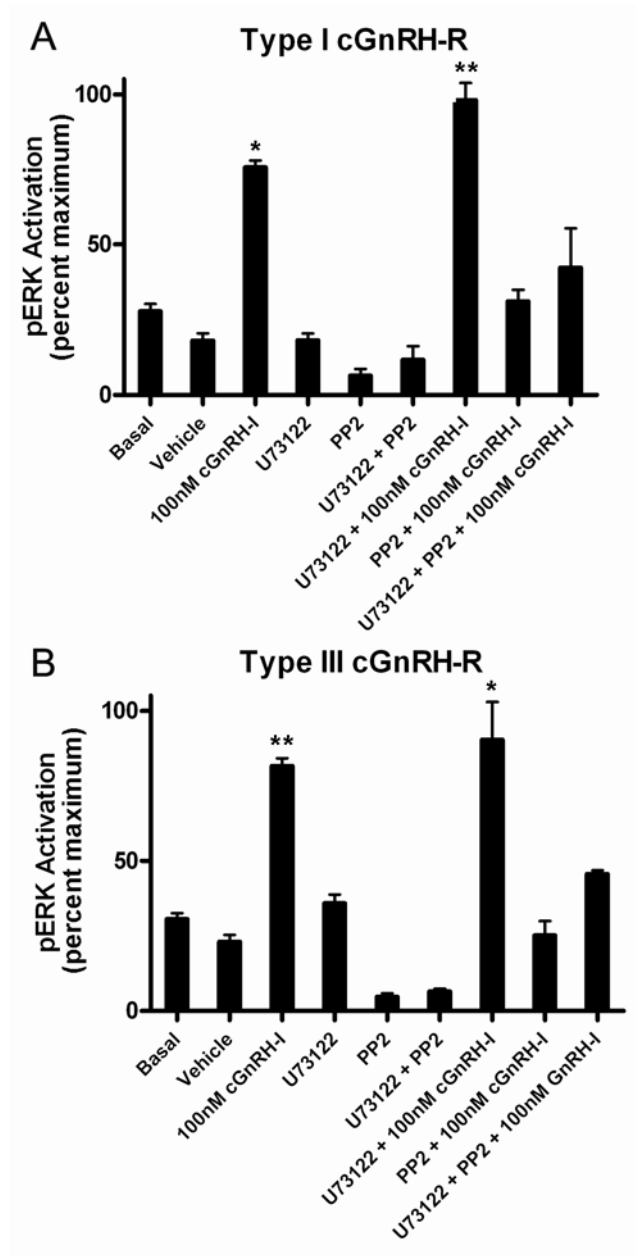


Fig. 5.5. The effects of PLC inhibitor (U73122) and c-Src inhibitor (PP2) on levels of pERK in (A) cGnRH-R-I and (B) cGnRH-R-III expressing cells. Data presented from four experiments performed in duplicate were quantified (using tERK immunoblot as a loading control) and the mean-fold elevation compared to control \pm S.E. The significance was calculated by applying a Student's t-test between the basal control and each individual stimulated time point. Values of $p < 0.05$ are represented by an asterisk * and $p < 0.01$ are represented by two asterisks ** and represent statistical significance compared to basal control.

5.4.4. Mutagenesis of the cGnRH-R-III SH3 binding motifs does not alter cGnRH-I induced pERK activation

Synthesis of cGnRH-R-III SH3 binding motif mutants in pcDNA 3.1/myc-His-A vector was performed as described in the methods section **Chapter 2-2.20**. The sequence of the SH3 binding motif mutants, i.e. mutagenesis of the target proline (P) codon to an alanine (A) codon was confirmed by DNA sequencing. The expression construct contained the exact sequence of the original cGnRH-R-III_1210f-3217r-pCDNA3.1/myc-His-A DNA but with the targeted mutated residues. No random mutations were generated during the reaction. A schematic representation of the cGnRH-R-III SH3 binding motif mutants, P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III is presented (**Fig.5.6**).

Figure 5. 6

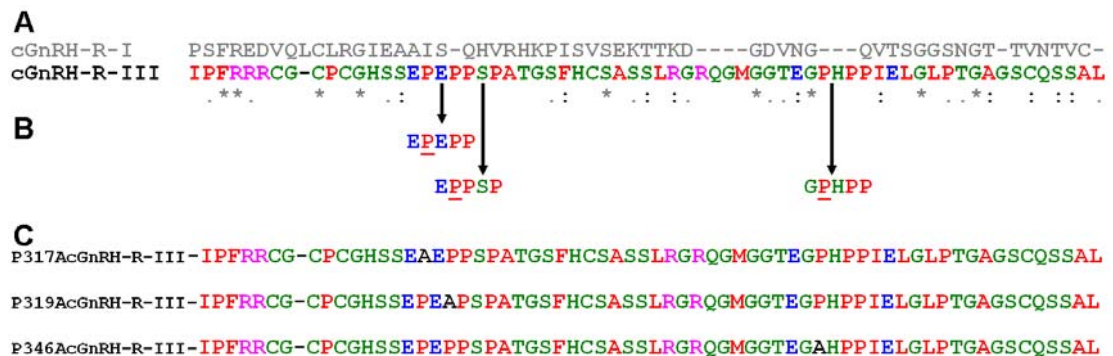


Fig. 5.6. Illustration of the cGnRH-R-III SH3 binding motif mutants. (A) The cytoplasmic-terminal tails of cGnRH-R-I (grey text) and cGnRH-R-III (coloured text), indicating that the putative SH3 binding motif in the cytoplasmic-tail of cGnRH-R-III (red text = small and hydrophobic, blue = acidic, magenta = basic, green = hydroxyl + amine + basic). (B) The putative SH3 binding motifs. (C) The SH3 binding motifs, P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III mutated by site directed mutagenesis, by substituting the proline (P) residue (red text) with an alanine (A) residue (black text).

No reduction or increase in the maximum His⁵-D-Tyr⁶-GnRH-I binding of P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III was observed when compared to the native cGnRH-R-III (**Fig. 5.7A-B**). Although a full displacement curve was not generated, the binding affinity can be assumed to be similar for P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III when compared to cGnRH-R-III as no difference in the displacement of ¹²⁵I-[His⁵-D-Tyr⁶]-GnRH-I with 10nM and 1µM cGnRH-I (**Fig. 5.7A**) or GnRH-II (**Fig. 5.7B**) was observed between receptor constructs. In accordance with observed higher ligand-binding affinity for GnRH-II compared to cGnRH-I at cGnRH-R-III (**Chapter 4-Fig.4.1**), the displacement of ¹²⁵I-[His⁵-D-Tyr⁶]-GnRH-I with 10nM and 1µM GnRH-II (**Fig. 5.7B**) was greater than that of 10nM and 1µM cGnRH-I (**Fig. 5.9A**) for all cGnRH-R-III SH3 binding motif mutants.

There were no significant differences between the maximal cGnRH-I induced pERK activation in the individual cGnRH-R-III SH3 binding motifs mutants (P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III) when compared to the native cGnRH-R-III at either 5 or 10 minute time-points. The data (**Fig. 5.8A-B**) are calculated as a fold response over non-stimulated pERK levels for each receptor construct transfected (**Fig. 5.8B**).

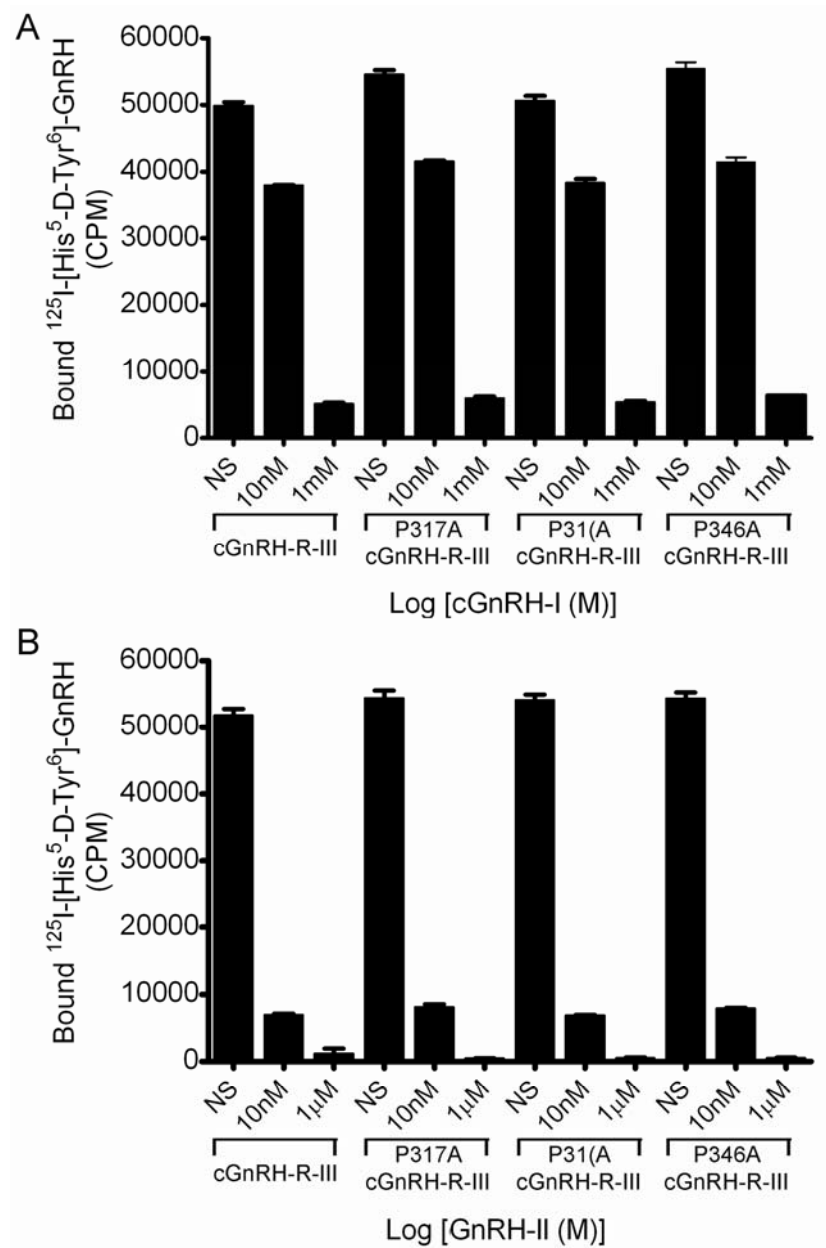
Figure 5. 7

Fig. 5.7. Mutagenesis of the individual SH3 binding motifs of cGnRH-R-III does not alter the cell surface receptor expression. Competitive displacement of ^{125}I -[His⁵-D-Tyr⁶]-GnRH with 10nM and 1μM of (A) cGnRH-I (B) and GnRH-II in COS-7 cells transiently transfected with cGnRH-R-III and SH3 binding motif mutants; P317A-cGnRH-R-II, P319A-cGnRH-R-III and P346A-cGnRH-R-III. The data presented is from three independent experiments each performed in triplicate.

Figure 5. 8

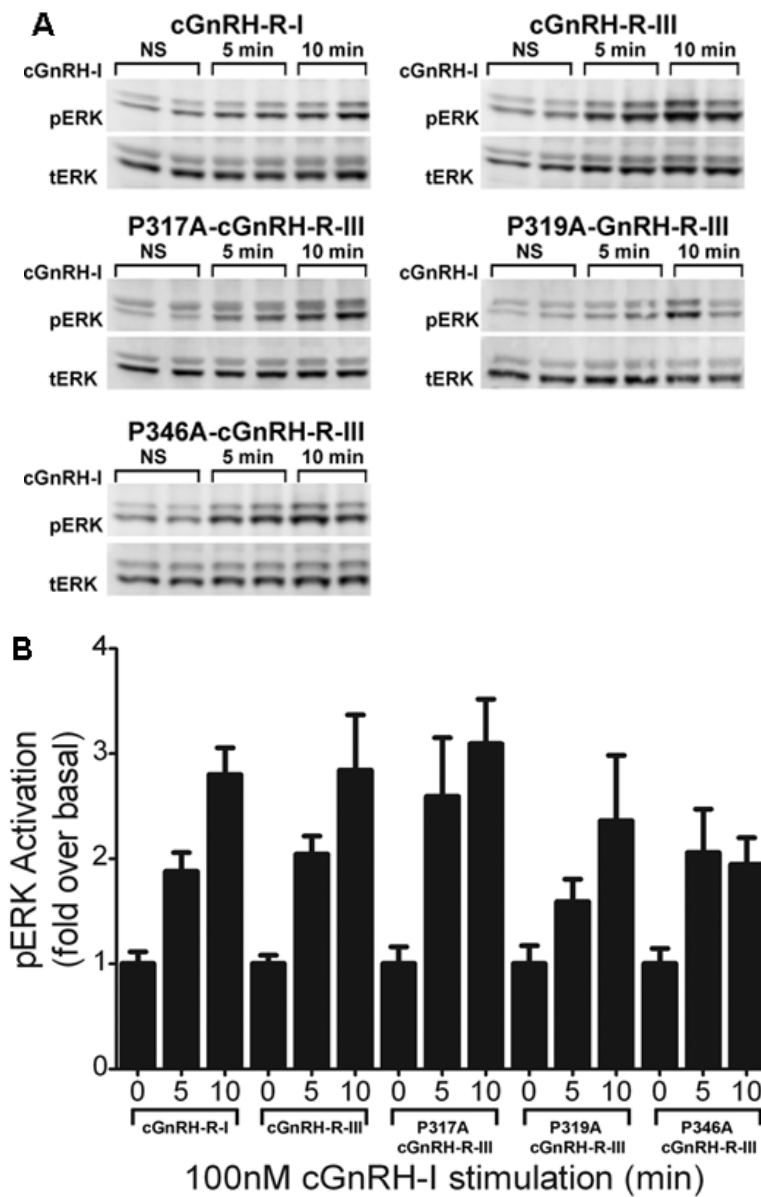


Fig. 5.8. Mutagenesis of the individual SH3 binding motifs of cGnRH-R-III does not alter cGnRH-I induced pERK activation. COS-7 cells expressing native chicken GnRH-R receptors or cGnRH-R-III SH3 binding motif mutants (P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III) were stimulated with 100nM cGnRH-I for 5 and 10 minutes or left non-stimulated as indicated. **(A)** Representative blots of the NP40 cell lysates are shown. **(B)** Data from at least three independent experiments performed in duplicate were quantified (using tERK immunoblot as a loading control) and a graph depicting the mean-fold over control \pm S.E. is presented below the blots. The statistical significance was calculated by applying a Student's t-test between cGnRH-R-I, P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III versus the native cGnRH-R-III at each specific time point (5 and 10 minutes). No statistical significant differences were detected.

5.4.5. Analysis of protein-protein interactions made by cGnRH-R-III or cGnRH-R-I

The sequences of the HA epitope-tagged cGnRH-R-I and cGnRH-R-III expression constructs generated as described in methods section (**Chapter 2: 2.20**) were confirmed by automated DNA sequencing and restriction enzyme digest. The expression construct contained the entire coding sequence and included a nucleotide sequence encoding a Kozak translation initiation sequence (GTT ATG) and a Hemagglutinin (HA) epitope-tag (TAT CCT TAT GAT GTT CCC AAC TAT GCT) immediately upstream of the usual receptor start codon through to the stop codon of either cGnRH-R-I or cGnRH-R-III cDNA sequences (**Fig. 5. 9**).

Critically for comparative studies, both HA epitope-tagged cGnRH receptors showed similar maximum binding (**Fig. 5.10A-B**), indicating that the HA epitope-tagged cGnRH-R receptors exhibit cell surface expression comparable with native cGnRH receptors. The binding affinity (measured as IC_{50}) of GnRH II for HA-cGnRH-R-I and HA-cGnRH-R-III were 0.43nM and 0.25nM respectively. Thus ligand-binding selectivity at HA-cGnRH-R-I and HA-cGnRH-R-III are comparable with those of the native cGnRH receptor homologs in respect to GnRH-II (**Chapter 4-Table 4.2**).

Figure 5. 9

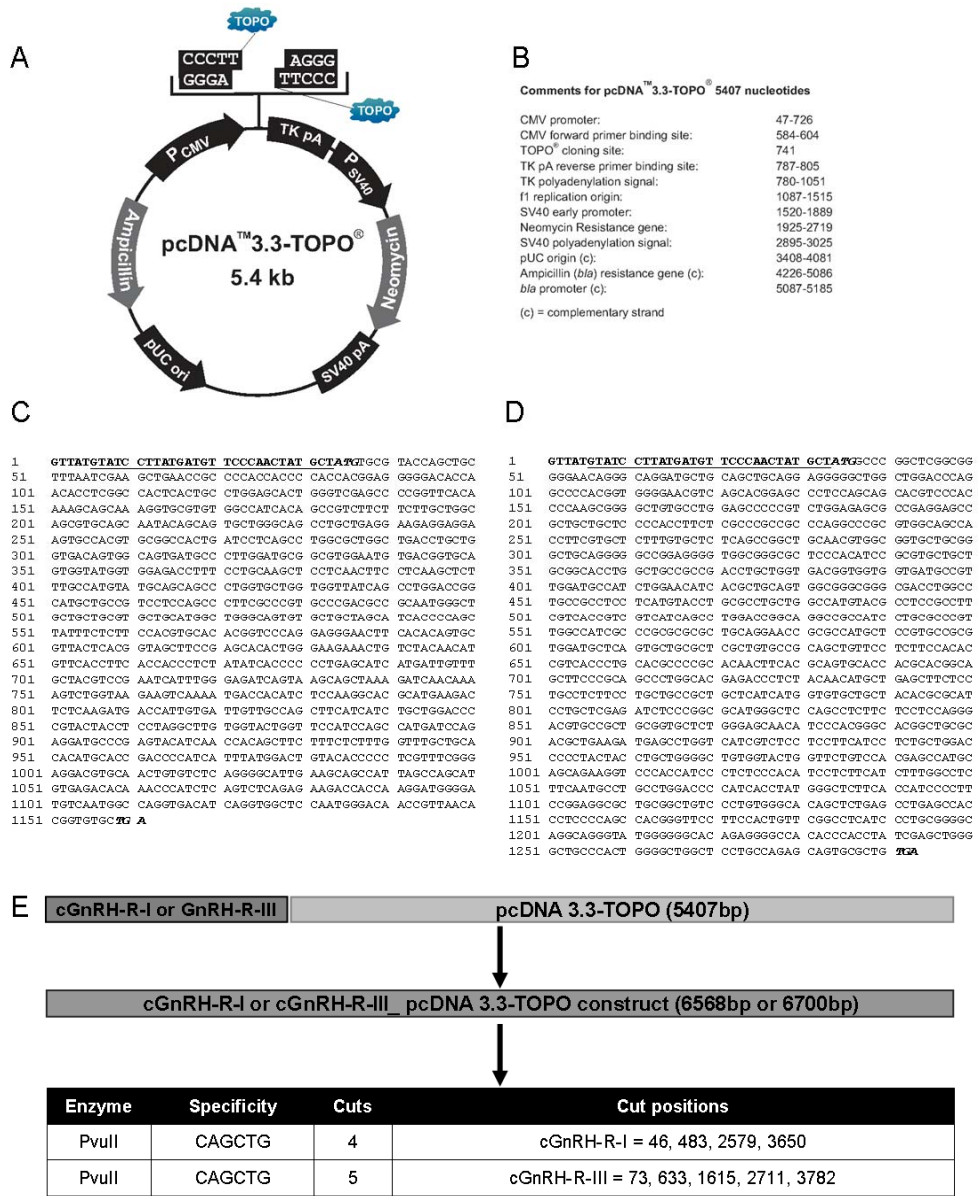


Fig. 5.9. Schematic representation of the construction of HA epitope-tagged cGnRH-R-I and cGnRH-R-III pcDNA 3.3 vector. (A) The pcDNA 3.3 vector. (B) The features of the pcDNA 3.3 vector (C) The cGnRH-R-I cDNA sequence, and (D), the cGnRH-R-III amplified with HA-tag primers (Table 2.1) The primer sequences used to amplify the original cDNA insert are annotated in bold, inclusive of the Kozak consensus (bold) and the HA epitope-tag (bold and underlined). The usual receptor translation start site (56-58bp) and the transcription termination site (1315-1318) are indicated in bold italics. (D) The restriction enzyme sites used to validate the integrity of the construct cloned by inserting the cGnRH-R-I or cGnRH-R-III epitope-tagged insert into the pre-linearised pcDNA3.3 vector. The base pair cut positions refer to the insert sequence.

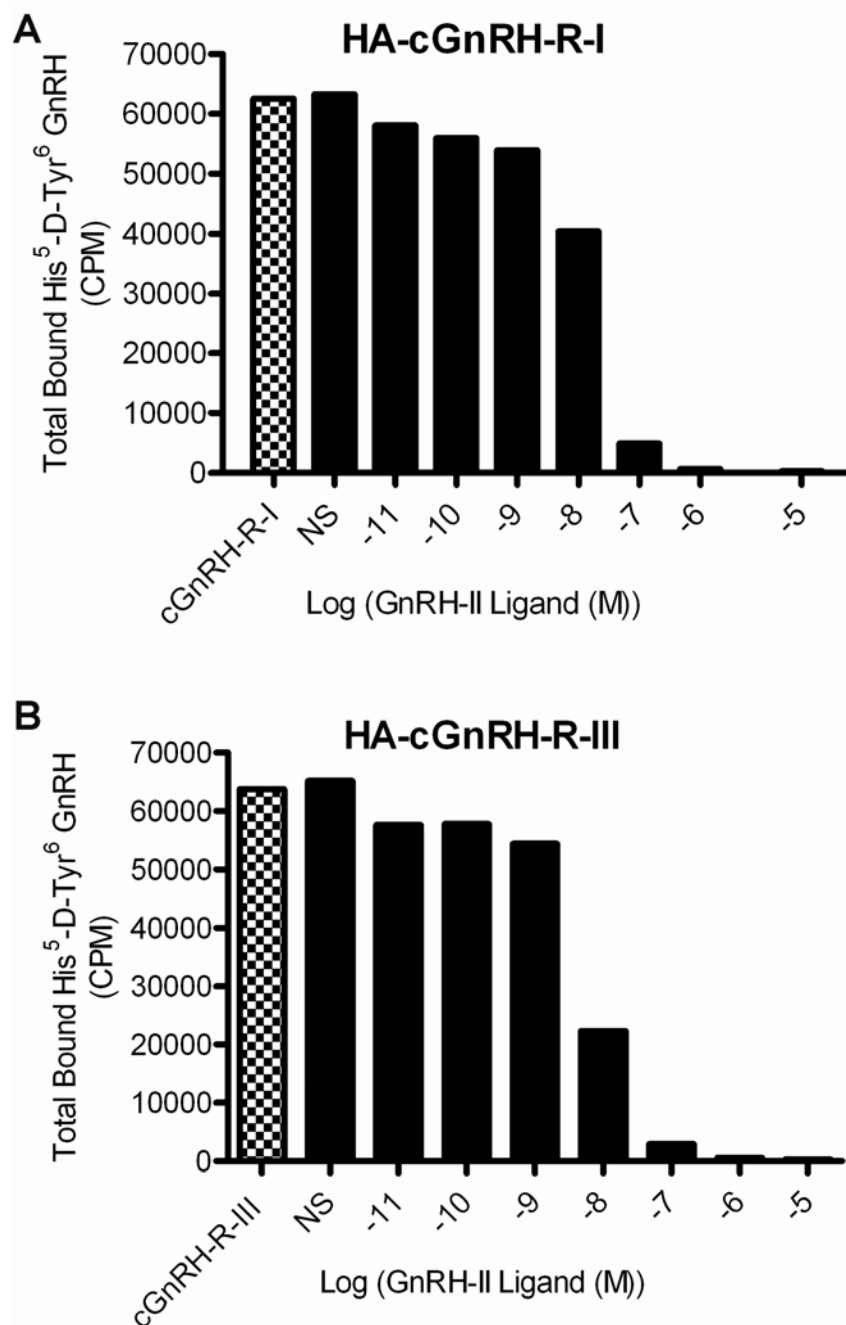
Figure 5. 10

Fig. 5.10. HA-cGnRH-R-I and HA-cGnRH-R-III have similar cell surface expression to native cGnRH-R-I and cGnRH-R-III. Competitive displacement of ^{125}I -[His⁵-D-Tyr⁶]-GnRH-I with serial dilutions (10^{-11}M to 10^{-5}M) of GnRH-II in COS-7 cells transiently transfected with (A) HA-cGnRH-R-I (filled columns) and (B) HA-cGnRH-R-III (filled columns) expression constructs. (A,B) The maximal binding of the native cGnRH-R-I and cGnRH-R-III (checked filled columns) is used as a measure of expression at the cell surface. The data presented is from one experiment performed in triplicate.

Attempts were made to optimise the immuno-precipitation of HA-GnRH-R-I and HA-cGnRH-R-III. It was possible to immuno-precipitate a human HA epitope-tagged GnRH-R construct (HA-hGnRH-R), although detection of HA-cGnRH-R-I and HA-cGnRH-R-III was not apparent (**Fig. 5.11**). The HA-hGnRH-R construct was kindly provided by Dr R. Forfar, MRC, HRSU, Edinburgh, for use as a positive control. In an attempt to verify the translation of the HA coding sequence of the chicken GnRH receptors, an immuno-blot was performed on NP40 cells lysates with anti-HA 3F10 antibody. However, due to the excessive number of bands representing non-specific binding of the antibody to the solubilised cell lysates, it was not possible to detect specific bands representing HA protein (**data not shown**).

An anti-cGnRH-R-III antibody was kindly provided by Prof. G. Bedecarrats, University of Guelph as part of collaborative work to validate the antibody and to detect expression of the cGnRH-R-III splice variants (**Chapter 3**). Detection of cGnRH-R-III in NP40 cell lysates of transiently transfected COS-7 cells expressing cGnRH-R-III by immuno-blotting with this anti-cGnRH-R-III antibody yielded three bands for both cGnRH-R-III and HA-cGnRH-R-III at approximately 40-45 kiloDaltons (**Fig 5.12**).

Attempts to immuno-precipitate the cGnRH-R-III and HA-cGnRH-R-III proteins produced by transient transfection of COS-7 cells using anti-cGnRH-R-III followed by immuno-blotting with either anti-cGnRH-R-III or anti-HA 3F10 yielded numerous non-specific bands (**data not shown**).

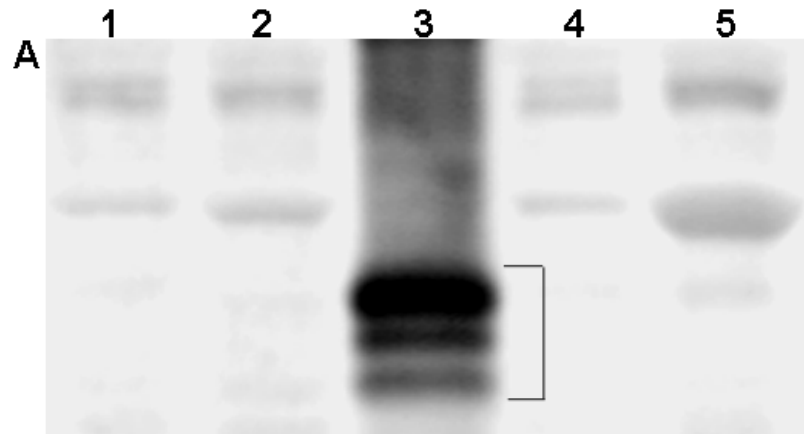
Figure 5. 11

Fig. 5.11. Immunoblot of HA epitope-tagged GnRH receptors immuno-precipitated with anti-HA-3F10. COS-7 cells were transiently transfected with HA epitope-tagged GnRH receptors and precipitated from NP40 solubilised cell lysates. The presence of the receptor in the precipitate was analysed using immuno-blotting with anti-HA-12CA5 antibody. 1 = HA-cGnRH-R-I, 2 = HA-cGnRH-R-III, 3 = HA-hGnRH-R, 4 = cGnRH-R-III, 5 = Non-transfected control (NTC). Bracket indicates receptor tagged protein.

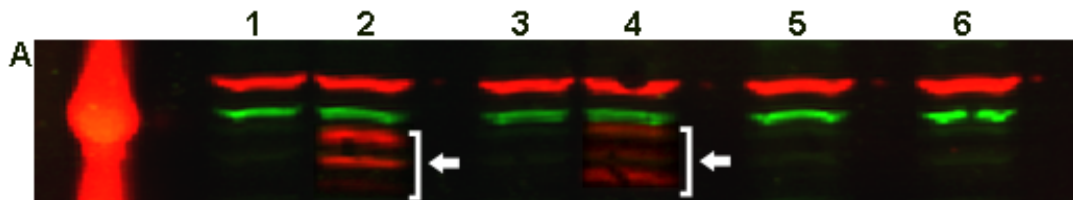
Figure 5. 12

Fig 5.12. Immunoblot of NP40 cell lysates of transiently transfected COS-7 cells expressing GnRH receptors. COS-7 cells were transiently transfected with HA epitope-tagged cGnRH receptor or un-tagged cGnRH receptor constructs and NP40 solubilised cell lysates prepared as described in the methods section 2.22. The presence of the receptor proteins in solubilised NP40 cell lysates was analysed using immuno-blotting with anti-HA-12CA5 (green) and anti-cGnRH-R-III antibody (red). 1 = cGnRH-R-I, 2 = cGnRH-R-III, 3 = HA-cGnRH-R-I, 4 = HA-cGnRH-R-III, 5 = HA-hGnRH-R, 6 = Non-transfected control (NTC). Arrowed brackets indicate detection of cGnRH-R-III tagged protein.

The membrane localisation of cGnRH-R-III and HA-cGnRH-R-III protein was quantified by isolating the membranes of COS-7 cells expressing cGnRH-R-III in

parallel with radioligand binding assays. Receptor expression detected by quantifying the maximal binding of His⁵-D-Tyr⁶-GnRH-I in whole cell radioligand binding assays showed that equal amounts of either cGnRH-R-III protein was expressed. However, the immuno-blots (**Fig. 5.13** and **Fig. 5.14**) showed detection of cGnRH-R-III but less protein expression of HA-cGnRH-R-III using anti-cGnRH-R-III antibody. HA-cGnRH-R-III was not detectable using anti-HA 3F10 for immuno-blotting, although HA-hGnRH-R was detectable using anti-HA 3F10 for immuno-blotting. Therefore, the number of cGnRH-R-III transfected COS-7 cells used to immuno-precipitate the cGnRH-R-III using anti-HA 3F10 antibody was increased in subsequent experiments. Immuno-blotting the immuno-precipitated proteins with anti-cGnRH-R-III indicated 3 bands specific for cGnRH-R-III, corresponding to a protein of approximately 40-45 kiloDaltons (**Fig 5.15.A-B**). However, immuno-blotting the immuno-precipitated samples with anti HA-3F10 yielded a number of non-specific bands (**data not shown**).

Figure 5. 13

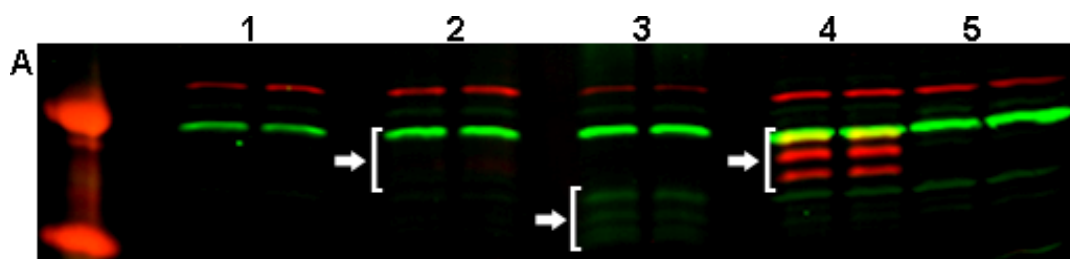


Fig 5.13. Immunoblot of cell membrane proteins from transiently transfected COS-7 cells expressing GnRH receptors. COS-7 cells were transiently transfected with HA epitope-tagged and native GnRH receptors and the membranes isolated by high speed centrifugation. The presence of the GnRH receptors in the isolated membranes was analysed using immuno-blotting with anti-HA-12CA5 (green) and anti-cGnRH-R-III anti-body (red). **1** = HA-cGnRH-R-I, **2** = HA-cGnRH-R-III, **3** = HA-hGnRH-R, **4** = cGnRH-R-III, **5** = Non-transfected control (NTC). Arrowed brackets indicate GnRH-R protein.

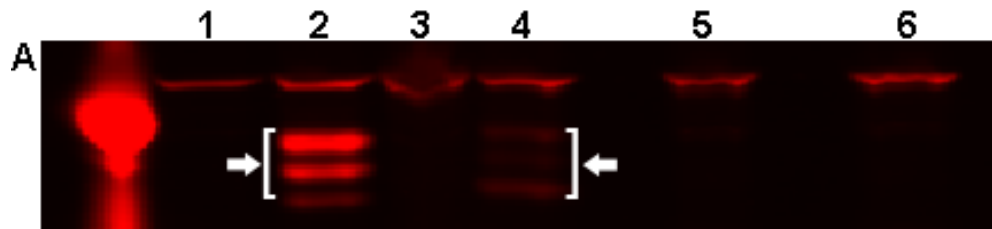
Figure 5. 14

Fig 5.14. Immunoblot of cell membrane proteins from transiently transfected COS-7 cells expressing GnRH receptors. COS-7 cells were transiently transfected with HA epitope-tagged and native GnRH receptors and the membranes isolated by high speed centrifugation. The presence of the GnRH receptors in the isolated membranes was analysed using immunoblotting with anti-cGnRH-R-III anti-body (red). **1** = cGnRH-R-I, **2** = cGnRH-R-III, **3** = HA-cGnRH-R-I, **4** = HA-cGnRH-R-III, **5** = HA-hGnRH-R, **6** = Non-transfected control (NTC). Arrowed brackets indicate cGnRH-R-III protein.

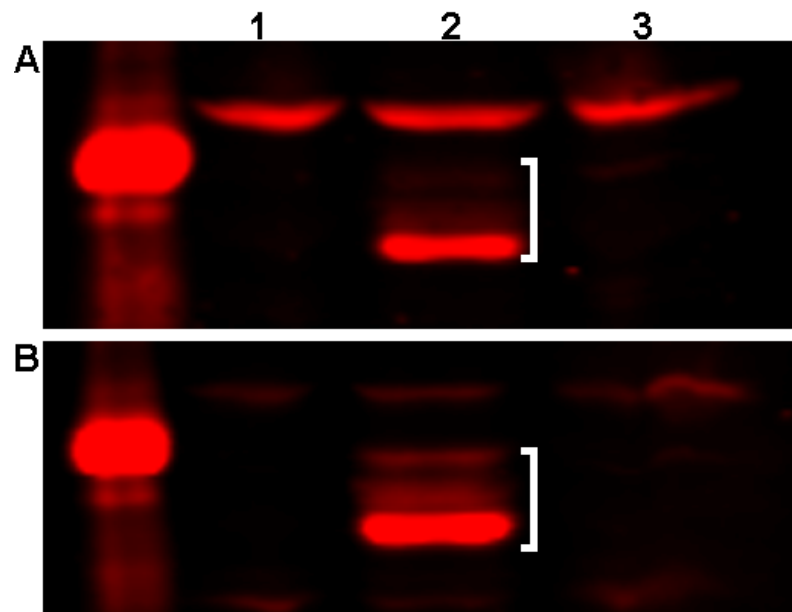
Figure 5. 15

Fig. 5.15. Immunoblot of HA epitope-tagged GnRH receptors immunoprecipitated with anti-HA-3F10. COS-7 cells were transiently transfected with HA epitope-tagged GnRH receptors and precipitated from NP40 solubilised cell lysates. The presence of the receptor in the precipitate was analysed using immunoblotting with anti-cGnRH-R-III anti-body (red). **1** = HA-cGnRH-R-I, **2** = HA-cGnRH-R-III, **3** = Non-transfected control (NTC). Brackets indicate cGnRH-R-III protein. (A,B) represent two occasions illustrating different intensities of cGnRH-R-III protein.

5.5. Discussion

cGnRH-I and GnRH-II stimulate inositol phosphate production in COS-7 cells expressing cGnRH-R-I and cGnRH-R-III (**Chapter 4 - Fig. 4.1B**) (Joseph et al., 2009; Shimizu and Bedecarrats, 2006; Sun et al., 2001b). Since inositol phosphate and DAG are generated by the recruitment of the $G\alpha_q$ protein and subsequent activation of PLC- β (Ruf et al., 2003; Stojilkovic et al., 1994), cGnRH-R-III and cGnRH-R-I can activate the Ca^{2+} and PKC signalling pathways which are the predominant physiological mediators of LH and FSH biosynthesis and secretion (Conn and Crowley, 1994; Sealfon et al., 1997). cAMP and PKC activation and Ca^{2+} are involved in the regulation of cGnRH-I induced LH secretion from chicken pituitary gonadotrophs (Bonney and Cunningham, 1977a; Bonney and Cunningham, 1977b; Davidson et al., 1987a; Davidson et al., 1987b; Johnson and Tilly, 1991; Luck and Scanes, 1980). However it was also shown that increases in intracellular Ca^{2+} is sufficient for secretion of stored LH, whilst elevated cyclic cAMP is not essential for LH secretion and PKC activates a variety of signalling molecules (Johnson and Tilly, 1991). The downstream signal transduction of Ca^{2+} and PKC and the factors translocated to the nucleus with subsequent transcriptional regulation of gonadotrophins in the chicken remain to be elucidated. GnRH release is probably pulsatile in birds and, in support of this view, chicken plasma LH levels are episodically modulated (Senthilkumaran et al., 2006; Vizcarra et al., 2004). Additionally, downstream signalling may be involved in sustaining LH production to meet the demands of pulsatile secretion. As PKC and Ca^{2+} mediate the activation of the MAPK cascade in pituitary gonadotrophs, (Dobkin-Bekman et al., 2006; Naor et al., 2000) it is feasible that the transcriptional regulation of the gonadotrophins in the chicken is mediated by the MAPK cascade and modulated via the type I and/or the type III cGnRH receptors. Research into signalling through the MAPK cascade via the chicken GnRH receptors subtypes has not been investigated to date but it may be expected to be similar to what has been established in mammals but perhaps with some species-specific differences. Three putative SH3 binding motifs were identified in the cytoplasmic C-terminal tail of cGnRH-R-III (**Chapter 3**), and it was

hypothesized that the type III GnRH receptor may differentially interact with SH3 domain-containing proteins through the SH3 binding motifs. In an attempt to elucidate the physiological roles of the receptor subtypes and attribute a differential functional capacity to cGnRH-R-III, the effects of the SH3 binding motifs in terms of activation of the MAPK signalling cascade were investigated in this chapter.

COS-7 cells do not endogenously express GnRH receptors and vehicle or GnRH treatment of non-transfected COS-7 cells did not affect levels of pERK (**data not shown**). Dose responses and time-dependent responses to cGnRH-I and GnRH-II were quantified in cGnRH-R-I and cGnRH-R-III expressing COS-7 cells to elucidate whether there were any differences in pERK activation via the two cGnRH receptor subtypes. The dose response of cGnRH-I and GnRH-II induced pERK activation via cGnRH-R-I and cGnRH-R-III clearly indicate that an increase in concentration of either cGnRH-I or GnRH-II results in an increase of the pERK activated by both cGnRH-I and cGnRH-R-III (**Fig. 5.1A-D**). cGnRH-I and GnRH-II induced pERK activation via cGnRH-R-I and cGnRH-R-III in a time-dependent manner with a maximal response observed at 15 minutes of stimulation (**Fig. 5.2A-D**). There is a clear decrease in pERK activation when cGnRH-R-I and cGnRH-R-III are stimulated with cGnRH-I and GnRH-II for 30 minutes when compared to the 15 minute stimulation (**Fig. 5.2A-D**). Both cGnRH-I and GnRH-II treatment resulted in pERK activation via cGnRH-R-I and cGnRH-R-III in a similar dose (**Fig. 5.1A-D**) and time (**Fig. 5.2A-D**) dependent manner despite the presence of three putative SH3 binding motifs identified in the cytoplasmic C-terminal tail of cGnRH-R-III. However, the G protein dependent activation of the MAPK cascade via PKC may mask any differences in Src mediated MAPK signalling via cGnRH-R-I and cGnRH-R-III or the effects elicited by SH3 domain binding proteins as previously suggested in a comparison between type I and type II GnRH receptors in which effects of β -arrestin mediated MAPK signalling were investigated (McArdle et al., 2002; Miller et al., 2000). Additionally, GnRH receptors interact with multiple G proteins in a 'cell-context' dependent manner and may form different signal transduction protein

complexes in these cell types (Dobkin-Bekman et al., 2006), as shown in studies where GnRH stimulated ERK activation is dependent on PKC, c-Src, dynamin, RAS and Ca^{2+} influx (Benard et al., 2001), or dependent on FAK and c-Src at focal adhesion complexes (integrins) mediated by Rac (Davidson et al., 2004) or alternatively activation of the EGFR resulting in c-Src activation and subsequent ERK activation mediated by Ras (Kraus et al., 2003). The existence of multiple and different MAPK protein complexes reflect properties of different cell types, gene promoters, species-specific effects and differences in cell culture conditions (Dobkin-Bekman et al., 2006; Karnoub and Weinberg, 2008; Naor, 2009; Omerovic et al., 2007). Perhaps therefore, it is necessary to compare the mechanism by which cGnRH-R-I and cGnRH-R-III signal to the MAPK cascade in more detail, prior to comparing the level of pERK activation elicited by the two receptors, as the two receptors may induce similar overall pERK activation, but they may signal to the MAPK cascade through different protein complexes. It is feasible that multiple and different MAPK protein complexes may couple to the two receptors resulting in pERK activation. The type III cGnRH-R may for instance predominantly signal through a protein complex which involves c-Src.

Therefore in an attempt to elucidate the process mediating pERK activation via cGnRH-R-I and cGnRH-R-III in COS-7 cells, the effects on pERK activation during inhibition of PLC and c-Src were investigated. In pituitary gonadotrophs a consensus signalling mechanism has emerged which activates the MAPK cascade, with $\text{G}\alpha_q$ activating PLC which generates DAG and IP3 which subsequently activate PKC and Ca^{2+} mobilisation, resulting in activation of ERK, JNK, and p38 (Dobkin-Bekman et al., 2006; Naor et al., 2000). Therefore, determining the dependency on PLC of the two cGnRH-Rs may inform on the $\text{G}\alpha_q$ dependency of ERK activation via the two cGnRH-Rs. Dependency on c-Src was also investigated, as it was anticipated that the two cGnRH receptors may mediate pERK activation through different second messenger pathways due to the SH3 binding motifs identified in the type III GnRH receptor which may differentially interact with SH3 domain-

containing proteins. Activation of the MAPK cascade is often dependent on c-Src interactions (Benard et al., 2001; Davidson et al., 2004; Gutkind, 1998; Kraus et al., 2003; Levi et al., 1998; Luttrell et al., 1999). PKC activated via $G\alpha_q$ can modulate the activity of non-receptor PTKs e.g. c-Src family kinases (Levi et al., 1998). In α T3-1, HEK293 and COS-7 cells GnRH stimulated ERK activation is c-Src dependent along with other second messengers (Benard et al., 2001; Davidson et al., 2004; Kraus et al., 2003). In α T3-1, JNK activation is also shown to be c-Src dependent (Levi et al., 1998). Therefore, determination of the dependency on c-Src, may potentially elaborate the function of the SH3 binding motifs in cGnRH-R-III.

Validation of the PLC inhibitor (U73122) action was performed by demonstrating that pre-incubation with U73122 effectively blocked accumulation of inositol phosphate production on 60 minutes of GnRH stimulation (**Fig. 5.3A-B**). Although the subsequent stimulations with cGnRH-I and GnRH-II to detect pERK activation were for 10 minutes in duration, stimulations required to measure inositol phosphate production were prolonged to 60 minutes (**Fig. 5.3B**) to clearly demonstrate an inhibition of inositol phosphate production. There was a clear increase in inositol phosphate production in response to cGnRH-I and GnRH-II via cGnRH-R-III when compared to the U73122 treated cGnRH-R-III transfected cells (**Fig. 5.3B**). The results demonstrate that despite an inhibition in inositol phosphate accumulation with U73122 with 60 minute GnRH stimulation (**Fig. 5.3B**) there was no inhibition of pERK activation via cGnRH-R-III and cGnRH-R-I in response to cGnRH-I and GnRH-II (10 minute stimulations) (**Fig. 5.4B-C and Fig. 5.5A-B**). This suggested that cGnRH-I and GnRH-II induced pERK via cGnRH-R-I and cGnRH-R-III independently of PLC. However, there was a small but significant increase ($p < 0.05$) in the accumulation of inositol phosphate production in response to GnRH-II stimulation via cGnRH-R-III transfected cells that were pre-treated with U73122 (**Fig. 5.3B**). Therefore, 20 μ M U73122 does not completely inhibit PLC. Additionally, the effects of U73122 inhibitor on PLC were only shown on 60 minutes of GnRH stimulation by measuring the production of inositol phosphate, whereas the

pERK was quantified on 10 minutes of GnRH stimulation. Therefore, the PLC inhibitor has not been shown to inhibit PLC in the time frame used for the pERK experiments. The effects of PLC inhibitor were compared alongside the effects of c-Src inhibition on GnRH-I induced pERK activation via cGnRH-R-I (**Fig 5.5A**) and cGnRH-R-III (**Fig. 5.5B**). The results confirm previous observations (**Fig 5.4B-C**) demonstrating that cGnRH-I induced pERK activation via cGnRH-R-I and cGnRH-R-III may be PLC independent. The cGnRH-I induced pERK activation via cGnRH-R-I and cGnRH-R-III is c-Src dependent (**Fig. 5.5A-B**). $G\alpha_q$ coupling primarily activates PLC which produces IP₃ which induces Ca^{2+} and DAG (Naor et al., 2000). As PLC and hence IP₃, Ca^{2+} and DAG are may not be not essential for pERK activation (**Fig. 5.4B-C and 5.5A-B**), the possible signal transduction mechanisms discussed thus far in GPCRs may not apply. These include mediation through (1) the free Ca^{2+} activating CaM kinase II which phosphorylates and inhibits RAS-GTPase-activating protein (RAS-GAP) inducing RAS and subsequently MAPK activation (Chen et al., 1998). (2) Ca^{2+} and DAG activated PKC or through Ca^{2+} /RAS-GEF and RAS activation leading to MAPK activation (Farnsworth et al., 1995; Naor et al., 2000; Nishizuka, 1992) (3) PKC activating c-RAF which is partially dependent on RAS resulting in stimulation of the ERK cascade (Kolch et al., 1993) (4) Ca^{2+} and PKC inducing activation of PTK (Zhang et al., 1996) which possibly facilitated by integrin interaction leads to MAPK activation (Naor et al., 2000). However, both receptors are coupled to $G\alpha_q$ (**Chapter 4 - Fig. 4.1B**) (Joseph et al., 2009; Shimizu and Bedecarrats, 2006; Sun et al., 2001b) and possibly $G\alpha_s$ (**Shimizu M et al., 2008**). GnRH induced pERK activation via cGnRH-R-I and cGnRH-R-III may be mediated by either G protein or the $G\beta\gamma$ complexes which can signal through PI3K to activate the MAPK cascade (Naor et al., 2000). Additionally, there may be residual PLC activity, as inositol phosphate production was not completely inhibited by application of PLC inhibitor (U73122) (**Fig. 5.3B**). Furthermore, pERK activation via cGnRH-R-I and cGnRH-R-III may be facilitated by other signalling components of the cGnRH-R-I and cGnRH-R-III signalling complex (Karnoub and Weinberg, 2008). pERK activation via cGnRH-R-I and cGnRH-R-III may be similar to the JNK activation observed in rat pituitary cultures, where it was shown to be PKC

independent and dependent on Ca^{2+} mobilisation (Mulvaney and Roberson, 2000; Mulvaney et al., 1999). As pERK activation via the two cGnRH receptors, is c-Src dependent (**Fig 5.5A-B**), it is more likely that the pERK activation via the two receptors is mediated in a fashion similar to HEK293 cells, where GnRH stimulation of GnRH receptor expressing cells results in ERK activation dependent on FAK and c-Src at focal adhesion complexes (intergrins) mediated by Rac (Davidson et al., 2004). Although, in COS-7 cells, $\text{G}\alpha_i$ affects EGFR, resulting in c-Src activation and subsequent ERK activation mediated by Ras and JNK activation mediated by PI3K (Kraus et al., 2003). This mechanism may possibly involve $\text{G}\alpha_q$ or $\text{G}\alpha_s$ coupling and may also be the mechanism in which the cGnRH-R-III mediates pERK activation. Or additionally, as in DU-145 cells, GnRH activation of ERK may involve, c-Src and EGFR trans-activation, which is mediated by the shedding of the heparin binding-EGF (HB-EGF) (Kraus et al., 2004).

As no receptor-subtype-specific differences in pERK activation were detected in terms of dose-response (**Fig. 5.1**), or time-course (**Fig5.2**), or inhibitor-probed mechanisms (**Fig. 5.5**), it was necessary to investigate the influence of SH3 binding motifs to further test the hypothesis that cGnRH-R-III has an additional capacity to signal through the MAPK signalling cascade through a direct interaction involving putative SH3 binding motifs (**Chapter 3**). Therefore mutagenesis of the SH3 binding motifs in the cGnRH-R-III cDNA receptor construct was performed to enable *in-vitro* studies (**Fig 5.6**). The SH3 binding motif mutants, P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III expressed in transfected COS-7 cells were compared, and the results showed that there are no changes in the maximal binding of His⁵-D-Tyr⁶-GnRH-I displacement with cGnRH-I and GnRH-II between the SH3 binding motif mutants and the native cGnRH-R-III (**Fig. 5.7A-B**). This demonstrated that the mutations did not alter the cell surface expression of the receptor. The displacement of His⁵-D-Tyr⁶-GnRH-I with 10nM and 1 μ M cGnRH-I and cGnRH-II demonstrate that the ligands have the same affinity to P317A-cGnRH-R-III, P319A-cGnRH-R-III, P346A-cGnRH-R-III compared to cGnRH-R-III (which

was as previously determined **Chapter 4-Fig. 4.1**). The features of pERK activation via P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III in response to cGnRH-I and GnRH-II were similar to both the native cGnRH-R-III and cGnRH-R-I (**Fig 5.8A-B**). Therefore, there appears to be no additional pERK activation via cGnRH-R-III as a result of direct coupling through the SH3 binding motifs. Capacity for further increases in pERK levels are possible in the COS-7 cell assay, as the maximum pERK levels in COS-7 cells transiently transfected with cGnRH-R constructs has been observed as high as 12 fold when compared to basal (**data not shown**). Therefore, the pERK assay did not obtain the maximum pERK levels feasible such that enhanced pERK activation would be detected if present. However, G protein dependent activation of the MAPK cascade may mask any differences in c-Src mediated MAPK signalling via cGnRH-R-I and cGnRH-R-III involving the SH3 binding motif mutants.

Therefore testing the hypothesis that cGnRH-III directly interacts with c-Src through the SH3 binding motifs was investigated. In order to test this hypothesis, the cGnRH-R-I and cGnRH-R-III receptor cDNA constructs were modified to encode an HA epitope-tag that would facilitate the immuno-precipitation of the receptors (**Fig 5.9**) and the protein complexes which they form. The HA epitope-tagged receptors did not exhibit any differences in receptor expression (**Fig 5.10**) in comparison with native cGnRH-R-I and cGnRH-R-III transfected cells (**Chapter 4 and Fig 5.7**). Additionally the ligand-binding selectivity at HA cGnRH-R-I and cGnRH-R-III (**Fig 5.10**) was comparable with that of the native cGnRH receptor homologs (**Chapter 4-Table 4.1**) in response to GnRH-II.

Initial attempts to immuno-precipitate the HA epitope-tagged chicken type I and type III GnRH receptors were unsuccessful despite the successful immuno-precipitation of a human HA epitope-tagged GnRH receptor used as a positive control (**Fig. 5.11**). This suggested that the methodology used was optimal for immuno-precipitation of the GnRH receptors, whilst the HA epitope-tag of the chicken GnRH receptors may

not have been efficiently translated. Using the anti-cGnRH-R-III antibody to immuno-blot NP40 cell lysates (**Fig. 5.12**), demonstrated that it is possible to detect the HA-cGnRH-R-III protein. In an attempt to enrich the HA-cGnRH-R-I and HA-cGnRH-R-III proteins, cell membranes were prepared by high speed centrifugation and immuno-blotted with anti-HA 3F10 and anti-cGnRH-R-III antibodies (**Fig. 5.13 & 5.14**). The detection of HA-hGnRH-R in enriched cell membrane protein samples, with anti-HA 3F10 antibody but not HA-cGnRH-R-I or HA-cGnRH-R-III (**Fig. 5.13**) indicated that the HA-epitope-tag of the chicken cDNA constructs was not being translated. However, the detection of cGnRH-R-III compared to HA-cGnRH-R-III in the cell membrane samples with anti-cGnRH-R-III antibody demonstrated that the HA-cGnRH-R-III protein was being translated but less protein was detectable when compared to the un-tagged construct. This observation did not correlate with the radioligand binding data, which showed that equal amounts of “tagged” and un-tagged receptors were being expressed in the transfected COS-7 cells (**Fig 5.10**), suggesting that the two proteins were being expressed (HA-cGnRH-R-III and cGnRH-R-III). This is possibly due to the design of the N-terminal HA-cGnRH-R-I and HA-cGnRH-R-III constructs (**Fig 5.9C-D**), where a Kozak translation start consensus and HA-epitope-tagged are inserted upstream and in addition to the usual translational start site of the cDNA encoding cGnRH-R-I and cGnRH-R-III. This would allow alternate translation from either the Kozak consensus or the physiological translational start site. Therefore to further facilitate the detection of HA-cGnRH-R-III, the number of cells transfected was increased prior to immuno-precipitation with anti HA-3f10 antibody and immuno-blotting with anti-cGnRH-R-III antibody (**Fig. 5.15**). Subsequently, several size forms of each HA-tagged receptor were detectable, allowing the composition of the cGnRH-R-III immuno-precipitate to be analysed in detail. However, attempts at detecting the HA-epitope tag of both cGnRH-R-I and cGnRH-R-III with anti-HA 3F10 antibody were not possible. Therefore, it was not possible to validate the immuno-precipitation of cGnRH-R-I as no anti-cGnRH-R-I antibody is available. It would therefore be impossible to make a complete comparison of the cGnRH-R-I and cGnRH-R-III immuno-precipitated protein complexes. It was concluded that it would be necessary

to redesign HA-epitope tagged cGnRH-R-I and cGnRH-R-III constructs, removing the physiological translational start site of cGnRH-R-I and cGnRH-R-III cDNAs prior to addition of the Kozak consensus and HA-epitope tag to ensure translation of only the cGnRH-R constructs encoding the HA-epitope tags. However, it is also possible that there may be a conformational change in the HA epitope tagged cGnRH-R proteins once biosynthesised in COS-7 cells that may inhibit binding to the anti HA antibody and thereby compromise immuno-precipitation.

In summary, the results of this chapter show that both chicken GnRH receptors induce phosphorylation of ERK in a similar manner in response to cGnRH-I and GnRH-II despite the three SH3 binding motifs in cGnRH-R-III. Both cGnRH-Rs potentially activate ERK independently of PLC but dependent upon c-Src. However, more research into the mechanism will be necessary, and possible effects of the inhibitor should be tested using Ca^{2+} assays which will enable verification of the inhibitor in the same time frame as the pERK experiments. Mutagenesis of the individual SH3 binding motifs of cGnRH-R-III suggested that the SH3 binding motifs in cGnRH-R-III do not influence MAPK activation when compared to the native cGnRH-R-III in a transient transfection assay. However, the SH3 binding motifs in cGnRH-R-III may recruit other protein-protein complex formations not tested in this thesis. **Therefore the SH3 binding motifs identified in cGnRH-R-III do not alter ERK activation when compared to cGnRH-R-I. The signal transduction mechanisms leading to ERK activation via cGnRH-R-I and cGnRH-R-III are probably similar. A method has been optimised to facilitate the immuno-precipitation of cGnRH-R-I and cGnRH-R-III in order to characterise the protein-protein complexes formed by these receptors.**

Chapter 6

General Discussion

5.6. Chapter Summaries

5.6.1. Cloning, Characterisation and Tissue-Specific Expression of Chicken Type III GnRH Receptor

The data presented in this chapter demonstrates that two GnRH receptor subtypes are expressed in the chicken (type I and type III). This was shown by RT-PCR amplification and subsequent cloning and sequencing of the cGnRH-R-III cDNA from broiler chicken anterior pituitary glands. Northern blot analysis indicated a single cGnRH-R-III transcript in the anterior pituitary gland, suggesting that the *in-vivo* expression of cGnRH-R-III splice variants previously identified by RT-PCR (but undetectable by Northern blotting) is relatively low. cGnRH-R-III was first designated as cGNRHR2 (Shimizu and Bedecarrats, 2006), whereas re-designation as cGnRH-R-III is more appropriate. This was demonstrated by phylogenetic analysis performed in this chapter. The type III GnRH receptor gene structure and amino acid sequence motifs were compared with cGnRH-R-I and the conservation of certain motifs suggests that the two cGnRH-R receptor homologs may have similar pharmacological profiles in relation to endogenous cGnRH ligands. Attempts to confirm the cellular localisation of cGnRH-R-III expression within pituitary gonadotroph cells using *in-situ* hybridisation proved unsuccessful. This may be because cGnRH-R-III mRNA is not expressed abundantly enough to be readily detected by *in-situ* hybridisation and it is possible that the cGnRH-R-III mRNA transcripts might not be concentrated in one particular subtype of pituitary cells, but may be spread over a number of different cell types. Real-time RT-PCR analysis in juvenile and sexually mature male and female broiler chicken tissue samples showed that cGnRH-R-III is expressed predominantly in the anterior pituitary gland. cGnRH-R-III mRNA expression in the anterior pituitary gland was 92.5 times greater than in the testes or ovary. A quantitative comparison of the expression of cGnRH-R-I and cGnRH-R-III demonstrated that the level of expression of cGnRH-R-III mRNA was greater than cGnRH-R-I mRNA in the median eminence, gonads and small intestine, and particularly so in the anterior pituitary where cGnRH-R-III

mRAN expression was ~1400 times that of cGnRH-R-I mRNA. No large differences in level of tissue-specific expression were detected for cGnRH-R-I mRNA, where expression in the testes and ovary was ~1.4 times greater than in the anterior pituitary. Perhaps the most interesting finding was the detection of a pronounced sex and age difference in cGnRH-R-III mRNA expression, with 2.7 fold higher pituitary transcript levels in sexually mature females compared to juvenile females. In contrast, 8.4 fold higher expression levels occurred in juvenile males compared to sexually mature males. The higher abundance of expression of cGnRH-R-III mRNA compared to cGnRH-R-I mRNA, and the correlation of cGnRH-R-III mRNA expression levels with reproductive status suggests that cGnRH-R-III is probably the major mediator of pituitary gonadotroph function, and that tissue-specific recruitment of this cGnRH-R isoform has occurred during evolution.

5.6.2. Pharmacological Characterisation of GnRH Agonist and Antagonist at Type I and Type III cGnRH Receptors and cGnRH-R-III Splice Variants

The data presented in this chapter included analysis of the *in-vitro* pharmacological properties of cGnRH-R-I and cGnRH-R-III, inclusive of ligand-binding and inositol phosphate production in response to endogenous ligands (cGnRH-I and GnRH-II) and a panel of GnRH analogs (sGnRH-III, mammalian antagonist 27, 135-18 and 135-25) in an attempt to identify a GnRH analog that may be applied to examine the differential effects of receptor subtypes on gonadotrophin synthesis and release. The results indicated that cGnRH-R-III has a higher ligand-binding affinity for GnRH-II than cGnRH-R-I (IC_{50} : 0.57 v 19.8 nM) and similar results were found for cGnRH-R-I, (IC_{50} : 0.51 v 10.8 nM). In accordance with the ligand-binding selectivity of cGnRH-R-III, GnRH-II was more potent at stimulating inositol phosphate production at cGnRH-R-III than cGnRH-R-I (EC_{50} : 0.8 v 4.38 nM). Similar results were found for cGnRH-R-I, (EC_{50} : 0.7 v 2.8 nM). Therefore, cGnRH-R-III, like cGnRH-R-I is capable of coupling to the $G\alpha_q$ protein and subsequently activating PLC, which

results in the generation of inositol phosphate and diacyl-glycerol, leading to the elevation of intracellular Ca^{2+} and PKC signalling pathways, the predominant mediators of LH and FSH biosynthesis. Mammalian antagonist 27 exhibited a large difference in antagonistic potency at cGnRH-R-I (IC_{50} : 2.3 nM) compared to cGnRH-R-III (IC_{50} : 351nM). Therefore, application of this synthetic peptide may allow delineation of receptor subtype function *in-vitro* and *in-vivo* in order to study the physiological role of the chicken type III GnRH receptor. The effect of the cGnRH-R-III splice variants on cGnRH-R-III receptor binding was also examined, and the results indicate that cGnRH-R-III_SV2 significantly affects cell surface expression of cGnRH-R-III when co-expressed with cGnRH-R-III, inferring that cGnRH-R-III splice variants may modulate cGnRH-R-III activity if over-expressed. Additionally, the ligand induced internalisation of both receptors was compared and results showed that the initial rate of receptor internalisation was faster for cGnRH-R-III than cGnRH-R-I ($26\%.\text{min}^{-1}$ v $15.8\%.\text{min}^{-1}$). It is possible that the spatial arrangement of amino acid residues in the carboxyl-terminal region affected by 8 additional residues in the carboxyl-terminal tail of cGnRH-R-III may contribute to the increased internalisation rate compared to cGnRH-R-I. Interestingly, cGnRH-I is thought to be the hypothalamic peptide responsible for regulating the pituitary gonadotroph, however both cGnRH-Rs possess higher ligand selectivity for GnRH-II and have a higher potency in inositol phosphate production with GnRH-II than with GnRH-I. These results suggest that evolutionary recruitment of ligand-receptor pairing for particular physiological processes does not necessarily correlate with ligand-binding affinity or potency of inositol phosphate production.

5.6.3. MAPK Signalling Activated via Type I and Type III cGnRH Receptors

The data presented in this chapter included investigations into whether three putative Src homology 3 (SH3) binding motifs present in the cytoplasmic C-terminal tail of cGnRH-R-III and not present in cGnRH-R-I may attribute a differential functional

capacity to cGnRH-R-III through interaction with SH3 domain-containing proteins. It was hypothesized that these motifs may enable differential activation of the MAPK signalling cascade. The results of studies performed in transfected COS-7 cells showed a 4-6 fold increase in ERK1/2 phosphorylation following activation of type I or type III cGnRH receptors within 10 minutes of cGnRH-I or GnRH-II stimulation, indicating that both receptors signal through the MAPK pathway to ERK in a similar time-dependent and dose-dependent manner. In an attempt to identify the protein complexes that couple to the two cGnRH receptors and result in MAPK activation, studies of the effects of pre-treatment with selected inhibitors were performed. Pre-treatment of these cells with phospholipase C (PLC) inhibitor or c-Src inhibitor showed that both cGnRH-Rs can activate ERK 1/2 independently of PLC but dependently upon c-Src at 10 minutes of stimulation. The influence of SH3 binding motifs was analysed by mutagenesis of the SH3 binding motifs in the cGnRH-R-III cDNA receptor construct. Subsequent evaluation of the ERK activation by SH3 binding motif mutants (P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III) in comparison with the native cGnRH-R-III and cGnRH-R-I was performed in transfected COS-7 cells. The data showed that ERK activation via P317A-cGnRH-R-III, P319A-cGnRH-R-III and P346A-cGnRH-R-III in response to cGnRH-I and GnRH-II was similar to both the native cGnRH-R-III and to cGnRH-R-I. Therefore, there is no evidence of an effect on ERK activation via direct coupling through the SH3 binding motifs in cGnRH-R-III. Characterisation of receptor protein-protein complexes isolated by immuno-precipitation of either cGnRH-R subtypes was investigated, facilitated by HA epitope-tagging both cGnRH-R-I and cGnRH-R-III (HA-cGnRH-R-I and HA-cGnRH-R-III). Several size forms of HA-cGnRH-R-III were detectable by western blotting and the composition of the immuno-precipitated protein complexes can be analysed in detail in future studies. The results obtained thus far indicate that the SH3 binding motifs identified in cGnRH-R-III do not alter ERK activation and cGnRH-R-III possibly does not exert a differential role in the regulation of LH and FSH transcription through the differential activation of ERK when compared to cGnRH-R-I. Other MAPK outputs

(p38 and JNK) were not investigated in this chapter and could be the focus of future investigations.

5.7. Concluding Discussion

During evolution GnRH isoforms have either been adopted for specialised physiological functions through gene duplication events and selection of certain motifs, or their roles have become redundant through residue mutations or deletions and subsequent silencing when their functions became redundant, in which case certain genes became pseudogenes or were completely lost during evolution (Millar et al., 2004; Okubo and Nagahama, 2008). Vertebrate GnRH-I members vary diversely in peptide sequence, particularly in teleost species. However, mammalian GnRH-I peptide is well conserved and is thought to be the ancestral form of GnRH-I (Kah et al., 2007; Okubo and Nagahama, 2008). This is supported by the identification of this same peptide in a basal chordate (amphioxus) (Chambery et al., 2009). The conservation of mammalian GnRH-I peptide sequence coincides with the loss of GnRH-II in several mammalian species. The pre-pro GnRH-II gene in some mammals is functionally compromised, although the retention of large sections of the GnRH-II gene in most mammals suggests that mammalian ancestors had a functional GnRH-II system (Stewart et al., 2009). Conversely, the lack of conservation of GnRH-I peptide sequence in non-mammalian vertebrates coincides with the retention of GnRH-II peptide. The GnRH-II decapeptide sequence is ubiquitously conserved in species from bony fish to humans, representing over 500 million years of evolution, suggesting it has an important physiological role (Millar, 2005). The selective pressure to conserve GnRH-II structure in vertebrates has been proposed to result from a co-ordinated evolutionary selection of residues required for ligand conformation and high affinity binding to the GnRH receptor (Pfleger et al., 2002). It is also possible that the GnRH gene which retained its function during evolution assumed the functional role of the pseudogene or deleted gene (Kah et al., 2007; Okubo and Nagahama, 2008). Alternatively, evolutionary loss of a GnRH isoform

may correlate with loss of functional requirement due to altered reproductive physiology. Of the three distinct paralagous forms of vertebrate GnRH peptides, (GnRH-I, GnRH-II and GnRH-III), all three forms co-exist only in species of the teleost lineage (Kah et al., 2007). The conservation of GnRH-III peptide sequence is also highly conserved, however, analysis of GnRH gene loci suggest that this gene may have been deleted during the early evolution of tetrapods prior to the divergence of amphibians, reptiles, aves and mammals (Okubo and Nagahama, 2008). The retention of GnRH-II and GnRH-III may correlate with diverse endocrine functions for GnRH (e.g. influences on growth hormone, prolactin and somatolactin production) and intra-gonadal functions (Foran and Bass, 1999; Kakizawa et al., 1997; Marchant et al., 1989; Weber et al., 1997). This coincides with an undeveloped hypophyseal portal system, direct innervation of the different types of pituitary gland by GnRH neurons (Oka and Ichikawa, 1990; Peter et al., 1990) and the differentiation of pituitary gland gonadotroph cell lineages in these animals.

There appears to be a functional refinement of GnRH peptide action during evolution which corresponds with refinement of reproductive strategy (development of pituitary, internal fertilisation, less eggs ovulated, investment of maternal care). In this refinement, GnRH forms may have been adopted for specialised functions or their roles may have become redundant. There is a correlation between the retention of GnRH peptide isoforms and the occurrence of GnRH receptor isoforms.

In some fish up to five GnRH receptors genes are retained in the genome (e.g. masu salmon) (Jodo et al., 2005), although only two isoforms of the receptor (type I and type III) exist in teleost fish based on phylogenetic analysis. Interestingly, unlike fish, amphibians and reptiles possess all three GnRH receptor isoforms (type I, type II and type III), as is seen in the bullfrog and the leopard gecko (Ikemoto and Park, 2007; Wang et al., 2001a). Only two receptor isoforms are functional in some mammals (type I and type II) (Stewart et al., 2009). In this thesis, a second GnRH

receptor was identified and characterised in the chicken, confirming the existence of only two receptors in birds, type I and type III GnRH receptors (Joseph et al., 2009; Shimizu and Bedecarrats, 2006). The organisation of the avian GnRH system is distinct in terms of retention of two GnRH ligands (GnRH-I and GnRH-II) and two GnRH receptors (GnRH-R-I and GnRH-R-III) (Morgan and Millar, 2004). The GnRH receptors expressed in fish and birds are similar (types I and III only), with both lacking a type II GnRH receptor isoform. Although, fish have three GnRH isoforms (GnRH-I, GnRH-II and GnRH-III), birds only have two (GnRH-I and GnRH-II). However, unlike amphibians and reptiles where three GnRH receptors exist (type I, type II and type III), birds do not possess the type II GnRH receptor isoform. Birds, amphibians and reptiles have two GnRH isoforms (GnRH-I and GnRH-II). In contrast to mammals, where up to two GnRH ligands and receptor isoforms exist (type I and type II), again birds differ in not possessing the type II GnRH receptor isoform. Type II and type III GnRH receptors are thought to have arisen by a gene duplication of a common ancestral gene (Ikemoto and Park, 2007; Millar, 2003; Millar, 2005), and it has been previously assumed that since only teleosts did not express type II receptors, the duplication event occurred after the divergence of the teleost and tetrapod lineages. However, since both birds and fish do not possess type II receptors, it is probable that the duplication occurred before the split, and that the type II GnRH receptors were lost on separate occasions in both birds and fish genomes. Therefore, studies into the GnRH systems in chickens will provide unique information to enable a better comprehension of the interplay between ligands and receptors in the control of avian reproduction and how GnRH systems evolved to assume their function in modern taxa. **It may also provide insights into the evident plasticity in utilisation of a particular GnRH receptor in particular tissues.** The approach used in this thesis to investigate this conundrum was to try to dissect the cellular and physiological functions of the two GnRH receptor subtypes isolated from the chicken (type I and type III).

In this thesis, plasticity in the spatiotemporal expression patterns of GnRH receptor subtypes was discovered in the chicken, with cGnRH-R-III being expressed predominantly in the anterior pituitary gland and with cGnRH-R-III expression being ~1400 times more abundant than cGnRH-R-I. Differential gene expression suggested that there is likely to have been evolutionary plasticity in pituitary utilisation of GnRH receptor subtypes for functional regulation of gonadotrophin production. No developmental or sex-specific differences or changes in expression were detected for cGnRH-R-I mRNA; however a pronounced reciprocal sex difference in pituitary cGnRH-R-III mRNA expression was detected. Higher cGnRH-R-III mRNA levels occur in adult females compared with adult males. In contrast, in juvenile chickens, higher cGnRH-R-III mRNA levels occurred in males compared to females. These observations lend further support for the existence of plasticity in evolutionary recruitment of GnRH receptor subtypes for regulation of pituitary gonadotrophin production. As no differences in ligand selectivity or potency in inositol phosphate production were detected between the two receptors in response to endogenous ligands, the results of pharmacological profiling suggest that receptor ligand selectivity measured *in-vitro* does not necessarily correlate with the *in-vivo* role of GnRH receptor isoforms nor does it determine the designation of ‘cognate’ receptors. Perhaps evolutionary recruitment of ligand-receptor pairing for particular physiological processes is not determined by selection according to the highest affinity for ligand binding or potency in inositol phosphate production. Thus delineation of ‘cognate’ ligand/receptor pairing requires the assessment of receptor protein expression in the target cell and assessment of ligand isoform delivery to that cell. It is plausible that cGnRH-R-III splice variants may modulate cGnRH-R-III activity and may be physiological regulators if expressed at sufficient levels, which would in turn affect the physiological function of the cGnRH-R-III. The increased internalisation rate of cGnRH-R-III compared to cGnRH-R-I may affect the longevity of the signalling cascades activated by cGnRH-R-III, in turn affecting LH and FSH gene transcription. The signal transduction mechanisms leading to ERK activation via cGnRH-R-I and cGnRH-R-III are probably similar; therefore the SH3

binding motifs identified in cGnRH-R-III do not alter ERK activation when compared to cGnRH-R-I.

In comparison with other species, some fish (e.g. catfish), amphibians (e.g. bullfrog and African clawed frog) and reptiles (e.g. leopard gecko), the chicken (bird), also shows species-specific recruitment of receptor subtypes and ligands within different tissues and there is also plasticity in the spatiotemporal expression patterns of GnRH receptor subtypes. Interestingly, the predominant receptor expressed in the pituitary of the catfish (Bogerd et al., 2002) is a type I, the African clawed frog (Troskie et al., 2000) is a type I (Troskie et al., 2000), the bullfrog (Wang et al., 2001a) is a type III and in the leopard gecko (Ikemoto and Park, 2007) is a type III. Despite the three GnRH receptor isoforms expressed in the amphibian and reptilian lineages (type I, II and III), there is no evidence for specialised recruitment of the type II receptor in the regulation of pituitary gonadotroph function. In contrast, fish and birds do not possess type II receptors. This suggests that type II receptors may have been retained for specialised functions in amphibians and reptiles and were not retained in birds or fish. Perhaps more detailed studies of GnRH-peptide hormone and GnRH-receptor isoform tissue-specific gene expression patterns can explain why different combinations of ligand and receptor have been retained in different vertebrate species. Such studies will require more immunohistochemistry, promoter function studies and isolation of clonal immortalised cells from brain and pituitary for examination of gene expression patterns *in-vitro*.

The reproductive physiologies of certain reptiles and birds are quite similar. They both generate a hierarchical order of pre-ovulatory follicles (Ikemoto and Park, 2007; Johnson and Leone, 1985). Interestingly, the leopard gecko (Ikemoto and Park, 2007) GnRH-I peptide hormone is identical in amino acid sequence to the chicken. The striking difference between these two species is the retention of a type II GnRH receptor in the reptiles and a loss in the birds, again suggesting the plasticity in utilisation of a particular GnRH receptor in a particular tissue. The type III receptor

is expressed exclusively in the anterior pituitary gland of the leopard gecko, and quantitative studies showed that the expression decreased by 88% after the egg-laying season compared to during the egg-laying season (Ikemoto and Park, 2007). Similarly like the leopard gecko, in the chicken, the type III GnRH receptor is expressed in the anterior pituitary, however, the expression is predominant (~1400 times more abundant) and coincides with the expression of the type I cGnRH receptor. Although changes of cGnRH-R-III mRNA were detected in the reproductive cycle of the birds, in a sex and age-dependent manner (Joseph et al., 2009). All three GnRH receptor forms were localised in the ovary of the leopard gecko, and type I GnRH receptor increased by 4 fold after the egg-laying season, whereas the other receptors did not show any changes in expression. Similarly in the chicken, there is a ~10 fold higher expression of the type III GnRH receptor in the ovary, compared to the type I receptor, however no changes in ovarian expression were tested at different physiological stages of the birds. There is also a distinct spatial expression pattern of the GnRH receptors in the ovary of the leopard gecko (Ikemoto and Park, 2007) and changes in expression among hierarchical follicles, although in this thesis it was not possible to detect cellular expression of cGnRH-R subtypes by *in-situ* hybridisation. Identifying if there is a direct effect of GnRH on the ovary via gonadal GnRH receptors would prove interesting for future studies. Developments in the understanding of the GnRH system in the reptile (leopard gecko) is more advanced than studies in the chicken thus far, with comparisons between ovarian and pituitary GnRH systems established (Ikemoto and Park, 2007). Further comparison of the avian and the reptilian GnRH system will provide a key understanding of the function of the type I, II and III GnRH receptors. However, more research would be necessary in the chicken in order to compare it with reptilian species. Research would facilitate understanding of the underlying question of the GnRH systems in terms of the plasticity in evolutionary recruitment of GnRH receptor subtypes for regulation of pituitary gonadotroph function and ovarian function and the correlation with reproductive physiologies. There is also promiscuity of receptor subtype activation *in-vitro* by different endogenous ligands and complex *in-vivo* interplay between ligands and receptors, which needs to be

considered, keeping in mind the potency of the ligands on the receptor subtypes and delivery of appropriate forms of GnRH isoforms to the target cells.

5.8. Future Work

5.8.1. Dissecting the cellular and physiological functions of cGnRH-R-I and cGnRH-R-III

Key to the understanding of the differential functional capacity of the two GnRH receptor isoforms is the characterisation of the receptor isoforms in different cell types, both in the pituitary and in the ovary. This will provide evidence for the effect of GnRH on specific cell types by delivery through the hypophyseal portal system and possibly through direct autocrine/paracrine effects in the gonads. Although *in-situ* hybridisation methods used in this thesis (**Chapter 3**) proved impossible to identifying pituitary cellular expression of GnRH receptor subtypes, it may be possible to employ the use of the cGnRH-R-III antibody as it was shown in this thesis (**Chapter 5**) to be quite specific for cGnRH-R-III. Employing double immunohistochemistry to detect the co-localisation of the cGnRH-R-III receptor subtypes in the gonadotrophs (LH and FSH) will provide understanding of the possible role of cGnRH-R-III in the control of LH or FSH gene transcription. In the ovary, characterisation of the cGnRH-R subtypes in hierarchical follicles and ovarian cell types will allow inference of functions of possible intra-gonadal effects of GnRH. Another possible method to identify the expression of the receptor subtypes in segregating populations of LH and FSH gonadotrophs using the laser capture technique that was attempted (**Chapter 3**). Although it was possible to detect LH stained gonadotrophs (**Chapter 3**), laser capture of pure gonadotrophs was impossible because the size limit for the micro-dissected area was greater than that of a single gonadotroph cell. It is plausible that this method could be applied *in-vitro* on dispersed pituitary cells.

As mentioned (**in Chapter 4**) mammalian antagonist 27 showed a 153-fold more potent antagonistic effect at cGnRH-R-I than cGnRH-R-III and it may be possible to utilise this antagonist to preferentially block chicken type I GnRH receptor function *in-vivo* in order to study the physiological role of the chicken type III GnRH receptor. However, as the peptide stock used to perform these experiments were depleted, additional stocks of the antagonist were sourced from Dr. Arie Katz laboratory at the University of Cape Town in South Africa. Unfortunately, once this new batch was tested, there were different pharmacological responses to those determined using the initial stock used for my experiments. Mass spectrometry analysis was performed, which showed that there were different degradation patterns between the two stocks of peptides, which may have attributed to their different pharmacological profiles at the two cGnRH receptor subtypes. Therefore, it was not possible to investigate the *in-vivo* effects of mammalian antagonist 27 in order to study physiological role of the chicken type III GnRH receptor. Identifying a different source of synthetic peptide 27, or an identical batch of peptide to the one was used in this thesis, would allow these studies to be performed, and should be a focus of future investigations.

The effects of oestrogen on cGnRH-R-I expression has been studied previously and shown to reduce mRNA levels in castrated juvenile cockrels (Sun et al., 2001a). In general, our observations (**Chapter 3**) suggest that the level of GnRH-R-III mRNA may be inversely related to the capacity of gonadotrophs to release LH in adult females (Joseph et al., 2009). It can be hypothesised that elevated levels of cGnRH-R-III mRNA in the adult female may be reflective of expression of membrane cGnRH-R-III receptor number and that increased levels of ovarian steroid or peptide hormones may act to diminish the LH response to GnRH at the gonadotroph, through down-regulation of intracellular signalling pathways. Therefore, understanding the effects of steroids (oestrogen and progesterone) on control of expression of cGnRH-R-I and cGnRH-R-III will provide an indication of the possible roles of GnRH receptors in the reproductive cycles of the birds. Additionally, immunoneutralisation

of inhibin in cockrels showed that there was accelerated puberty and hindered age-related sexual senescence in these birds (Satterlee et al., 2006). It is feasible that inhibin has an inhibitory effect on gonadotrophin biosynthesis (Dunn I. C. et al., 2009). Activin which is expressed in folliculo stellate cells may have a role in modulating GnRH-R expression (Kaiser et al., 1992; Rispoli and Nett, 2005). Therefore, identifying the effects of inhibin and or activin on the expression of receptor subtypes would provide a further understanding of the exact molecular mechanisms in which they affect gonadotrophin biosynthesis.

Although it was not possible to differentiate between the receptors in terms of their pharmacological response to endogenous ligands (**Chapter 4**), a statistically significant difference in terms of ligand-induced internalisation rates was identified with cGnRH-R-III internalising at a faster initial rate than cGnRH-R-I (**Chapter 4**). It would be interesting to identify if these differences in internalisation rates affected the longevity of the signalling cascades activated by the two cGnRH receptor isoforms, with potential effects on LH and FSH gene transcription. cGnRH-R-III_SV2 significantly affected the cell surface expression of full-length cGnRH-R-III when co-expressed in COS-7 cells, inferring that cGnRH-R-III splice variants may modulate cGnRH-R-III activity if over-expressed (**Chapter 4**), however, the effects of co-expression of type I and type III GnRH receptor isoforms was not investigated. Interestingly, co-transfection of leopard gecko cDNA receptor isoform constructs was shown to elicit a distinct pharmacology compared to single isoform transfection (Ikemoto and Park, 2007). As cGnRH-R-I and cGnRH-R-III are both expressed in the pituitary gland and may be expressed in identical cell types, it would be interesting to determine the effects of co-expression of cGnRH-R isoforms in relative quantities similar to those expressed in the pituitary gland to determine the effects on pharmacological properties and cellular responses.

The hypothesis that three putative Src homology 3 (SH3) binding motifs present in the cytoplasmic C-terminal tail of cGnRH-R-III and not present in cGnRH-R-I may

attribute a differential functional capacity to cGnRH-R-III through interaction with SH3 domain-containing proteins may be further investigated as the method for immuno-precipitation has been optimized (**Chapter 5**). Several size forms of HA-cGnRH-R-III were detectable by western blotting and the composition of the immuno-precipitated protein complexes can be analysed in detail in future studies (**Chapter 5**). The results obtained thus far indicate that the SH3 binding motifs do not alter ERK activation of cGnRH-R-III when compared to cGnRH-R-I (**Chapter 5**), however, they may affect other MAPK outputs (p38 and JNK), and should be the focus of future investigations.

In-vitro GnIH stimulation of cockerel pituitary fragments reduced the common gonadotrophin alpha and the FSH beta subunit mRNA levels, although it failed to impact upon LH beta mRNA levels (Ciccone et al., 2004). Additionally, there is a reciprocal relationship in the expression of cGnRH-R-III and cGnIH-R during the reproductive cycles in chickens (Bedecarrats et al., 2009). Characterising possible cross-talk between GnIH and GnRH receptor signalling may prove beneficial in understanding the molecular mechanisms of the control of reproduction by GnIH. Use of a cAMP responsive element (CRE) luciferase reporter construct showed that in cGnRH-R-I and cGnRH-R-III transfected cells there is an increase in CRE-luc activity on GnRH stimulation (Shimizu M et al., 2008). In contrast, activation of the cGnIH-R reduced the cGnRH-I induced activation of CRE-luc in a dose-dependent manner, and this effect is also dependent on receptor expression ratio (Shimizu M et al., 2008). It has also been reported that in sheep pituitary cells, GnIH can abrogate GnRH-induced ERK phosphorylation (Sari et al., 2009). cGnRH-I induced activation of the cGnRH-R-III and cGnRH-R-I and leads to ERK phosphorylation (**Chapter 5**), and determining if the effect of GnIH on GnRH-induced CRE activation and ERK phosphorylation are interrelated may be the focus of future investigations.

Both GnRH and GnIH have been shown to impact upon gonadotrophin gene expression. However, it is not clear whether cGnRH-R isoforms are involved independently or differentially, and if GnIH-R signalling directly acts on target gene promoters or merely interacts with GnRH-Rs signalling. Thus to go beyond signalling, it would be of great interest to characterize gonadotrophin subunit gene promoters. This could be achieved by generating reporter constructs encoding chicken LH-beta and FSH-beta as well as the common alpha subunit promoters fused to luciferase. This construct could then be transfected alongside cGnRH-R isoforms and GnIH-R and any effect on gene expression could be monitored following GnRH and GnIH stimulation.

One ambitious way to examine GnRH receptor subtype function in pituitary gonadotrophs would be to isolate clonal gonadotroph cell lines. This could be achieved by immortalisation of embryonic pituitary cell cultures with (FDH/LH-beta promoter-SV40-TAG or FSH/LH beta-hTERT promoter constructs) with subsequent examination of which receptors are endogenously expressed in clonal cell lines. Alternatively, employing the application of lentiviral SI-RNA constructs to knock down GnRH receptor isoform expression in chickens would provide information on the role of the GnRH-R isoforms in the control of reproduction.

5.8.2. Understanding evolutionary recruitment of GnRH receptor isoforms

With the near completion of a number of genome sequencing projects it will become increasingly possible to compare the syntenic gene loci of GnRH peptide precursor genes and receptor isoform genes. This will provide greater understanding of possible evolutionary mechanisms in which GnRH systems were recruited and their correlation with reproductive strategies. Of particular interest will be a comparison of the Chicken, Zebra Finch, and Anole Lizard genomes. Both the Zebra Finch and Anole Lizard preliminary genome assemblies have been released (Ensembl) however; complete construction of the genomes is ongoing.

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The actual residues and the conversion to the ‘Ballesteros numbering convention’ for the residues attributed the number 50. This will enable derivation of any desired Ballesteros number from the alignment above. The number in the brackets indicates the actual residue number in the respective sequence. **See Table Appendix 1.1** for ‘Ballesteros numbering’ of transmembrane helices AA’s.

Table Appendix 1. 1

Transmembrane helix	Ballesteros numbering	Human GnRH -R	Chicken GnRH-R-I	Chicken GnRH-R-III
1	1.50	Asn ^{1.50(53)}	Asn ^{1.50(60)}	Asn ^{1.50(83)}
2	2.50	Asn ^{2.50(87)}	Asp ^{2.50(87)}	Asp ^{2.50(112)}
3	3.50	Arg ^{3.50(139)}	Arg ^{3.50(139)}	Arg ^{3.50(164)}
4	4.50	Trp ^{4.50(164)}	Trp ^{4.50(162)}	Trp ^{4.50(190)}
5	5.50	Pro ^{5.50(223)}	Pro ^{5.50(216)}	Pro ^{5.50(245)}
6	6.50	Pro ^{6.50(282)}	Pro ^{6.50(273)}	Pro ^{6.50(307)}
7	7.50	Pro ^{7.50(320)}	Pro ^{7.50(312)}	Pro ^{7.50(346)}

Table Appendix 1. 2

Ballesteros and Weinstein 'Numbering Conversion' for cGnRH-R-I, cGnRH-R-III and hGnRH-R-I. In each column the residues are ordered according to the multiple sequence alignment in **Fig. Apen.1**. The NH₂ terminal of each receptor is shaded in yellow, the transmembrane domains are shaded in blue, the extracellular loops in grey and the intracellular loops in pink. The C-terminal cytoplasmic tail domains of the cGnRH-R isoforms are shaded in green. The 'Ballesteros and Weinstein Number' for each transmembrane domain is shown, and can be completed by inserting the actual residue number from the respective receptor in brackets after the 'Ballesteros and Weinstein Number' as shown in **Table Appen. 1**. The AA in the loops, are numbered according to the proximity of the transmembrane boundary, which determines which identifier is used (Ballesteros and Weinstein, 1995).

Chicken	Chicken	Human	Ballesteros
GnRH-R-I	GnRH-R-III	GnRH-R-I	Number
	1 M		
	2 A		
	3 R		
	4 L		
	5 G		
	6 G		
	7 G		
	8 T		
	9 G		
	10 Q		
	11 D		
	12 A		
1 M	13 A		
2 C	14 A		
3 V	15 A		
4 P	16 G		

5 A	17 G		
6 A	18 G		
7 L	19 W	1 M	
8 I	20 L	2 A	
9 E	21 D	3 N	
10 A	22 P	4 S	
11 E	23 G	5 A	
12 P	24 P	6 S	
13 P	25 T	7 P	
14 H	26 V	8 E	
15 H	27 G	9 Q	
16 P	28 N	10 N	
17 T	29 V	11 Q	
18 T	30 S	12 N	
19 E	31 T	13 H	
20 G	32 E	14 C	
21 D	33 P	15 S	
22 T	34 S	16 A	
23 N	35 S	17 I	
24 T	36 S	18 N	
25 S	37 T	19 N	
26 A	38 S	20 S	
27 T	39 H	21 I	
	40 P	22 P	
	41 K		
	42 R		
	43 G		
	44 C		
	45 A		
	46 W		
	47 S		
	48 P		
	49 R		
	50 L		
	51 E		

28 H	52 S		
29 C	53 A		
30 L	54 E	23 L	
31 E	55 E	24 M	
32 H	56 P	25 Q	
33 W	57 L	26 G	
34 V	58 L	27 N	
35 E	59 L	28 L	
36 P	60 P	29 P	
37 R	61 T	30 T	
38 F	62 F	31 L	
39 T	63 S	32 T	
40 K	64 P	33 L	
41 A	65 A	34 S	
42 A	66 A	35 G	
43 K	67 Q	36 K	1.33
44 V	68 A	37 I	1.34
45 R	69 R	38 R	1.35
46 V	70 V	39 V	1.36
47 A	71 A	40 T	1.37
48 I	72 A	41 V	1.38
49 T	73 T	42 T	1.39
50 A	74 F	43 F	1.40
51 V	75 V	44 F	1.41
52 F	76 L	45 L	1.42
53 F	77 F	46 F	1.43
54 L	78 V	47 L	1.44
55 L	79 L	48 L	1.45
56 A	80 S	49 S	1.46
57 A	81 A	50 A	1.47
58 C	82 G	51 T	1.48
59 S	83 C	52 F	1.49
60 N	84 N	53 N	1.50
61 T	85 V	54 A	1.51
62 A	86 A	55 S	1.52

63 V	87 V	56 F	1.53
64 L	88 L	57 L	1.54
65 G	89 R	58 L	1.55
66 S	90 A	59 K	1.56
67 L	91 A	60 L	1.57
68 L	92 G	61 Q	
		62 K	
		63 W	
		64 T	
		65 Q	
		66 K	
69 R	93 G	67 K	
70 K	94 R	68 E	
71 R	95 R	69 K	
	96 G	70 G	
	97 G	71 K	
72 R	98 G	72 K	
73 K	99 R	73 L	
74 C	100 S	74 S	
75 H	101 H	75 R	
76 V	102 I	76 M	
77 R	103 R	77 K	
78 P	104 V	78 L	
79 L	105 L	79 L	2.42
80 I	106 L	80 L	1.23
81 L	107 R	81 K	2.44
82 S	108 H	82 H	2.45
83 L	109 L	83 L	2.46
84 A	110 A	84 T	2.47
85 L	111 A	85 L	2.48
86 A	112 A	86 A	2.49
87 D	113 D	87 N	2.50
88 L	114 L	88 L	2.51
89 L	115 L	89 L	2.52
90 V	116 V	90 E	2.53

91 T	117 T	91 T	2.54
92 V	118 V	92 L	2.55
93 A	119 V	93 I	2.56
94 V	120 V	94 V	2.57
95 M	121 M	95 M	2.58
96 P	122 P	96 P	2.59
97 L	123 L	97 L	2.60
98 D	124 D	98 D	2.61
99 A	125 A	99 G	2.62
100 A	126 I	100 M	2.63
101 W	127 W	101 W	2.64
102 N	128 N	102 N	2.65
103 V	129 I	103 I	2.66
104 T	130 T	104 T	
105 V	131 L	105 V	
106 Q	132 Q	106 Q	
107 W	133 W	107 W	
108 Y	134 R	108 Y	
109 G	135 A	109 A	
110 G	136 G	110 G	
111 D	137 D	111 E	
112 L	138 L	112 L	
113 S	139 A	113 L	
114 C	140 C	114 C	
115 K	141 R	115 K	
116 L	142 L	116 V	3.27
117 L	143 L	117 L	2.28
118 N	144 M	118 S	2.29
119 F	145 Y	119 Y	3.30
120 L	146 L	120 L	3.31
121 K	147 R	121 K	3.32
122 L	148 L	122 L	3.33
123 F	149 L	123 F	3.34
124 A	150 A	124 S	3.35
125 M	151 M	125 M	3.36

126 Y	152 Y	126 Y	3.37
127 A	153 A	127 A	3.38
128 A	154 S	128 P	3.39
129 A	155 A	129 A	3.40
130 L	156 F	130 F	3.41
131 V	157 V	131 M	3.42
132 L	158 T	132 M	3.43
133 V	159 V	133 V	3.44
134 V	160 V	134 V	3.45
135 I	161 I	135 I	2.46
136 S	162 S	136 S	2.47
137 L	163 L	137 L	3.48
138 D	164 D	138 D	3.49
139 R	165 R	139 R	3.50
140 H	166 Q	140 S	3.51
141 A	167 A	141 L	
142 A	168 A	142 A	
143 V	169 I	143 I	
144 L	170 L	144 T	
145 Q	171 R	145 R	
146 P	172 P	146 P	
	173 L	147 L	
	174 A	148 A	
147 F	175 I	149 L	
148 A	176 A	150 K	
149 R	177 R	151 S	
150 A	178 A	152 N	
151 R	179 R	153 S	4.39
152 R	180 C	154 K	4.40
153 R	181 R	155 V	4.41
154 N	182 N	156 G	4.42
155 G	183 R	157 Q	4.43
156 L	184 A	158 S	4.44
157 L	185 M	159 M	4.45
158 L	186 L	160 V	4.46

159 R	187 R	161 G	4.47
160 A	188 A	162 L	4.48
161 A	189 A	163 A	4.49
162 W	190 W	164 W	4.50
163 L	191 M	165 I	4.51
164 G	192 L	166 L	4.52
165 S	193 S	167 S	4.53
166 V	194 A	168 S	4.54
167 L	195 A	169 V	4.55
168 L	196 L	170 F	4.56
169 A	197 A	171 A	4.57
170 S	198 V	172 G	4.58
171 P	199 P	173 P	4.59
172 Q	200 Q	174 Q	4.60
173 L	201 L	175 L	4.61
174 F	202 F	176 Y	4.62
175 L	203 L	177 I	4.63
176 F	204 F	178 F	4.64
177 H	205 H	179 R	4.65
178 V	206 T	180 M	4.66
179 H	207 V	181 I	
180 T	208 T	182 H	
181 V	209 L	183 L	
182 P	210 H	184 A	
183 G	211 A	185 D	
	212 P	186 S	
		187 S	
		188 G	
		189 Q	
		190 T	
184 G	213 H	191 K	
185 N	214 N	192 V	
186 F	215 F	193 F	
187 T	216 T	194 S	
188 Q	217 Q	195 Q	

189 C	218 C	196 C	
190 V	219 T	197 V	
191 T	220 T	198 T	
192 H	221 H	199 H	
193 G	222 G	200 C	
194 S	223 S	201 S	
195 F	224 F	202 F	
196 R	225 P	203 S	
197 A	226 Q	204 Q	
198 H	227 P	205 W	
199 W	228 W	206 W	
200 E	229 H	207 H	
201 E	230 E	208 Q	5.35
202 T	231 T	209 A	5.36
203 V	232 L	210 F	5.37
204 Y	233 Y	211 Y	5.38
205 N	234 N	212 N	5.39
206 M	235 M	213 F	5.40
207 F	236 L	214 F	5.41
208 T	237 S	215 T	5.42
209 F	238 F	216 F	5.43
210 T	239 S	217 S	5.44
211 T	240 C	218 C	5.45
212 L	241 L	219 L	5.46
213 Y	242 F	220 F	5.47
214 I	243 L	221 I	5.48
215 T	244 L	222 I	5.49
216 P	245 P	223 P	5.50
217 L	246 L	224 L	5.51
218 S	247 L	225 F	5.52
219 I	248 I	226 I	5.53
220 M	249 M	227 M	5.54
221 I	250 V	228 L	5.55
222 V	251 C	229 I	5.56
223 C	252 C	230 C	5.57

224 Y	253 Y	231 N	5.58
225 V	254 T	232 A	5.59
226 R	255 R	233 K	
227 I	256 I	234 I	
228 I	257 L	235 I	
229 W	258 L	236 F	
230 E	259 E	237 T	
231 I	260 I	238 L	
232 S	261 S	239 T	
233 K	262 R	240 R	
234 Q	263 R	241 V	
235 L	264 M	242 L	
	265 G	243 H	
	266 S	244 Q	
	267 S		
	268 L		
	269 F		
236 K	270 S	245 D	
237 I	271 S	246 P	
238 N	272 R	247 H	
239 K	273 D	248 E	
240 S	274 V	249 L	
241 L	275 P	250 Q	
242 V	276 L	251 L	
243 R	277 R	252 N	
244 S	278 C	253 Q	
245 Q	279 S	254 S	
246 N	280 G	255 K	
247 D	281 S	256 N	
248 H	282 N	257 N	
249 I	283 I	258 I	
250 S	284 P	259 P	
251 K	285 R	260 R	
252 A	286 A	261 A	
253 R	287 R	262 R	

254 M	288 L	263 L	
255 K	289 R	264 K	
256 T	290 T	265 T	
257 L	291 L	266 L	
258 K	292 K	267 K	6.35
259 M	293 M	268 M	6.36
260 T	294 S	269 T	6.37
261 I	295 L	270 V	6.38
262 V	296 V	271 A	6.39
263 I	297 I	272 F	6.40
264 V	298 V	273 A	6.41
265 A	299 S	274 T	6.42
266 S	300 S	275 S	6.43
267 F	301 F	276 F	6.44
268 I	302 I	277 T	6.45
269 I	303 L	278 V	6.46
270 C	304 C	279 C	6.47
271 W	305 W	280 W	6.48
272 T	306 T	281 T	6.49
273 P	307 P	282 P	6.50
274 Y	308 Y	283 Y	6.51
275 Y	309 Y	284 Y	6.52
276 L	310 L	285 V	6.53
277 L	311 L	286 L	6.54
278 G	312 G	287 G	6.55
279 L	313 L	288 I	6.56
280 W	314 W	289 W	6.57
281 Y	315 Y	290 Y	6.58
282 W	316 W	291 W	6.59
283 F	317 F	292 F	
284 H	318 C	293 D	
285 P	319 P	294 P	
	320 R		
286 A	321 A	295 E	
287 M	322 M	296 M	

288 I	323 Q	297 L	
289 Q	324 Q	298 N	
290 R	325 K	299 R	
291 M	326 V	300 L	
292 P	327 P	301 S	
293 E	328 P	302 D	
294 Y	329 S	303 P	
295 I	330 L	304 V	7.34
296 N	331 S	305 N	7.35
297 H	332 H	306 H	7.36
298 S	333 I	307 F	7.37
299 F	334 L	308 F	7.38
300 F	335 F	309 F	7.39
301 L	336 I	310 L	7.40
302 F	337 F	311 F	7.41
303 G	338 G	312 A	7.42
304 L	339 L	313 F	7.43
305 L	340 F	314 L	7.44
306 H	341 N	315 N	7.45
307 T	342 A	316 P	7.46
308 C	343 C	317 C	7.47
309 T	344 L	318 F	7.48
310 D	345 D	319 D	7.49
311 P	346 P	320 P	7.50
312 I	347 I	321 L	7.51
313 I	348 T	322 I	7.52
314 Y	349 Y	323 Y	7.53
315 G	350 G	324 G	7.54
316 L	351 L	325 Y	7.55
317 Y	352 F	326 F	7.56
318 T	353 T	327 S	7.57
319 P	354 I	328 L	7.58
320 S	355 P		
321 F	356 F		
322 R	357 R		

323 E	358 R		
324 D	359 R		
325 V	360 C		
326 Q	361 G		
327 L	362 C		
328 C	363 P		
329 L	364 C		
330 R	365 G		
331 G	366 H		
332 I	367 S		
333 E	368 S		
334 A	369 E		
335 A	370 P		
336 I	371 E		
337 S	372 P		
338 Q	373 P		
339 H	374 S		
340 V	375 P		
341 R	376 A		
342 H	377 T		
343 K	378 G		
344 P	379 S		
345 I	380 F		
346 S	381 H		
347 V	382 C		
348 S	383 S		
349 E	384 A		
350 K	385 S		
351 T	386 S		
352 T	387 L		
353 K	388 R		
354 D	389 G		
	390 R		
	391 Q		
	392 G		

	393 M		
355 G	394 G		
356 D	395 G		
357 V	396 T		
358 N	397 E		
359 G	398 G		
	399 P		
	400 H		
	401 P		
360 Q	402 P		
361 V	403 I		
362 T	404 E		
363 S	405 L		
364 G	406 G		
365 G	407 L		
366 S	408 P		
367 N	409 T		
368 G	410 G		
369 T	411 A		
	412 G		
370 T	413 S		
371 V	414 C		
372 N	415 Q		
373 T	416 S		
374 V	417 S		
375 C	418 A		
	419 L		

Appendix B: Supporting data for cGnRH-R-III protein expression

Since submission of this thesis, there has been some unpublished data that has become available on cGnRH-R-III protein expression (McFarlane et al., in preparation). The finding in this thesis that cGnRH-R-III cDNA showed that it is predominantly expressed in the anterior pituitary, ~1400 times more abundant than cGnRH-R-I cDNA and the pronounced sex and age difference existed, with higher pituitary transcript levels in sexually mature females versus juvenile females in contrast to, higher expression levels occurring in juvenile males compared to sexually mature males is based on the assumption that mRNA is translated into protein in proportional quantities. However, cGnRH-R-III protein expression appears to correlate with reproductive stages and showed that levels remain low until peak sexual activity is reached (McFarlane et al., **in preparation**). (**Fig. Appendix 1.2 and 1.3**). No research has been performed to compare the protein expression levels of cGnRH-R-I and cGnRH-R-III. The translation of mRNA into protein is dependent on a number of variables which may explain why the assumption that mRNA is translated into protein in proportional quantities may be presumptive. These include: RNA transport (Nakielny et al., 1997) and mRNA degradation (Ross, 1995).

H. McFarlane, **N. T. Joseph**, S.R. Maddineni, R.Ramachandran and G. Bedecarrats. Development, validation and utilisation of a novel antibody specific to the type III chicken gonadotrophin-releasing hormone receptor. (*In preparation*)

Permission has been granted by the authors (listed above) for inclusion of this data and figures (Appendix 1.2 and 1.3) in this thesis.

Figure Appendix 1. 2

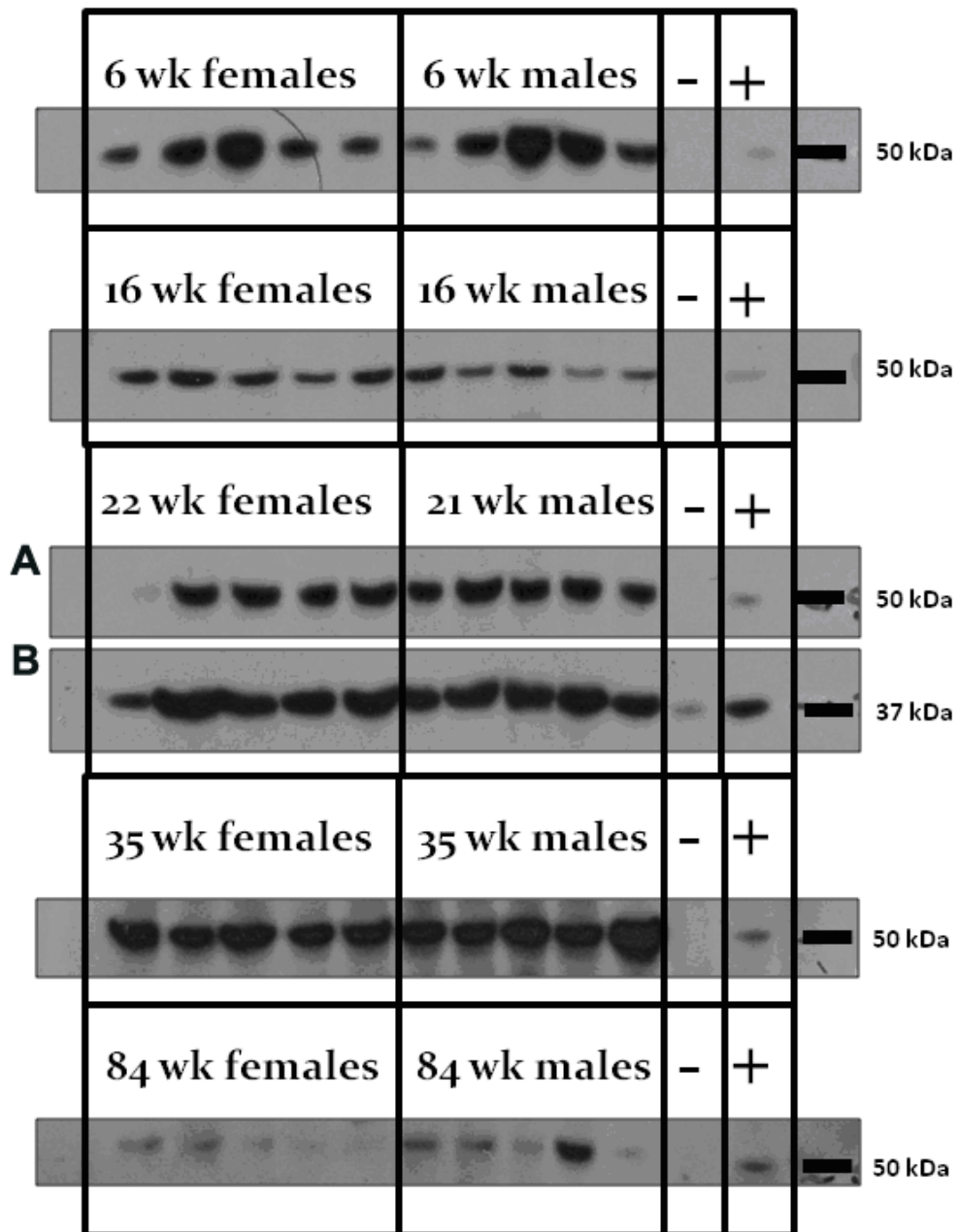


Fig. Appendix 1.2. Representative immunoblots of individual pituitary gland samples with cGnRH-R-III antibody. The positive control (+) corresponds to a pooled pituitary sample which was used as inter-blot calibrator, whereas a skeletal muscle sample was used as negative control (-). The cGnRH-R-III protein immunoreactive band at 50kDa in all samples except the negative tissue was used for quantification (A). A representative (21 wk age group) hybridization with GAPDH antiserum is shown in panel (B).

Figure Appendix 1. 3

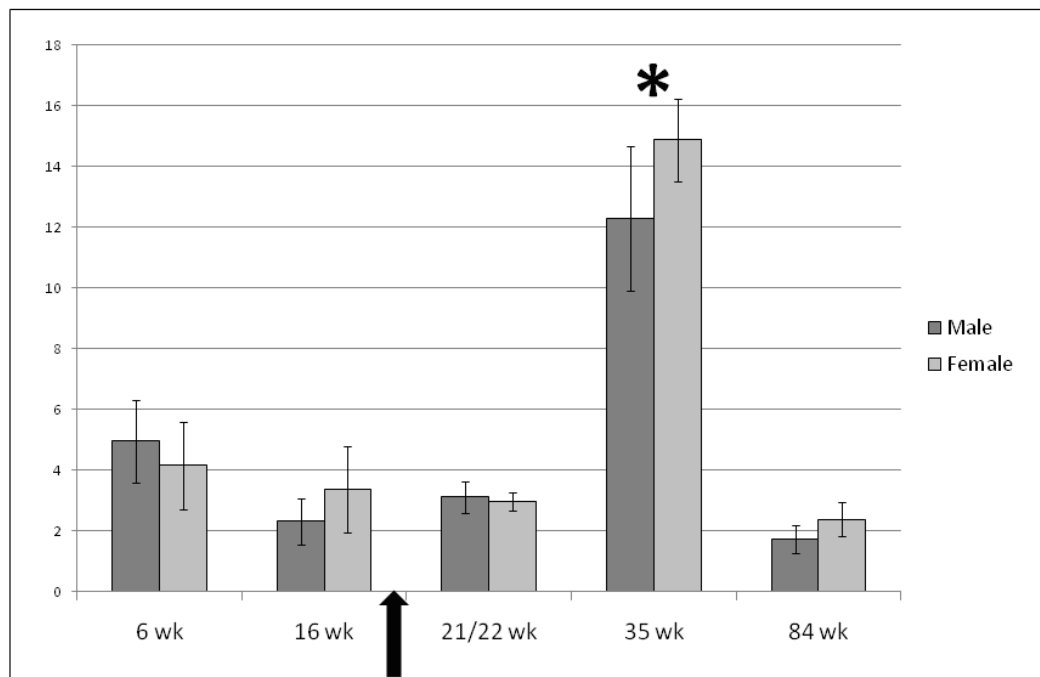


Fig. Appendix 1.3. Changes in cGnRH-R-III protein levels in chicken pituitary glands at varying reproductive stages. Values for individual samples were normalized for GAPDH protein and corrected with the inter-blot calibrator (pooled sample). Arrow indicates time of photostimulation (17 wk: males, 18 wk: females). Asterisk represents statistical significance ($p < 0.0001$).

Appendix C: Publication

The majority of the data presented in Chapter 3 and 4 of this thesis has been published in a paper in *Journal of Endocrinology*:

N. T. Joseph, K. Morgan, R. Sellar, D. McBride, R. P. Millar and I. C. Dunn. (2009). The chicken type III GnRH receptor homologue is predominantly expressed in the pituitary, and exhibits similar ligand selectivity to the type I receptor. *Journal of Endocrinology* **202**:179-190

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