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Interaction Between GABA, GnRH and Activin A in the Goldfish Neuroendocrine Brain

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Interaction between GABA, GnRH and Activin A in the Goldfish Neuroendocrine Brain

Kristin Le Saux-Farmer

Thesis submitted to the
Faculty of Graduate and Postdoctoral Studies
in partial fulfillment of the requirements for the
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Abstract

The neurotransmitter gamma-aminobutyric acid (GABA) stimulates the release of luteinizing hormone (LH) by enhancing gonadotropin-releasing hormone (GnRH) release in the goldfish, *Carassius auratus*. Activin A is another protein that stimulates the release of LH. Activin A also stimulates the release of GnRH from the rat hypothalamus, but this effect has never been shown in fish. Using real-time RT-PCR, we have shown that an injection of baclofen, a GABA_B receptor agonist, into sexually mature goldfish stimulates the expression of activin β A subunits in the telencephalon and sGnRH in the hypothalamus. Baclofen also inhibits the expression of that activin receptor IIB and IB in the hypothalamus. Immunocytochemical studies show that activin β A subunits and activin receptors are localised in the olfactory bulb, telencephalon, thalamus, hypothalamus and optic tectum. Activin receptors are colocalised with GnRH fibres in the hypothalamus. This study has provided further insight into the role of activin as a neuroendocrine factor controlling reproduction in the goldfish brain.

Résumé

Le neurotransmetteur acide gamma-aminobutyrique (GABA) stimule la sécrétion de l'hormone lutéotrope (LH) en augmentant la sécrétion de l'hormone libératrice des gonadotropines (GnRH) chez *Carassius auratus*. L'activine A est une autre protéine qui stimule la sécrétion de LH. L'activine A stimule aussi la sécrétion de GnRH de l'hypothalamus des rats, mais cet effet n'a pas encore été démontré chez les poissons. En utilisant le RT-PCR en temps réel, nous avons montré que l'injection du baclofène, un agoniste des récepteurs GABA_B, dans des cyprins dorés sexuellement matures stimule l'expression de la sous-unité activine β A dans le télencéphale et de la sGnRH dans l'hypothalamus. Le baclofène inhibe aussi l'expression du récepteur de l'activin type IIB et IB dans l'hypothalamus. Des études d'immunohistochimie ont montré que les sous-unités d'activine β A et les récepteurs de l'activine sont localisées dans le bulbe olfactif, le télencéphale, le thalamus, l'hypothalamus, et le toit optique. Les récepteurs sont colocalisés avec le GnRH dans des fibres dans l'hypothalamus. Cette étude nous donne plus d'information à propos du rôle de l'activine en tant que facteur neuroendocrinien contrôlant la reproduction dans le cerveau des cyprins dorés.

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Table of Contents

Abstract	i
Résumé	ii
Acknowledgments.....	iii
List of Tables	vi
List of Figures.....	vii
List of Abbreviations.....	viii

Rationale and Hypothesis.....	1
-------------------------------	---

Chapter 1: General Introduction

1.1. Control of Gonadotropin release.....	5
1.1.1. <i>Gonadotropins</i>	5
1.1.2. <i>Gonadotropin releasing hormone</i>	6
1.1.3. <i>Dopamine</i>	7
1.1.4. <i>Gamma aminobutyric acid</i>	8
1.1.5. <i>Glutamate</i>	10
1.1.6. <i>Norepinephrine</i>	11
1.1.7. <i>Sex steroids</i>	11
1.2. Activins.....	12
1.2.1. <i>Activins subunits</i>	12
1.2.2. <i>Activin receptors</i>	14
1.2.3. <i>Smad proteins</i>	15
1.2.4. <i>Inhibin and follistatin</i>	16
1.2.5. <i>Activin in tissue repair</i>	18
1.2.6. <i>Activin in behaviour</i>	18
1.2.7. <i>Activin in development</i>	19
1.2.8. <i>Activin in growth</i>	21
1.2.9. <i>Activin in mammalian reproduction</i>	21
1.2.10. <i>Activin in non-mammalian reproduction</i>	24
1.3. Conclusions.....	29

Chapter 2: Effects of the GABA_B agonist baclofen on activin subunits and activin receptor mRNA in the goldfish brain

2.1. Introduction.....	32
2.2. Materials and Methods.....	35
2.2.1. <i>Experimental protocol and RNA extraction</i>	35

2.2.2.	<i>LH assay</i>	36
2.2.3.	<i>Real time RT-PCR</i>	37
2.2.4.	<i>Statistical Analysis</i>	38
2.3.	Results	39
2.3.1.	<i>Effect of a GABA_B receptor agonist on LH level</i>	39
2.3.2.	<i>Effect of baclofen on gene expression in the telencephalon</i>	39
2.3.3.	<i>Effect of baclofen on gene expression in the hypothalamus</i>	39
2.4.	Discussion	40

Chapter 3: Localisation of activin subunits in the brain and possible interaction of activin and GnRH

3.1.	Introduction	56
3.2.	Materials and Methods	58
3.2.1.	<i>Animal and Tissue preparation</i>	58
3.2.2.	<i>Antibodies</i>	59
3.2.3.	<i>Immunohistochemistry</i>	60
3.2.4.	<i>Double labelling immunohistochemistry</i>	61
3.3.	Results	62
3.3.1.	<i>Activin βA subunit location in the goldfish brain</i>	62
3.3.2.	<i>ActRII localisation in the goldfish brain</i>	63
3.3.3.	<i>Double labelling of actRII and GnRH</i>	63
3.4.	Discussion	65

Chapter 4: General Discussion

4.1.	Activin in the brain	82
4.2.	Future research	87
	References	91
	Appendix	105

List of tables

Table 2.1. Sequences of primers used for real time reverse transcriptase polymerase chain reaction.....	38
Table A1. Gonadosomatic index (GSI) from the male goldfish used in chapter 2.....	105
Table A2. Gonadosomatic index (GSI) from the female goldfish used in chapter 2.....	106

List of figures

Figure 1.1. Multifactorial control of luteinizing hormone (LH) release from the gonadotrope cells of the pituitary in goldfish.....	31
Figure 2.1. Gonadosomatic index for control and baclofen treated male and female goldfish...	50
Figure 2.2. Serum LH levels in goldfish after single i.p. injections of baclofen.....	51
Figure 2.3. Relative mRNA levels of the activin subunits β A and β B, activin receptors types IB, IIA, and IIB, sGnRH and cGnRH-II in the telencephalon.....	52
Figure 2.4. Relative mRNA levels of the activin subunits β A and β B, activin receptors types IB, IIA, and IIB, sGnRH and cGnRH-II in the hypothalamus.....	54
Figure 3.1. Schematic representation of successive rostrocaudal transverse sections of goldfish brain showing the distribution of activin β A subunits cells.....	74
Figure 3.2. Brightfield micrographs of transverse sections of goldfish pituitary stained with activin β A subunits.....	75
Figure 3.3. Brightfield micrographs of transverse sections of goldfish brain stained with activin β A subunits.....	76
Figure 3.4. Schematic representation of successive rostrocaudal transverse sections of goldfish brain showing the distribution of activin receptor type II subunits cells.....	78
Figure 3.5. Brightfield micrographs of transverse sections of goldfish brain stained with actRII.....	79
Figure 3.6. Fluorescent micrographs of transverse sections of goldfish brain stained with actRII and GnRH.....	80
Figure 3.7. Fluorescent micrographs of transverse sections of goldfish pituitary stained with actRII and GnRH.....	81
Figure 4.1. Schematic representation of the hypothetical pathway by which GABA stimulates the release of GnRH in the neuroendocrine brain of sexually mature goldfish.....	90

List of abbreviations

AC - Anterior Commissure
ActRII - Activin receptor type II
AMPA - S- α -amino-3-hydroxy-5 methyl-4-isoxazolepropionic acid
cAMP - cyclic adenosine monophosphate
cGnRH-II - chicken GnRH-II
DA - Dopamine
DAB - 3-3'-diaminobenzidine
DBH - Dopamine β -Hydroxylase
Dm - Area Dorsalis Telencephali Medialis
E₂ - Estradiol
EGF - Epidermal Growth Factor
FSH - Follicle Stimulating Hormone
GABA - γ -aminobutyric acid
GABA-T - GABA Transaminase
GAD - Glutamic Acid Decarboxylase
GH - Growth Hormone
GnRH - Gonadotropin-Releasing Hormone
GPCR - G-protein coupled receptor
GSI - Gonadosomatic Index
GtH-I - Gonadotropin-I
GtH-II - Gonadotropin-II
hCG - Human Chorionic Gonadotropin
i.p. - Intraperitoneal
IGF-I - Insulin-like Growth Factor I
IPSP - Inhibitory Postsynaptic Potential
LH - Luteinizing Hormone
LHRH - Luteinizing Hormone Releasing Hormone
mLF - medial longitudinal fasciculus
mPOA - Medial Preoptic Area
NDL - Nucleus Dorsolateralis Thalami
NDLI - Nucleus Diffusus Lobi Inferioris
NDM - Nucleus Dorsomedialis Thalami
NDTL - Nucleus Diffusus Tori Lateralis
NE - Norepinephrine
NE - Nucleus Entopeduncularis
NIL - Neurointermediate Lobe
NMDA - N-methyl-D-aspartic acid
NOS - Nitric Oxide Synthase

NPGa - Nucleus Preglomerulosis pars anterioris
NPO - Nucleus Preopticus
NPP - Nucleus Preopticus Periventricularis
NPY - Neuropeptide Y
NRL - Nucleus Recessus Lateralis
NSE - Neuron Specific Enolase
NT - Nucleus Tenia
NVM - Nucleus Ventromedialis
OIT - Olfactory Tract
OT - optic tract
OTec - Optic Tectum
PBS - Phosphate Buffered Saline
PKA - Protein Kinase A
PPD - Proximal Pars Distalis
sGnRH - salmon GnRH
T - Testosterone
TGF β - Transforming Growth Factor β
TH - Tyrosine Hydroxylase
Vd - Area Ventralis Telencephali Dorsalis
Vv - Area Ventralis Telencephali Pars Ventralis

Rationale and Hypothesis

Reproduction is essential for the survival of a species. Universally, the reproductive process in vertebrates involves the release of the gonadotrophic hormone follicle stimulating hormone (FSH) and luteinizing hormone (LH) from the pituitary gland in the brain in order to cause sperm and egg development and release, essential for reproduction. In order for this to occur however, proteins known as activins are also released from the gonads (testes and ovaries) to stimulate the release of FSH (Vale et al., 1986; Ling et al., 1986a; Ling et al., 1986b). Activins have subsequently been shown to be implicated in many different areas of the reproductive axis, with roles in the gonads, in the pituitary, and in the brain.

Although primarily produced in the gonads, activins are also produced within the pituitary where they have been shown to play a role similar to that of gonadotropin-releasing hormone (GnRH) by causing the release of the gonadotropin hormones (MacConell et al., 1998). It is known that there are three types of activin, activin A, activin B and activin AB. While most studies have focused on the effects of activin A in the pituitary, a few other studies have noted similar roles for activin B. They both stimulate the release of FSH from the pituitary, and they also stimulate an increase in FSH β and LH β expression in the pituitary. Further evidence of their critical role in release of gonadotropin hormones was seen in a mouse pituitary cell line model where follistatin, an activin binding protein which antagonises the effects of activin, blocked the GnRH-stimulated expression of LH β and FSH β subunits (Persanetti et al., 2001).

Activin's actions also appear to occur in another critical reproductive area: the brain. In the mammalian brain, follistatin and activin subunits are located in close proximity to GnRH fibres in the hypothalamus, indicating a possible regulatory role of the activins on the release

of GnRH from the mammalian hypothalamus (MacConell et al., 1996; MacConell et al., 1998). Moreover, *in vitro* studies show that activin A stimulates the expression and release of GnRH from a GnRH-releasing neuronal cell line and from explanted rat hypothalamus (Calogero et al., 1998; MacConell et al., 1999).

To date, very few studies have focused on the role of activin in the fish brain. Activin β A subunits have only been localised in the brain of one fish, the thin lipped grey mullet, *Liza ramada*. This study found cells containing activin β A subunits in the telencephalon, midbrain tegmentum and cerebellum (Mousa and Mousa, 2003). GnRH cells have also been localised in the preoptic area of the telencephalon of goldfish and masu salmon (Amano et al., 1991; Kim et al., 1995), thus suggesting the potential for activin A-stimulated GnRH release. A study by Martyniuk et al. (2007a) showed that an exposure to a γ -aminobutyric acid (GABA)_B receptor agonist baclofen increases the expression of the activin β A subunit through the GABA_B receptor in the hypothalamus and in the telencephalon of sexually recrudescing female goldfish, *Carassius auratus*. Thus, circumstantial evidence exists that activins are stimulated by GABA and likely play a central role in the reproductive cycle with functions not only in the gonads, but also in the pituitary and in the brain in mammals.

The goal of this Masters research was to study the role of activin A and B in the reproductive axis in goldfish. The goldfish brain is an ideal model to study this in that the brain resembles that of other cyprinid fish and thus the findings can be generalized to fish that are important commercially. Goldfish are easily accessible fish and are excellent models for neuroendocrine research since their pituitary is directly innervated by neurons from the brain (reviewed by Trudeau, 1997). We hypothesised that activin is involved in the pathway by which GABA stimulates the release of LH from the pituitary. We studied the effects of a

GABA_B receptor agonist on the expression of the activin signalling system, which includes the activin subunits and activin receptors. Specifically, it is hypothesized that GABA would stimulate the expression and release of activin A and activin A would subsequently stimulate the release of GnRH from the hypothalamus into the pituitary.

The first part of the study focused on the effects of an intraperitoneal (i.p.) injection of a GABA_B receptor agonist, baclofen, on the expression of genes along the activin signalling pathway and on the expression of the two forms of GnRH found in the goldfish brain. The mRNA expression of activin subunits, activin receptors and GnRH was studied in the hypothalamus and telencephalon of sexually mature male and female goldfish following baclofen injection. We predicted that since GABA affects the expression of activin β A in the hypothalamus and in the telencephalon in sexually recrudescing goldfish, similar changes would be expected in the sexually mature goldfish (Martyniuk et al., 2007a). We examined other parts of the activin signalling system that could also be affected by GABA_B receptor stimulation by measuring expression levels of activin receptors type IB, type IIA and type IIB. Since we expect that activin subunit expression will increase in response to GABA, we would expect that the expression of activin receptors will remain unchanged or even be inhibited in order to regulate the levels of activin signalling.

The second part of the study focussed on studying the location of activin subunits within the neuroendocrine regions of the goldfish brain using immunohistochemistry. This aspect of the study is novel and has not previously been done in the goldfish brain. If, as is stated in our hypothesis, activins stimulate the expression and release of GnRH from the goldfish brain, activin subunits or activin receptors should be colocalised with GnRH cell bodies or fibres within the brain or pituitary. To this end, we investigated whether activin receptors were, in

fact, colocalised with GnRH in the goldfish neuroendocrine brain and in the pituitary using the technique of fluorescent immunohistochemistry. This finding could offer a glimpse into the potential role of activin not only in reproduction but also in other pathways, such as growth.

In the general introduction, I will begin by reviewing the control of reproduction by the gonadotropin hormones and describe known hormones and neurotransmitters which control the release of the gonadotropins. Next, I will review activins, how they are controlled, and illustrate their various roles within vertebrates, with an emphasis on the previously identified role of activins in reproduction.

Chapter 1

General Introduction

1.1. Control of gonadotropin release

1.1.1. Gonadotropins

The gonadotropic hormones, luteinizing hormone (LH) and follicle stimulating hormone (FSH) are the major regulatory hormones in the reproductive axis in vertebrates. In fish, LH and FSH were previously called gonadotropin-I (GtH-I) and gonadotropin-II (GtH-II). However, due to the similar structure to the gonadotropic hormones in mammals, they were renamed FSH and LH, respectively (Qu erat et al., 2000). LH and FSH are both heterodimers of an α and a β subunit. The α subunit is common to both LH and FSH. The β subunit is what differentiates FSH from LH, and they are named FSH β and LH β , respectively. Both hormones are synthesized and released from the gonadotropes in the anterior pituitary.

In mammals, the gonadotropes that release LH and FSH are randomly distributed within the anterior pituitary. In contrast, in fish, the gonadotropes are grouped together in one distinct region of the pituitary known as the *proximal pars distalis* (PPD). Another significant difference between mammals and fish is how these cells are innervated. In mammals, the brain and pituitary are connected by a median eminence, a network of capillaries into which hormones and neurotransmitters are released from the hypothalamus and subsequently transported into the pituitary via the bloodstream. In fish, rather than a network of capillaries, the pituitary is directly connected to the brain by nerve fibres whose cell bodies originate in the hypothalamus and release their hormones and neurotransmitters directly onto the cells which they are meant to innervate (Trudeau et al., 2000).

In mammals, LH and FSH have distinct roles in vertebrate reproduction. LH stimulates steroid synthesis and subsequent release of steroid hormones from the gonads. In female mammals, LH stimulates ovulation and subsequently stimulates the development of the corpus luteum, which then stimulates the synthesis and release of progesterone. In male mammals, LH is responsible for sperm release and spermiation (Norris, 1997). FSH in females is involved in the first phase of the reproductive cycle, stimulating follicular growth and maturation. In males, FSH stimulates spermatogenesis (Norris, 1997).

In fish, FSH and LH appear to have similar roles both serving to stimulate steroidogenesis and gonadal growth. What is dissimilar however, is the distinctly different patterns of release of FSH and LH from the pituitary over the course of the reproductive cycle. Goldfish, *Carassius auratus*, are fish that reproduce annually, and in the Northern hemisphere, they are sexually mature during the months of April and May (Trudeau, 1997). LH is released more abundantly in sexually mature fish, and its levels decrease following ovulation in May (Trudeau, 1997).

Given the important role of FSH and LH in controlling the reproductive axis, their release is very tightly controlled by many hormones and neurotransmitters (see Figure 1.1. for summary).

1.1.2. Gonadotropin-releasing hormone

Gonadotropin-releasing hormone is a 10 amino acid hypothalamic neuropeptide and is the primary regulator of gonadotroph function. Most vertebrates have more than one form of GnRH (Somoza et al., 2002). The two forms of GnRH that exist in goldfish are salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II). The two forms of GnRH in goldfish differ by their signal transduction pathways; cGnRH-II is dependent on Ca^{2+} entry through a calcium

channel, whereas sGnRH is dependent on the mobilisation and metabolism of arachidonic acid (Trudeau, 1997). Studies on the locations of GnRH show different roles for the two forms of GnRH in salmon due to their locations in different brain parts: sGnRH is involved in the regulation of reproduction whereas cGnRH-II is involved in neuromodulation (Amano et al., 1991).

1.1.3. Dopamine

Neurotransmitters also modulate the release of GnRH from the brain. The only neurotransmitter that has been identified to date as having an inhibitory role in the goldfish reproductive axis is dopamine (DA). DA is a neurotransmitter synthesised from tyrosine by the enzyme tyrosine hydroxylase (TH) (Popesku et al., 2008). Two different types of DA receptors exist within the brain, namely D1 and D2. D1 receptors stimulate cyclic adenosine monophosphate (cAMP) production, whereas D2 receptors inhibit cAMP production (Popesku et al., 2008).

In the pituitary, DA inhibits the release of LH in two ways: DA directly inhibits the release of LH from the gonadotrophs of the pituitary and DA also inhibits the GnRH-stimulated LH release. In fact, the LH-stimulated ovulation is due to an inhibition of DA release, indicating the importance of DA in the reproductive axis as a regulator of LH release. Additionally, DA can also inhibit the release of LH by inhibiting the synthesis of γ -aminobutyric acid (GABA), another neurotransmitter that is involved in reproduction (Trudeau, 1997, Trudeau et al., 2000). Finally, DA also regulates the number of gonadotroph cells in the goldfish pituitary. After injections of a dopamine antagonist, resperine, immunohistochemistry determined that the number of gonadotropes was increased by nearly 300% (Osornio et al., 2004).

DA is also involved in the negative feedback of sex steroids on LH release. When levels of circulating sex steroids were increased in circulation by injecting fish with estradiol, there was a subsequent increase in the number of DA receptors (Levavi-Sivan et al., 2006). An increase in the levels of DA receptors increases DA signalling, which subsequently increase the level of DA inhibition on LH release from the pituitary.

1.1.4. Gamma-aminobutyric acid

Gamma-aminobutyric acid (GABA) is one of the neurotransmitters that causes an increase in LH release from the goldfish pituitary. Despite its stimulatory role in reproduction in goldfish, GABA causes hyperpolarisation in cells once it binds to its receptors, which is why it often functions as an inhibitory neurotransmitter in the brain.

GABA is synthesized from glutamate by the enzyme glutamic acid decarboxylase (GAD), and degraded into succinic semialdehyde by GABA transaminase (GABA-T). Multiple forms of GAD exist in vertebrates. Two of these forms are GAD₆₅ and GAD₆₇ (named according to their molecular weights of 65 and 67 kDa, respectively). GAD₆₅ and GAD₆₇ are coded for by two different genes that originated from a gene duplication which occurred 400-500 million years ago (Bosma et al., 1999). There is also a third GAD that was discovered in the deep sea fish, the armoured grenadier *Coryphaenoides (Nematonurus) armatus* and subsequently in goldfish named GAD3 (Bosma et al., 1999; Larivière et al., 2002). This third isoform has not been widely studied, and therefore its significance is not known. The expression of GAD3 does not change over the course of the goldfish reproductive cycle whereas the expression does change for the other two forms of GAD. This implies that GAD3 might not be involved in reproduction compared to the other two isoforms (Larivière et al., 2005).

There are two primary types of GABA receptors in vertebrates. The GABA_A receptor is a pentamere of different GABA_A receptor subunits. This pentamere forms a chloride channel, making the GABA_A receptor an ionotropic receptor. When the receptor is activated by GABA or by an agonist, an inhibitory postsynaptic potential (IPSP) is created, which is characterized by a rapidly activating current that degrades quickly. GABA_A receptors are also present on non-synaptic membranes and exhibit tonic inhibitory communication properties (Macdonald et al., 2004). The second GABA receptor is the GABA_B receptor. The GABA_B receptor is a G-protein coupled receptor (GPCR) class C, and is therefore a metabotropic receptor. The GABA_B receptor system is coupled to Ca²⁺ and K⁺ channels. The GABA_B receptor is a heterodimer of the GABA_{B1} and the GABA_{B2} subunits. The GABA_{B2} subunit has many functions: it is involved in the shuttling of the GABA_{B1} subunit to the cell surface, it increases the affinity to GABA agonists, and it is required for normal activation of the G-protein that is associated to the receptor (Couve et al., 2004, Pin et al., 2004). Binding of GABA to the GABA_B receptor causes an increase in K⁺ conductance, and thus causes a slow IPSP (Nicoll et al., 2004).

GABA regulates the expression of genes that are involved in its own signal transmission, including GAD, GABA-T, and the receptor subunits. An increase in GABA signalling causes a decrease in the expression of the GABA_A β4 subunit in the hypothalamus, and a decrease in the GABA_A β2 subunit in the telencephalon. As well, the mRNA expression of GAD₆₅ and GABA-T are decreased in the telencephalon, and the expression of GAD₆₇ and GABA-T are decreased in the hypothalamus (Martyniuk et al., 2005; 2007a). GABA also regulates the levels of dopamine by decreasing the levels of tyrosine hydroxylase in the telencephalon (Martyniuk et al., 2007a).

GABA plays an important role in reproduction. In mammals, GABA has a dual action on the release of LH and GnRH. It has been shown to both stimulate and inhibit the release of both hormones. In immortalised hypothalamic neurons, the GABA_A receptor agonist stimulated the release of GnRH, and the GABA_B receptor agonist inhibited the GABA_A-stimulated release of GnRH (Favit et al., 1993). The hypothalamic neurons have both the GABA_A and the GABA_B receptors present on their membrane, therefore the stimulation and inhibition from GABA occurs from direct action on the neurons (Favit et al., 1993). In fish, GABA stimulates the release of LH from the pituitary through both the GABA_A and the GABA_B receptors in sexually regressed and in sexually mature fish (Martyniuk et al., 2007a; Trudeau et al., 1993b). The stimulation of LH release from the pituitary is partially mediated by the stimulation of GnRH release by GABA (Trudeau, 1997). This stimulation is primarily done through the GABA_A receptor. Thus, GABA not only affects GnRH but can also directly stimulate the release of LH from the pituitary.

1.1.5. Glutamate

Interestingly, glutamate, a precursor to GABA is converted into GABA by GAD, and also has an effect on the release of gonadotropins from the pituitary. The effect of glutamate appears to be mediated through the N-methyl-D-aspartic acid (NMDA) receptor and through the S- α -amino-3-hydroxy-5 methyl-4-isoxazolepropionic acid (AMPA) receptor (Trudeau et al., 2000; Trudeau, 1997).

In mammals, glutamate is likely indirectly stimulating the release of LH by first stimulating the release of other intermediate hormones and neurotransmitters such as GnRH, neuropeptide Y (NPY), or catecholamines. In fish, a GnRH antagonist blocks this glutamate-stimulated release of LH from the pituitary, indicating that the glutamate stimulation of LH

release is likely caused by an increase in GnRH release, but the mechanism still remains unclear and requires further study in fish (Trudeau et al., 2000).

1.1.6. Norepinephrine

Other neurotransmitters resulting from the GABA and dopamine synthesis pathways can also affect the release of gonadotropins from the pituitary. Norepinephrine (NE) is a metabolite of dopamine, and it is converted from DA to NE by the enzyme dopamine β -hydroxylase (DBH). The function of NE in the reproductive axis has not been as widely studied. There is a suggestion from one study however, that NE can have an effect on the release of the gonadotropin hormones (Chang and Peter, 1984). They showed that unlike DA, NE stimulated the release of LH from the pituitary of sexually regressed goldfish, but not from the pituitary of sexually mature goldfish. This difference indicates that circulating sex steroids are likely mediating this effect. Sex steroids do not have an effect on the steady state levels of NE, but they affect the turnover rates of NE differentially depending on the sex steroids and the reproductive stage of the fish. Estradiol (E_2) had more of an effect than testosterone (T) on the NE turnover rates in both sexually regressed and sexually recrudescing fish, decreasing NE turnover rates in sexually regressed fish and increasing NE turnover rates in sexually recrudescing fish (Trudeau et al., 1993d).

1.1.7. Sex steroids

The sex steroids, T and E_2 can also have an effect on GnRH-stimulated LH release. When implanted with T and E_2 , the response of the gonadotroph cells of the pituitary to sGnRH and cGnRH-II was potentiated. The sex steroids only affected the GnRH-stimulated LH release,

and not basal LH release. The DA turnover rates were increased, which increased the DA inhibition of LH release (Trudeau et al., 1993a; 1993c).

T, E₂, and progesterone also regulate the responses to the different hormones and neurotransmitters. T and E₂ change the turnover rates of NE (Trudeau et al., 1993d). Both T and E₂ create both negative and positive feedback loops within the brain. The positive feedback loop is caused by high levels of E₂ to stimulate ovulation in females, which causes an increase in LH. The negative feedback loop is caused by T and E₂ at all other times to directly and indirectly decrease the release of LH and FSH. T decreases the synthesis of GABA, therefore exhibiting negative feedback on the reproductive axis. Conversely, E₂ increased the rate of GABA synthesis thus creating the positive feedback loop on the reproductive axis (Trudeau et al., 1993c).

In mammals, sex steroids potentiate the glutamate-stimulated LH release. Progesterone-stimulated LH release is also mediated through the NMDA receptors (Carbone et al., 1992). This does not seem to be the case in sexually regressed goldfish though, as sex steroids had no effect on the LH release stimulated by the NMDA receptor (Trudeau et al., 1993a).

1.2 Activins

1.2.1. Activin subunits

Activin is a protein that belongs to the activin/inhibin family that is part of the transforming growth factor β (TGF β) superfamily. Activins were originally discovered and isolated from ovarian extracts. Functionally activins are known as substances that are able to stimulate the release of FSH from the pituitary (Vale et al., 1986; Ling et al., 1986b; MacConell et al., 1998).

Two types of activin subunits (the α and the β subunits) exist in the activin/inhibin family. There is one form of α subunit and two forms of β subunits (activin subunit β A and activin subunit β B). Inhibins and activins are dimers of these two types of subunits. Inhibins are a heterodimer of an α and a β subunit. A dimer with a β A subunit will form inhibin A, and a dimer with a β B subunit will form inhibin B. Activins are dimers of two β subunits. Two β A subunits form activin A, two β B subunits form activin B, and a β A and a β B subunit form activin AB.

Both the activin β A and the activin β B subunits are well conserved across vertebrate species. Goldfish activin β A has a protein sequence of 116 amino acids, with an 18 amino acid deletion compared to the mammalian activin protein (Yam et al., 1999). The activin β B subunit also has a protein sequence of 116 amino acids (Ge et al., 1997). The amino acid sequence of β B is better conserved than the sequence of activin β A. The sequence of activin β A is 78% identical to both the amino acid sequence from human and *Xenopus*, and the sequence of β B subunits is 94% and 96% identical to the amino acid sequence from human and *Xenopus* respectively (Ge et al., 1993a; 1997). The high level of sequence conservation across vertebrates of each subunit indicates a fundamental biological function for both subunits. The β A and β B subunits in zebrafish are not similar to each other though, having only 56% amino acid identity to each other (Wang and Ge, 2003c).

In mammals, activin β A subunits have been found to be located most commonly in the ovaries, but also in testes, brain, thyroid, pancreas, and bone marrow (Wada et al., 1996). In goldfish, activin β A subunits have been found in the ovaries, testes, brain, and liver (Yam et al., 1999b).

Within the gonads of goldfish, activin β A and β B were found in the cytoplasm of previtellogenic cells of the ovaries and in the follicular cell layer. In the testes, the two β subunits are found in the interstitial cells. The α subunits were found in the cytoplasm of previtellogenic cells of the ovaries and in the spermatozoa in the testes (Ge et al., 1993b).

Previously, the localisation of activin β A subunit mRNA or protein in the goldfish pituitary was difficult. However, some investigators have found the activin β A subunits in the somatotrophs of goldfish (Ge and Peter, 1994). The α subunit is localised in the nerve fibres of the intermediate lobe in the pituitary (Yuen and Ge, 2003; Ge and Peter, 1994). In the pituitary of *Xenopus*, activin β A subunits have been localised in the somatotroph cells, the thyrotroph cells and the gonadotroph cells (Uchiyama et al., 1996).

1.2.2. Activin receptors

There are two types of activin receptors in vertebrates: activin receptor type I (a 50-60 kD protein) and activin receptor type II (a 70-80 kD protein). They are transmembrane kinases with a single cross-membrane domain (Ge et al., 1997). In mammals, there are two forms of each type of activin receptor: activin receptor type I forms are named activin receptor type IA and type IB, and activin type II receptor forms are named activin receptor type IIA and type IIB. Goldfish have both forms of the activin type II receptor, but only have one form of activin type I receptor, the activin receptor type IB. In goldfish, the activin receptor type IIB is a 509 amino acid protein (Garg et al., 1999).

Neither activin receptor type I nor activin receptor type II is sufficient to transmit the activin signal alone to the cell to activate transcription, both being required to transmit the activin signal. Activin receptor type II first binds the activin β subunit. The presence of the

second β subunit on the activin protein allows the activin receptor type II to recruit, bind and phosphorylate the activin receptor type I. The binding of the activin receptor type I activates the complex, to start the downstream signalling which involves Smad proteins (Müller et al., 2006, Ge et al., 1997).

1.2.3. Smad proteins

Since activin receptors are membrane receptors, there must be downstream signalling in order to stimulate target gene transcription. Downstream signalling for activins uses the Smad protein family. There are three different types of Smad proteins within the Smad protein family; R-Smads are the receptor regulated Smads which interact with the receptors; co-Smads interact with the R-Smads for binding DNA; and inhibitory Smads. The latter group either interact with the receptors to stop phosphorylation of the R-Smads, interfere with the R-Smad/Smad4 complex, or recruit E3 ligase to degrade the receptor (Attisano et al., 2001). The R-Smads that are involved in activin signalling are Smad2 and Smad3, the co-Smad is Smad4, and the inhibitory Smad is Smad7 (Lau and Ge, 2005).

Smad proteins are also well conserved across vertebrates. Goldfish Smad2 has a 468 amino acid sequence that is 99% and 95% identical to *Danio rerio* and mammalian Smad2 sequences, respectively. Goldfish Smad3 has a 422 amino acid sequence which is 97% and 93% identical to *Danio rerio* and mammalian sequences, respectively. Goldfish Smad4 proteins have a 505 amino acid sequence with 91% and 83% identities to carp and mammalian sequences, respectively. Finally, goldfish Smad7 protein has a 377 amino acid sequence with 98% and 73% identities to *Danio rerio* and mammalian sequences, respectively (Lau and Ge, 2005). Similarly to the activin subunits, there is a strong evolutionary

conservation across vertebrate species indicating an essential role of Smad proteins in cell signalling.

When the receptor complex is activated, activin receptor type I phosphorylates either Smad2 or Smad3. R-Smads also bind FoxH1 before binding Smad4 and DNA. Smad4 functions to stabilize the complex so that gene transcription can be activated (Attisano et al., 2001). In mammals, Smad3 is sufficient for activin signalling and will bind to Smad4, whereas Smad2 plays a smaller role (Coss et al., 2005, Suszko et al., 2005). This has not been studied in fish, but in all probability, the same Smad proteins are used.

1.2.4. Inhibin and follistatin

The effects of activin can also be antagonised and otherwise regulated by other proteins within vertebrates known as inhibin and follistatin.

Inhibin (the other member of the activin/inhibin family), is a heterodimer of the activin/inhibin α subunit and either the β A or the β B subunit. An α and a β A subunit form inhibin A, and an α and a β B subunit form inhibin B. Inhibin antagonises the effects of activin by blocking the activin receptor complex. The shape of the inhibin α subunit enables the binding to the coreceptor, betaglycan which then enables inhibin to bind to the activin receptor II. However, due to the lack of a second β subunit, the activin receptor type II cannot recruit the activin type I receptor, thereby preventing downstream signalling and eventually the release of gonadotropins. Inhibin has the opposite action to that of activin, inhibiting the release of FSH from gonadotroph cells in the pituitary. It was recently shown that inhibin B is a much more potent inhibitor to the release of FSH than inhibin A, and this difference is possibly due to a separate coreceptor for inhibin B (Makanji et al., 2009).

Follistatin, an activin binding protein has also been shown to antagonise the effects of activin in goldfish pituitary cell culture (Yuen and Ge, 2004). Follistatin is often found in the same areas in which the activin subunits or the activin receptors are found including the gonads and in the pituitary thereby allowing it to regulate activin's function in these areas (Wada et al, 1996, MacConell et al., 1996; MacConell et al., 1998; Kaiser et al., 1992).

In the gonads of goldfish, activin is found in the follicle cells, and follistatin and the activin receptors are found in the oocytes, supporting the oocytes as the site of action for activin (Wang and Ge, 2003b). The levels of follistatin mRNA and protein in the gonads, in the pituitary and in the brain are also regulated by both gonadotropins and activin itself to regulate the action of activins within the reproductive axis. Human chorionic gonadotropin (hCG), a gonadotropin hormone produced in the human ovaries, increases the expression of follistatin mRNA in the goldfish ovarian follicle *in vitro* (Wang and Ge, 2003a). This increase in follistatin mRNA would allow a larger amount of activin to be bound, limiting the amount of available activin in the follicles. Activin B increases follistatin expression in goldfish pituitary cell cultures. Interestingly, T and E2 both also increase follistatin expression in the pituitary, without affecting activin β B levels (Cheng et al., 2007). This particular data provides very interesting insight into an important role for follistatin in regulating activin's action within the reproductive axis, and hints at an alternate method of regulating activin signalling without changing the expression of activin subunits.

In mice, there are three isoforms of follistatin that differ at the level of exon 6. The shortest isoform, FST288, lacks exon 6, whereas the longest isoform, FST315, has exon 6 in its entirety. The third isoform, FST303, only has a part of exon 6. FST288 appears to be more suited for autocrine function within tissues whereas FST315 would be used for endocrine

functions. Follistatin knockout mice show that follistatin and activin are necessary for development as they do not survive to adulthood. On the other hand, mice that only have FST288 develop normally, but they have a reduced fertility due to a reduced number of tertiary and preovulatory follicles when the mice are sexually mature. In mice, therefore, activin appears to play a crucial role in reproduction (Kimura et al., 2010).

1.2.5. Activin in tissue repair

In addition to vertebrate reproduction, activins also appear to have other diverse functions in tissue repair. Activin's role in tissue repair has been demonstrated in the rat amygdala. Rat amygdala cultures that were exposed to activin A had a reduced number of atrophied neurons as well as an increase in the length and branching of the neuritic processes in these cells (Trudeau et al., 1997). Another study showed that there is a strong induction of activin β A subunit expression immediately following a neuronal injury. Subsequently, the same group showed that the expression of activin A was increased as a neuroprotective factor involved in the basic fibroblast response in protecting neurons against injury (Tretter et al., 1996; 2000). Thus, there is early evidence that activin is a factor involved in neuronal growth or repair.

1.2.6. Activin in behaviour

A study by Torii et al. (1993) found activins in the arcuate nucleus, and in the nucleus of the solitary tract, both areas important in behaviour. In this study, rats that have been fed diets deficient in protein and in the amino acid lysine were found to have suppressed activin A secretion which appeared to correlate with changes in feeding behaviour. The investigators concluded that activin A appeared to be a neurotrophic factor in the rat brain that is utilized in changing feeding behaviour. Activin would allow for an adjustment in their feeding habits to

be able to ingest the specific amino acid that they are lacking, such as lysine, as was the case in this study.

1.2.7. Activin in development

The role of activins and inhibins in embryonic development has been studied in many different animal models including the African clawed frog, mice, rats, and human embryonic stem cells. Activin subunits have been identified in developing embryos, perhaps implying a potentially important function in various developmental processes. Furthermore, activins are known to be involved in organogenesis (such as in mesoderm induction) in mammals and in lower vertebrates such as the African clawed frog, *Xenopus laevis* (Dohrmann et al., 1993; Feijen et al., 1994; Roberts and Barth, 1994; Smith et al., 2007). Their specific involvement in neural development has been supported by finding them in the developing brain of mice and frogs (Dohrmann et al., 1993; Feijen et al., 1994). In rats, Roberts and Barth (1994) found that activin A is preferentially involved in organogenesis more than activin B. In embryonic stem cells, the inhibition of the activin/Nodal pathway by Lefty is necessary for neuroectoderm development (Smith et al., 2007).

Knockout mice models have clearly demonstrated the relative importance of both activin β A and β B in other reproductive outcomes. Activin β A knock-out mice show developmental problems such as a failure to suckle, and as a consequence, they die prematurely. Activin β B knock-out mice are viable and fertile, but they show a longer gestational period and an inability to nurse their young, implicating activin B in the regulation of reproduction. This appears to validate the conclusion by Roberts and Barth (1994) that activin β A is more important in development than activin β B.

Other investigators induced a mutation in which activin β B is expressed in the position of activin β A. These mice were viable and fertile, but they were smaller than the wild-type mice. The mutant mice had a reduction in bone growth that stemmed from a reduction in thickness at the growth plate in bones and a lack of apoptosis which allows for mineralisation. As well, they had lower levels of insulin-like growth factor I (IGF-I) which indicates a link between activin signalling and IGF-I signalling during development (Brown et al., 2003).

Inhibin appears to be more important in the regulation of the development of the fetal reproductive system than in any other developing system. In fact, in developing mice, the mRNA of the activin α subunit is mostly found in the developing reproductive tissues such as the pituitary, testes and ovaries. Activin β A and β B subunits are also found in these tissues, indicating that both inhibin A and inhibin B could be regulating the development of the reproductive system (Roberts and Barth, 1994; Feijen et al., 1994).

1.2.8. Activin in growth

In addition to being involved in tissue repair and in development, activins are also involved in the regulation of growth in general. In goldfish and in the African clawed frog, activin subunits were found in the growth hormone (GH)-releasing cells of the pituitary, the somatotroph cells. In *Xenopus*, activin subunits are also found in the gonadotrophs and thyrotroph of the pituitary as well as in the somatotroph cells (Ge and Peter, 1994; Uchiyama et al., 1996). Therefore, activins can act as local autocrine or paracrine factors in the pituitary and influence the release of GH. In fact, in goldfish pituitary cell culture, activin A increased the release of GH by 200% compared to control (Ge and Peter, 1994).

In the teleost fish including goldfish, African catfish, and tilapia, activins A and B are involved in regulating body size, and more specifically, muscle mass. This regulation likely occurs by inhibiting excessive muscle growth by limiting protein content (Carpio et al., 2009).

1.2.9. Activin in mammalian reproduction

Early studies attesting to the role of activin in mammalian reproduction was the demonstration of the presence of activin in porcine follicular fluid which stimulated the release of FSH from the pituitary. It was activin A that was specifically first found to stimulate the release of FSH (Vale et al., 1986; Ling et al., 1986a). Although less is known about its role in non-mammalian vertebrates, in mammals, activin's role in reproduction has been widely studied and affects the reproductive axis in the brain, the pituitary and the gonads.

Roberts and Barth (1994) found that inhibins and activins are an important part of the development of the rat foetal reproductive system. In adults, the role of activins in the female gonads has been more widely compared to their role in the male gonads. In the female gonads, the activin β A subunits are found mostly in the follicle cells (Dohrmann et al., 1993). Activin receptor type II mRNA and proteins are also found in the gonads. The mRNA for both of the activin receptor type II forms are found in the uterus of female mice. Activin receptor type IIB mRNA is more widely distributed than activin receptor type IIA mRNA. The activin receptor type IIA mRNA is found in the cumulus cells and in oocytes whereas activin receptor type IIB mRNA is found in the uterus, ovary and extragonadal tissue (Wu et al., 1994). The activin receptor type II protein was found in all stages of oocytes, in the corpus luteum, as well as a weak signal in the granulosa cells in the rat gonads (Cameron et al.,

1994). The Smad proteins, necessary for activin signal transmission, are found in the nuclei of the somatic cells in the ovarian stroma (between clusters of germ cells), in the somatic cells (intermingled with germ cells), and in the nuclei of pre-granulosa cells surrounding oocytes. Of note, the Smad proteins show changes in mRNA expression levels during the reproductive cycle, indicating their co-dependence with the activin system in the reproductive cycle. In this model, their expression is increased almost 2-fold over the gestational period (Coutts et al., 2008).

In males, the mRNA of activin type IIB receptors are found in the epididymus, the testes and the extragonadal tissue of mice (Wu et al., 1994). In male rats, activin receptor type II protein was found in tubule sections, in spermatids and in spermocytes. The type IIB isoform was found in Leydig cell clusters and in the interstitial cells (Cameron et al., 1994).

As stated previously, in addition to its role in the gonads, activin also has an important role in regulating pituitary function. The first study involving activin A in the pituitary showed that application of porcine ovarian extracts increased FSH release from the pituitary, but not LH release, and that the protein that was causing this increased release was activin A (Vale et al., 1986; Ling et al., 1986a). Moreover, another study exposed adult male rats to an intracerebroventricular infusion of activin A which caused an increase in LH serum levels, but not serum levels of FSH (MacConell et al., 1998). This supports their function, at least in some species, in stimulating FSH release.

Activin A not only affects the release of FSH and LH, but it also regulates their mRNA expression levels. Activin A increases the mRNA expression of both the α and the β subunits of the FSH protein in the pituitary of mice (Gore et al., 2005; Huang et al., 2001; Persanetti et

al., 2001). Activin A also stimulates the mRNA expression of the LH β subunit in the pituitary of mice after one day in a static culture of pituitary cells. This activin signalling depends mainly on the activity of Smad3 and very little on the activity of Smad2 (Coss et al., 2005; Suszko et al., 2005). It is well known that GnRH also stimulates the mRNA expression of the gonadotropin subunits. This stimulation is partly dependant on activin signalling since follistatin blocked GnRH in the pituitary of mice (Huang et al., 2001; Persanetti et al., 2001). Thus the mechanism of activin activity to cause gonadotropin hormone release probably depends on the expression of mRNA.

Furthermore, there is evidence that activin is also involved in the release of GnRH from the mammalian brain. *In situ* hybridisation studies on the rat brain have shown that parts of the activin signalling system are located in areas that are correlated with regions that express GnRH mRNA (MacConell et al., 1996; 1998). Additional studies have also shown that activin A stimulates the release of GnRH protein and the expression of GnRH from a GnRH cell line and from explanted rat hypothalamus (Calogero et al., 1998; MacConell et al., 1999). A study with activin receptor type II knockout mice showed that activin receptor type II signalling was not necessary for GnRH expression and release in the brain. This study by Kumar et al. (2003) also showed that activin receptor type II signalling was important for the transcription and biosynthesis of FSH. Inhibin, on the other hand, was important for biosynthesis and secretion of FSH. This effect of activin A has yet to be shown in non-mammalian species.

The *in situ* hybridisation study by MacConell et al. (1996) that found follistatin in areas that are correlated with GnRH also found follistatin mRNA in other areas that are related with other aspects of reproduction. Follistatin mRNA was found in the solitary tract nucleus and in the paraventricular nucleus, areas which are involved in the oxytocin pathway. Activin is

involved in this pathway as well, which mediates the milk-ejection reflex, and follistatin is present to regulate the role of activin.

Activin receptor type II knockout mice provide fascinating information on how activins affect another facet of reproduction, that is, reproductive behaviour. Reproductive behaviour, which includes the control of mounting, copulation, intromission and ejaculation, is mediated by changes in nitric oxide synthase (NOS) activity in the medial preoptic area (mPOA). These knockout mice show changes in many aspects of their reproductive behaviour which decreases their chances of reproductive success. These changes include delays in the initiation of copulation, mounting and intromission as well as an increase in the latency of mount, intromission and ejaculation. In these mice, no hormonal changes were observed, and the only significant difference that was found to explain these behavioural changes was a reduced NOS activity in the mPOA (Ma et al., 2005).

1.2.10. Activin in non-mammalian reproduction

In lower vertebrates such as fish and amphibians, the role of activin in reproduction has been less studied compared to the considerable evidence presented earlier that exists in mammals. Some earlier mammalian studies have been somewhat comparable to those done in both fish and in frogs. As such, using *in situ* hybridisation and immunohistochemistry, the location of activin subunits in the gonads and in the pituitary of non-mammalian vertebrates has been documented. To date however very little is known about the role of activin in the brain of goldfish.

In goldfish, the ovarian granulosa cells appear to be the major source of activin production. In *Xenopus* (Dohrmann et al., 1993) and in goldfish (Ge et al., 1993b), activin β A

and β B subunits are found in the follicle cells of the ovaries, but not in the oocytes. The activin/inhibin α subunit is found in the cytoplasm of previtellogenic ovaries. The staining of the β subunits in the follicle cells is very strong in the cytoplasm of the previtellogenic ovaries, and staining progressively decreases as the ovaries mature. Two hypotheses exist to explain this, either the subunits are stored in the previtellogenic ovaries and are released later on to stimulate the release of gonadotropins, or the subunits are stored in the previtellogenic ovaries, are diluted as the ovaries grow, and are passed on to the embryos to stimulate their growth and development (Ge et al., 1993b; 1997).

As for the activin receptors within the reproductive axis of goldfish, the highest level of expression seen previously in goldfish is in the ovary. The largest amount of staining of activin receptor type IIB is in the oocytes, and like the staining of the β subunits in the follicle cells, the amount of staining is inversely proportional to the maturational stage. There is no staining in the follicle cells or in the interstitial tissues, unlike the activin β subunits (Garg et al., 1999). Follistatin is expressed in both the ovaries and in the follicle cells, but the staining was much stronger in the oocytes, where it likely regulates the role of activin. The different location of the activin proteins, activin receptors and follistatin indicates that activin could have a role in transmitting signals from the follicle cells to the oocytes (Ge, 2005).

The location of activins and inhibins has also been examined in the testes of goldfish where two forms of activin β subunits and the α subunits are also found. The α subunit has very strong staining in the spermatozoa, but is not found in the interstitial tissue. On the other hand, the β A and β B staining is only found in the interstitial tissue (Ge et al., 1993b). From these immunohistochemistry results, it would appear that the gonads in male and female goldfish produce more activins than inhibins (Ge et al., 1997).

Activins act in a manner analogous to the gonadotropin hormones in the gonads: they increase the maturational competence of the oocytes and stimulate final oocyte maturation in fish. Activins promote oocyte maturation *in vitro* and this is achieved by a change in gene transcription (Pang and Ge, 1999). The *in vivo* effects are the same as the *in vitro* effects, with the exception that both transcription and protein synthesis are affected by activin A and B to stimulate oocyte maturational competence (Pang and Ge, 2002a).

Other factors have also been identified that stimulated the growth of the female gonads in the same pathway as activin. Two of these factors are epidermal growth factor (EGF) and TGF α . EGF and TGF α time- and dose-dependently stimulate oocyte maturation with activin operating downstream of these two factors. Both EGF and TGF α stimulate the expression of the activin β A subunit and activin receptor type II in the gonads (Pang and Ge, 2002c). Activin is involved in the hCG pathway which induces oocyte maturation (Ge, 2005). Activins also regulate the levels of certain steroid hormones in the female gonads. Activin B and TGF- β decrease both basal T production and hCG-stimulated T production in early vitellogenic follicles, whereas E₂ production was not affected. In these same follicles, activin B also decreased the production of the pheromone 17,20 β -P (Calp et al., 2003). This multitude of roles of both forms of activin demonstrates their crucial role in regulating reproduction within the gonads.

Studying the daily variations in activin β A subunits, activin β B subunits and follistatin mRNA expression in zebrafish has revealed separate roles for the two forms of activin in the regulation of the reproductive cycle. Activin β A subunit and follistatin expression increases during the preovulatory period whereas the expression of the activin β B subunit declines during the preovulatory period, but increases at ovulation. Therefore, it appears that activin A

promotes growth during the preovulatory period. Conversely, activin B has more of a tonic role during the preovulatory period and later stimulates ovulation (Ge, 2005).

In summary, the actions of activins are regulated in all areas of the reproductive axis by different factors, whose actions either control the expression and release of activins, or modify the levels of the proteins that control activin action (such as levels of inhibin and follistatin). The levels of activin β A and β B subunits in the gonads are also carefully controlled by gonadotropin hormones. In the gonads of both goldfish and zebrafish, hCG stimulates the expression of both the activin β A subunit as well as the expression of the activin receptor type IIA. Conversely, hCG suppresses the expression of activin subunit β B in zebrafish. The stimulation of activin β A subunit expression by hCG and the inhibition of activin β B subunit expression by hCG are both mediated through cAMP signalling. However, hCG accomplishes this differential regulation by employing a different pathway downstream of the cAMP signalling. The stimulation of β A is a part of a protein kinase A (PKA)-dependent pathway whereas the inhibition of β B is a part of a PKA-independent pathway (Pang and Ge, 2002b; Wang and Ge, 2003c).

There is emerging evidence that the activin system is also involved in controlling the reproductive axis at the level of the pituitary in goldfish, although the evidence for this is not as robust. It is known that activin mRNA and proteins are present in the pituitary of many different types of fish and frogs. In goldfish, activin subunits are present in the somatotroph cells of the pituitary. The study showed that the staining of the β A subunit was much more intense than the staining of the β B subunit. Inhibin α subunits stained only in the neurointermediate lobe, suggesting that the goldfish pituitary mainly produces activin (Ge and Peter, 1994). In the thin lipped grey mullet, *Liza ramada*, the β B subunit stained the pituitary

very strongly in the pars intermedialis, and the β A subunit was found in nerve fibres close to somatotroph and gonadotroph cells (Mousa and Mousa, 2003). In the frog, the β B subunit has strong staining in the anterior lobe in thyrotroph, gonadotroph, and somatotroph cells (Uchiyama et al., 1996). The close association of the activin subunits and the cells producing and releasing gonadotropins points to a possible autocrine and paracrine role for both activin A and activin B in the pituitary.

The effects of activins on pituitary gonadotropin expression and secretion that were demonstrated in mammals have also been demonstrated in goldfish. Activin B treatment of pituitary cell cultures decreases the mRNA expression of LH β after 48 hours (Yam et al., 1999a). In goldfish, activin A and inhibin A both stimulate LH release from a pituitary cell culture, and this release is independent from the GnRH-stimulated release of LH. Unfortunately, no assay to specifically measure serum FSH levels in goldfish is available at this time so the effect of activin on FSH release is not known.

In the same way that hormones in the gonads regulate activin production, hormones such as DA also regulate activin production within the pituitary. It is in a comparable manner to DA that inhibits the release of LH directly and inhibits the release of GnRH-stimulated LH release from the pituitary, DA also slightly reduces activin- and inhibin-stimulated LH release from the goldfish pituitary (Ge et al., 1992).

Currently, very little is known about the location of activins in the brain of non-mammalian vertebrates in order to elucidate more fully their function in the regulation of reproduction. One study by Mousa and Mousa (2003) studied the location of activin subunits in the brain of the fish *Liza ramada*. They located activins in some of the major

neuroendocrine areas (activin β A subunits were found in neurons in the telencephalon). Although it is known that GABA can cause changes in activin β A subunit expression in the hypothalamus and in the telencephalon of sexually regressed goldfish (Martyniuk et al., 2007a), to date the location of activin subunits has never been studied in the goldfish brain, nor in the brain of any other fish. The amplitude of the roles of activin in reproduction of fish is therefore not fully elucidated.

1.3. Conclusions

This general introduction presents a review of the release of LH and FSH from the pituitary in vertebrates and the roles of activins in the reproductive system. The release of FSH and LH is crucial to the reproductive system since these two hormones drive the maturation of the gonads, ovulation and steroidogenesis. Activins, particularly activin A, is known to stimulate the release of FSH and LH.

The release of LH and FSH is mediated by hormones and neurotransmitters that are in turn regulated by activin. The prime example of such action is the release of GnRH which is controlled in part by activin. Other hormones and neurotransmitters, such as GABA and DA, in turn control the expression of activin subunits (Martyniuk et al., 2007, Popesku, 2009). How GABA affects the activin signalling pathway however is not clearly known. Whether GABA would increase activin signalling by increasing the expression of activin receptors or by directly increasing the expression of the activin subunits remains to be seen. It is also not known whether activin A then stimulates the expression of other hormones such as GnRH downstream.

As shown in this review, activins are also involved in other vital functions including growth, neuronal tissue repair, all pathways which imply substantial central nervous system association. Locating these subunits in the goldfish brain could provide valuable insight into where, in the central nervous system, activin controls these functions. Using the technique of immunohistochemistry to reveal the sites of activin in the brain is of crucial importance into fully understanding activin and its role in the teleost brain, and further appreciating the neural control of reproduction.

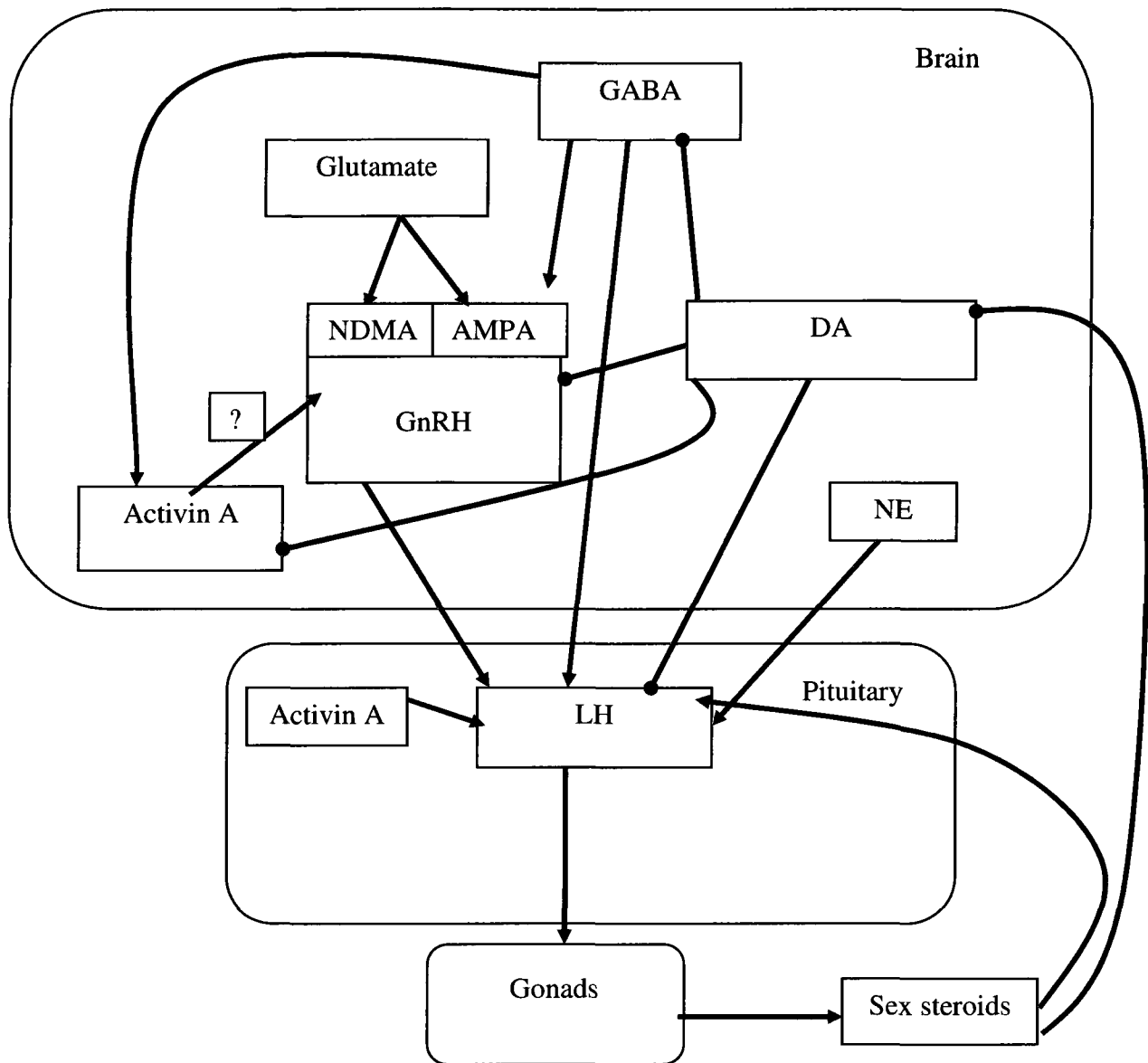


Figure 1.1. Multifactorial control of luteinizing hormone (LH) release from the gonadotrope cells of the pituitary in goldfish. GnRH represents both sGnRH and cGnRH. Dopamine (DA) is the only inhibitory neurotransmitter identified to date. LH is released from the gonadotrophs in the pituitary into the circulation, and will stimulate the release of sex steroids from the gonads. These sex steroids will then feedback on to reproductive axis to better control it. GABA, γ -aminobutyric acid; NMDA, N-methyl-D,L-aspartic acid; AMPA, S- α -amino-3-hydroxy-5-methyl-4-isoxazolepropionic acid; NE, norepinephrine. →: stimulation; ●: inhibition. Adapted from Trudeau, 1997.

Chapter 2

Effects of the GABA_B receptor agonist baclofen on activin subunits and activin receptor mRNAs in the goldfish brain

2.1. Introduction

Gamma-aminobutyric acid (GABA) is a major stimulatory neurotransmitter in the reproductive axis in the goldfish. The main action of GABA in the reproductive axis is to stimulate the release of LH from the gonadotropes of the pituitary. It does this in two ways: firstly by direct action of GABA on the gonadotropes and secondly by stimulating the expression and release of other hormones and factors from the brain and the pituitary. GABA stimulates the release of LH in teleost fish at all reproductive stages including sexually regressed, sexually recrudescing and sexually mature fish. In goldfish this stimulation mainly occurs through the ionotropic GABA_A receptor, and to a lesser extent through the metabotropic GABA_B receptor (Trudeau et al., 1993b; Martyniuk et al., 2007a).

The release of LH from the pituitary is mainly controlled by GnRH. It is GABA however that controls the expression and release of GnRH from the neuroendocrine brain. Most vertebrates have multiple forms of GnRH (Somoza et al., 2002), goldfish have two forms: salmon GnRH (sGnRH) and chicken GnRH-II (cGnRH-II). Both forms of GnRH are found in the same areas of the goldfish brain: in the transitional area between the olfactory nerve and the olfactory bulb (the terminal nerve ganglion), in the medial olfactory tract, in the ventral telencephalon, in the preoptic nucleus, and in the ventrolateral hypothalamus. In these areas, although both types are present, cells containing sGnRH are more abundant than the cells containing cGnRH-II. Conversely, cGnRH-II-containing cells are more concentrated in the

midbrain tegmentum of goldfish (Kim et al., 1995). In masu salmon, contrary to their localisation in the goldfish brain, sGnRH is not localised in the same areas as cGnRH-II. Cells containing sGnRH in salmon are found in the same areas as they are in goldfish, whereas cells containing cGnRH-II in salmon are only present in the midbrain tegmentum. The localisation of sGnRH and cGnRH-II in the brain of the masu salmon leads to the hypothesis that different functions for each type of GnRH. Since sGnRH is located in the telencephalon and in the hypothalamus, the areas for neuroendocrine regulation of the reproductive axis (and cGnRH-II is absent from these areas), sGnRH is likely involved in the regulation of reproduction and neuromodulation, whereas cGnRH-II likely only has a role in neuromodulation (Amano et al., 1991). A similar difference in function was observed in goldfish. In sexually regressed goldfish, only sGnRH stimulated the expression of the GTH α subunit and the LH β subunit, whereas cGnRH-II had no effect. However, in sexually mature goldfish, both sGnRH and cGnRH-II stimulated the expression of the GTH α subunit and the LH β subunit (Trudeau, 1997).

GABA has also been shown to affect the brain mRNA expression of another reproductive axis hormone, activin A. Activins has been implicated in reproduction in mammals, in amphibians, and in fish. Activins stimulate oocyte development and maturation in the gonads, the expression and release of gonadotropins from the pituitary, and the expression and release of GnRH from mammalian hypothalamic explants (Pang and Ge, 2002a; Ge et al., 1992; MacConell et al., 1999). GABA increased the expression of the activin β A subunit 3- to 4-fold in the hypothalamus and in the telencephalon in sexually regressed goldfish from September only through action on the GABA_B receptor (Martyniuk et al., 2007a).

Activins have two types of transmembrane kinase receptors that interact to transmit activin signalling to the cell to stimulate gene transcription through the actions of Smad proteins, the activin type I receptor and the activin type II receptor. There is one form of the activin receptor type I in goldfish, and two forms of activin receptor type II (type IIA and type IIB). It is unclear whether the two forms of the activin receptor type II have distinct roles or distribution within the brain or within the rest of the reproductive axis. Activin proteins will bind to either of the two forms of activin receptor type II, which will then recruit and bind the activin receptor type IB (Ge, 2005). This receptor dimer will then activate the remainder of the downstream signalling which involves the Smad proteins.

We hypothesized that activin partially mediates the GABA stimulatory effect on serum LH levels. Therefore, we predicted that a GABA_B receptor agonist, baclofen, will result in an increase in activin subunit expression in the neuroendocrine brain concomitantly with an increase in LH serum levels. We also predicted that if activin was mediating the effect of GABA on LH through the GnRH signalling system, baclofen exposure will result in an increase in the expression of one or both of the forms of GnRH. We aimed to demonstrate the effect of baclofen on the mRNA expression of activin subunits β A and β B, and activin receptors type I, type IIA, type IIB, and on the two forms of GnRH, sGnRH and cGnRH-II in the telencephalon and in the hypothalamus of sexually mature male and female goldfish. Finally, we sought to compare our results to the 4-fold increase in activin β A expression in sexually regressed goldfish in the telencephalon and hypothalamus after a 6 hour exposure to baclofen (Martyniuk et al., 2007a). This would potentially identify seasonal differences in the effects of baclofen on the expression of activin subunits since their study was done on sexually regressed fish whereas our study used sexually mature fish. Goldfish are sexually

regressed during the month of August and early September. They have low levels of GH and LH and have very low gonad weight due to having ovulated in May. Sexually mature fish, on the other hand, have high levels of LH and GH and very high gonad weight as they are ready for spawning season (Trudeau, 1997). We expect that we will observe seasonal differences since sexually mature fish might be more sensitive to changes in hormones and neurotransmitter levels in the reproductive axis due to higher levels of hormones that prime the fish for reproduction. Thus we would expect to see either a stronger increase in activin subunit expression or a change in where the activin subunits are being expressed to more specifically stimulate ovulation.

2.2 Materials and Methods

2.2.1 Experimental protocol and RNA extraction

Goldfish were purchased from a commercial supplier (Aleong's International Inc, Mississauga, ON, Canada) in May 2008. They were allowed to acclimatize in $18^{\circ}\text{C} \pm 1^{\circ}\text{C}$ water on a simulated natural photoperiod. They were fed standard fish pellet food (Martin Mills, Elmira, ON, Canada). Fish were anaesthetized with MS222 (Tricaine methanesulfonate; Sigma Aldrich, St-Louis, MN, USA) in water before all handling procedures.

In early June, sexually mature male and female goldfish were injected with a GABA_B receptor agonist, baclofen (Sigma Aldrich, St-Louis, MN, USA) intraperitoneally (i.p.). In total, 13 to 19 fish were used in each treatment group. The sexual maturity was confirmed by calculating the gonadosomatic index (GSI) (gonad weight/body weight x 100). The gonadosomatic index (GSI) of the control female goldfish was $14\% \pm 1$ and was not

significantly different from the GSI of treated female goldfish which was $13\% \pm 1$. The GSI of the control male goldfish was $3\% \pm 0.2$ was similar to the GSI of the treated male goldfish $3\% \pm 0.2$. However, the GSI of female goldfish for that time of year was 4.3-fold higher than the GSI of the male goldfish, a significant difference ($P > 0.05$) (Figure 2.1). The injection dose was $10 \mu\text{g/g}$ body weight, with an injection volume of $1 \mu\text{L/g}$. Baclofen was dissolved in 0.6% saline; control fish were injected with the same volume of saline. There were no significant differences in body weight between control and treatment fish and male and female fish. The average weight of goldfish was 21.77g. Blood was collected from the caudal vein 5 hours after the injection, left overnight at 4°C , after which red blood cells were removed by centrifugation to leave serum available for analysis. Whole brains were removed from the fish 5 hours after the injection, immediately placed on ice, and the hypothalamus and the telencephalon were extracted immediately. The hypothalami and telencephali were then flash frozen on dry ice. Hypothalami and telencephali were each pooled (2 per tube) to ensure adequate amounts of RNA.

Total RNA was extracted using the Qiagen Mini Plus kit (Qiagen, Valencia, CA, USA) according to the manufacturer's protocol. RNA concentrations were measured using the Agilent Nanodrop ND-1000.

2.2.2 *LH assay*

Serum LH levels were measured by the Chang lab according to Peter et al. (1984). Student's unpaired t-test was used to determine if there were significant differences in LH levels between control and treatment animals. The detection limit of the LH assay is 0.32 ng/mL, and the specific binding for LH is 40.6%.

2.2.3 *Real-time RT-PCR*

First Strand cDNA synthesis was performed using 1 µg of total RNA in a final volume of 10 µL, with 1 µL of each dNTPs and random primers. The mixture was then heated to 65°C for 5 minutes, and then quick chilled on ice. The contents were collected by brief centrifugation. 4 µL of 5X First Strand Buffer and 2 µL 0.1 M DTT were then added. The mixture was then heated to 25°C for 2 minutes. 1 µL of Superscript II was then added. The mixture was then incubated for 10 minutes at 25°C, 50 minutes at 42°C and then the reaction was inactivated by heating at 70°C for 15 minutes and stored at -20°C until used.

Primers for real-time RT-PCR were created with Primer 3 (<http://frodo.wi.mit.edu/>). They were created such that they would be ideally 20 bp in length; amplify segments 100-200 bp in length; and have a melting temperature of around 60°C. After RT-PCR, the products were sequenced to confirm the sequence that was amplified. All primers specifically amplified the target gene. The primer sets used are reported in Table 2.1.

The total reaction volume was 25 µL and reagent concentrations were: approximately 50 ng cDNA, 1X PCR Buffer, between 2.5 and 3.0 mM of MgCl₂ (depending on the primer set), 0.2 mM dNTPs, between 150-600 nM of each forward and reverse primers (depending on the primer set), 0.25X SYBR Green I dye, 0.10 µM ROX, and 1.25 U HotStar Taq. Real time RT-PCR was run on the Stratagene MX3000. The thermocycler parameters used were: enzyme was activated at 95°C for 15 minutes, followed by 40 cycles of 15 seconds of denaturation at 95°C, annealing for 5 seconds at 59°C to 60°C, elongation for 30 seconds at 72°C and data acquisition for 8 seconds at 80°C. This was followed by denaturation at 95°C for 1 minute,

and the creation of a dissociation curve from 55°C to 95°C at a rate of increase in temperature of 1°C every 30 seconds.

Standard curves were created with a 1 in 5 serial dilution (1:5 – 1:15 625) of cDNA from the subject tissue. Each sample represented pooled brain parts of two fish. Samples were diluted 1:80. Results were analysed using the MX4000 software. Standard curves were considered acceptable if the R² value was above 0.85, and if the efficiency was between 90% and 110%.

Table 2.1. Sequences of primers used for real time reverse transcriptase polymerase chain reaction.

Amplicon	Forward primer (5' to 3')	Reverse primer (3' to 5')
Activin βA	TTT AAG GAC ATC GGG TGG AG	TGA TTG ATG ACG GTG GAA TG
Activin βB	GAT GGA AAA GCG TGT GGA GT	CAG GAA TGG ACG GTG TGA G
Activin receptor IB	TCA CAT ACC ACC TTC CTC ATC T	TAT CTA CGC TTT GGG ACT GG
Activin receptor IIA	ACA CAG AGA CGG CCA CAA AC	TCA AAC CTC AAA GCC AGA CC
Activin receptor IIB	CGA CTT CAA CTG CTA CGA CAG A	GAC GGG CAA CAA GAG AAC AC
sGnRH	CTG GTC ATA CGG TTG GCT TC	CAT CAG CAT CCA CTT CAT TCA C
cGnRH-II	TAC GAT TCC TCA GAG GTT TCA G	CAT CCA GCA CTA TTG TCT TCA G

2.2.4 Statistical analysis

Samples from both sexes of control and treated fish from one tissue were run at the same time. Therefore, the means for control and treated fish for both males and females were analysed using a two-way ANOVA. However, we were not able to compare the gene

expression from the hypothalamus and the telencephalon since they were not run at the same time.

2.3 Results

2.3.1 Effect of a GABA_B receptor agonist on LH levels

Baclofen, a GABA_B receptor agonist, significantly elevated LH levels in both male and female goldfish in June ($P < 0.05$). Baclofen increased the mean serum LH levels in male goldfish by 2.3-fold. The mean serum LH levels in female goldfish were increased 1.8-fold following the i.p. injection of baclofen (Figure 2.2). There were no significant differences between mean LH levels in males and females in June.

2.3.2 Effect of baclofen on gene expression in the telencephalon

Five hours following injection of baclofen, activin β A mRNA levels were significantly elevated in the telencephalon of male and female goldfish ($P < 0.05$). In males, activin β A mRNA levels were increased 4-fold in the telencephalon whereas the levels of activin β A mRNA expression were significantly increased 3.6-fold in females (Figure 2.3a).

Baclofen did not significantly affect the mRNA levels of activin β B, activin receptor IB, activin receptor type IIA, activin receptor type IIB, sGnRH or cGnRH-II in the telencephalon of either male or female goldfish (Figure 2.3b-g).

2.3.3 Effects of baclofen on gene expression in the hypothalamus

The mRNA levels of activin receptor type IB were significantly decreased 2-fold in the hypothalamus of female goldfish ($P < 0.05$). The levels of activin receptor type IB mRNA were not significantly changed in the hypothalamus in male goldfish (Figure 2.4c). The

mRNA levels of activin receptor type IIB were significantly decreased 2.6-fold in the hypothalamus of male goldfish ($P < 0.05$). There were no significant differences between the mRNA levels of activin receptor type IIB in the hypothalamus of control and baclofen-injected female goldfish (Figure 2.4e). Baclofen significantly increased the expression of sGnRH 2.9-fold in the hypothalamus of male goldfish ($P < 0.05$). The mRNA expression of sGnRH was increased similarly 2-fold in the hypothalamus of female goldfish, but this did not reach statistical significance (Figure 2.4f). The mRNA expression of cGnRH-II in the hypothalamus was significantly higher in control males than in control females ($P > 0.05$). However, following the i.p. injection of baclofen, the mRNA levels in males was reduced 14-fold compared to control to levels that were not significantly different from the mRNA levels found in female goldfish. There were no changes in the mRNA expression of cGnRH-II in females (Figure 2.4g).

Baclofen did not significantly affect the gene expression of activin subunit βA or βB in the hypothalamus of male or female goldfish. It also did not affect the gene expression of activin receptor type IIA in the hypothalamus in male or female goldfish (Figure 2.4a, b, d).

2.4 Discussion

One of the main objectives of this study was to examine the effects of a GABA_B receptor agonist on the activin signalling system in the neuroendocrine brain of goldfish. Others had previously reported that GABA stimulates the expression of the activin βA subunit through the GABA_B receptors, but not through the GABA_A receptors in sexually regressed goldfish (Martyniuk et al., 2007a). In that study, the stimulation occurs 6 hours post-injection in both the hypothalamus and in the telencephalon. For this reason, we repeated a similar experiment

with sexually mature goldfish, and examined the expression of the activin subunits in sexually mature goldfish 5-hours post-injection with the GABA_B receptor agonist.

We also investigated the expression of the three types of activin receptors to determine the effects of GABA on the activin signalling system. Finally, in order to relate this to the expression of other reproductive hormones, we assessed the expression of two forms of GnRH, sGnRH and cGnRH-II. Two previous studies have shown that GABA increases serum LH levels through both the GABA_A and the GABA_B receptor (Trudeau et al., 1993b; Martyniuk et al., 2007a). As expected, an i.p. injection of baclofen increased serum LH levels 2-fold after 5 hours in the sexually mature male and female goldfish as compared to control goldfish. This increase in LH release confirms the baclofen stimulation in the treated goldfish.

The expression of several of the actors in the activin signalling system was examined in the two neuroendocrine regions of the brain, namely the hypothalamus and the telencephalon. Interestingly, there was a differential regulation of the activin subunits, of the activin receptors and of the two forms of GnRH between the hypothalamus and the telencephalon. In the telencephalon, baclofen only changed the expression of the activin β A subunit. The 4-fold stimulation in activin β A subunit expression in male and female goldfish mirrors what was shown by Martyniuk et al. (2007a). In that study, baclofen caused a 4-fold increase in the expression of activin β A in both the telencephalon and the hypothalamus of sexually regressed female goldfish. This demonstrated a seasonal difference in the response of activin β A expression to baclofen injections. Prior work however, did not explicitly demonstrate the change in expression of the activin β A subunit in the brain over the course of the reproductive cycle. Our study together with the study by Martyniuk et al. (2007a) shows that there could be a difference in the expression of the activin subunits in each of the different reproductive

stages in goldfish, and this should be studied further, looking at the levels of activin β A over the course of the reproductive cycle. As in the study by Martyniuk et al (2007), there was no change in the expression of the activin β B subunit in either the hypothalamus or in the telencephalon following an i.p. injection of baclofen. As well, as will be discussed in chapter 3, we were unable to localise the activin β B subunit in the goldfish brain by immunohistochemistry. It is possible that the activin β B subunit is not found in the goldfish brain, and it is therefore likely that activin B and activin AB do not play an important role in regulating the reproductive axis within the brain. Activin B may play a much more important role within the gonads, where a specific role has been established for activin B in zebrafish (Ge, 2005).

Although baclofen only affected the expression of one gene (activin β A) studied in the telencephalon, it appeared that baclofen had significant effects on the expression of many genes in the hypothalamus. Baclofen caused a 2.6-fold decrease in activin receptor IIB expression in male goldfish, and a 2-fold decrease in the expression of activin receptor IB in female goldfish. Baclofen also caused 2.9-fold increase in the expression of sGnRH and a 14-fold decrease in the expression of cGnRH-II in male goldfish. If the goal of GABA is to increase activin signalling, we would have expected the expression of activin receptors to increase. However, the decrease in activin receptor expression contrasts with the strong increase in activin β A subunit expression. The expression of activin receptors could be decreased in response to the increase in activin β A subunit or sGnRH expression in order to maintain homeostasis.

Our study showed important differential regulation within different areas of the brain and between sexes. For example, the activin subunit expression is changed in the telencephalon

whereas the expression of the receptors and GnRH is changed in the hypothalamus. In this way, the modulation of the expression of subunits and receptors in the neuroendocrine brain appears similar to their expression in the gonads of goldfish. In the goldfish gonads, activin subunits are expressed exclusively in the follicle cells whereas the activin receptors and follistatin are only expressed in the oocytes. This observation on activin subunit and receptor localisation in the gonads leads to the hypothesis that activin acts as a paracrine factor in the gonads to transmit signals from the follicle cells to the oocytes (Ge, 2005). Therefore, a similar hypothesis could be made about activin's role within the brain. Activin A could be a neuroendocrine factor that transmits signals from the telencephalon to the hypothalamus. This is supported by the fact that the expression of both forms of GnRH is only changed in the hypothalamus.

The regulation of the two forms of GnRH provides insight into the roles of sGnRH and cGnRH-II in reproduction. GABA typically stimulates reproduction in goldfish. We would therefore expect the expression of GnRH to be stimulated by baclofen treatment. We therefore hypothesize that sGnRH is more involved in reproduction than cGnRH-II. A similar hypothesis had previously been proposed by Amano et al. (1991) concerning the roles of sGnRH and cGnRH-II in the masu salmon.

The sex differences that were observed are also very intriguing. The expression of activin receptor type IB is only inhibited in the hypothalamus of female goldfish, and the expression of activin receptor IIB is only inhibited in the hypothalamus of male goldfish. This type of sex difference in gene expression in the brain has not often been reported in goldfish. One study by Larivière et al. (2005) reported one such sex difference in the expression of GAD65 and GAD67 in the goldfish brain. The study investigated the expression of GAD65 and GAD67

over the course of the reproductive cycle and the effect of different sex steroids on the expression of GAD65 and GAD67. Progesterone stimulated the expression of both forms of GAD in females in May, when fish are sexually mature, but progesterone had no effect on their expression in males. This is an interesting comparison to our study as the sex differences in GAD expression also occurred in the hypothalamus of sexually mature goldfish. However, despite the difference in the regulation of activin receptor gene expression in males and females in our study, the same effect is obtained: a reduction in activin signalling. There is also a difference in the regulation of sGnRH and cGnRH-II between males and females since there is only a significant change in mRNA levels in the hypothalamus of male goldfish. Since it is in these fish that the expression of activin receptor IIB is changed, it is possible that the sex difference accounts for the difference in regulation of GnRH expression. However, there was a 2-fold increase in the expression of sGnRH in females. However, this result did not reach significance, possibly due to large interindividual variation. We therefore cannot draw robust conclusions on the effect of the sex difference. Additionally, serum LH levels were similarly increased in both males and females, indicating a stimulation of reproduction in both sexes. Therefore, the difference in the regulation of activin receptors between males and females likely reflects two different ways of achieving the same endpoint.

GABA and activin have many similar actions within the brain and pituitary in relation to stimulation of reproduction. This leads us to our hypothesis that activin is involved in the pathway by which GABA stimulates the release of GnRH. Activin and GABA both act to stimulate the release of gonadotropins from the pituitary. Activin A stimulates the release of FSH from the mammalian pituitary (Vale et al., 1986; Ling et al., 1986a). Activin A also stimulates the release of LH from goldfish pituitary cells (Ge et al., 1992). Unfortunately, due

to the lack of an assay specific to FSH, we cannot directly measure the effects of activin A on the release of FSH. An indirect measure of this however is to measure the mRNA expression of the FSH β subunit in the pituitary as this could reflect how FSH is released. GABA also stimulates the release of LH from the pituitary, and it does so directly. In our study, we have shown that GABA stimulates the release of LH through the GABA $_B$ receptors. We did not collect the pituitaries from the goldfish, and were therefore not able to test the effects of baclofen on the gene expression of LH β or FSH β subunits. Baclofen significantly elevated the serum LH levels from 1.7-fold in females to 2.3-fold in males, with no significant differences between the serum LH levels of males and females in either control or in treated fish. This effect of GABA on LH release has also been shown in other studies. GABA stimulates the release of LH in goldfish through both the GABA $_A$ and the GABA $_B$ receptors. A GABA $_A$ receptor agonist caused a 3-fold increase in serum LH levels in sexually regressed fish 6 hours post-injection (Martyniuk et al., 2007a). Since GABA $_B$ receptors also modulate the expression of the activin signalling system and activin also has similar effects to GABA in the pituitary, activin A could be working downstream of GABA to stimulate the release of LH.

There is further evidence for activin signalling downstream of GABA. GABA regulates the expression and release of GnRH in mammals and in fish. In mammalian studies it appears that activin is also a regulator of GnRH expression and release. GABA stimulates and inhibits the release of GnRH from an immortalised hypothalamic cell line. In GnRH neurons, GABA appears to stimulate the release of GnRH through the GABA $_A$ receptor, and inhibit the release of GnRH through the GABA $_B$ receptor (Favit et al., 1993). However, our study has shown that GABA stimulates the expression of sGnRH mRNA through the GABA $_B$ receptor in the hypothalamus of goldfish. The apparent difference between mammals and fish on the action

of the GABA_B receptor on GnRH could be due to differences in the species, or due to differences in the regulation of gene expression as opposed to the regulation of protein release.

Additionally, there is increasing evidence in mammals that activin stimulates the expression and release of GnRH from the mammalian hypothalamus. Immunohistochemical studies in the rat brain have shown that follistatin, the activin binding protein, has a similar distribution to GnRH, in some areas of the rat brain. The areas of similar distribution are the dorsal and ventral areas of the lateral nucleus. This similar distribution may indicate a possible interaction between GnRH and the activin signalling system (MacConell et al., 1996). A second study found that activin subunits are present in close proximity to GnRH fibres in the rat brain (MacConell et al., 1998). Activin A also stimulates and inhibits the expression of GnRH mRNA in a time-dependent manner in a GnRH cell line. The expression of GnRH is stimulated after a 6 hour exposure to activin A, inhibited after 12 hours of exposure, and once again stimulated after 48 hours of exposure. Activin modulates the expression of GnRH by modulating the activity of the GnRH reporter gene in the neuronal cell line (MacConell et al., 1999). This multiphasic response to activin A could possibly also partially explain how GABA can both stimulate and inhibit the expression of GnRH. In addition to stimulating the expression of GnRH from the GnRH cell line, activin A stimulated the secretion of GnRH protein from the GnRH cell line as well as from rat hypothalamic explants. This stimulation is inhibited by both inhibin and testosterone (MacConell et al., 1999; Calogero et al., 1998). However, the actions of activin on GnRH have not yet been shown in fish. Our study does show that the expression of sGnRH is stimulated in the hypothalamus, which is the same area in which the expression of activin receptors is affected.

Therefore, this data would support our hypothesis that activin A acts as a neuroendocrine factor in the brain by transmitting signals from the telencephalon to the hypothalamus. Activin A could be regulating the expression of sGnRH by transmitting the GABA signal from the telencephalon to the hypothalamus.

The GABA and activin signalling pathways are both regulated by some of the same factors, providing further support to our hypothesis that activins work downstream of GABA. Some of the factors that regulate GABA are sex steroids that are released from the gonads. Prior work has shown that testosterone and progesterone decrease the rate of GABA synthesis whereas E₂ increases the rate of GABA synthesis in sexually regressed goldfish (Trudeau et al., 1993b). Sex steroids also modulate the levels of GAD proteins over the course of the reproductive cycle of goldfish. In May, when the goldfish are sexually mature, progesterone decreases GAD65 and GAD67 expression in the hypothalamus of females, and testosterone and progesterone both decrease GAD65 and GAD67 expression levels in the telencephalon of males (Larivière et al., 2005). This decrease in GAD expression will decrease the rate of GABA synthesis which will ultimately affect LH release. The interaction between activin A and sex steroids has not been studied as extensively as the interaction between sex steroids and other hormones and neurotransmitters of reproductive axis, but a study by Calogero et al. (1998) found that testosterone suppressed the activin A-stimulated GnRH release from rat hypothalamic explants, indicating that testosterone modulates the action of activin in the brain, and this could also be true for the other sex steroids, and merits further research.

Another factor that regulates both the GABA signalling system and the activin signalling system is DA, the major inhibitory neurotransmitter on the release of gonadotropin hormones from the brain. DA acts on many different hormones and neurotransmitters to inhibit the

release of LH, including GABA and activin A. DA inhibits the GABA_A receptor-stimulated release of LH from the pituitary (Trudeau et al., 1993b). In addition, DA also inhibits GABA neuronal activity (Trudeau et al., 2000). Recently, it was also shown that DA inhibits the expression of activin β A subunits in goldfish. A 5 hour exposure to an i.p. injection of a DA D1 receptor antagonist increased the mRNA expression of activin β A subunits in the hypothalamus and in the telencephalon, 10-fold and 4-fold, respectively. Similar results were obtained when the D1 receptor antagonist was combined with AMPA, which specifically activates the AMPA receptor, a glutamate receptor. A D2 receptor antagonist had no effect on the expression of the activin β A subunit, and neither antagonist had any effect on the expression of the activin β B subunit (Popesku, 2009). This reinforces the hypothesis that activin A signals downstream from GABA in the neuroendocrine brain. It also provides further evidence that activin AB and activin B might not be involved in the regulation of the reproductive axis in the brain.

In conclusion, the activin signalling system in the brain of goldfish is affected by GABA, and this GABA regulation is sex-dependent, season-dependent, and location-dependent in the neuroendocrine brain. The expression of the activin β A subunit is stimulated by GABA whereas the expression of the activin receptors is inhibited by GABA, and this inhibition could be a result of negative feedback from an increase in activin β A or from an increase in sGnRH. While the expression of the activin β A subunit is stimulated in the telencephalon, the expressions of activin receptors and cGnRH-II are inhibited in the hypothalamus and the expression of sGnRH is stimulated in the hypothalamus. This provides an indication that activin A is a neuroendocrine factor that transmits signals from the telencephalon to the hypothalamus. Furthermore activin is likely to be acting downstream of GABA to regulate the

expression of GnRH in the hypothalamus, as is seen in mammals. If this is true in fish, we would expect that activin receptors would be in close proximity to GnRH cells or fibres in the goldfish brain, as is noted in the mammalian brain. The location of activin A protein and activin receptors in the goldfish brain is unknown. If the location is shown this could further elucidate the potential interaction between activin A and GnRH.

Figure 2.1 Gonadosomatic index for control (white bars) and baclofen (black bars) treated male and female goldfish. Gonadosomatic index is obtained from the percentage of gonad weight over body weight.

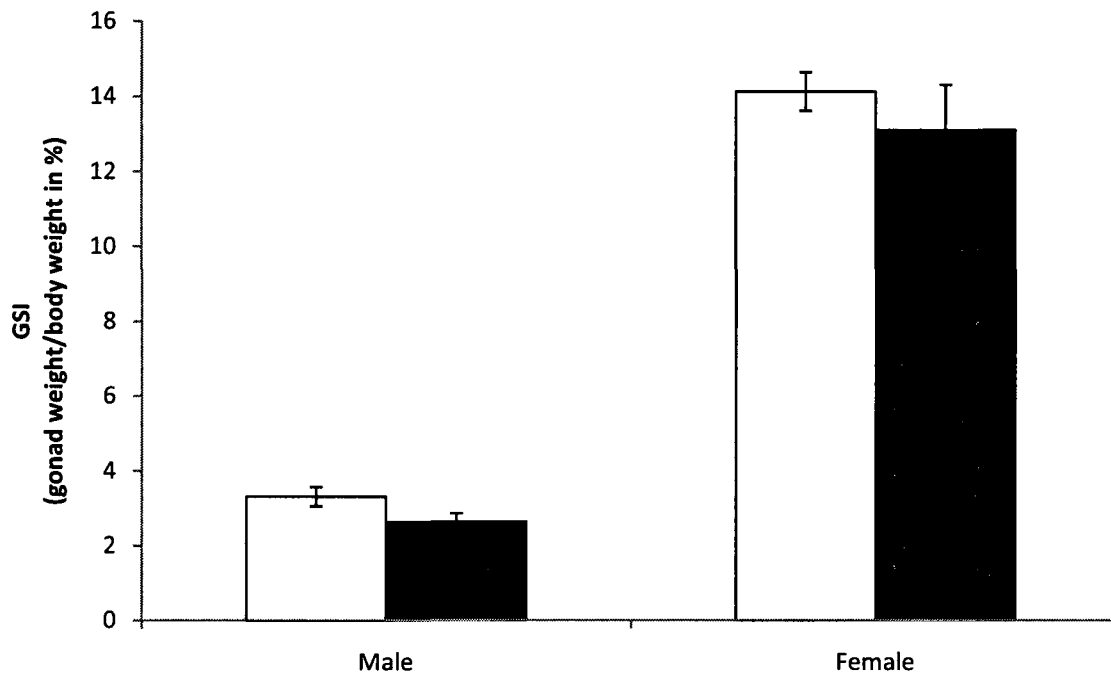


Figure 2.2. Serum LH levels in goldfish after single i.p. injections of baclofen. Mean (\pm SEM) serum LH levels are presented for males and females (n=13-19). Control and treated LH levels were compared by unpaired t-test, significant differences between control (white bars) and treatment (black bars) are marked with an asterisk (P<0.05).

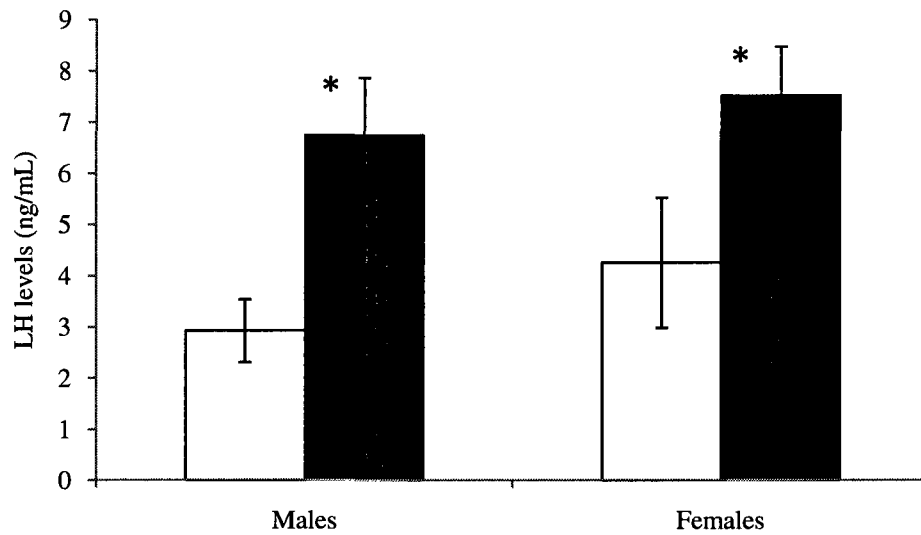
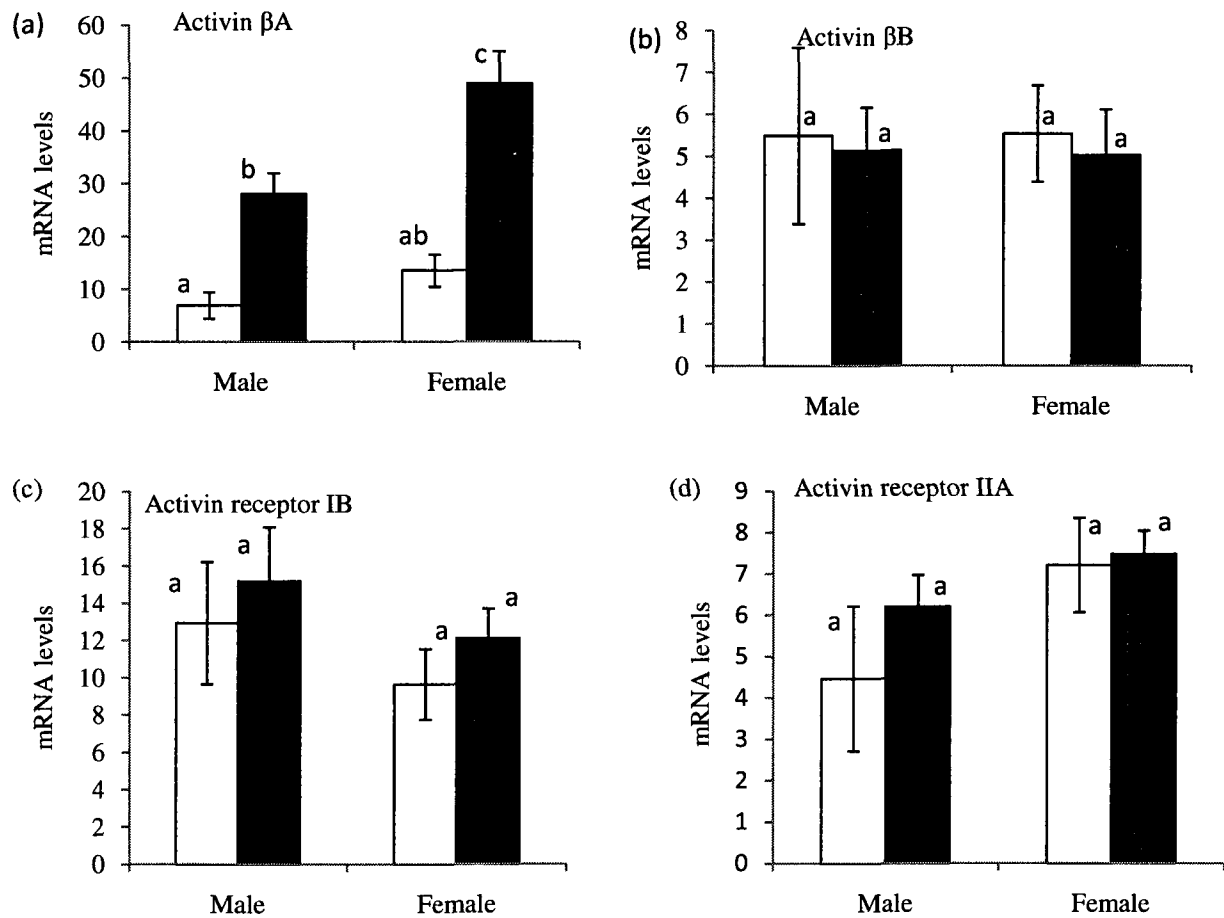


Figure 2.3. mRNA levels of the activin subunits β A and β B, activin receptors types IB, IIA, and IIB, sGnRH and cGnRH-II in the telencephalon. Controls (white) and treatment (black) are shown for males and females in the telencephalon. Control and treated animals and males and females were compared by a two-way ANOVA, significant results between control and treatment are denoted by different letters (a, b or c) ($P < 0.05$).



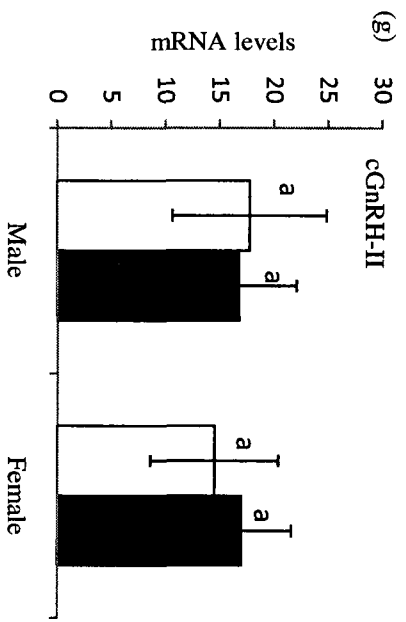
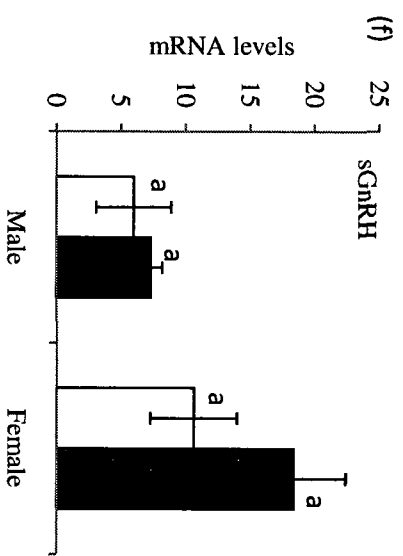
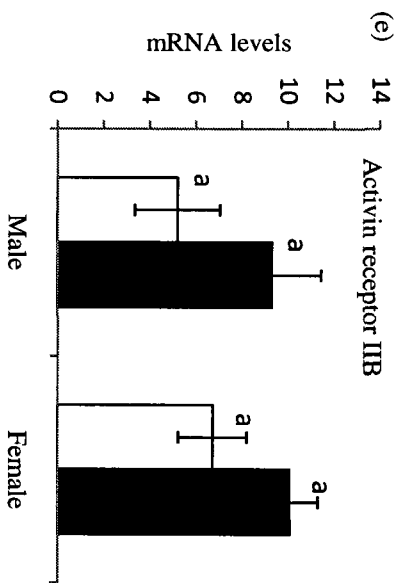
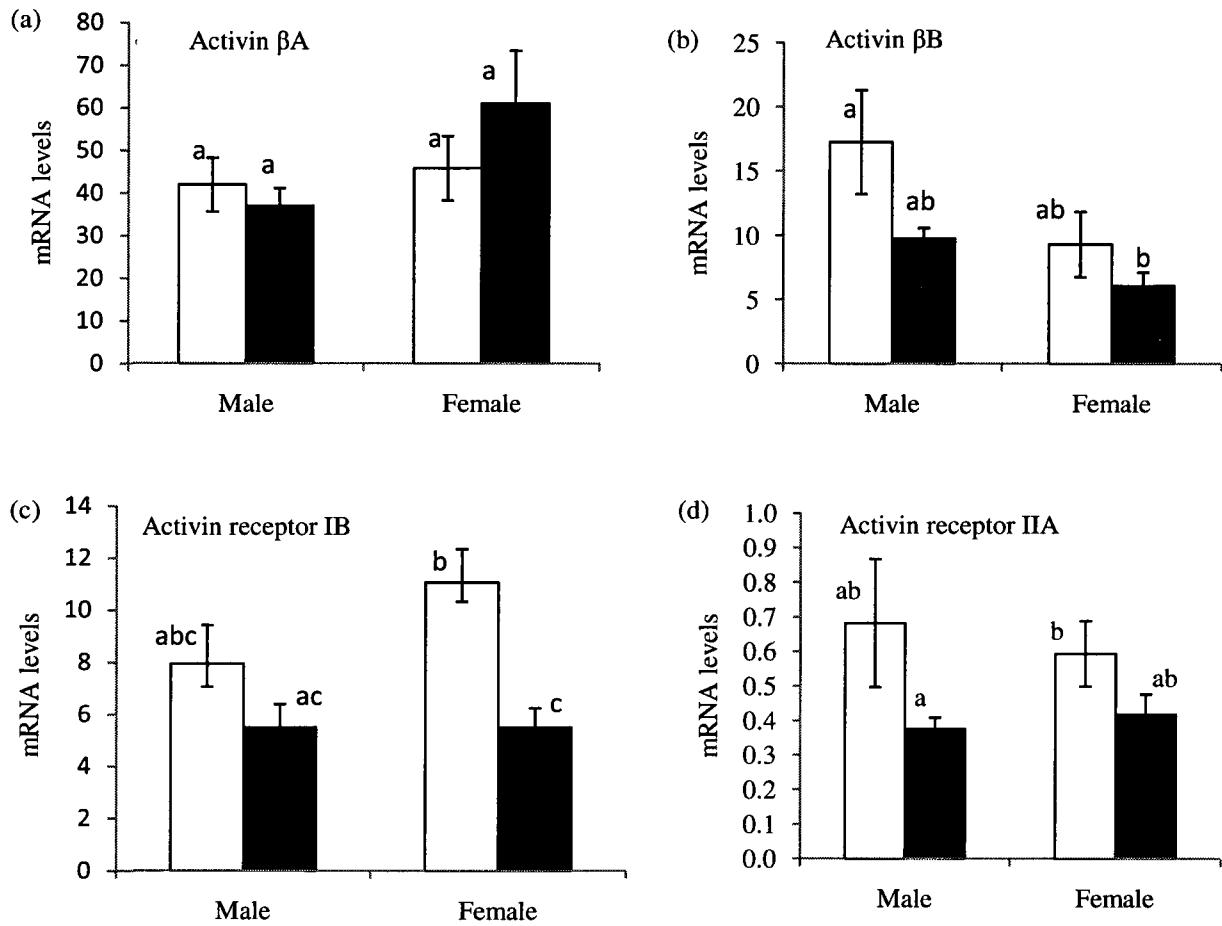
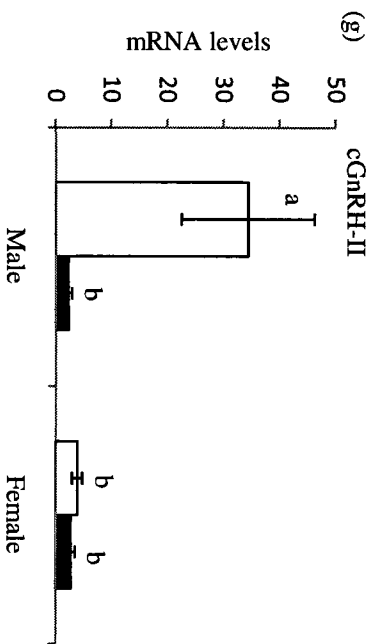
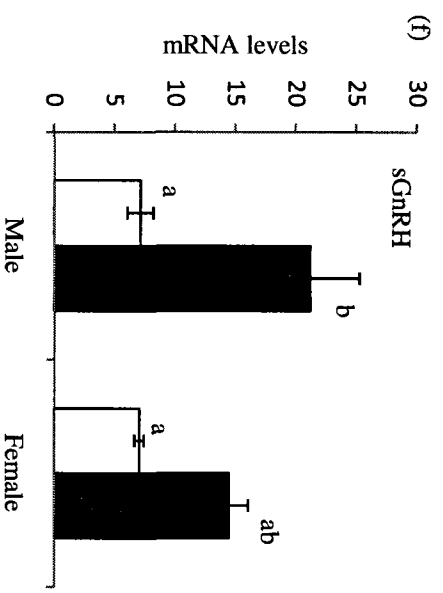
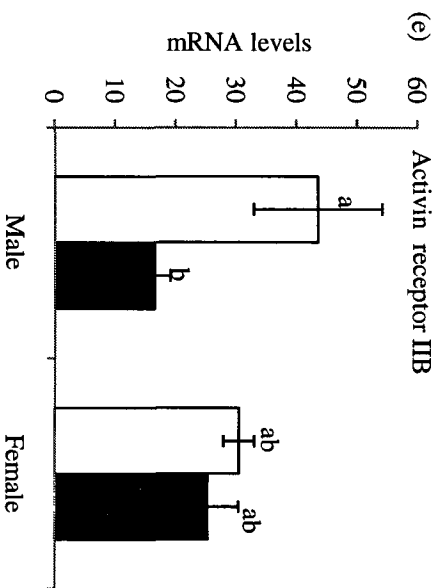


Figure 2.4. mRNA levels of the activin subunits β A and β B, activin receptors types IB, IIA, and IIB, sGnRH and cGnRH-II in the hypothalamus. Controls (white) and treatment (black) are shown for males and females in the hypothalamus. Control and treated animals and males and females were compared by a two-way ANOVA, significant results between control and treatment are denoted by different letters (a, b or c) ($P < 0.05$).





Chapter 3

Localisation of activin subunits in the brain and possible interaction of activin and GnRH

3.1. Introduction

One important finding resulting from studies on mammals and cell lines is that activin A and GnRH interact with each other in various ways within the brain. Activin A and GnRH interact at the level of the hypothalamus where activin A stimulates the release of GnRH (Calogero et al., 1998; MacConell et al., 1999). The first indication that activin can play a role in regulating reproductive hormones in the brain was from immunohistochemical studies indicating that follistatin, the activin binding protein, is found in the olfactory regions, neocortex, septum, basal ganglia, amygdala, thalamus, hypothalamus, and brainstem. Another protein that is also known to be localised in the septal area in the rat brain is GnRH (MacConell et al., 1996), implicating activin as a possible mediator of GnRH activity in this area. Colocalisation experiments have shown that neurons expressing follistatin protein also express GnRH protein (MacConell et al., 1998). This is a strong indication that there is a likely interaction between the activin signalling system and GnRH (MacConell et al., 1996; 1998). Activin β A and β B subunits in the rat brain are localised in the medial septum, the horizontal limb of the diagonal band, and the medial preoptic area where GnRH neurons are known to be localised. Although they do not appear to be localised in the same cells, activin β A subunits are found in close association to cells expressing GnRH mRNA. The GnRH cell bodies are surrounded by fibres containing activin β A subunits (MacConell et al., 1998). Subsequent studies have shown that activin A stimulates the expression and the release of

GnRH from rat hypothalamic explants and from a GnRH neuronal cell line (Calogero et al., 1998, MacConell et al., 1999).

There are two forms of GnRH that are found in the brain of goldfish, sGnRH and cGnRH-II. Both forms of GnRH are found in the same areas in the goldfish brain. Cells containing sGnRH and cGnRH-II are found in the terminal nerve ganglion, in the medial olfactory tract, in the ventral telencephalon, in the preoptic nucleus and in the ventrolateral hypothalamus. sGnRH was generally more abundant than cGnRH-II (Kim et al., 1995). Fibres that contain sGnRH and cGnRH-II are found throughout the brain.

Although the mRNA of activin subunits is known to be expressed in the brain of goldfish, the localisation of the activin mRNA or of the activin proteins has never been studied. Activin subunits have only been localised in the brain of one fish, the thin lipped grey mullet, *Liza ramada*. The study by Mousa and Mousa (2005) looked at the location of the activin β A subunit in the brain of the grey mullet. The activin β A subunits are localised in cells in the telencephalon, in the midbrain tegmentum, and in the cerebellum in the brain of the grey mullet. No information however is known about which nuclei these cells are located in for each of the different brain parts or where these cells project to within the brain. However, since we know that GnRH cells are present in the preoptic area of the telencephalon in fish, there is a possibility that cells or fibres containing activin subunits which were found in the telencephalon are located in or around the preoptic area. This would indicate a possible interaction between activin and GnRH in the telencephalon. In the studies that have colocalised the activin signalling system and GnRH in the rat brain, only follistatin and activin β A subunits were localised with GnRH in the brain (MacConell et al., 1996; 1998). The colocalisation of activin receptors and GnRH in the goldfish has never been reported

before in the literature. This would seem to provide direct evidence that activins can regulate the expression or release of GnRH.

Many parallels exist between the regulation of activin A in mammals and non-mammalian vertebrates in the pituitary and in the gonads. We therefore hypothesize that the regulation of GnRH by activin A in the brain will also exist in fish. Thus, activin receptors should be localised with GnRH cells in the brain. For this reason, we used immunohistochemistry to localise activin β A subunits and activin receptors type II (ActRII) in the neuroendocrine brain of goldfish. We also performed fluorescent double-labelling experiments to determine if ActRII and GnRH were located in similar areas in the neuroendocrine brain of goldfish.

3.2. Materials and methods

3.2.1. Animal and tissue preparation

Goldfish were purchased from a commercial supplier (Aleong's International Inc. Mississauga, ON, Canada) in June and September 2009. They were allowed to acclimatize at $18 \pm 1^\circ\text{C}$ water on a simulated natural photoperiod for two weeks. They were fed standard fish pellet food (Martin Mills, Elmira, ON, Canada). Fish were anaesthetized with MS222 (Tricaine methanesulfonate; Sigma Aldrich, St-Louis, MN, USA) before all handling procedures.

Fish were first anaesthetized with MS222, opened ventrally to expose the heart and then perfused through the heart with 0.6% saline until the gills were completely cleared of blood. This provided an indication that the brain was also cleared of blood. Following this, the fish were perfused with Bouin's fixative. The fish were then sacrificed by spinal section, and the

brain, pituitary, and gonads were removed. The pituitaries were left in Bouin's fixative for 1 hour and the brain and the gonads were left in Bouin's fixative overnight. Following this, the brain, gonads and pituitaries were transferred to two or three changes of 70% ethanol and left in the refrigerator overnight. Through a series of graded ethanol changes, the tissues were dehydrated, and subsequently embedded in paraffin (Stobl-Mazzulla et al., 1991). Sections of 4-5 μm were cut using the microtome and placed on Fisherbrand Superfrost slides (Fisher Scientific, Ottawa, ON, Canada). Three male and six female sexually regressed fish were used for analysis (August to October). The gonadosomatic index (GSI) of each fish was measured, and the average GSI for both male and female fish was 3.34%.

3.2.2. Antibodies

Rabbit antisera against human anti-cyclic activin/inhibin βA (81-113)- NH_2 was kindly provided by Drs W Vale and J Vaughan (The Salk Institute, La Jolla, California). This antibody was previously validated in goldfish ovaries by Ge et al. (1993b) and in goldfish pituitary by Ge and Peter (1994). An affinity purified antibody to actRII, rabbit anti-TyrGly mActRII (482-494) (Code #334-118-ET) was also kindly provided by Drs W Vale and J Vaughan (The Salk Institute, La Jolla, California). Activin βA subunit and actRII were both sequenced and it was found that the epitope against which the antibodies were designed was highly conserved between mammals and goldfish. A mouse anti-mammalian luteinizing hormone releasing hormone (LHRH) antibody was obtained from Dr Matozaki (Gunma University, Maebashi, Gunma, Japan). This LHRH antibody recognises both sGnRH and cGnRH-II forms (Murakami et al., 1991). Neuron specific enolase (NSE) (Dako Scientific, Mississauga, ON, Canada) was used to identify neurons within the goldfish brain. Alexa Fluor 488 goat anti-rabbit IgG antibody and Alexa Fluor 594 goat anti-mouse IgG antibody

(Invitrogen, Burlington, ON, Canada) were used as secondary antibodies for the double labelling experiment.

3.2.3. Immunohistochemistry

Sections were deparaffinised and hydrated by graded alcohol changes. Tissues were incubated for 45 minutes with 0.3% H₂O₂ in water to deactivate any endogenous peroxidase activity. They were then washed in two changes of phosphate buffered saline (PBS) for 10 minutes each. After 30 minutes in a blocking solution of 5% milk in PBS 1X with 0.3% Triton 100X, the sections were incubated overnight in a humidified chamber with primary antibody. The antibody for activin β A was diluted at 1:750 with the blocking solution. The antibody for actRII was diluted at 1:1000 with the blocking solution. The antibody for NSE was diluted at 1:100 with the blocking solution. Negative controls were performed by omitting the primary antibody. As a positive control, pituitary sections were used, as activin localisation has previously been reported in these tissues (Ge and Peter, 1994). The following day, sections were rinsed and immunostaining was performed with the LSAB2 System-HRP (Dako Scientific, Mississauga, ON, Canada) based on the biotin-streptavidin-peroxidase complex. Briefly the sections were incubated with the biotinylated-secondary antibody for 35 minutes at room temperature, then were washed (two 10 minutes changes) with PBS and subsequently incubated with streptavidin-HRP for 30 minutes at room temperature. After another wash in PBS 1X, the tissues were incubated in a solution of 0.05% 3-3'-diaminobenzidine (DAB) (Sigma), and 0.01% H₂O₂. The tissues were then washed with water, incubated in hematoxylin for 30 seconds, washed in water, and incubated for 1 second in an alcohol-acid solution. After another wash in water, the tissues were dehydrated and mounted using Canada Balsam (Fisher Scientific, Ottawa, ON, Canada) for microscopic

observation. Anatomical localization in the forebrain was determined by comparing with the goldfish brain atlas (Peter and Gill, 1975). The nomenclature was based on Peter and Gill (1975) and Braford and Northcutt (1983) as previously reported by Canosa et al. (2004). Protein was considered to be localised in a specific section if it was found in the same area in the brain of 4 different goldfish.

3.2.4. Double labelling immunohistochemistry

Co-localisation of actRII and GnRH was performed. The tissue sections were deparaffinised and hydrated. After 30 minutes in a blocking solution of 5% milk and 0.1% Triton X100, the slides were incubated overnight in a humidified chamber with the primary antibodies. The antibody for actRII was used at a concentration of 1:250, and the antibody for GnRH was used at a concentration of 1:500. As a negative control, the primary antibody was omitted. After a wash in PBS, the sections were incubated in a humidified chamber at 37°C with fluorescent secondary antibody for 2 hours. The secondary antibodies were both used at a concentration of 1:100. After this, the sections were washed and dehydrated, and finally mounted using Vectastain (Vector Laboratories, Burlington, ON, Canada) for microscopic observation. Anatomical localization in the forebrain was determined by comparing with the goldfish brain atlas (Peter and Gill, 1975). The nomenclature was based on Peter and Gill (1975) and Braford and Northcutt (1983) as previously reported by Canosa et al. (2004). Protein was considered to be localised in a specific section if it was found in the same area in the brain of 4 different goldfish.

3.3. Results

3.3.1. Activin β A subunit location in the brain

For our positive controls, the activin β A subunit was localised in cell bodies in the proximal pars distalis (PPD) of the pituitary, as was reported in Ge and Peter (1994) (Figure 3.2).

The location of activin β A and β B subunits within brain nuclei has never been identified in goldfish. In this study, we have determined in which cells and fibres the activin β A subunits are localised within the forebrain. We also attempted to localise the activin β B subunits, but the staining for these subunits was very weak and we were therefore not able to determine their location.

Activin β A subunits were found in cell bodies in the olfactory bulb (Fig 3.3A), in the telencephalon, in the thalamus, in the optic tectum, and in the hypothalamus of the forebrain of goldfish (Figure 3.1). In the telencephalon, cell bodies were located in the *nucleus tenia* (NT), in the *area ventralis telencephali pars ventralis* (Vv) (Fig 3.3C), and in the *nucleus preopticus* (NPO) (Fig 3.3B). In the thalamus, the cell bodies containing activin β A subunits were found in the *nucleus dorsolateralis thalami* (NDL), in the *nucleus dorsomedialis thalami* (NDM) (Fig 3.3D), in the *nucleus ventromedialis* (NVM) (Fig 3.3G), in the *nucleus recessus lateralis* (NRL) (Fig 3.3E-F), and in the *nucleus diffusus lobi inferioris* (NDLI). Cells containing activin β A subunits were also found in the optic tectum (OTec) (Fig 3.3I) in areas that bordered the cerebellum. In the midbrain tegmentum, activin β A subunits were localised in cell bodies in the medial longitudinal fasciculus (mLF) (Fig 3.3H). Certain sections were stained with NSE to determine where neurons were localised in the brain. This staining

revealed that neurons of the NDM stained for NSE (Fig 3.3J), which indicated that the activin β A in the NDM are in neurons.

Activin β A subunits were found in fibres throughout the forebrain (Fig 3.1). The fibres were found in the olfactory bulb, in the olfactory tract, in the optic tract (OT), in the Vv, in the anterior commissure (AC) (Fig 3.3L), in the *nucleus preopticus periventricularis* (NPP), in the *nucleus entopeduncularis* (NE) (Fig 3.3K), in the NDLI, in the *nucleus diffusus tori lateralis* (NDTL) (Fig 3.3N), in the *nucleus preglomerulosus pars anterioris* (NPGa) (Fig 3.3M), in the NRL, and in the cerebellum.

3.3.2. ActRII localisation in the goldfish brain

ActRII was also localised in the goldfish brain by immunohistochemistry (Fig 3.4). Cell bodies immunoreactive for actRII were localised in the olfactory bulb (Fig 3.5A), in the Vv (Fig 3.5B), in the AC (Fig 3.5C), and in the NDM (Fig 3.5D) and NDL. There were indications that actRII were found in the NDLI of the hypothalamus, but this data was not conclusive.

Fibres containing actRII were found in many different areas of the brain (Fig 3.4). There were fibres in the olfactory bulb, in the olfactory tract (OIT), in the *area dorsalis telencephali medialis* (Dm) (Fig 3.5G), in the Vv (Fig 3.5H), in the *area ventralis telencephali dorsalis* (Vd) (Fig 3.5E), in the NPO, in the AC (Fig 3.5I), in the NDL, in the NRL, and in the OTec (Fig 3.5F).

3.3.3. Double labelling of actRII and GnRH

We used fluorescent immunohistochemistry to determine if actRII was localised in close proximity to GnRH protein in the brain and in the pituitary. In all stained sections, actRII

staining was moderate, with high background, and was considered positive if it was above background, as compared to negative control slides.

In the pituitary, the actRII and GnRH proteins were localised in similar areas throughout the pituitary. The GnRH fibres overlapped with actRII fibres in the neurointermediate lobe (NIL) (Fig 3.7C), but in the nearby PPD, the GnRH fibres were not near any ActRII staining (Fig 3.7A). However, in other areas of the NIL, GnRH fibres that did not overlap with actRII were in close association with cell bodies that appeared to contain actRII (Fig 3.7B).

ActRII containing cell bodies were abundant throughout the olfactory bulb, but no GnRH containing cell bodies were found in the olfactory bulb. However, in the fibres that were exiting the olfactory bulb, GnRH fibres were found to be interspersed with the actRII fibres (Fig 3.6A).

It was more difficult to localise actRII and GnRH in the brain as the actRII fibres and cells were much scarcer than they were in the pituitary. There were two areas in the telencephalon that showed actRII and GnRH to be in close proximity. In the Vv, there were many cell bodies containing actRII, and GnRH fibres were found in that same area (Fig 3.6B). In the NPP, cell bodies containing GnRH were found with actRII fibres in close proximity (Fig 3.6C). However, in the NPO, where the largest amount of GnRH cell bodies was found, there was no evidence of actRII near these cells (Fig 3.6D).

In the hypothalamus, fibres that stained with actRII antibody and fibres that stained with the GnRH antibody were interspersed amongst each other in the NRL (Fig 3.6F). The fibres that stained positively for actRII were more abundant than those that stained for GnRH. There

were certain fibres that stained for both actRII and GnRH protein, indicating that both proteins were present on that particular fibre.

3.4. Discussion

For the first time, we have localised the activin β A subunits and the actRII in the goldfish neuroendocrine brain. The activin β A subunits were localised in cell bodies and fibres in the olfactory bulb, telencephalon, thalamus, hypothalamus, midbrain tegmentum, optic tectum, and cerebellum. Although we saw clear staining with the activin β A subunit antibody in the brain, the staining for the activin β B subunit was very weak throughout the brain. The amount of staining was too weak to be able to make any conclusive observations about the localisation of the activin β B subunits. The study by Mousa and Mousa (2003) also attempted to localise the activin β B subunit in the brain of *Liza ramada*, but noted that there was staining for the activin β B subunit was absent. A study by Ge and Peter (1994) on the localisation of the activin and inhibin subunits in the pituitary of goldfish also reported that the staining for the activin β B subunit in the goldfish pituitary was weaker than the staining for the activin β A subunit. This data appears to indicate that there are relatively few activin β B subunits present within the brain and pituitary of goldfish, and that therefore activin B and activin AB may not be important for the control of reproduction in the brain, and may have a more important role in other reproductive organs such as the gonads. In human adult testes, the staining for the activin β B subunit was much stronger than the staining for the activin β A subunit. This provides evidence to the fact that activin B has a bigger role in the gonads than in the brain or in the pituitary. It is also possible that the β B antibody has a low affinity for the goldfish protein, and thus staining is weak. In this case, the levels of activin β B might be higher than originally thought. However, the real time RT-PCR data which show low

expression levels of activin β B genes. It would therefore seem that activin B does not in fact have an important role in reproduction in the goldfish brain.

The location of activin β A subunit cells and fibres in the goldfish brain poses an interesting question. The most anterior activin β A-containing cells in the telencephalon are in the VI, Vv, and NPO. However, there are activin β A fibres more anterior to this in the Vv and in the OIT. It is therefore likely that these fibres originate from the cells that are located in the olfactory bulb or from the cells in the Vv in fibres that project anteriorly. However, this hypothesis will require confirmation using tract-tracing methods. Additionally, there were many activin β A subunits that were localised in non-neuroendocrine regions of the brain, including in the midbrain tegmentum and the thalamus. Activin has been shown to have many non-reproductive functions like in tissue repair (Trudeau et al., 1997, Tretter et al., 1996) and in behaviour (Torii et al., 1993), and this is potentially the function of activin present in these regions.

The location of actRII was very similar to the location of the activin β A subunit in the goldfish brain. Both the activin β A subunit and actRII were localised in cells in the olfactory bulb, the Vv, the NPO and the AC of the telencephalon, in the NDM and NDL of the thalamus, and in the NRL of the hypothalamus. This is expected as the activin protein would need to be in the same area as its receptor to transmit signal. However, there were many areas where the activin β A subunit was localised but actRII was not, such as in the cerebellum and the NDTL. This could be because the activin receptors in these areas are in a concentration that is too low for detection. Interestingly, the areas in which activin β A and actRII were both localised are areas that are relevant to our hypothesis. For instance, they were both found in the NPO in the telencephalon, and in the NRL in the hypothalamus. It is also in the NRL in

the hypothalamus that actRII and GnRH fibres were co-localised. Localising activin, its receptor and GnRH in the same nucleus provides evidence for our hypothesis that activin may stimulate the release of GnRH. There were indications that cell bodies and fibers containing actRII were localised in the NDLI, which is a nucleus in which activin β A subunits were found, but these results were not conclusive as they were only found in 2 of the brains tested, without conclusive staining in the others. We were not able to obtain an antibody to the activin receptor type IB. However, the two types of activin receptors must work together in order to transmit activin's signal, therefore activin receptor type IB must be localised in the same areas as actRII.

Only one other attempt has been made to localise the activin β A subunits in the brain of fish. The activin β A subunits were localised in cells in the telencephalon, midbrain tegmentum and cerebellum in the thin lipped grey mullet, *Liza ramada* (Mousa and Mousa, 2003). This is therefore similar to the results that we found. In goldfish, activin β A cells were also found in the telencephalon and in the midbrain tegmentum in goldfish. However, the cerebellum of the thin lipped grey mullet contained activin β A cells, whereas the cerebellum of the goldfish contained activin β A fibres and no cells. There were no indications as to where the fibres containing activin β A subunits were localised, or in which nuclei the cells were localised in the grey mullet. Our study therefore was intended to be more complete. The activin β A subunits were found in cells in many more areas in the goldfish brain than in the thin lipped grey mullet like in the olfactory bulb, the thalamus, the hypothalamus, and the optic tectum.

Many studies have been done on the localisation of activin β A subunits in the mammalian brain. A study on GnRH cells that originated in the human olfactory epithelium

indicated that activin β A mRNA is synthesized in these cells (Florio et al, 2000). Multiple other studies have been done to localise the activin β A subunits and follistatin in the rat brain. Activin β A subunits were reported to be localised in fibres that projected from the brainstem to the hypothalamus in the rat brain. Fibres containing activin β A subunits were localised throughout the hypothalamus in rats in nuclei that included the preoptic area (MacConell et al, 1998). The preoptic nucleus is found in the telencephalon in goldfish, and activin β A fibres and cells were located in and around the NPO in the goldfish brain. This is where many GnRH neurons are also localised in the goldfish brain (Fig 3.6D). This indicates a possible interaction between activin A and GnRH within the preoptic area in goldfish.

To examine whether there is a possibility of an interaction between activin A and GnRH within the brain, we repeated a similar experiment to the one done by MacConell et al. (1998) in which the activin β A subunit and follistatin were co-localised with GnRH in the rat brain. In our study, we examined a possible co-localisation of actRII and GnRH. This type of co-localisation should give us an indication of where activin is directly acting on GnRH. ActRII was found to be co-localised with GnRH proteins only in specific areas of the goldfish brain. In the fibres leaving the olfactory bulb, there were many fibres containing actRII, mixed with a few GnRH fibres. The fibres that stained for actRII were much more abundant than the fibres staining for GnRH. Interestingly, actRII cell bodies were abundant in the olfactory bulb but there were very few GnRH cells and they were not near the cells that stained for actRII. Therefore, activin is likely not stimulating the release or expression of GnRH in the olfactory bulb. In the NPP of the telencephalon, fibres containing actRII were found near cells containing GnRH proteins. Since actRII are located near the GnRH cells, activin could be stimulating the expression of GnRH mRNA or it could be stimulating the release of GnRH

protein from the cells. Another area which showed co-localisation of actRII fibres and GnRH fibres was the NRL of the hypothalamus. In this area, the fibres were scattered amongst each other, and some GnRH fibres overlapped the actRII fibres. As was observed in the olfactory bulb, the actRII fibres were more abundant than the GnRH fibres. It is possible that in this area, actRII is located on or near the GnRH fibres and activin could therefore stimulate the release of GnRH from the fibres. No GnRH cell bodies were found in that area of the brain, therefore it is possible that activin only stimulates the release of GnRH, and not its expression.

The study by MacConell et al. (1998) that examined the co-localisation of activin β A subunits and GnRH as well as the co-localisation of follistatin and GnRH focussed on their localisation within the hypothalamus of rats. They showed activin A fibres and GnRH fibres were often in the same area and crossed each other in the hypothalamus. These results indicate that the activin signalling system interacts with GnRH signalling in the rat brain. This signalling could either be activin A stimulating the expression and release of GnRH or GnRH stimulating the expression and release of activin A. Studies have been done to test both of these potential interactions in mammals. In rats, activin A stimulates the mRNA expression and release of GnRH from a neuronal cell line and from hypothalamic extracts in a time- and dose-dependent manner (MacConell et al., 1999; Calogero et al., 1999). Activin A changes the activity of the GnRH reporter in the cell line (MacConell et al., 1999). Since our study shows the co-localisation of actRII and GnRH proteins, activin A could also stimulate the expression and release of GnRH from the goldfish neuroendocrine brain in the areas where co-localisation was observed. Evidence also exists for GnRH-stimulated activin A release in human olfactory epithelium (Florio et al., 2000). Our study does not provide any evidence for this since we did not attempt to co-localise the activin β A subunit and GnRH. However,

activin β A subunits were found in the NPO and in the NPP where GnRH cell bodies are localised, as well as both activin β A subunits and GnRH proteins being localised in the olfactory bulb. This indicates that a similar interaction to that which was found in human olfactory epithelium is possible within the goldfish brain. Further experiments would need to be done to test this further.

Activin β A subunits were previously reported in the pituitary of goldfish by Ge and Peter (1994). We observed staining in the same areas of the pituitary, confirming the specificity of the immunoreaction. The pituitaries were examined for the double staining experiments to determine if activin A also stimulated the release of GnRH within the pituitary. There were many clusters of fibres in the NIL which showed staining for both actRII and GnRH. It would appear that activin could interact with GnRH within these fibres. GnRH fibres were also located in areas that showed no actRII staining such as in the PPD. This could indicate that perhaps activin and GnRH only interact in specific parts of the pituitary and not in others. However, it is also possible that the actRII were not present in a high concentration, and therefore were below the detection limit of the immunohistochemistry. There is an indication in the mouse L β T2 pituitary cell line and in a mouse pituitary cell culture that there is an interaction between activin and GnRH in the pituitary. Follistatin partially inhibits the GnRH-stimulated FSH β and LH β subunits expression implicating activin in the GnRH stimulation of gonadotropin subunit expression in the pituitary (Persanetti et al., 2001). A hypothesis has been postulated that activins increase the mRNA and protein expression of GnRH receptors within the pituitary (Huang et al., 2001). This interaction between activin and GnRH in the pituitary would be interesting to examine in goldfish as well. Since activin appears to be working downstream of GnRH in the pituitary, the co-localisation of actRII and GnRH would

not demonstrate this. A co-localisation of activin and GnRH or activin and GnRH receptors within the pituitary would more accurately show whether GnRH stimulates the release of activin A or if activin stimulates the expression of GnRH proteins. This could be why the only co-localisation of GnRH and actRII that was observed was in fibres in the NIL, and not in cell bodies.

Activin β A subunits were localised in specific nuclei in the telencephalon, thalamus and hypothalamus in the goldfish brain. Studies on the localisation of other hormones and neurotransmitters provide insight into other systems that activin could be interacting with. Many other hormones and enzymes from the reproductive axis are located in the same areas as activin in the brain, such as GAD mRNA, GABA-T mRNA, nitric oxide synthase (NOS) and kisspeptin genes mRNA. NOS is another enzyme involved in the reproductive axis that is also located in the same nuclei as activin and actRII. NOS synthesizes nitric oxide (NO) which controls reproductive behaviour such as copulation, mount, intromission and ejaculation in mice (Ma et al., 2005). Though this role for NOS has never been shown in fish, NOS also appears to regulate the biosynthesis of GnRH in fish (Jadhao and Malz, 2003). NOS is also located in the NPP and in the NRL of catfish (Jadhao and Malz, 2003), both nuclei in which activin subunits and receptors were found in goldfish. Activins could therefore also interact with NO to change reproductive behaviour in goldfish, or interact with NO to affect GnRH biosynthesis.

Kisspeptin has been identified as a protein that is a regulator of GnRH neuronal activity in mice and rats. Kisspeptin is coded for by two proteins, kiss1 and kiss2. The mRNA for kiss1 and kiss2 was localised by *in situ* hybridisation in the brain of medaka. Kiss1 is found in the NPPv (Mitani et al., 2010), which is also where activin subunits were found. On the other

hand, *kiss2* is found in the NRL (Mitani et al., 2010), like the actRII protein. The localisation of other reproductive hormones in the same nuclei as activin strengthens the role of activin in the reproductive axis.

GAD mRNA is found in the Vv and NRL, and GABA-T is found in the NRL and in the NDLI (Martyniuk et al., 2007b). These are all areas in which activin β A or the actRII were localised. The presence of GABA synthesis and degradation in these areas indicates the presence of GABA in the same nuclei in which activin is located, providing further support for our hypothesis that activin is involved in the pathway by which GABA stimulates the release of GnRH.

Activin has also been implicated in the regulation of growth in fish (Ge and Peter, 1994; Carpio et al., 2009). Two hormones involved in the regulation of growth are galanin and somatostatin. Galanin stimulates food intake in mammals and in fish, and stimulates the release of LH and GH from the pituitary of mammals and fish. Galanin's precursor, preprogalanin, is found in the NRL in the hypothalamus (Unniappan et al., 2004). The localisation of actRII in the NRL as well could implicate activin in the regulation of galanin. Interestingly, activin β A subunits and actRII are also located in the same nuclei where somatostatin binding is observed. Somatostatin is the GH inhibitory hormone. Somatostatin binds to the NPP in the telencephalon of goldfish, to the NDM and NDL in the thalamus, and to the NDLI in the hypothalamus (Cardenas et al., 2000). Activin has opposing actions to that of somatostatin in the brain as somatostatin inhibits GH release and activin stimulates the release of GH (Ge and Peter, 1994). Therefore, activin could not interact with somatostatin, or somatostatin could have inhibitory actions on the expression or release of activin. However these two studies lend support to the role of activin in the regulation of growth in vertebrates.

This study shows, for the first time, that the activin β A subunit is located in many areas throughout the goldfish neuroendocrine brain in which it can influence reproduction. As well, the colocalisation of fibres containing actRII and GnRH in the NRL of the hypothalamus indicate an interaction in this area, which reflects what has previously been shown in cell lines and rat hypothalamic extracts. This provides evidence that supports our hypothesis that activin stimulates the expression and release of GnRH from the goldfish brain. Even though we were not able to colocalise actRII with GnRH cell bodies in the NPO or NPP, it is still possible that there is an indirect action of activin A on GnRH in this area since activin β A subunits were localised in both the NPO and in the NPP.

Figure 3.2. Brightfield micrographs of transverse sections of goldfish pituitary. Sections were stained with activin β A subunit antibody. Brown color indicates positive staining. Arrows indicate positively stained cells and arrowheads indicate positively stained nerve fibres. PPD: proximal pars distalis. NIL: neurointermediate lobe. Scale bar: 150 μ m

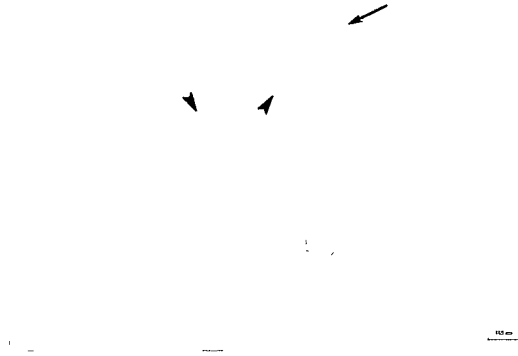
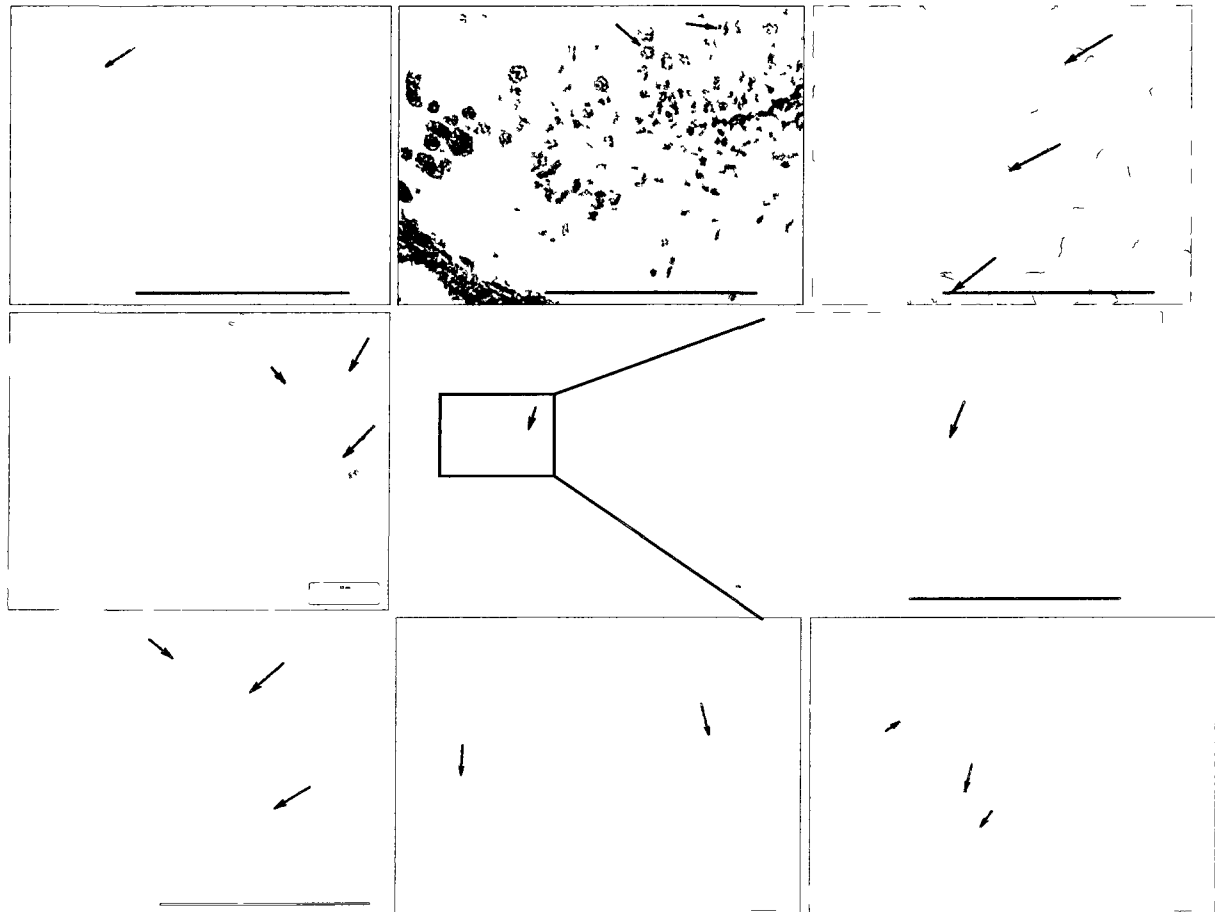
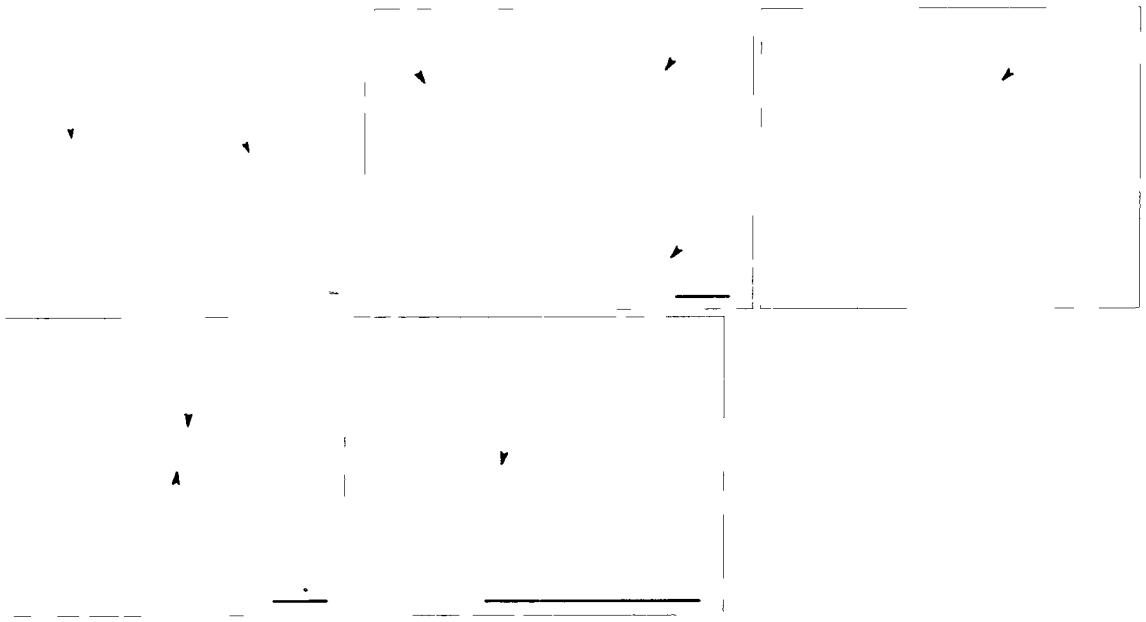


Figure 3.3. Brightfield micrographs of transverse sections of goldfish brain. Sections A-I and K-N were stained with activin β A subunit antibody and section J was stained with NSE antibody. Brown color indicates positive staining and blue staining indicates nuclei stained with haematoxylin. Arrows indicate positively stained cells. A: olfactory bulb. B: NPO. C: Vv. D: NDM. E and F: NRL. G: NVM. H: mlF. I: OT. J: NDM. K: EN. L: AC. M: NPGa. N: NDTL. Scale bars: 150 μ m





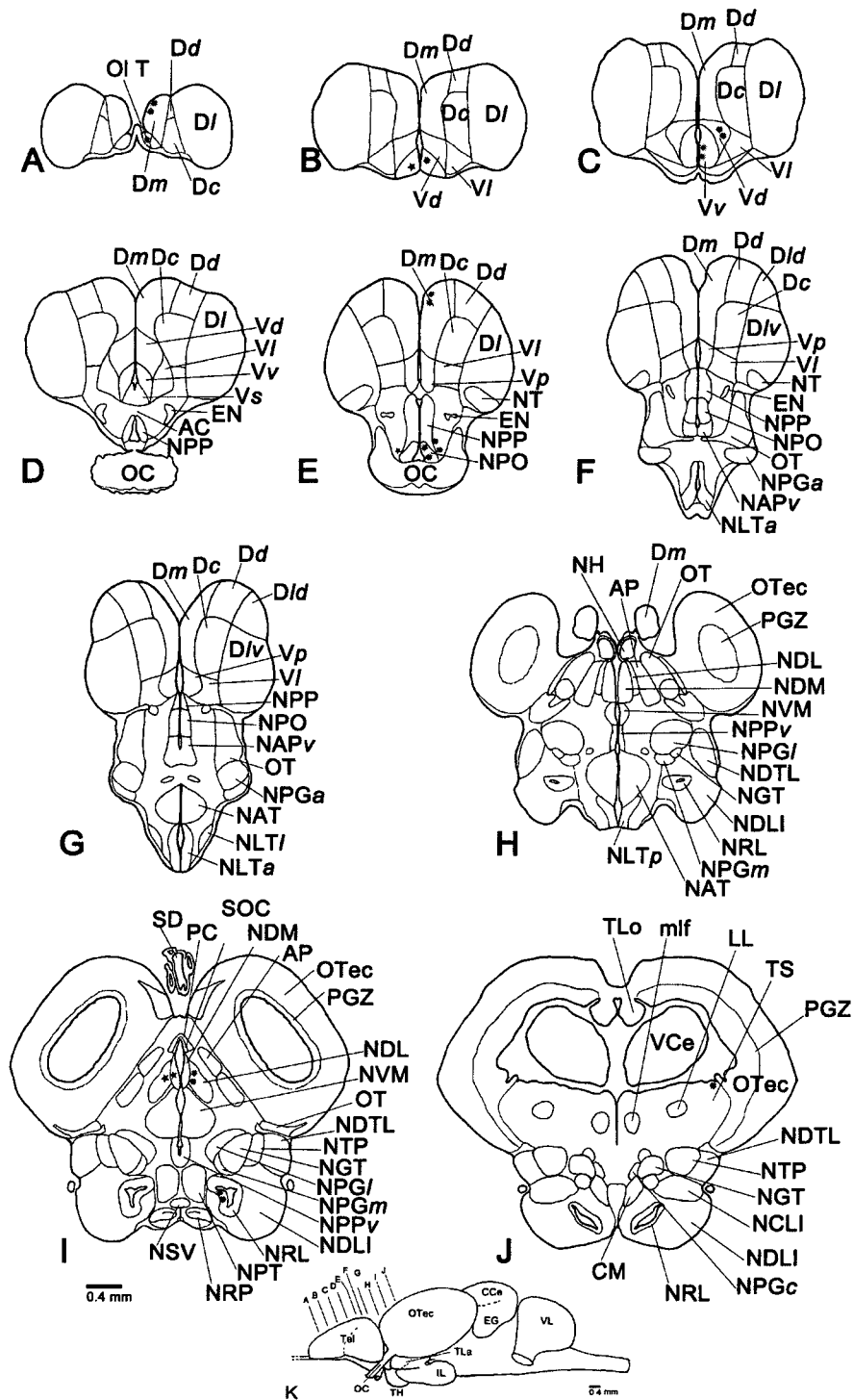


Figure 3.4. Schematic representation of successive rostrocaudal transverse sections of goldfish brain showing the distribution of activin receptor type II subunits cell bodies (★ on the left) and fibres (● on the right). K. Lateral view showing the level of the sections. Adapted from Canosa et al., 2004. Scale bars = 0.4 mm.

Figure 3.5. Brightfield micrographs of transverse sections of goldfish brain. Sections were stained with activin receptor type II subunit antibody. Brown color indicates positive staining and blue staining indicates nuclei stained with haematoxylin. Arrows indicate positively stained cells and arrowheads indicate positively stained fibres. A: olfactory bulb. B: Vv. C: AC. D: NDM. E: Vv and Vd. F: OTec. G: Dm. H: Vv. I: AC. Scale bars = 150 μ m

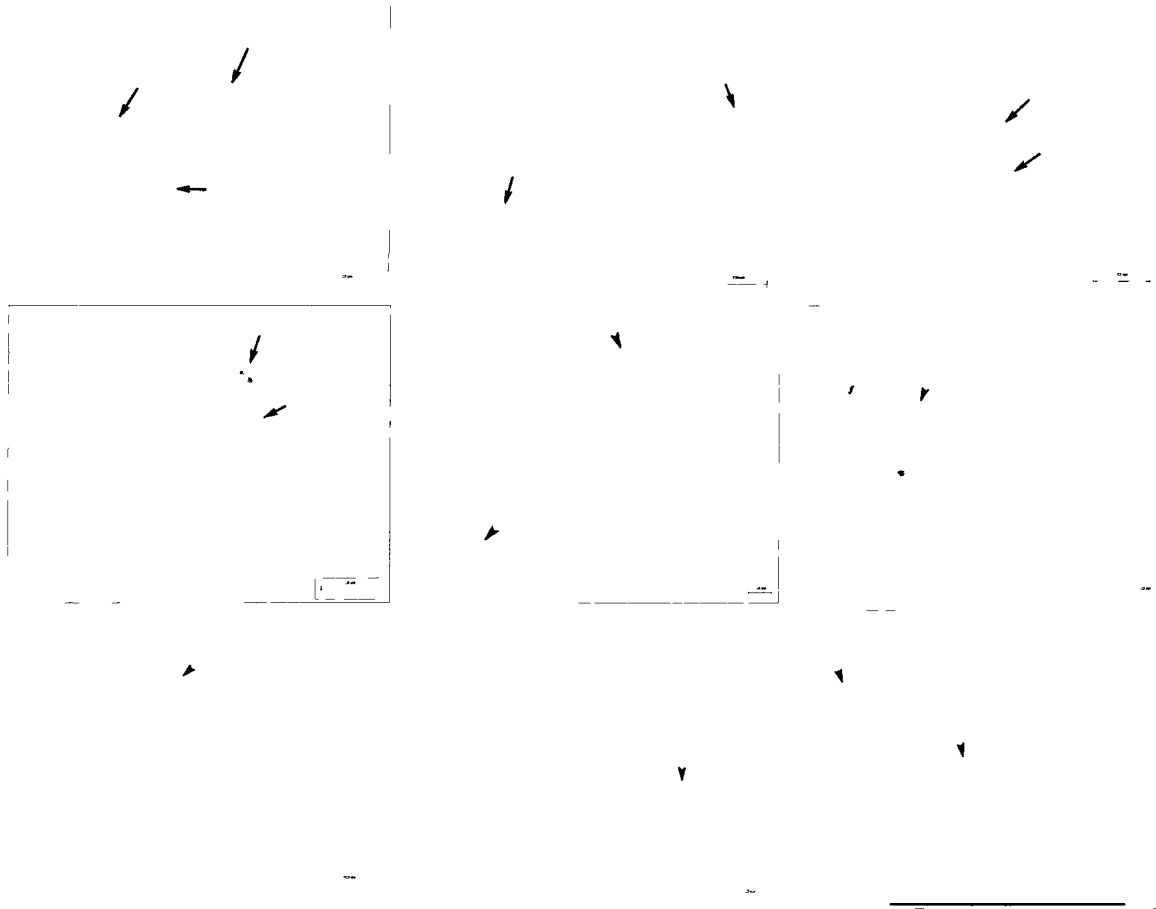


Figure 3.6. Fluorescent micrographs of transverse sections of goldfish brain. Sections were stained with activin receptor type II antibody (green, marked by arrowheads) and with GnRH antibody (red, marked by arrows). A: Positive fibres GnRH and ActRII in the olfactory bulb. B: Positive ActRII cells and GnRH fibres in the Vv of the telencephalon. C: Positive GnRH cells and ActRII fibres in the NPP of the telencephalon. D: Positive GnRH cells in the NPO. E: Large view of hypothalamus with F highlighted. F: Positive GnRH and ActRII fibres in the NRL of the hypothalamus. Scale bars = 50 μ m

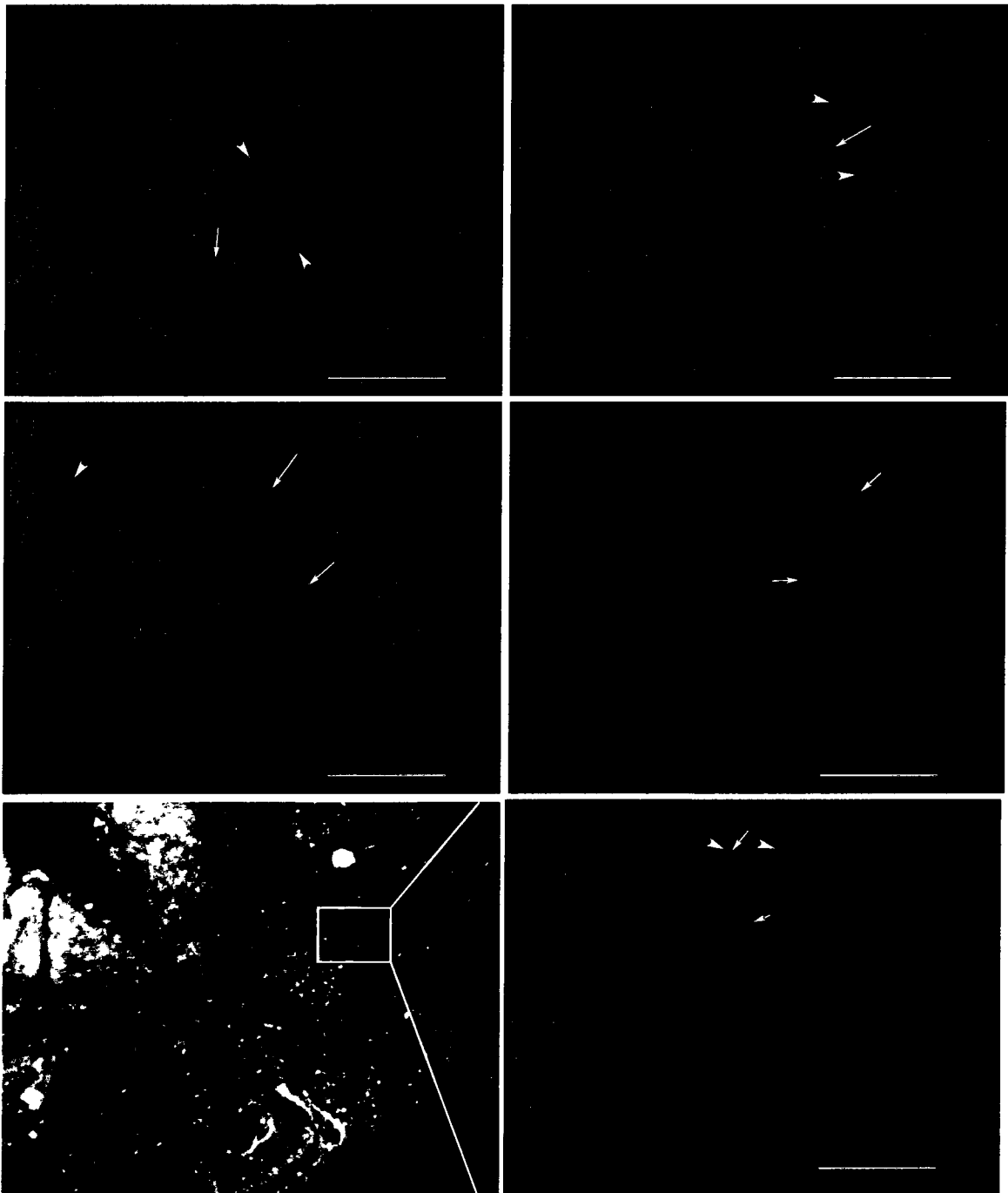
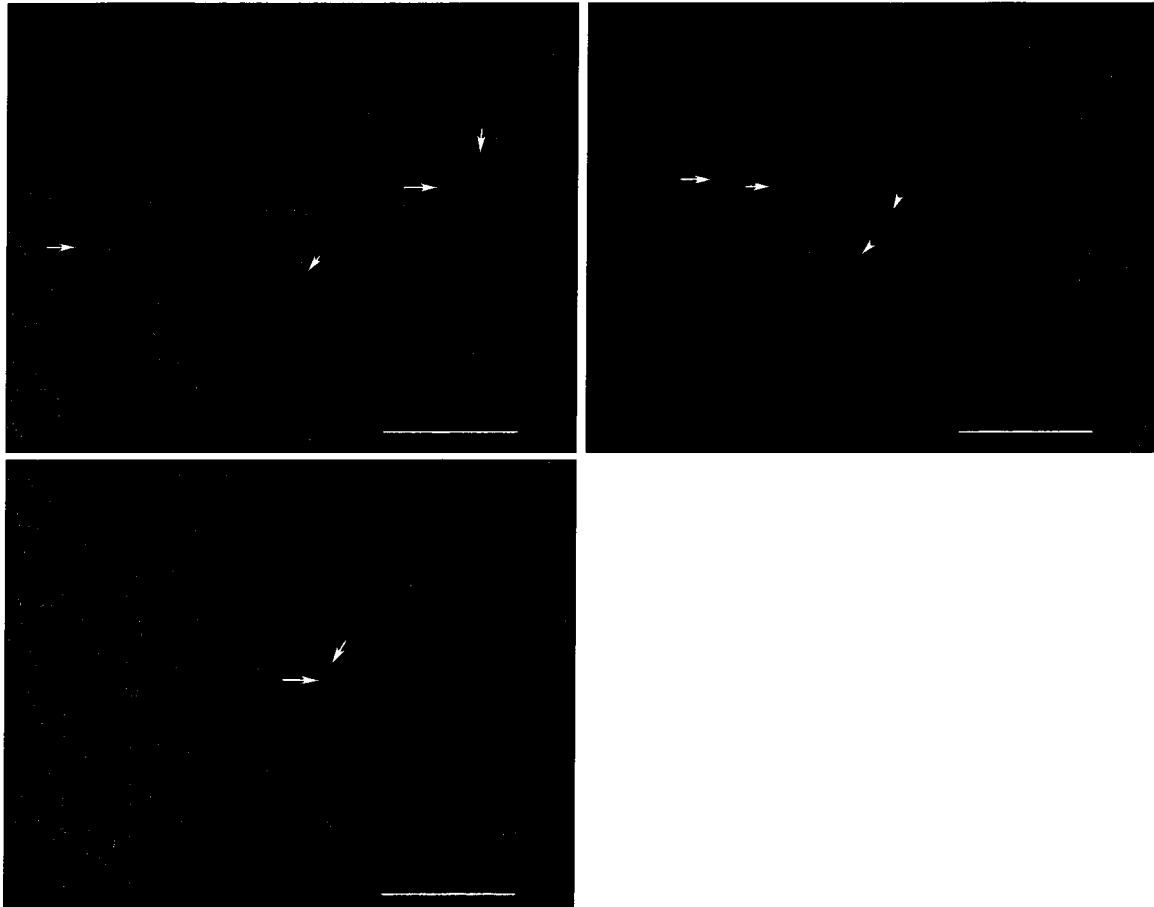


Figure 3.7. Fluorescent micrographs of transverse sections of goldfish pituitaries. Sections were stained with activin receptor type II antibody (green, marked by arrowheads) and with GnRH antibody (red, marked by arrows). A: Positive ActRII and GnRH fibres in the PPD and in the NIL. B: Positive ActRII cells and GnRH fibres in the NIL. C: Positive GnRH and ActRII fibres in the NIL. Scale bars = 50 μ m



Chapter 4

General Discussion

4.1 Activin in the brain

During the course of this Masters project, two separate experiments were performed to elucidate the role of activin A in regulating reproduction within the brain. Our hypothesis is that activin is involved in the pathway by which GABA stimulates the release of GnRH from the goldfish neuroendocrine brain. According to our hypothesis, GABA would stimulate the release of activin A in the brain, which would subsequently stimulate the release of GnRH from the brain.

Prior knowledge concerning the interaction of activin and GnRH in the brain comes from studies on rat brains, neuronal cell lines and rat brain extracts. In the rat brain, activin β A subunits were co-localised with GnRH proteins in fibres in the hypothalamus (MacConell et al., 1998) thus suggesting an interaction between the two hormones. Further work delineated that in this model, activin A stimulates the expression and the release of GnRH in both rat hypothalamic extracts and in neuronal cell lines (Calogero et al., 1998; MacConell et al., 1999). In goldfish, the GABA_B receptor agonist, baclofen has been shown to stimulate the expression of activin β A subunit in the hypothalamus and telencephalon of sexually regressed goldfish (Martyniuk et al., 2007a). This information applied to our model allowed us to put forward the following predictions:

Since baclofen stimulated the expression of activin β A subunit in sexually regressed goldfish, baclofen should also stimulate the expression of activin β A subunit in sexually mature goldfish.

If activin is involved in controlling reproduction in goldfish, its cell bodies should be localised in the neuroendocrine areas of the goldfish brain, that is, the telencephalon and the hypothalamus in proximity to the GnRH cells.

Our initial experiment involved examining the effect of a GABA_B receptor agonist on the mRNA expression of several members of the activin signalling pathway and the two forms of GnRH present in goldfish. We determined that baclofen increased the expression of the activin β A subunit in the telencephalon, and decreased the expression of the activin receptors IB in females and type IIB in males in the hypothalamus. Additionally, the expression of sGnRH was also increased by GABA in the hypothalamus. This led us to hypothesize that activins could function as neuroendocrine factors transmitting the GABA signal from the telencephalon to the hypothalamus. Since stimulation of the GABA_B receptors increased both the expression of activin and sGnRH, this experiment mildly supported our hypothesis that activin is involved in the GABA-stimulated release of GnRH.

The next study involved localising the activin β A subunits and the activin receptors type II in the goldfish brain by immunohistochemistry. Activin β A subunits and activin receptors type II were localised in cell bodies and fibres throughout the forebrain in the olfactory bulb, telencephalon, thalamus, hypothalamus and midbrain. A few activin β A cell bodies and activin receptor type II fibres were found in the preoptic nucleus in the telencephalon, which is where GnRH cell bodies are found (Kim et al., 1995). In order to study the possible interaction of activin A and GnRH in this area and to support our hypothesis that activins stimulate the expression or release of GnRH, we attempted to co-localise the activin receptors with GnRH proteins in goldfish brain. We found no evidence of co-localisation of the activin receptors and GnRH in the preoptic nucleus. Indeed, we found no evidence of co-localisation

of GnRH cell bodies and activin receptors anywhere in the goldfish forebrain. Activin receptor fibres were located near GnRH cells in the NPP of the telencephalon, but were not found on the GnRH cells. The only co-localisation of activin receptors and GnRH that was observed was in the NRL in the hypothalamus. In this instance, both of the proteins were localised in fibres in this nucleus. This experiment therefore supports our hypothesis about a direct action of activin A on GnRH. This experiment supports the idea that activin may stimulate the release of GnRH in the hypothalamus. Since we were not able to co-localise actRII and GnRH cell bodies in the NPO or NPP, it is possible that activin is unable to stimulate the expression of GnRH. However, it is also possible that the concentration of actRII in the NPO was too low to be detected by fluorescent staining.

Although cells containing activin β A subunits were found in both the telencephalon and in the hypothalamus, the expression of activin β A was only affected by baclofen in the telencephalon. This could mean that only the activin β A cells in the telencephalon are involved in reproduction. Similarly, although cells containing activin receptor type II were found in the telencephalon and in the hypothalamus, only the mRNA in the hypothalamus was decreased by baclofen injection. Similarly to activin β A, it is possible that only the activin receptors type II cells in the hypothalamus are involved in the control of reproduction. Activin A appears to be acting as a neuroendocrine factor in the brain by mediating a potential GABA-dependent pathway from the telencephalon to the hypothalamus where it stimulates the expression or the release of GnRH. The decrease in activin receptor type IB and IIB expression poses an interesting question about the meaning of this effect of baclofen on activin receptors. The expression of activin receptors could be decreased when activin subunit or GnRH expression increases, or when GABA signalling increases in order to maintain

homeostasis. This could mean that the role of the activin receptors in control of reproduction would be to control activin signalling in order to maintain homeostasis. The postulated mechanism would involve decreasing the expression of the activin receptors when the expression of activin β A or GnRH increases in the brain or when GABA levels increase (Figure 4.1).

This regulation of activin subunit and receptor expression by baclofen could also have non-reproductive roles. GABA and activin both have non-reproductive roles which include the regulation of neuronal growth during development and in the adult brain. During development, GABA neurons mature earlier than others and appear to control the development of the rest of the neuronal system (Akerman and Cline, 2007, Sernagor et al, 2010). Similarly, activin subunits are also involved in neural development (Dohrmann et al, 1993, Feijen et al, 1994). GABA also regulates neuritic growth in the adult brain (Sernagor et al, 2010). Activin also has been shown to play a role in stimulating an increase in length and branching of primary cultures of rat amygdala neurons in vitro (Trudeau et al, 1997). Therefore, the control that GABA has over the expression of activin could also have non-reproductive roles which include regulating neuronal development.

Over the course of the two experiments, intriguing data arose with respect to activin B. Baclofen did not affect the expression of the activin β B subunits following injection in sexually mature goldfish despite increasing activin β A subunit expression. Baclofen also had no effect on the expression of the activin β B subunit in sexually regressed goldfish (Martyniuk et al, 2007a). In addition, we were not able to convincingly localise the activin β B subunit in the goldfish neuroendocrine brain since staining was very weak. This difficulty in localising the activin β B subunit was also noted in the study by Mousa and Mousa (2003) in

the brain of *Liza ramada* and by Ge and Peter (1994) in the goldfish pituitary. This data leads us to conclude that that activin B is not present within the brain of goldfish, and that activin B may possibly play a much larger role elsewhere such as in the gonads where it is important in other models.

It is of note that the only area of the goldfish brain that showed any co-localisation of GnRH proteins and actRII was in the NRL of the hypothalamus. Interestingly, it is also in the hypothalamus that baclofen changed the expression of activin receptor type IB and IIB, and sGnRH. Since we were able to measure mRNA levels of these proteins in the hypothalamus, this implies that in this area there are cells expressing these proteins. However, the GnRH proteins and activin receptors were co-localised in fibres and not in cell bodies. In fact, no cell bodies expressing actRII were localised conclusively in the hypothalamus of goldfish nor were there GnRH cell bodies found in the goldfish hypothalamus. One possible explanation for this is that there are few actRII cells and GnRH cells in the hypothalamus, and that the concentration of protein within the cell was too low for detection. Nevertheless, our studies provide some support for our hypothesis that activin stimulates the release of GnRH from the hypothalamus. Activin was shown to stimulate the release of GnRH from the explanted rat hypothalamus (Calogero et al., 1998), and the same appears to be true in the hypothalamus of goldfish due to the colocalisation of actRII and GnRH proteins.

One other aspect that remains to be explored here was the control of activin signalling by follistatin. Previous investigators found in goldfish pituitary cell culture that T and E₂ regulated activin signalling by increasing the level of follistatin expression without any effect on activin β B expression, effectively decreasing free activin and decreasing activin signalling (Cheng et al., 2007). In our first study, we observed that activin receptor expression was

decreased in the hypothalamus following baclofen injection. The expression of follistatin could also be examined by real-time RT-PCR to see how it is affected by an increase in baclofen. Further research could study the expression of follistatin and specifically examine its role in regulating activin signalling in the brain.

We were not able to find any differences between males and females with the localisation or abundance of activin receptors type II cells in the goldfish brain despite finding that baclofen decreases the level of activin receptor type IB and IIB in the goldfish hypothalamus. Although we did not have any antibodies to the activin receptor type IB, it is likely that these receptors are localised in similar cells as activin receptor type II since both receptors must work together to transmit signal. One difference between the expression study and the immunohistochemistry study was the sexual maturity of the fish. The expression study was done on sexually mature goldfish whereas the localisation study was done on sexually regressed goldfish. It is possible that a difference in activin receptor cell abundance might be observable between sexually regressed and mature goldfish, or post-baclofen injection which might reflect the data found in our expression study.

4.2. Future research

Although we have determined many important aspects of activin function and localization, many questions concerning the role of activin in the release of GnRH from the neuroendocrine brain and in the pituitary remain unanswered. Our immunohistochemical studies provide a suggestion of an interaction between activin and GnRH in the hypothalamus of goldfish. The role of activin A in the expression and release of GnRH in goldfish could then be elucidated further using hypothalamic explants similar to prior experiments in rats.

Although the existence of such an interaction was not conclusive, if such an interaction exists, it is likely that activin A would only stimulate the release of GnRH and not its expression as the activin receptors were not localised on GnRH cells. Using cell lines or explanted hypothalamus such a study could indicate if the expression of activin receptors is decreased due to increased GABA signalling or due to the increase in activin A concentration. The hypothalamic expression of follistatin following activin A exposure could also be studied to examine how it responds to increases in activin A concentration to regulate activin signalling.

The interaction between activin A and GnRH in the olfactory bulb should also be investigated further. It appeared that many of the fibres in the ventral part of the telencephalon originated from those cells in the olfactory bulb, particularly for the actRII, indicating the potential importance of the protein synthesized in the cells of the olfactory bulb. We did not, however, look at the expression of activin β A and activin receptor type II in the olfactory bulb. It could be interesting to do this to see if there are any changes in the mRNA expression of the activin β A subunit and activin receptor type II. GnRH cells are also found in the goldfish olfactory bulb, and this could lead to an interaction between GnRH and activin A. The GnRH present in the olfactory bulb of goldfish does not regulate ovarian development and ovulation, but might have more of a neuromodulatory role (Kobayashi et al., 1994). This possible interaction between activin A and GnRH in the olfactory bulb would be all the more interesting because most of the sGnRH in the goldfish brain originates from the olfactory bulb, with the exception of the GnRH in the NPO and in the hypothalamus. Activin has also been shown to have roles in reproduction other than in regulating the release of LH and FSH from the pituitary, which includes regulating reproductive behaviour (Ma et al., 2005). GnRH has previously been shown to stimulate the release of activin A from olfactory epithelium

(Florio et al., 2000). GnRH could also stimulate the release of activin A from the olfactory bulbs in goldfish. Therefore, another similar study could also be performed in which GnRH is applied to explanted olfactory bulbs to test if GnRH stimulates the release of activin A from the olfactory bulbs in goldfish.

Understanding the role of activin is essential to fully understand the complex control of reproduction that occurs within the brain. The proposed studies would be useful to further clarify the precise role that activin plays in the reproductive system in the brain and pituitary of goldfish. In conclusion, this Masters project has furthered our knowledge of how GABA affects the activin signalling system, leading to a hypothesis of a new neuroendocrine role for activin A in the brain. Our colocalisation study indicates that activin could stimulate the release of GnRH from the hypothalamus. Optimally, these observations would justify further confirmation, perhaps by studying explanted goldfish hypothalami.

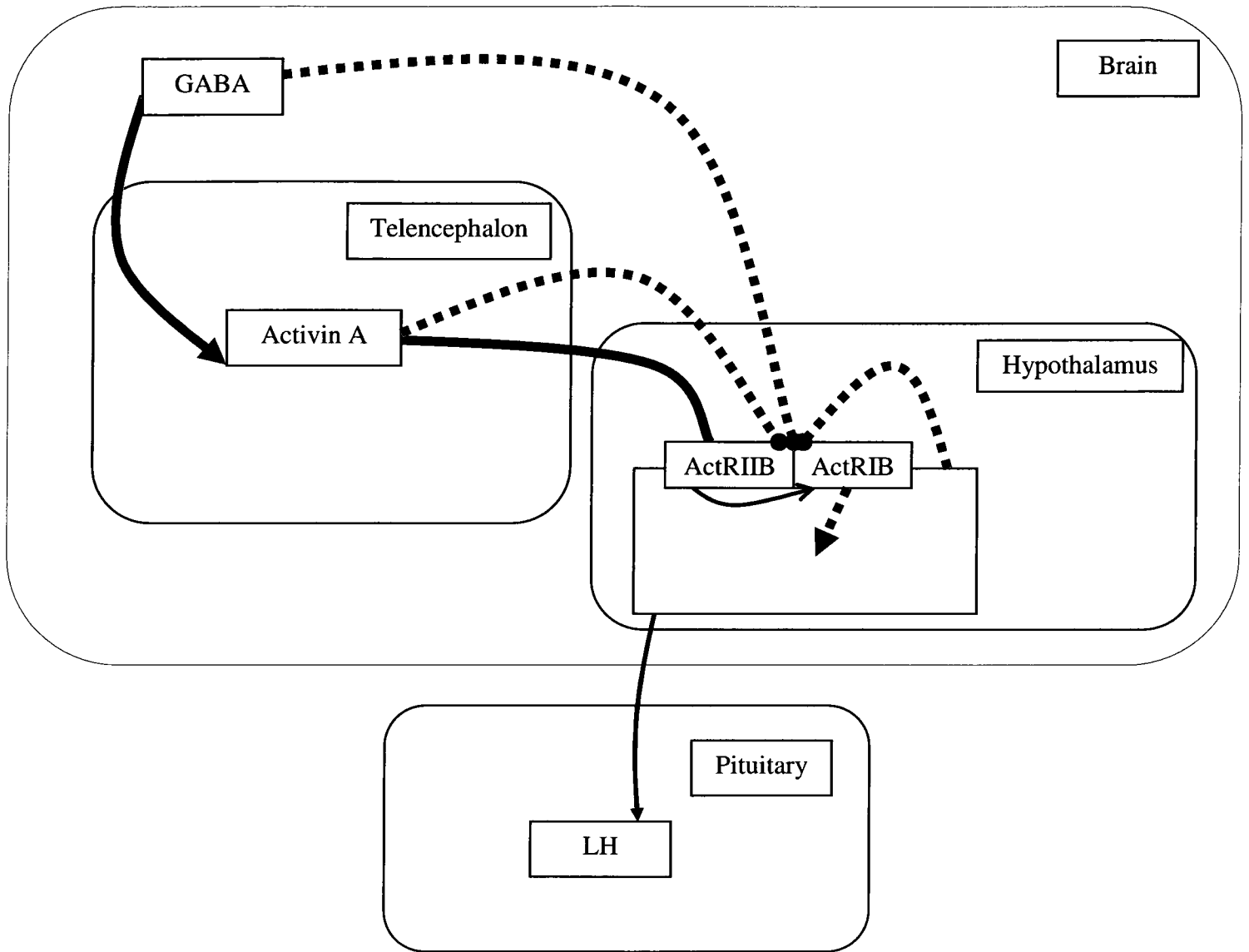


Figure 4.1. Schematic representation of the hypothetical pathway by which GABA stimulates the release of GnRH in the neuroendocrine brain of sexually mature goldfish. GABA stimulates the expression of activin β A subunits in the telencephalon. Activin A proteins then stimulate the expression or release of GnRH from the hypothalamus through action on the activin receptors. Either GABA or Activin A decrease the expression of the activin receptors type IIB (ActRIIB) or type IB (ActRIB). GnRH is then transferred from the hypothalamus to the pituitary through nerve fibres where it stimulates the release of LH. See text for further explanation. \rightarrow : stimulation. \bullet : inhibition. --- : transfer via neurons. \rightarrow : recruits. Dashed lines indicate speculative interactions.

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Appendix

Table A1. Gonadosomatic index (GSI) from the male goldfish used in chapter 2. Body weight and gonad weight were measured at the time the fish were killed. GSI was calculated from (gonad weight/body weight) * 100.

Treatment	Sex	Body Weight (g)	Gonad Weight (g)	GSI (%)
Control	Male	20.1	0.96	4.8
Control	Male	19	0.45	2.4
Control	Male	20.3	0.44	2.2
Control	Male	24.9	0.58	2.3
Control	Male	26.6	1.54	5.8
Control	Male	24.8	0.98	4.0
Control	Male	22.8	0.99	4.3
Control	Male	19.6	0.61	3.1
Control	Male	21.9	0.60	2.7
Control	Male	28.7	1.04	3.6
Control	Male	25.9	0.69	2.7
Control	Male	18.2	0.31	1.7
Control	Male	28.7	0.82	2.9
Control	Male	21.4	0.62	2.9
Control	Male	19.6	0.78	4.0
Control	Male	24.9	1.02	4.1
Control	Male	21.02	0.61	2.9
Baclofen	Male	21.88	0.55	2.5
Baclofen	Male	27.21	0.52	1.9
Baclofen	Male	16.93	0.49	2.9
Baclofen	Male	22.4	0.69	3.1
Baclofen	Male	26.17	0.71	2.7
Baclofen	Male	16.25	0.24	1.5
Baclofen	Male	16.56	0.55	3.3
Baclofen	Male	21.75	0.87	4.0
Baclofen	Male	18.07	0.34	1.9
Baclofen	Male	18.4	0.52	2.8
Baclofen	Male	15.9	0.57	3.6
Baclofen	Male	20.96	0.96	4.6
Baclofen	Male	19.62	0.73	3.7
Baclofen	Male	19.85	0.44	2.2
Baclofen	Male	22.63	0.63	2.8
Baclofen	Male	19.8	0.48	2.4
Baclofen	Male	23.4	0.56	2.4
Baclofen	Male	19.5	0.16	0.8
Baclofen	Male	17.4	0.18	1.0

Table A2. Gonadosomatic index (GSI) from the female goldfish used in chapter 2. Body weight and gonad weight were measured at the time the fish were killed. GSI was calculated from (gonad weight/body weight) * 100.

Treatment	Sex	Body Weight (g)	Gonad Weight (g)	GSI (%)
Control	Female	24.84	3.30	13.3
Control	Female	20.01	2.84	14.2
Control	Female	23.1	2.73	11.8
Control	Female	25.72	4.04	15.7
Control	Female	14.15	2.09	14.8
Control	Female	21.56	2.85	13.2
Control	Female	27.21	3.75	13.8
Control	Female	26.08	3.00	11.5
Control	Female	23.2	2.81	12.1
Control	Female	13.85	2.01	14.5
Control	Female	22.45	3.77	16.8
Control	Female	26.31	4.00	15.2
Control	Female	19.06	3.47	18.2
Control	Female	19.1	2.41	12.6
Baclofen	Female	20.4	2.13	10.4
Baclofen	Female	20.2	3.50	17.3
Baclofen	Female	26.8	4.22	15.7
Baclofen	Female	17.2	2.45	14.2
Baclofen	Female	20.9	0.39	1.9
Baclofen	Female	29.5	5.81	19.7
Baclofen	Female	25.5	3.80	14.9
Baclofen	Female	24.05	3.57	14.8
Baclofen	Female	20.54	2.54	12.4
Baclofen	Female	22.35	2.33	10.4
Baclofen	Female	18.7	2.13	11.4
Baclofen	Female	18.3	2.73	14.9
Baclofen	Female	27.4	3.34	12.2