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Christopher Joseph Martyniuk
AUTEUR DE LA THÈSE / AUTHOR OF THESIS

Ph.D. (Biology)
GRADE / DEGREE

Department of Biology
FACULTÉ, ÉCOLE, DÉPARTEMENT / FACULTY, SCHOOL, DEPARTMENT

GABA Action and Sex Steroid Feedback in the Goldfish (carassius auratus) Neuroendocrine Brain

TITRE DE LA THÈSE / TITLE OF THESIS

Vance Trudeau
DIRECTEUR (DIRECTRICE) DE LA THÈSE / THESIS SUPERVISOR

CO-DIRECTEUR (CO-DIRECTRICE) DE LA THÈSE / THESIS CO-SUPERVISOR

EXAMINATEURS (EXAMINATRICES) DE LA THÈSE / THESIS EXAMINERS

John Arnason

Steve Perry

Hamid Habibi

William Willmore

Thomas Moon

Gary W. Slater
Le Doyen de la Faculté des études supérieures et postdoctorales / Dean of the Faculty of Graduate and Postdoctoral Studies

GABA action and sex steroid feedback in the goldfish (*Carassius auratus*) neuroendocrine brain

by

Christopher Joseph Martyniuk

Thesis submitted to the Faculty of Graduate and Postdoctoral Studies, University of Ottawa, in partial fulfillment of the requirements for the Ph.D. Degree in the Ottawa-Carleton Institute of Biology.

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Dedicated to

Ruth Ann and Oliver Jack Martyniuk

Acknowledgements

"To raise new questions, new possibilities, to regard old problems from a new angle, requires creative imagination and marks real advance in science"
- *Albert Einstein*

"An optimist laughs to forget, a pessimist forgets to laugh"
- *anonymous*

It is true, and although I fancy myself an optimist, there were as expected some times I forgot to laugh. It is in these times, however, that your family, partner, friends, and colleagues make all the difference...and then you laugh and continue on again. There are a number of people who I must thank. I know it is cliché, but truly, without their support the path ahead would have been very difficult indeed.

I think it most appropriate to first thank Dr. Vance Trudeau, my mentor and supervisor for the last 5 years. I know that few have had the opportunities that I have had in your lab. I am certain when I say that there is nothing more I could have wanted from my doctorate degree. I would also like to thank my Ph.D. advisory committee, Drs. Cris Martin, Tom Moon, and Michael Poulter, for their direction, guidance, and stimulating conversations and Dr. Tom Finger with assistance with the in situ hybridization technique. Thank you also to Guy Drouin and Stephane Aris-Brosou for the GABA receptor subunit evolution analysis.

I would also like to thank everyone in the Trudeau lab, past and present, too many to mention. What a great experience to have met so many interesting people! Especially Nat and Max, both of you with me from the beginning. JP, what more can I say than "wings...done". Susanna and Vicki, and well, Dolly Parton, always fun. I also thank Huiling Xing, Kate Crump, and Emily Gerrie with assistance with the microarray production and analysis.

My family: Greg and Denise and Olli, Brian and Trina, Steph and Matt, and Bud. This is the second time most of you have made it into an acknowledgement section! We have had to endure a lot as a family and I am proud of you all. Thank you for all the great times we have had over these years, especially the most recent ones here in Ottawa. Canada Day would certainly not have been the same. I hope you are as proud of your oldest brother as I am of you. To my fiancée, April. Thank you for all your love and support. We met here during our graduate degrees and started our wonderful life together!

And finally Dad and Mom, again as I embarked on another project, destined to write a second thesis, you supported me more than ever, emotionally (financially) and everything in between. I dedicate this thesis to my Mom and nephew, both taken from us far too soon. Even though you are not here in body, you are in my heart always. I will cherish the values you have both taught me in our short time together.

Abstract

The inhibitory amino acid neurotransmitter γ-aminobutyric acid (GABA) has a significant role in vertebrate reproduction. In teleost fish, GABA will stimulate the release of LH from the pituitary during certain periods of gonadal development. This thesis characterizes the mRNA transcript distribution of the enzymes that are responsible for the synthesis (glutamic acid decarboxylase) and degradation (GABA-transaminase) of GABA in the brain of the goldfish and shows that regions containing estrogen receptors and reproductive neuropeptides also contain high amounts of GABA-related transcripts. I show that GABAergic synaptic transmission may be modulated by GABA via genomic effects on GABA receptor subunits. In order to better understand GABA stimulated LH release in fish, I studied GABA agonist action on gene expression. This experiment showed that GABA agonists modulate GABA synthesis and degradation differentially. I also show that a rapid and pronounced increase in activin βα transcription in neuroendocrine tissues via $GABA_B$ receptors and $GABA_A$ mediated changes in tyrosine hydroxylase mRNA are putative mechanisms by which GABA mediates pituitary LH release in fish. Lastly, I outline the development of a goldfish brain enriched cDNA microarray to study estrogen feedback in the neuroendocrine brain. Male goldfish exposed to the xenoestrogen ethinylestradiol (EE2) had significantly reduced gonads and circulating sex steroids. Microarray and real-time RT-PCR showed that brain aromatase, secretogranin-III, and interferon-related developmental regulator 1 (IFDR-1) were altered after EE2 treatment. This thesis 1) further characterizes the GABAergic system in goldfish using different approaches to gain a better understanding of the role of GABA in neuroendocrine

function in fish 2) identifies evolutionary conserved patterns of gene expression changes after GABA and estrogenic treatment in fish and 3) demonstrates that the goldfish microarray is a useful tool to study the effects of endocrine disrupting chemicals on neuroendocrine function.

Résumé

Le neurotransmetteur inhibiteur acide γ-aminobutyrique (GABA) joue un rôle significatif dans la reproduction des vertébrés. Chez les poissons téléostéens, GABA stimule la sécrétion de LH de la glande pituitaire durant certaines périodes du développement des gonades. Cette thèse caractérise la distribution de transcrits d'ARNm des enzymes responsables de la synthèse (acide glutamique décarboxylase) et de la dégradation (GABA-transaminase) de GABA dans le cerveau de poissons rouges et démontre que les régions qui contiennent des récepteurs d'oestrogène et des neuropeptides reproducteurs contiennent aussi des niveaux élevés de transcrits reliés à GABA. Je démontre que la transmission synaptique GABA-ergique peut être modulée par GABA via des effets génomiques sur les sous-unités du récepteur GABA. Afin de mieux comprendre comment GABA stimule la sécretion de LH chez les poissons, j'ai étudié l'action d'agonistes de GABA sur l'expression des gènes. Cette expérience a démontré que les agonistes de GABA modulent différentiellement la synthèse et la dégradation de GABA. Je montre aussi qu'une augmentation rapide et prononcée de la transcription d'activine βα dans les tissus neuroendocriniens via les récepteurs $GABA_B$ et les changements d'ARNm de tyrosine hydroxylase médiés par $GABA_A$ sont des mécanismes putatifs par lesquels GABA médie la sécrétion de LH chez les poissons. Dernièrement, je décris brièvement le développement d'un microréseau fabriqué d'ADNc provenant de cerveaux de poissons rouges pour étudier la rétroaction d'oestrogène dans le cerveau neuroendocrinien. Les poissons rouges mâles exposés au xénooestrogène éthinyloestradiol (EE2) démontraient une réduction significative de la taille des gonades et des niveaux de stéroïdes sexuelles circulant dans le serum. Des

analyses par microréseau à base d'ADN et RT-PCR en temps réel ont identifiés que l'aromatase, la sécrétogranine-III, et l'interferon-related developmental regulator 1 (IFDR-1) étaient altérés après traitement à EE2. Cette thèse 1) caractérise en détail le système GABAergique du poisson rouge en utilisant différentes approches pour mieux comprendre le rôle de GABA dans la fonction neuroendocrine chez les poissons 2) identifie des patrons conservés évolutionnaires de l'expression des gènes dans les poissons après traitements à GABA et l'oestrogène et 3) démontre que le microréseau à base d'ADN de poisson rouge est un outil utile pour étudier les effets des dérégulateurs endocriniens sur la fonction neuroendocrine.

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LIST OF ABBREVIATIONS

5HT - 5 hydroxytryptamine
AC - anterior commissure
ACH- acetylcholine
CCK - cholecystokinin
cDNA - complimentary DNA
CNS - central nervous system
CRF – cortisol releasing factor
DA - dopamine
Dc - dorsalis telencephali pars centralis
Dl - dorsalis telencephali pars lateralis
Dm - dorsalis telencephali pars medialis
E2 - 17β-estradiol
EDCs - endocrine disrupting chemicals
EE2 - 17α-ethinylestradiol
ER - estrogen receptor
GABA - gamma-aminobutyric acid
GAD - glutamic acid decarboxylase
GABA-T - gamma aminobutyric acid transaminase
GAPDH - Glyceraldehyde-3-phosphate dehydrogenase
GAT - gamma aminobutyric acid transporter
GnRH - gonadotropin releasing hormone
GSI - gonadosomatic index
GTH - gonadotropin hormone
GVG - gamma-vinyl GABA
HPG - hypothalmic-pituitary-gonadal axis
ISH – in situ hybridization
i.p. - interperitoneal
LH - luteinizing hormone
MT - midbrain tegmentum
NE - norepinephrine
NAH - nucleus anterioris hypothalami
NAT - nucleus anterior tuberis
NAP_V - nucleus anterioris periventricularis
NDLI - nucleus diffuses lobi inferioris
NLT(i) - nucleus lateral tuberis pars inferioris
NLT(p) - nucleus lateral tuberis pars posterioris
NPGI - nucleus preglomerulosus pars lateralis
NPO - nucleus preopticus
NPP, nucleus preopticus periventricularis
NPY - neuropeptide Y
NRL - nucleus recessive lateralis
NVM - nucleus ventromedialis thalami
OD - ornithine decarboxylase
RT PCR – reverse transcriptase polymerase chain reaction

Sg-III – secretogranin-III
SNAP25 - synaptic vesicle associated protein 25
SSADH - succinic semi-aldehyde dehydrogenase
T - testosterone
Vd - ventralis telencephali pars distalis
Vl - ventralis telencephali pars lateralis
Vs - area ventralis telencephali pars supracommissuralis
Vv - area ventralis telencephali pars ventralis

Thesis outline and rationale

The amino acid neurotransmitter γ-aminobutyric acid (GABA) plays a significant role in mediating reproduction in vertebrates. GABA stimulates the release of luteinizing hormone (LH; also called GTH-II in fish), a hormone that promotes steroidogenesis and gametogenesis in the gonads, from the anterior pituitary. The primary objectives of my thesis were to investigate the underlying molecular mechanisms involved in the stimulatory effect of GABA on reproduction in the goldfish and to develop the first brain enriched cDNA microarray for a teleost fish to use as a tool to study 17β-estradiol (E2) feedback in the neuroendocrine brain.

This thesis is organized into chapters based on 1) describing the distribution of enzymes that synthesize and degrade GABA in the goldfish (i.e. glutamic acid decarboxylase; GAD and GABA transaminase; GABA-T) 2) an updated phylogenetic analysis of GABA receptor evolution in vertebrates 3) studying the autoregulation of GABA synaptic transmission by GABA 4) testing new hypotheses to identify the mechanisms of GABA stimulated LH release and 5) developing a brain derived cDNA microarray to study E2 feedback in the CNS. This thesis has characterized the GABAergic system in goldfish using different approaches to gain a better understanding of the role of GABA in neuroendocrine function and reproduction.

Chapter 2 describes the mRNA distribution of GAD65, GAD67, and GABA-T mRNA in the brain of the goldfish. Until this study, GAD65 and GAD67 mRNA distributions had not been described in a teleost fish using specific riboprobes. In addition, GABA-T mRNA distribution to the best of our knowledge had not been described for any vertebrate using *in situ* hybridization. The objectives were to describe

GAD and GABA-T mRNA expression in the brain and to identify whether brain regions differed in GAD isoform and GABA-T mRNA expression. It was determined that GAD mRNA and GABA-T mRNA are localized in similar areas of the brain and that there is a high amount of GAD positive cells in regions of the brain that contain GnRH (gonadotropin-releasing hormone) neurons, further evidence that GABA has a role in the control of reproduction.

In order to study the modulation of genes in the CNS by GABA, I first cloned a number of partial sequences for genes involved in GABA synaptic transmission, many of which had not been cloned in fish at the time (see Chapter 4). It was necessary to verify gene sequences using phylogenetic analysis to be certain of the identity of the gene. With the dramatic increase in gene sequence information in taxa such as early chordates, fish, and birds, it was decided to perform a more complete and updated phylogenetic analysis of the GABA receptor subunit family in vertebrates to better understand the evolutionary history of this diverse gene family. Chapter 3 provides an updated evolutionary history of the GABA receptor subunit family. This study showed that GABA receptor subunits ε and θ underwent diversifying evolution in the mammalian lineage, and discuss the possible implications of the evolution of these mammalian specific receptor subunits in context of neuroendocrine function.

This thesis investigated putative molecular mechanisms of GABA stimulated LH release and E2 feedback in the CNS. Before a large-scale analysis of gene expression in the brain was done, we used reverse northern blotting to study GABA action in the brain. The objectives were to focus on a small number of genes that were involved in GABA synaptic transmission (e.g. GABA receptor subunits) and reproduction (e.g. estrogen

receptor alpha; ERα and GnRH). This study (Chapter 4) found that increasing GABA levels in the brain resulted in a down-regulation of $GABA_A$ receptor subunits. Increasing GABA levels also mediate the transcriptional response of the neuropeptide cortisol releasing factor, CRF. This study suggests that the autoregulation of GABA receptor subunits by GABA may be a conserved evolutionary mechanism in vertebrates, possibly to reduce tonic GABAergic inhibition.

To better understand the mechanisms underlying GABA stimulated LH release, I hypothesized that GABA may alter the expression of genes in the neuroendocrine brain involved with GABA turnover and reproduction. These genes included GAD65, GAD67, GABA-T, activin βa and βb, salmon GnRH (sGnRH), and tyrosine hydroxylase (TH). The role of these factors in stimulating LH release will be explained in detail elsewhere. Chapter 5 describes a study in which $GABA_A$ and $GABA_B$ receptors were differentially activated by GABA agonists muscimol and baclofen. GAD65 and GAD67 steady state mRNA was decreased after muscimol and baclofen treatment, respectively and both agonists decreased GABA-T mRNA. This suggests that GABA may regulate its synthesis and turnover differentially through both GABA receptor types. Activin βα mRNA, a potent stimulator of GnRH, was up-regulated ~ 3-4 fold by baclofen in both the hypothalamus and telencephalon. This is hypothesized to be a putative mechanism by which GABA stimulated LH release occurs in the goldfish. A model is presented in the final chapter to address how LH release may be stimulated by GABA in fish.

A major advance in studying neuroendocrine processes in fish was the development of a goldfish brain enriched cDNA microarray. This was a collaborative effort, beginning with members of our laboratory and growing to include bioinformatics,

ecological, and physiological expertise from both academic and government laboratories. A microarray is an ordered arrangement of probe DNA (cDNA in the present case) fixed to a solid surface. The cDNA on the microarray is hybridized with labelled cDNA from both a control and experimental sample simultaneously. Relative dye intensity is used to determine whether a transcript is differentially regulated between the control and treatment groups. Thus, global expression patterns of multiple genes can be determined under a number of physiological conditions (e.g. drug action, hormone cascades, chemical exposures) and a "signature" gene expression profile produced. This approach provided information on novel genes regulated by E2 in the goldfish brain. Real-time RT (reverse transcriptase) PCR was used in conjunction with the cDNA array data to validate gene expression. Microarrays are hypothesis generators, shedding light on the complex molecular and biochemical pathways induced or depressed by a treatment. New hypotheses about the function of known or uncharacterized genes can be proposed based on expression profile similarity to other genes with known function. Most exciting is that the expression profile of multiple genes can be used to identify groups of genes that are involved in a physiological/molecular response that share temporal expression patterns or "synexpression" groups (Niehrs and Pollet, 1999). Leonhardt et al. (2000) determined that changes in gene expression of metabolic machinery (i.e. enzymes, transporters) in GABAergic and glutamatergic neurons were correlated with the preovulatory gonadotropin surge in rats. On a global scale, expression profiling has identified groups of genes transcribed at various stages in central nervous system development in the rat (Wen et al., 1998). This approach does not only provide basic information on gene discovery and function but has broad applications in both pharmaceutical research (e.g.

determine the effects of a drug on a global scale) and toxicological research (e.g. mechanisms of disruption of biological function by toxins).

Chapter 6 describes a brain enriched goldfish cDNA microarray (www.auratus.ca) and demonstrates the use of expression profiling technology in studying the effects of pharmaceuticals on brain function in aquatic organisms. Considerable attention has been given in the literature to the disruption of the endocrine system by endocrine disrupting chemicals (EDCs). These compounds mimic endogenous hormones and can interfere with the homeostasis of the organism. More recently, some pharmaceutical products found in the environment have been identified as EDCs. 17α-Ethinylestradiol (EE2), a potent synthetic estrogen used widely in birth control pills and hormone replacement therapy, is one such pharmaceutical. There is strong evidence that EE2 disrupts the reproductive system in fish. For example, in female zebrafish, exposure to EE2 results in decreased spawing and egg production (Van den Belt et al., 2003) and in male zebrafish, exposure to low concentrations of estrogens results in decreased gonad size and elevated vitellogenin (egg yolk precursor protein) production (Fenske et al., 2005). The objectives of this study were to evaluate the effects of EE2 exposure in the neuroendocrine brain of male goldfish. I used two doses of EE2: a lower dose of 0.1 nM EE2 (30 ng/L) that was comparable to levels found in water systems in North America and Europe, and a higher dose of 1.0 nM EE2 (300 ng/L) that represented the highest concentration of EE2 that has been detected at the source of waste water treatment plants. We found that 1) gonad size of male fish were reduced in the high EE2 group, 2) sex steroids were decreased in both treatment groups, 3) the two doses showed largely different gene profiles in the hypothalamus, 4) aromatase expression, the enzyme that converts testosterone (T) into

E2, was induced in the high treatment group only, and 5) secretogranin-III (Sg-III) transcription, a gene important in the transport of neurotransmitters at the synapse, was modulated by EE2. In the final chapter, I compare these results to a study currently ongoing in collaboration with Ph.D. students Vicki Marlatt and Dapeng Zhang using silastic implants of E2 for a period of 1 and 7 days.

Hypotheses

1) GABA modulates the transcription of genes involved in GABA synaptic transmission (GABA transporters, GABA receptor subunits) and reproduction (activins, GnRH, ERα, etc.) (Chapters 4 and 5).

2) E2 modulates the transcription of genes involved in GABA synaptic transmission (Chapter 6).

3) E2 and GABA will regulate similar pathways in the CNS (i.e. cross-talk between GABA and E2 to control reproduction) (Chapter 4 and 6).

Chapter 1

GENERAL INTRODUCTION

1.1. The amino acid neurotransmitter GABA

GABA and glutamate are the two most abundant amino acid neurotransmitters in the vertebrate CNS. Approximately half of all synapses in the rat suprachiasmatic, arcuate, supraoptic, and paraventricular nuclei are immunoreactive for GABA (Decavel and van den Pol, 1990). GABA is considered to be the major inhibitory neurotransmitter in vertebrates whereas glutamate is considered to be the major stimulatory neurotransmitter. Included in the multiple roles of this abundant neurotransmitter, GABA plays a significant role in neuroendocrine function, for example, mediating the reproductive (Feleder et al., 1999; Trudeau et al., 2000c) and stress (Tizabi and Calogero, 1992) axis. However, despite the fact that GABA is largely involved in neuronal inhibition, studies have shown that GABA can also have an excitatory role in selective processes. In the mammalian neonatal brain, GABA has been shown to act as both an inhibitory and stimulatory neurotransmitter (Chen et al., 1996) and stimulates neuronal growth and migration (reviewed in Lujan et al., 2005). In the context of reproduction, GABA stimulates GnRH neurons in rats (Moenter and Defazio, 2005) and in teleost fish, GABA stimulates the release of LH from the pituitary during certain periods of the reproductive cycle (Sloley et al., 1992; Trudeau et al., 1993b). The role of GABA in teleost reproduction will be addressed in detail in section 1.2.

1.1.1. GABA metabolism

A general schematic of GABA synthesis and degradation is depicted in Figure 1.1. GABA is synthesized from glutamate by a single step reaction, achieved through the rate-limiting enzyme GAD. GAD exists throughout the major vertebrate taxa as two isoforms, GAD65 and GAD67. However, there has been a third GAD isoform (GAD3) identified in both the grenadier (Bosma et al., 1999) and goldfish (Larivière et al., 2002). The GAD isoforms are thought to have arisen by a genome duplication event prior to the branching of the teleost lineage approximately 400 million years ago and the presence of GAD3 in some vertebrates suggest that an ancestral GAD may have undergone two phases of gene duplication events (Larivière et al., 2002).

GABA is present in both neurons and glial cells of the CNS. In neurons of the rat, GAD65 is thought to be the predominant isoform responsible for synthesizing GABA that is released at the synaptic cleft (i.e. synaptic pool) and GAD67 is responsible for maintaining the GABA metabolic pool (Erlander and Tobin 1991; Martin and Rimvall 1993). To further support the hypothesis of differential roles of GAD65 and GAD67 in the vertebrate brain, each isoform shows differences in temporal and spatial expression (Esclapez et al., 1993, 1994; Maqueda et al., 2003) and regulation of transcription (Szabo et al., 2000; Bosma et al., 2001).

GABA is degraded by the enzyme GABA-T to succinic acid semialdehyde. Unlike the GAD isoforms, GABA-T is a mitochondrial gene involved in the GABA shunt, metabolizing excess GABA in both glial and neuronal cells. Further degradation by the enzyme succinic semi-aldehyde dehydrogenase (SSADH) forms succinate, an intermediate of the Krebs cycle and cellular respiration. A second pathway, the

ornithine-putrescine pathway, is also utilized to synthesize GABA. Ornithine, a metabolite produced during the urea cycle, is converted to putrescine by the rate-limiting enzyme ornithine decarboxylase. The ornithine-putrescine pathway has a prominent role in the synthesis of GABA in embryonic tissues when GAD65 and GAD67 are not yet present or detectable (Eliasson et al., 1997).

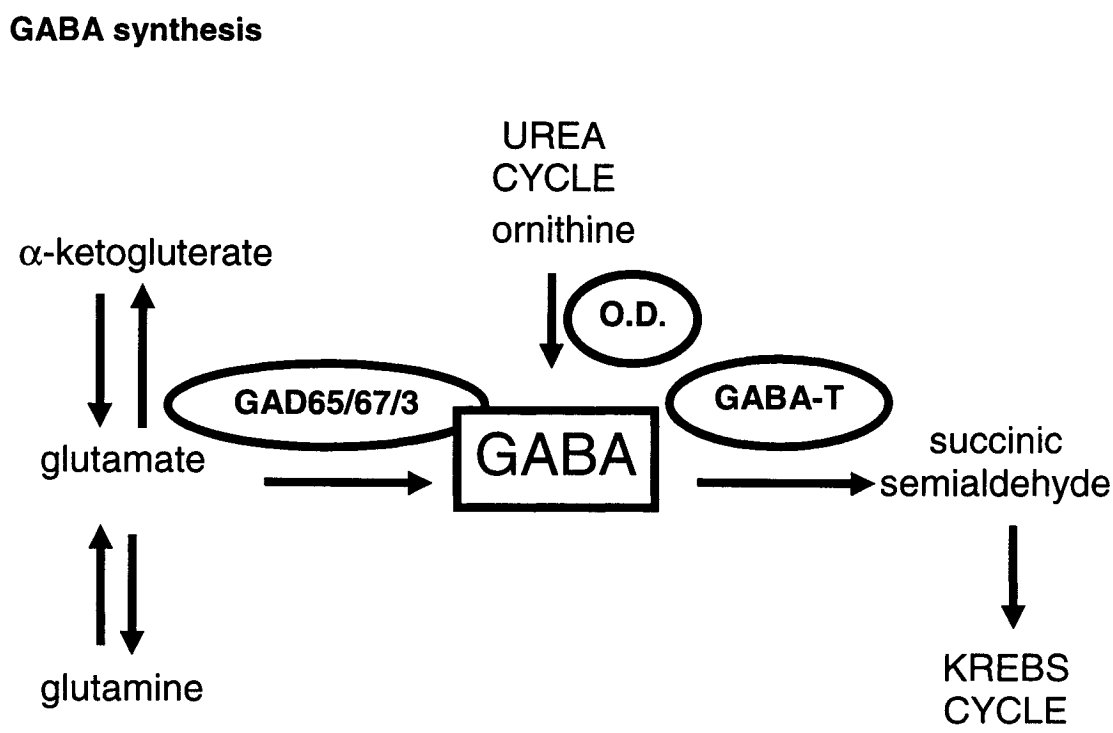


Figure 1.1. The major pathways of GABA synthesis and degradation. Enzymes with a significant role in GABA production and degradation are also depicted. GAD, glutamic acid decarboxylase; GABA-T, GABA-transaminase; OD, ornithine decarboxylase.

1.1.2. GABAergic signalling through membrane bound receptors

GABAergic signalling is via membrane bound receptors. To date, there are three known GABA receptors (designated A, B, and C) in the vertebrate CNS. The predominant model for the ionotropic $GABA_A$ receptor is a ligand gated, pentameric receptor which form a Cl^- channel composed of various subunits (α, β, γ, δ, ε, θ, π) (reviewed in Macdonald and Olsen, 1994; Costa et al., 2002). The $GABA_A$ receptor contains allosteric regulatory binding sites for psychotropic drugs (e.g. benzodiazepines, ethanol, and barbiturates) that modulate receptor function and convulsant drugs (e.g. picrotoxin) that inhibit receptor activity. Some neurosteroids and anaesthetics can also bind to the $GABA_A$ receptor to alter GABA-mediated transmission (Maitra and Reynolds, 1998).

Similar to the $GABA_A$ receptors, the $GABA_C$ receptors form Cl^- channels. However, these receptors form homo-oligomers containing a single family of subunit (ρ subunits) (Bormann and Feigenspan, 1995; Koulen et al., 1998). $GABA_C$ receptors show a more limited distribution in the CNS compared to the other GABA receptor subtypes and are predominantly localized to the retina and spinal cord (Koulen et al., 1998). The ionotropic $GABA_A$ and $GABA_C$ receptors are involved in rapid GABA synaptic transmission.

The $GABA_B$ receptor is a metabotropic receptor that is coupled to downstream Ca^{2+} and K^+ channels by G-proteins and second messengers, therefore they are slower acting (Bormann, 1988; Kardos et al., 1994). The variation in sensitivity and function of GABA receptors is primarily dependent upon the subtype composition (Whiting et al., 1997). Chapter 3 provides additional details on GABA receptor structure and evolution .

1.1.3. GABA Transporters

Plasma membrane transporters modulate the action of a neurotransmitter through neurotransmitter reuptake by the presynaptic axon and surrounding glial processes. Thus, the number and efficiency of membrane transporters will determine the duration a neurotransmitter is retained in the extracellular synapse. GABA transporters (GAT) are Na^+/Cl^--dependent membrane transporters responsible for the uptake and distribution of GABA. Four members of the GAT transporter family have been identified from mammalian tissues, each characterized by different tissue distribution and pharmacology. In the rat, GAT-1 is predominantly found in neurons and astrocytes of the cerebral cortex and preoptic area (Minelli et al., 1995a). GAT-2 is predominantly expressed in non-neuronal ependymal cells and the cortical parenchyma while GAT-3 is found in the cortex and is exclusively expressed in distal astrocytic processes (Conti et al., 2004). There is evidence that astrocytes expressing GAT-3 are in close physical proximity to GABAergic axons and this plays an important role in the termination and regulation of synaptic transmission (Minelli et al., 1995b). The fourth GABA transporter, a betaine-glycine transporter, is less well studied and is pharmacologically different than the other GAT transporters. Figure 1.2. depicts a generalized GABAergic synapse in the CNS.

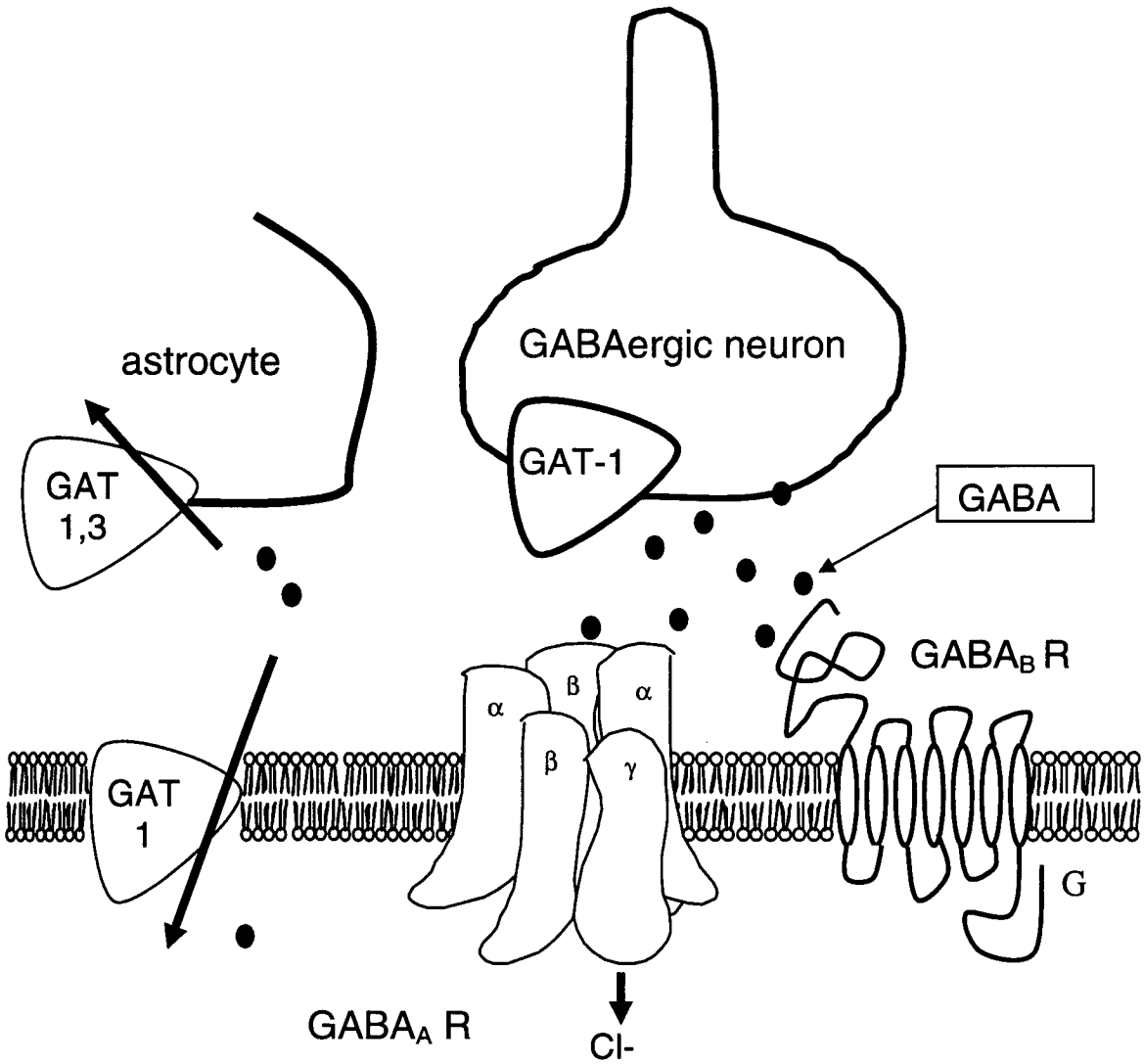


Figure 1.2. Generalized schematic depicting a GABAergic synapse depicting both ionotropic (GABA-A) and metabotropic (GABA-B) receptors as well as GABA transporters (GAT 1 and 3).

1.2. GABA and reproduction

1.2.1. Neuroendorine control of reproduction

The control of reproduction in vertebrates involves the integration of neural and hormonal systems at multiple molecular and physiological levels. The decapeptide GnRH directly stimulates the release of LH from the anterior pituitary. In fish, LH is also known as GTH-II but in this thesis, GTH-II will be referred to as LH. LH release in fish is influenced by a number of other neuropeptides and amines (reviewed in Trudeau, 1997). For example, norepinephrine (NE) has a slight stimulatory effect on LH release in sexually regressed goldfish and cholecystokinin (CCK) has a stimulatory effect on LH release in sexually mature goldfish. Other factors that stimulate LH release include serotonin (5 hydroxytryptamine; 5HT) and neuropeptide Y (NPY) (Trudeau, 1997) whereas the catecholamine dopamine (DA) inhibits LH release. The main target of LH is the gonad where it stimulates gametogenesis and steroidogeneis. Sex steroids that are produced, for example E2 and T, feedback to the CNS and pituitary to mediate reproduction. It should be mentioned that goldfish, the model teleost used for this thesis, are seasonal reproducers and are sexually inactive or regressed between early June and September until late October–early November when gonadal development begins. Goldfish have the highest concentration of E2 and T and are sexually mature in late March to early May.

Teleost fish provide an ideal model to study vertebrate neuroendocrine function, specifically the effects of GABA on reproductive processes. Bony fish lack the hypothalamo-pituitary portal system and because of this, the anterior pituitary is directly innervated by the hypothalamic neurons that synthesize neuropeptides and

neurotransmitters, including GABA (Peter et al. 1990; Trudeau 1997). This direct innervation of the anterior pituitary is thought to be functionally equivalent to the median eminence in mammals. The anterior pituitary of the goldfish and other teleost fish is also different from that of mammals because it shows regionalized cellular distribution (Kah et al., 1992). This means that gonadotrophs, somatotrophs, and other cells are grouped in specific regions of the anterior pituitary.

1.2.2. GABA also stimulates reproduction in teleosts

GABA stimulates reproduction by affecting the release of LH from the anterior pituitary (Figure 1.3.). GABA-stimulated LH release has been shown in teleost fish such as rainbow trout (Mañanos et al. 1999), Atlantic croaker (Joy et al., 1999), common carp (Roelants et al. 1990), and the goldfish (Sloley et al., 1992; Kah et al., 1992; Trudeau et al., 1993b, 1997). Interperitonial (i.p.) injections of GABA into sexually regressed or recrudescent (i.e. seasonal gonadal re-growth) goldfish results in a significant increase in LH (Kah et al., 1992). Furthermore, *in vivo* i.p. injections of γ-vinyl glutamate (GVG), a reversable inhibitor of GABA-T that increases GABA concentration in the brain, results in significant increases in serum LH in early stages of gonadal development (Sloley et al., 1992; Trudeau et al., 1993b). This effect on pituitary LH release is seasonally dependent and GABA is inhibitory at different stages of sexual maturation (Khan and Thomas 1999). GABA appears to have its largest influence on LH release in the early stages of gonadal development (Kah et al. 1992; Trudeau et al. 1993b).

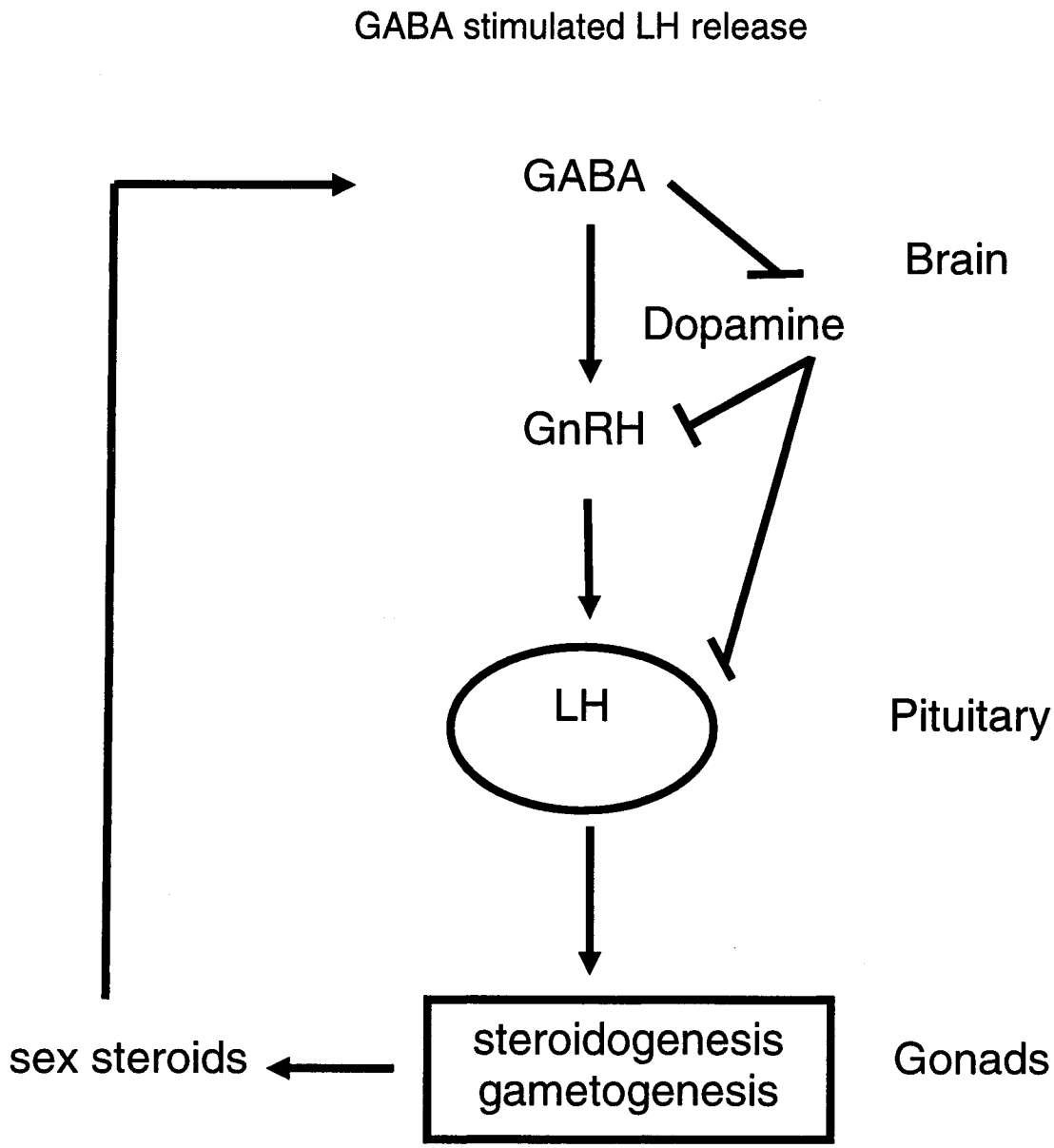
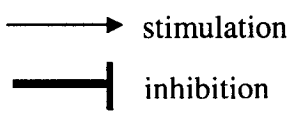


Figure 1.3. A general overview depicting the role of GABA in the reproductive hypothalamic-pituitary-gonadal axis. GABA stimulates GnRH release, a potent stimulator of LH. GABA will also inhibit dopamine to enhance LH release. LH stimulates steriodogenesis and gametogenesis and sex steroids produced in the gonads feedback to the hypothalamus and pituitary to modulate the HPG axis.



1.2.3. Mechanism of GABA stimulated LH release

It is thought that GABA stimulated LH release is achieved indirectly through both the stimulation of GnRH and the inhibition of DA (Figure 1.3) (Trudeau et al., 1993b,d). In the goldfish, there are two GnRH isoforms, sGnRH and chicken GnRH-II (cGnRH-II) (Rosenblum et al., 1994). The GnRH isoforms, products of separate genes, show differential expression and are under different regulatory control in the CNS. The actions of GABA on LH release are hypothesized to be via GnRH release, enhanced activity of GnRH, and, to a lesser extent, directly on gonadotrophs (Trudeau et al. 2000c). DA will inhibit GnRH release by the DA type–1 receptor and LH release by the DA type-2 receptor. GABA also stimulates the release of GnRH through the inhibition of the catecholamine DA, which inhibits hypothalamic GnRH release (Kah et al. 1992; Trudeau et al. 1993d, 1997). This inhibition may involve direct mechanisms through the DA type 2-like receptors (Peter et al. 1986) or by indirect mechanisms through the inhibition of hypothalamic GnRH neurons into the pituitary (Yu and Peter 1990; Yu et al. 1991). However, current evidence suggests that the main mechanism for GnRH release is through GABA stimulation because DA antagonists and DA synthesis inhibitors have been shown not to block, but actually enhance, GnRH release (Trudeau et al. 1993b,d).

1.3. Sex steroid feedback on the GABA system in teleosts

Sex steroids feedback to both the hypothalamus and pituitary to regulate the process of sexual maturation and reproduction. In general, T potentiates the stimulatory effect of GABA stimulated LH release and E2 inhibits GABA stimulated LH release during early gonadal development in the goldfish (Kah et al., 1992; Trudeau et al.,

1993a,c). E2 inhibition on LH release is most likely achieved by increased dopaminergic inhibition of GABA and GnRH neurons, although the mechanism by which E2 decreases the stimulatory effects of GABA on LH release are not yet clear. Sex steroids can modulate GABA action in the goldfish forebrain by differential temporal (e.g. reproductive state) and spatial (e.g. tissue localization) expression of biosynthesis/degradation enzymes, receptor subunits, and transporter proteins. Bosma et al. (2001) investigated the expression of GAD65 and GAD67 mRNA in the sexually regressed goldfish hypothalamus and telencephalon after sex steroid treatment. The expression of both GAD65 and GAD67 in the male goldfish decreased significantly in response to either E2 or T whereas only GAD65 responded to steroid treatment in females. Furthermore, this effect was tissue specific in that the decrease of GAD65 and GAD67 in males was only observed in the hypothalamus whereas the increase of GAD65 in females was observed in both tissues studied. Larivière et al. (2005) showed recently that T and progesterone (P4) decrease GAD65 and GAD67 mRNA in the telencephalon of sexually mature male goldfish. These studies provide evidence that the sex steroid feedback on GABA involves modulation of GAD that is dependent upon the reproductive state of the animal. E2 can also influence the mRNA expression of both GABA receptors (Herbison and Fénelon, 1995) and GATs (Herbison et al., 1995) in mammals. This has yet to be shown in teleost fish but remains a plausible mechanism for the modulation of GABA stimulated LH release by sex steroids.

Chapter 2

Distribution of GAD and GABA-T mRNA in the brain of the goldfish

2.1. Introduction

GABA is one of the most abundant and widespread inhibitory neurotransmitters in the vertebrate brain. GABAergic projections heavily innervate the basal ganglia leading to the thalamus and the cerebral cortex. GABA is also abundant in the globus pallidus and is implicated in the etiology of Parkinson's disease (Maneuf et al., 1994) and the cerebellum contains a large number of GABA producing cells that project to the motor cortex.

GABA plays a significant role in the neuroendocrine control of reproduction. Previous quantitative analysis indicates that half (49%) of all synapses in the rat suprachiasmatic, arcuate, supraoptic, and paraventricular nuclei of the rat hypothalamus are immunoreactive for GABA (Decavel and van den Pol, 1990). GABA has both inhibitory and stimulatory effects on vertebrate reproduction by modulation of pituitary luteinizing hormone (LH) release through multiple mechanisms (Trudeau et al., 2000c; Jackson and Kuehl, 2002). GABA has a predominant stimulatory effect on LH release in post-spawning goldfish (Kah al., 1992; Trudeau et al., 1993b) and trout (Mañanos et al., 1999). In Atlantic croaker, stimulatory and inhibitory effects of GABA have been observed on LH release post-spawning (Khan and Thomas, 1999). Moreover, the effects of GABA on LH release vary with stage of sexual maturation and GABA synthesis is a target for sex steroid feedback in mammals and fish (Herbison, 1998; Trudeau et al., 1993a, 2000c).

GABA is synthesized from glutamate by a single step reaction, achieved through the rate-limiting enzyme GAD. GAD exists throughout the vertebrate taxa as two major isoforms, GAD65 and GAD67, thought to have arisen by a genome duplication event prior to the emergence of cartilaginous fishes approximately 450 million years ago (Larivière et al., 2002). There exists other GAD-like cDNAs in fish (Larivière et al., 2002) and genome queries reveal additional GAD-like sequences in mammals (Trudeau, V., unpublished). However, whether these are true glutamate decarboxylases remains unknown because their full length coding sequences and functions have not been determined. The GAD65 and GAD67 isoforms are the products of two independently regulated genes that show differences in structure, cellular distribution and dependence upon the cofactor, pyridoxal 5' phosphate or PLP (Kaufman et al., 1991). In addition, knockout studies in mice suggest that the GAD isoforms have different functions (Asada et al., 1997; Kash et al. 1997). In GABAergic cells, GAD65 protein is more highly localized to nerve terminals and is associated with the GABA synaptic pool whereas GAD67 is more evenly distributed throughout the cell and primarily synthesizes the GABA metabolic pool. Esclapez et al. (1993, 1994) reported that GAD67 mRNA labelling appeared more robust than GAD65 mRNA labelling in Purkinje and stellate cells of the cerebellar cortex and in the olfactory bulb of rats. While the differential distribution of GAD65 and GAD67 mRNAs and proteins are relatively well described in some mammalian models, there are no detailed reports for the distributions of both GAD mRNAs in adult non-mammalian brains, although there is one report on GAD expression in embryonic zebrafish (Martin et al., 1998). The first study in adult fish by Anglade et al. (1999) reported distribution of GAD65 but the *in situ* probe was relatively short and

directed against the most conserved part of the trout GADs. While it is likely that it was specific for GAD65, some cross-reactivity with GAD67 mRNA can not be discounted. The central distribution of GAD in the lamprey *Petromyzon marinus* has been described (Reed et al., 2002; Root et al., 2005) but agnathans have a single highly divergent form of GAD (Larivière et al., 2002) and thus it is difficult to compare to gnathostome species with multiple GADs (i.e., all other vertebrates). Other immunocytochemical localization studies in lungfish (Trabucchi et al., 2000) and frogs (Tonon et al, 1992) also do not distinguish between the different GAD forms. Thus, given the abundance and importance of GABA and the lack of data on the differential distribution of GAD65 and GAD67 in non-mammalian vertebrates, we investigated this in the adult goldfish brain.

Of major importance in the control of GABAergic neurotransmission is the degradation of GABA to succinic semialdehyde by GABA-T and subsequent actions of SSDH to produce succinate. Unlike the GAD isoforms, GABA-T is encoded by a single mitochondrial gene and is present on the inner mitochondrial membranes of both neuronal and glial cells (Bedoya et al., 1988). Using reverse northern blots, Jeon et al. (2000) reported that GABA-T is not only highly expressed throughout the human brain, for example, in the cerebral cortex and hypothalamic regions, but is detectable in peripheral organs, such as the liver and kidneys. Sherif et al. (1991) reported that the activity of GABA-T is sexually dimorphic in human and rat brain but it is unknown whether the expression of GABA-T mRNA is regulated differentially throughout the reproductive cycle. Male rats appeared to have a higher activity of GABA-T than females. However, GABA-T is physiologically relevant to reproduction. A single injection of the antiepileptic vigabatrin (a GABA-T inhibitor; γ-vinyl-GABA) increases

GABA levels in the hypothalamus provokes prolonged release of LH and elevates serum testosterone levels in male goldfish (Sloley al., 1994). To our knowledge there are no reports on the distribution of GABA-T mRNA in the vertebrate brain. Moreover, the spatial relationship between GAD and GABA-T distributions is to date unknown.

This study describes the localization of GAD65, GAD67, and GABA-T mRNA in the forebrain and midbrain of the goldfish using non-radioactive in situ hybridization. Data are presented for the telencephalon and hypothalamus, two tissues important for behaviour and neuroendocrine control in teleost fish. We also report on the distribution of GAD65, GAD67, and GABA-T mRNA in the midbrain tegmentum, optic tectum, and cerebellum and compare total enzyme activity of GAD and GABA-T in different brain regions. This study is the first to report a significant difference in the expression of GAD mRNA in the nucleus recessus posterioris of the hypothalamus, with GAD65 mRNA staining more pronounced than GAD67.

2.2. Materials and Methods

2.2.1. Experimental animals

Common goldfish were obtained from a commercial supplier (Aleong's International Inc, Mississauga, ON, Canada) in April 2003 and allowed to acclimatize over several months to 18 °C under a natural stimulated photoperiod. Fish were fed and maintained on standard flaked goldfish food. Handling and sample protocols were done as per the animal care protocol. Before dissection of brain, goldfish were anesthetised using MS222 (3-aminobenzoic acid ethyl ester). Tissue for in situ hybridization (ISH) was obtained from 17 goldfish (7 males; 10 females).

2.2.2. Cloning of goldfish GABA-T probe sequence: first strand cDNA synthesis

Common goldfish were anesthetized and whole brain rapidly dissected and frozen on dry ice. Whole brains were homogenized on ice and total RNA extracted using TRIzol reagent® (Invitrogen Life Technologies, Carlsbad, CA, USA) as per the manufacturer's protocol. First-strand cDNA synthesis was done with approximately 3 µg of mRNA from goldfish whole brain in a reaction tube containing 1 µl oligonucleotide dT (Invitrogen) in a final volume of 10 µl DEPC treated water. The reaction components were heated to 70 °C for 10 minutes and quickly chilled on ice. After a brief centrifugation, 4 µl 5X reaction buffer, 2 µl 0.1M DTT, 1 µl 10mM dNTPs, and 1 µl RNAse inhibitor (Promega, Madison, WI, USA) was added, gently mixed, and heated at 42 °C for 2 minutes. 1 µl Superscript (Invitrogen) was added and the reaction was allowed to continue at 42 °C for 50 minutes. The reaction was inactivated at 70 °C for 15 minutes and stored at –20 °C.

2.2.3. GABA-T degenerate primer design, polymerase chain reaction (PCR), cloning, and sequencing

Mammalian and non-mammalian nucleic acid sequence information for GABA-T was obtained from the National Center for Biotechnology Information (NCBI) and aligned using ClustalW (EMBL-EBI; http://www.ebi.ac.uk/clustalw/). Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design gene specific or degenerate primers. Primer sequences were as follows; GABA-T F' (5' to 3') ATC CTG CCA CCT GAG AAC TT and GABA-T R' (5' to 3') CCC AKC CAS GTG

TTG AAG AT. PCR amplification was done using 1-2 µl cDNA template in 36.3 µl PCR water, 5 µl 10X PCR reaction buffer, 1.5 µl $MgCl_2$ (50mM), 1 µl dNTPs (10mM), 2 µl forward and reverse primers (10mM), and 0.2 µl Taq® Polymerase (Invitrogen). Initial denaturation occurred at 95 °C for 4 minutes followed by 35 cycles at 95 °C for 30 seconds, 56 °C for 45 seconds, and 72 °C for 1 minute. Amplification product was ligated directly into TOPO® II vector (Invitrogen). One Shot TOP 10® chemically competent *E. coli* were transformed. Plasmids containing amplicon were purified with the Wizard® Plus SV Minipreps DNA Purification System (Promega). Sequencing was done at Canadian Molecular Research Services (CMRS; Ottawa, Canada).

2.2.4. cRNA probe synthesis

Partial sequences for GAD65 and GAD67 were obtained from the following primers; GAD65 F' (5' to 3') TGCCAGCCAATGATCTCC; GAD65 R' (5' to 3') GCTGGGTTTCGATTCAGC; GAD67 F' (5' to 3') CAGACAGCTCCAGGTTGAA; GAD67 R' (5' to 3') GGAGATTATCCTGTCGCCTTT. GAD amplicons were subcloned into the TOPO® II vector (Invitogen) and used to synthesize probes using SP6 and T7 promoters. The GAD65 riboprobe was 800 bp (bp position 131-931; AF045594), GAD67 riboprobe was 680 bp (bp 57 – 737; AF149833), and the GABA-T riboprobe was 754 bp (DQ287923). ClustalW alignment of the partial gene sequences used to synthesize riboprobes revealed that there was 14 % nucleotide sequence similarity between GAD65 and GAD67. Both sense and antisense riboprobes were synthesized using the TOPO-GAD transcription vector. Approximately 1 µg linearized template was incubated at 37 °C in 4 µl 5x transcription buffer, 2 µl of digoxygenin RNA labelling mix

(Roche, Indianapolis, IN USA), 1 µl RNAsin (Promega), and 2.5 µl SP6 or T7 enzyme (Invitrogen) to a final volume adjusted to 22 µl with DEPC water for 2 hours. The synthesized RNA probes were precipitated at –20 °C overnight in 2.5 µl 4M LiCl and 75 µl cold 100% ethanol. Riboprobes were centrifuged at 12,000 RPM for 15 minutes and resuspended in 70% ethanol. The pellet was spun again at 12,000 RPM for 5 minutes and dried for an additional 5 minutes before being resuspended in 40 µl DEPC water. The concentration of the digoxygenin labeled riboprobes was evaluated using a reverse dot blot and the size of the synthesized riboprobes checked on a denaturing 1% agarose gel.

2.2.5. Tissue Preparation and ISH

Goldfish were anesthetized with MS222 and either perfused with Ringer's solution followed by 4% paraformaldehyde/0.1M saline phosphate buffer (PBS; pH=7.4) or the whole brain was rapidly dissected and placed in an ice cold RNAase-free solution of 4% paraformaldehyde/0.1M PBS solution containing 20% sucrose for 1 hour. All brains were further fixed in RNAase-free 4% paraformaldehyde/0.1M PBS solution containing 20% sucrose overnight. Whole brains were washed in 0.1M PBS (2 x 5 minutes) before being embedded in cryoprotectant O.C.T. and frozen at –20 °C. Transverse and sagittal sections (30-50 µm) were cut on a cryostat at -20 °C and collected in succession onto PermaFrost® Plus slides (VWR, West Chester, PA USA).

Tissue sections were removed from -20 °C and allowed to adhere to the slides at room temperature for approximately 15 minutes. Sections were post-fixed in 4% paraformaldehyde/1X PBS solution for 5 minutes. After washing (3 x 10 minutes) in 1X PBS buffer containing 0.1% Tween® 20 (Sigma, Oakville, ONT CAN) (1X PBST

buffer), sections were treated with proteinase K (20 μg/ml) in PBST or 50 mM Tris buffer with 0.5 M EDTA for 15 minutes at room temperature. Sections were washed (2 x 10 minutes) in PBST to stop the reaction, refixed in 4% paraformaldehyde/1X PBS (1x 5 minutes), and rinsed again in 1X PBST (2 x 10 minutes). Sections were quickly dipped in DEPC and left to dry for up to 30 minutes at room temperature.

Hybridizations were performed in a hybridization chamber at 58 °C in hybridization buffer, 0.3 ng/μl Dig-labeled sense or antisense GAD and GABA-T riboprobes, 250 μg/ml Poly A RNA, and 250 μg/ml salmon sperm. Hybridization buffer (100 μl) consisted of 50% deionized formamide, 5X hybridization salts (20X stock consisted of 3 M NaCl, 100mM EDTA, and 100mM Pipes; pH = 6.8), 1X Denhardt's solution, 10% SDS, and 5% dextran sulphate. Slides were covered with Parafilm® and left to hybridize for approximately 16 hours (overnight). Sections were washed the following day in 20X SSC (3 x 10 minutes) at 60 °C, 0.2X SSC (3 x 10 minutes) at 60 °C, 50% 0.2X SSC and 50% 0.1M PBST (1 x 10 minutes) at room temperature, and 0.1M PBST (2 x 10 minutes).

Following washes, sections were processed for the immunohistochemical detection of the digoxigenin label. Sections were incubated in a pre-incubation buffer for 2 hours to reduce non-specific binding. The buffer consisted of 1% sheep serum, 2 mg/ml bovine serum albumin, 0.3% Triton X-100 (Sigma) in 0.1M PBS (approximately 200 ul/slide). The slices were then incubated in the same buffer with anti-digoxigenin FAB fragments-alkaline phosphatase (sheep, Roche) diluted 1/1000 in the buffer. The sections were covered and incubated overnight at 4 °C. Sections were washed the following day in 0.1M PBST buffer (3 x 10 minutes) at room temperature and quickly

rinsed in DEPC. Slices were then pre-incubated in colouration buffer (2 x 5 minutes) at room temperature. The colouration buffer consisted of 100mM Tris buffer pH=9.5, 50mM $MgCl_2$, 100mM NaCl, and 0.1% Tween® 20 in sterile water (approximately 300 µl/slide). Slices were then incubated in the same colouration buffer with 3.5 µl/ml of BCIP stock solution (Roche) and 4.5 µl/ml of NBT stock solution for 2-3 hours or until colour was sufficient at room temperature. The reaction was stopped with distilled water and the sections were washed in 0.1M PBS buffer (3 x 10 minutes). Tissue was quickly refixed in 4% paraformaldehyde/1x PBS for storage, slides were dried, and coverslipped with Cryoseal® (VWR).

2.2.6. GAD and GABA-T enzymatic activity

The telencephalon and hypothalamus of female goldfish (n=10) were dissected into anterior, posterior, medial, and lateral sections. Tissue was collected and pooled (2-3 individuals/sample). Similarly, the midbrain region, optic tectum, cerebellum, hindbrain, and whole brain were dissected and measured for enzyme activity.

GAD activity was measured using a radiometric method previously described by Snedden et al. (1996), with modifications that are published in Sardana et al. (2006). The production of radiolabelled CO_2 was used to determine the activity of total GAD (Figure 2.1.). GABA-T activity for each sample was measured in triplicate in a high throughput spectrophotometric assay (modified from Jung et al. 1977) using 96-well plates. Samples were first homogenized in 10 mM dipotassium hydrogen orthophosphate, K_2HPO_4 (pH 6.8) buffer containing 20 % glycerol, 0.13 % Triton-X 100, 0.1 mM glutathione, 0.1 mM pyridoxal-5'-phosphate and 1 mM disodium EDTA. Samples were centrifuged at 2000 *g*

for 20 minutes at 4 °C and the supernatant was used as the enzyme source. Each well contained 40 μL of homogenate (buffer blanks) and 180 μL of incubation medium (100 mM potassium pyrophosphate, 5 mM α-ketogluarate, 4 mM nicotinamide adenine dinucleotide (NAD), 3.5 mM 2-mercaptoethanol and 0.01 mM pyridoxal phosphate). The plate was pre-incubated for 15 minutes at 30 °C. 10 mM (20 μL of 115 mM) GABA (H_2O blanks) was then added and incubated again for 15 minutes. The rate of the reaction was determined by measuring NADH production (Figure 2.1.) at 340 nm for 10 minutes within the linear range. The GABA-T inhibitor γ-vinyl-GABA completely blocked this reaction, confirming our previous observations on goldfish GABA-T both *in vivo* (Trudeau et al., 1993b; Fraser et al., 2002) and *in vitro* (Trudeau et al., 2000c).

2.3. Results

2.3.1. Cloning goldfish GABA-T

A gene fragment of 754 bp for GABA-T was cloned using degenerate primers designed from both fish (zebrafish) and mammalian sequences (rat; human). This gene fragment had 87% nucleotide similarity with zebrafish GABA-T (BC045433) and 81% nucleotide similarity with human GABA-T (NM_000663) based on information in the NCBI database. This fragment represented approximately 1/3 of the coding region of this mitochondrial gene based on the zebrafish GABA-T sequence (2190 bp). The deduced amino acid is 92% identical to zebrafish GABA-T and 78% identical to the human GABA-T.

2.3.2. GAD65, GAD67, and GABA-T mRNA distributions

Following sectioning, adjacent brain sections were hybridized with GAD65, GAD67, and GABA-T riboprobes. The following tissue distribution description of the GAD isoforms and GABA-T proceeds from the rostral telencephalic region to the cerebellum. Nomenclature of brain regions follows that of Peter and Gill (1975). Staining for enzyme mRNA was observed in both male and female sexually regressed goldfish. Hybridizations with sense riboprobes resulted in the absence of staining for all three enzymes. Figure 2.2. is a composite diagram of mRNA expression of GAD and GABA-T, showing the major sites of GABA synthesis and degradation in the goldfish brain.

2.3.3. Telencephalic Regions and Preoptic Area

Both GAD65 and GAD67 positive cells were detected in all areas of the telencephalon and showed a similar distribution pattern (Figure 2.2.A,B). Figure 2.3.A shows the distribution of GAD67 mRNA in the telencephalic region. I detected a dense population of both GAD65 and GAD67 positive cells in the area ventralis telencephali pars ventralis (Vv), pars supracommissuralis (Vs) and pars distalis (Vd). The majority of GAD labelling in the telencephalon was located medially, along the anterior commissure and was more sparse in the area ventralis telencephali pars lateralis (Vl). Sporadic staining of GAD65 and GAD67 positive cells was also detected in the area dorsalis telencepali pars dorsalis (Dd) in addition to the area dorsalis telencephali pars medialis (Dm) and lateralis (Dl). Similarly, the areas dorsalis telencephali pars centralis (Dc) and lateralis (Dl) exhibited sparse to low staining of GAD isoforms. GABA-T positive cells

were also detected throughout the telencephalon (Figure 2.3.B). Unlike the GAD isoforms, GABA-T mRNA appeared to be more uniformly distributed in both the ventral and dorsal regions of the telencephalon.

The preoptic area of the brain contained heavy staining for GAD65, GAD67 and GABA-T (Figure 2.2.C,D). GAD65, 67, and GABA-T mRNA was detected heavily in the nucleus preopticus periventricularis (NPP) and nucleus preopticus (NPO), densely situated around the anterior commissure (AC). Staining became more diffuse moving away from the medial ventricles. Staining was absent for GAD65, GAD67, and GABA-T in the optic chiasma. The anterioris periventricularis (NAP_V), which contains the suprachiasmatic and ventromedial nuclei, also contained GAD65, and GAD67 positive cells. No difference in expression or abundance was apparent in the preoptic area between the GAD isoforms or GABA-T.

2.3.4. Hypothalamus and Inferior Lobe

GAD65, GAD67, and GABA-T positive cells were present throughout the hypothalamus, being detected in all areas in the anterior to posterior regions. Staining for GAD65 appeared strong in the nucleus recessus posterioris (NRP) of the hypothalamus, in contrast to GAD67 which showed limited staining in that region (Figure 2.4.A.B). GABA-T showed moderate staining in the NRP (Figure 2.4.C). In general, heavy staining for the GADs and GABA-T was observed around the nucleus recessus lateralis (NRL) with staining becoming more scattered in the dorsal areas of the hypothalamus and pons inferior lobe (Figure 2.2.E.F, 2.4.A-C). Staining for riboprobes became more diffuse moving away from the NRL into the nucleus diffusus lobi inferioris (NDLI)

(Figure 2.5.A.B.). Scattered GAD and GABA-T positive cells were also detected in the nucleus anterioris hypothalami (NAH), nucleus lateral tuberis (NLT (i) and (p)), nucleus anterior tuberis (NAT), and nucleus preglomerulosus pars lateralis (NPGI).

2.3.5. Midbrain, Optic Tectum, and Cerebellum

I detected moderate staining of the GADs in the area surrounding the interpeduncular nucleus (Figure 2.6.A) and in the torus longitudinalis (Figure 2.6.B). GAD65 and GAD67 were detected in both the anterior and posterior regions of the cerebellum, and cells appeared more diffuse when compared to the optic tectum and hypothalamic regions. Large Purkinje cells and Golgi type II cells in the granule cell layer of the cerebellum were stained for both GAD65 and GAD67. This study detected no staining in the molecular layer of the cerebellum for either GAD isoform or GABA-T.

The third layer of the periventricular grey zone (PGZ) in the optic tectum contained heavily stained cells for all riboprobes (Figure 2.2.G). Figure 2.7.A and 2.7.B show the staining for GAD65 and GABA-T respectively. Large GAD positive cell bodies were stained in the peripheral region but were sparsely distributed when compared to the PGZ. Lighter staining for the GADs and GABA-T mRNA was present in the central zone and the outer dorsal layer of the optic tectum. Staining for the GADs and GABA-T mRNA in the midbrain tegmentum was more sparse than in the hypothalamus and telencephalon.

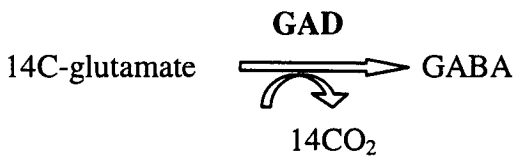
I would like to point out that differences in tissue expression of vertebrate GAD isoforms in previous studies, as well as the present study, are dependent upon a number of factors including sex, season, and age of the individual. In addition, technical

limitations, such as probe concentration and length can influence the signal intensity. The authors direct the reader to Maki et al. (1997) for more details about the technical limitations of using the digoxygenin-labelling system.

2.3.6.. Total GAD and GABA-T Enzyme Activity

Enzyme activities for GAD and GABA-T were detected in all brain regions examined. Variation in specific enzyme activity was high among pools of individuals. Total GAD activity was moderate in the telencephalon and hypothalamic regions when compared to the other brain regions examined (Figure 2.8.A). The optic tectum contained the highest activity of total GAD when compared to other brain regions. Total GAD activity was lowest in the cerebellum. GABA-T activity was also variable between brain regions. GABA-T was higher in the anterior region of the telencephalon when compared to the posterior region (Figure 2.8.B). There was no notable difference in GABA-T activity in different regions of the hypothalamus. Similar to total GAD activity, GABA-T was high in the optic tectum and low in the cerebellum.

A)



B)

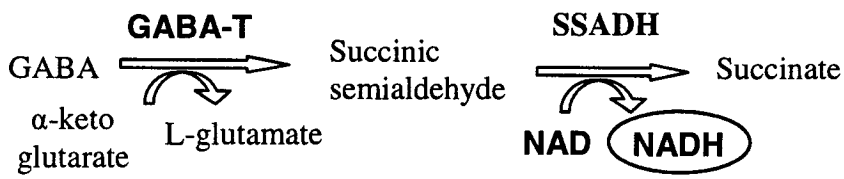
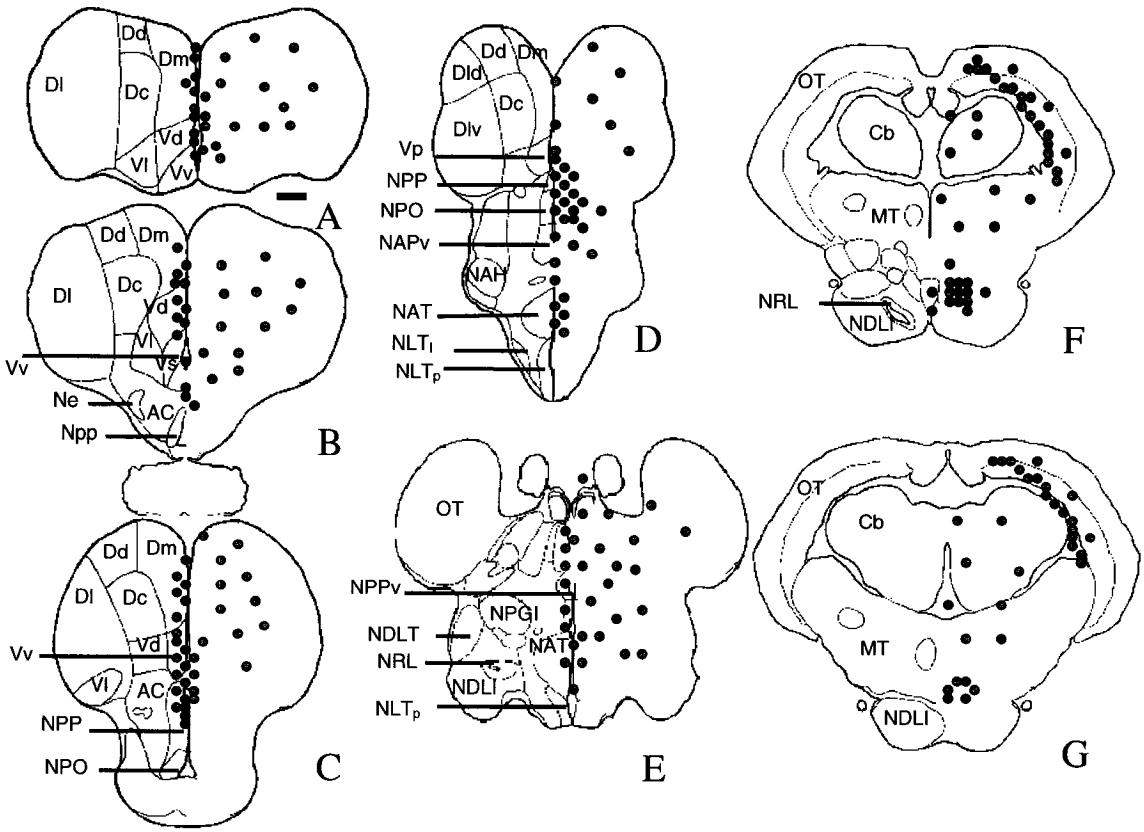


Figure 2.1. Total GAD activity is measured from the production of radiolabelled CO_2 (A) and GABA-T activity is measured by the production of NADH (absorbance of 340 nm) (B).

Figure 2.2. Composite summary of the distribution of GAD and GABA-T mRNA expression in the goldfish brain, moving from anterior to posterior regions. Dots represent the intensity of staining in brain areas. Intervals between sections (mm) are as follows; A-B (0.9); B-C (0.7); C-D (0.4); D-E (0.4); E-F (0.5); F-G (0.6). Scale (0.2 mm) shown in A. Figures not drawn to scale. AC, anterior commissure; Dc, area dorsalis telencephali pars centralis; Dl, area dorsalis telencephali pars lateralis; Dm, area dorsalis telencephali pars medialis; MT, midbrain tegmentum; NAH, nucleus anterioris hypothalami; NAT, nucleus anterior tuberis; NAP_V, nucleus anterioris periventricularis NDLI, nucleus diffuses lobi inferioris; NLT (i) nucleus lateral tuberis pars inferioris; NLT (p) nucleus lateral tuberis pars posterioris; NPGI, nucleus preglomerulosus pars lateralis; NPO, nucleus preopticus; NPP, nucleus preopticus periventricularis; NRL, nucleus recessive lateralis; NVM, nucleus ventromedialis thalami; Vd, ventralis telencephali pars distalis; Vl, ventralis telencephali pars lateralis; Vs, area ventralis telencephali pars supracommissuralis; Vv, area ventralis telencephali pars ventralis.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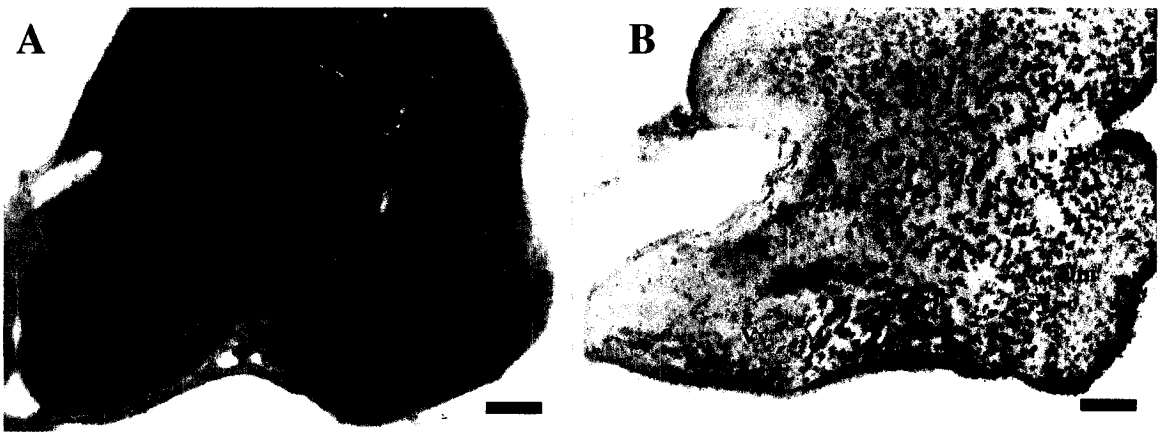


Figure 2.3. Transverse section of the telencephalon of A) GAD67 and B) GABA-T. The majority of GAD positive cells in the telencephalon were located medially, along the anterior commissure and were more sparse in the area ventralis telencephali pars lateralis (Vl). Sporatic staining of GAD positive cells was also detected in the area dorsalis telencepali pars dorsalis (Dd) in addition to the area dorsalis telencephali pars medialis (Dm) and lateralis (Dl). Scale bar represents 100 μM.

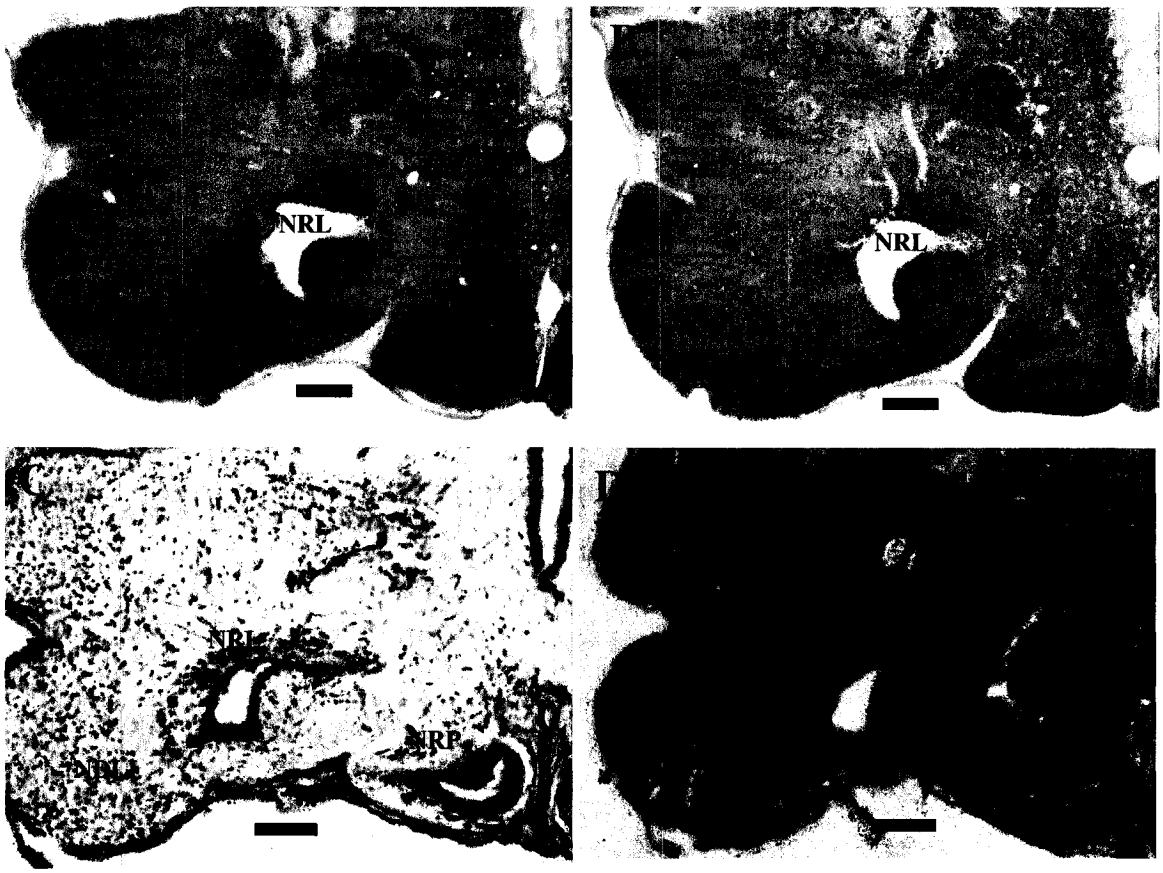


Figure 2.4. Transverse section of the inferior lobe of the hypothalamus of A) GAD65 B) GAD67 and C) GABA-T. Note the heavy staining around the nucleus recessive lateralis (NRL). Staining for riboprobes becomes more scattered in the nucleus diffusus lobi inferioris (NDLI). GAD65 positive cells (A) are more dense than GAD67 positive cells (B) in the nucleus recessus posterioris (NRP). Panel D is the sense probe for GABA-T showing no specific staining. Scale bar represents 100 μM.

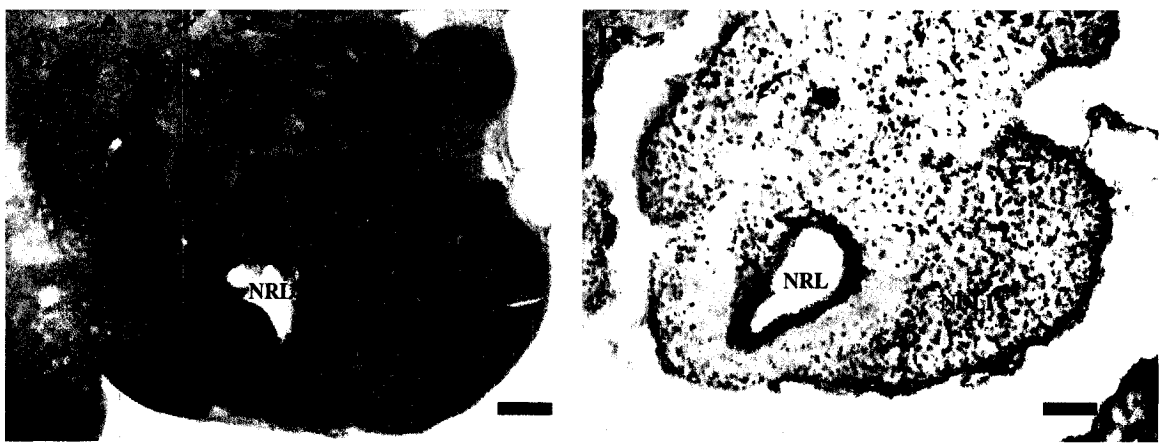


Figure 2.5. Transverse section of the inferior lobe of the hypothalamus of A) GAD65 and B) GABA-T. Scale bar represents 100 μM.

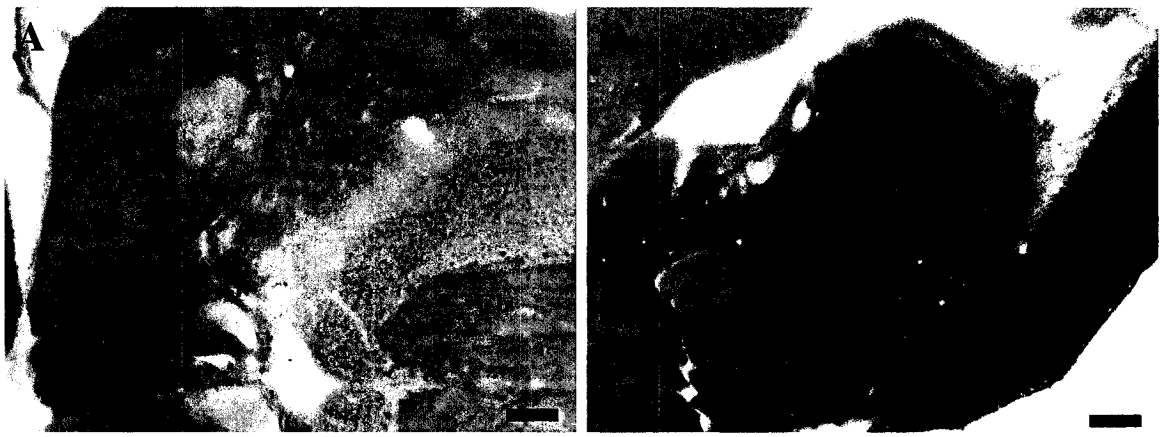


Figure 2.6. Transverse sections in the midbrain and posterior regions of the brain of GAD65 positive cells showing the A) interpeduncular nucleus (IP) and cerebellum (Cb) and B) torus longitudinalis (TL) and optic tectum (OT). Scale bar represents 100 μM.

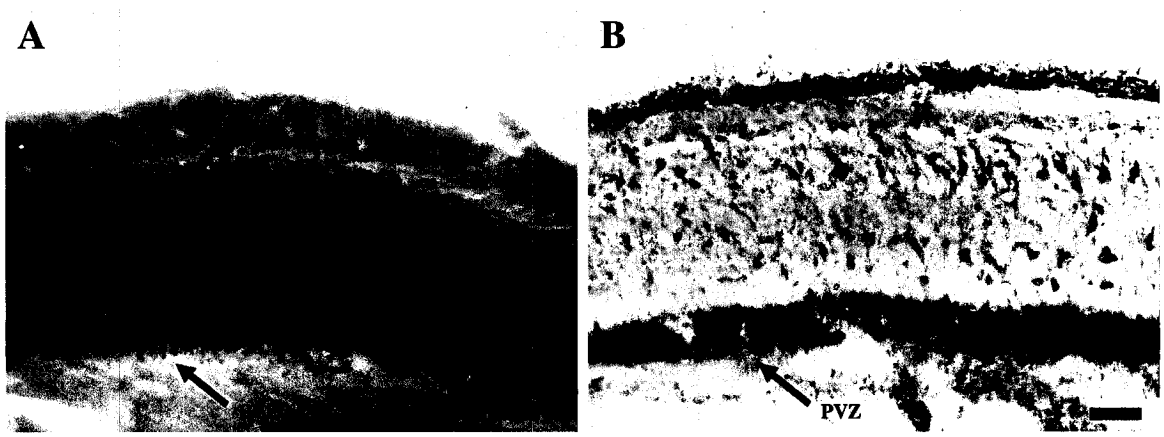


Figure 2.7. Transverse section through the optic tectum showing the distribution of A) GAD65 and B) GABA-T. Note the strong labeling for enzyme mRNA in the third layer of the periventricular grey zone (PGZ) of the optic tectum. Scale bar represents 25 μM.

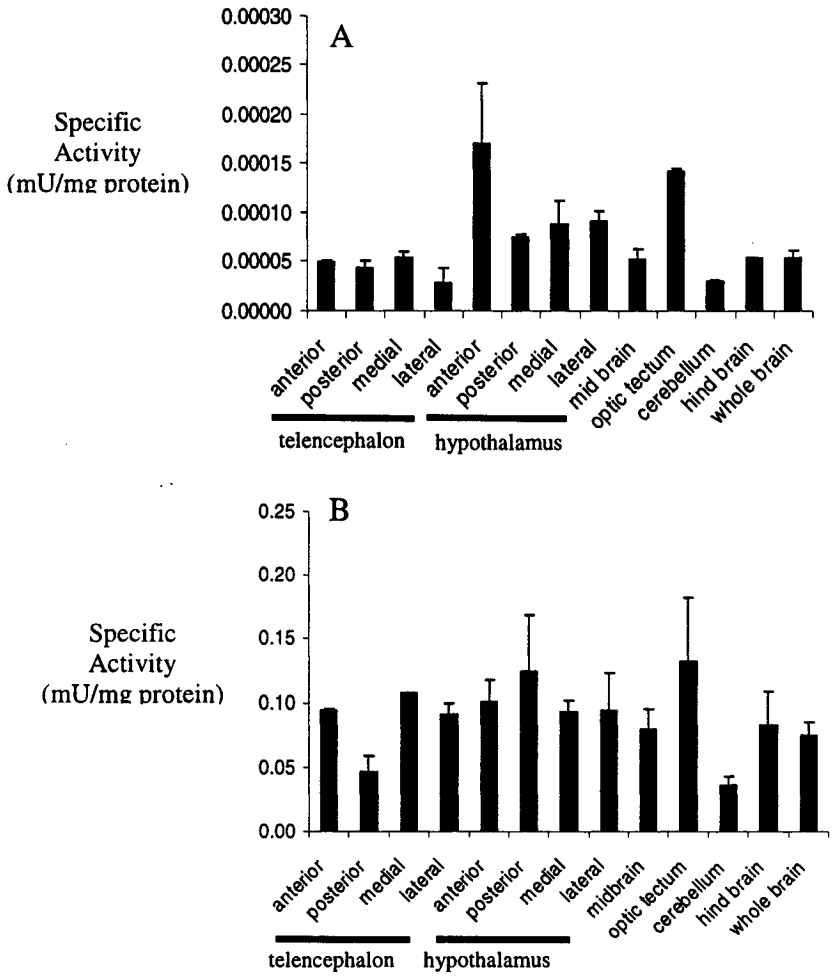


Figure 2.8. Specific activity of A) total GAD and B) GABA-T in the goldfish brain, as determined by the radiometric and spectrophotometric enzyme assays. Each bar represents the mean of 2-3 pooled samples ± standard error. mU = nmoles/minute.

2.4. Discussion

I provide the first detailed description in a non-mammal vertebrate of the distribution of mRNA encoding enzymes involved in GABA synthesis (GADs) and degradation (GABA-T) in the forebrain and midbrain. This study found that GAD65, GAD67, and GABA-T positive cells were ubiquitous throughout the brain and showed overlapping distributions. Previous studies in adult teleost fishes report an abundance of GABAergic neurons in the telencephalon and hypothalamus (Anglade et al., 1999; Trabucchi et al., 2000). Ekström and Ohlin (1995) showed that in embryonic three spined stickleback (*Gasterosteus aculeatus L.*), GABA immunoreactive neurons are first detected after 51 hours post-fertilization in the ventral regions of the forebrain and after 72 hours, GABA positive cells are detected in preoptic areas as well as the dorsal and caudal regions of the hypothalamus. GABA positive cells appear in the cerebellum and optic tectum after 124 hours of development. In the zebrafish, GAD65 and GAD67 mRNA is detectable 24 hours post-hatch, each sharing a similar distribution in the ventral telencephalon and midbrain region (Martin et al., 1998).

2.4.1. GAD expression in the goldfish forebrain

In the telencephalon, the majority of GAD positive cells were located along the anterior commissure. In the goldfish, Martinoli et al. (1990) reported an abundance of GABAir cells found along the medial fissure, with a reduction of GABAir cells moving towards the posterior lobes of the telencephalon. In the rainbow trout (*Oncorhynchus mykiss*), Anglade et al. (1999) showed very similar GAD expression in the telencephalon and hypothalamus using a 270 bp riboprobe with 90% sequence to GAD65. This

includes the Vv and Vd of the telencephalon, an area that receives afferent connections from regions such as the olfactory bulb, dorsal telencephalic area, suprachiasmatic nuclei, and periventricular nucleus and sends efferent connections to regions such as the preoptic region, paracommissural nucleus, posterior dorsal thalamus, tuberal hypothalamus and interpeduncular nucleus (Rink and Wullimann, 2004).

Teleost fishes are different from mammals because they lack a portal blood system and there is direct innervation of the anterior pituitary (Peter et al., 1990). The POA, located in the teleost telencephalon, and the mediobasal hypothalamus are major sites for neuroendocrine control (Anglade et al., 1993; Kah et al., 1993). This morphological feature provides a unique opportunity to study the effect of direct neural inputs on hypophysiotropic processes. Morphological studies also show that GABAergic nerve fibres are abundant in the anterior pituitary and directly innervate gonadotrophs and somatotrophs in the pars distalis of the pituitary; GABA respectively stimulates and inhibits the release of LH and growth hormone from these cells (Kah et al., 1987; Kah et al., 1992; Trudeau et al. 2000b). In the telencephalon, there was heavy staining of GAD in the pars ventralis. Moreover, using electrical stimulation and patch-clamp electrophysiology, we have shown a direct monosynaptic GABAergic projection from Vs-Vv to the ventral preoptic region, a neuroendocrine area containing GnRH and dopamine neurons that innervate the pituitary (Trudeau et al., 2000c). Electrolytic lesions in the Vs-Vv region impairs reproductive behavior in male goldfish by blocking the initiation of spawning and spawning consistency in these animals was negatively correlated with the volume of Vs-Vv destruction (Kyle and Peter, 1982). This impairment may be a result of a loss of GABAergic input to the POA.

The close association of GABA and GnRH neurons in the mammalian and fish brain is well known (Gonzalez-Martinez et al., 2001; Amano et al., 2002; Peter et al, 2003). Additionally, GnRH and GABA neurons have been co-localized in areas surrounding the third ventricle in the sea lamprey (*Petromyzon marinus*) (Reed et al., 2002; Root et al., 2005). GABA stimulates the release of GnRH from nerve terminals in isolated goldfish pituitary fragments (Kah et al., 1992) and also from preoptic anterior hypothalamus tissue slices in red seabream (*Pagrus major*) (Senthilkumaran et al., 2001). Injection of the GABA metabolism inhibitor γ-vinyl-GABA raises brain GABA and stimulates LH release in association with a decrease in tissue sGnRH levels, which was interpreted as increased GnRH release (Sloley et al., 1994). However, I have also shown using a similar experimental approach that increasing GABA levels in the goldfish brain does not significantly modulate GnRH mRNA expression in the telencephalon or hypothalamus (Chapter 4; Martyniuk et al., 2005). Thus, GABA may be important for the regulation of release rather than synthesis of GnRH in fish. An important question remaining is the relative importance of GAD65 versus GAD67 in the synthesis of GABA in neuroendocrine neurons regulating LH release. Previous work has partially addressed this question. Neurochemical depletion of DA, the main inhibitor of LH, release leads to increased goldfish hypothalamic GABA synthesis which is correlated with increase GAD67 mRNA but not GAD65 mRNA levels. Now that GAD distributions are established, it may be possible to use somatic gene transfer to inhibit the GADs (Martres et al., 1998; Trudeau et al., 2005) by local injection of isoform specific GAD antisense constructs and determine the effects on LH release.

2.4.2. GAD65 mRNA staining is more abundant than GAD67 in the nucleus recessus posterioris recessus posterioris

We observed that the NRP of the posterior hypothalamus consistently showed a stronger signal for GAD65 staining than for GAD67 staining. Since GAD65 preferentially localizes to axon terminals rather than cell bodies, the high relative levels of GAD65 in these neurons may indicate a corresponding tendency of the enzyme to be located in the synaptic terminals of these neurons rather than in the perikaryon. There is good evidence that the NRL and NRP of the hypothalamus are regions important for the control of reproduction in teleost fishes. For example, in the male catfish, there are relatively high levels of aromatase activity, the enzyme that facilitates local production of neuroestrogen in the NRL and surrounding areas (Timmers et al., 1987). Recently, Hawkins et al. (2005) report in the Atlantic croaker that there are significant differences in estrogen receptor (ERs) isoform expression in the forebrain. For example, ER-beta b, but not ER-beta a (previously called ER gamma), labelling is detected in the magnocellular neurons of the POA, nucleus posterior tuberis (NPT), the NRP and cerebellum. Dopamine-producing cells are also located in areas high in GAD and GABA-T staining. Dopamine is the major inhibitory neurotransmitter in the teleost brain and is a potent inhibitor of LH release (reviewed in Trudeau et al., 2000). Dopamine-immunoreactive cell bodies are present in hypothalamic nuclei of teleosts such as the the NPP, the NLT, the NRL and NRP (Corio et al., 1991), suggesting that there is a close association between GABA and dopamine producing cells in these regions. The medial POA, an area important for sexual behaviour and neuroendocrine function, receives axonal projections from neurons in areas such as the NPP, NRL and NRP as shown in

hime salmon (*Oncorhynchus nerka*) (Shiga et al., 1985). These regions, especially the NPP (both GAD isoforms) and the NRP (GAD65) are high in GAD positive cells. Sex steroids are known to influence GAD transcription in the goldfish (Larivière et al., 2005) and whether GAD65 expression is predominantly driven by the ER-beta b in the NRP is not known. The interaction between the GADs, ERs, and dependence upon local neuroestrogen synthesis in the NRL and NRP warrants further investigation.

This study is the first to report a difference in GAD expression in the vertebrate NRP. However, differences in the regional distribution of the two GADs in other brain areas have been reported previously. In the developing zebrafish embryo, the level of GAD65 mRNA in the nucleus of the medial longitudinal fasciculus was reported to be higher than GAD67 transcripts (Martin et al., 1998). There was also a complete lack of GAD65 positive cells in the rostral hindbrain of embryonic zebrafish which showed staining for GAD67 during this period. Esclapez et al. (1993, 1994) reported that GAD67 mRNA labelling appeared more robust than GAD65 mRNA labelling in Purkinje and stellate cells of the cerebellar cortex and in the olfactory bulb of rats.

2.4.3. GAD expression in the goldfish midbrain and hindbrain

I detected staining in the Purkinje cells and Golgi II cells of the granule layer of the cerebellum. Wuenschell et al. (1986) reported similar staining in the mouse cerebellum with a GAD riboprobe, with staining localized to Purkinje, Golgi II, stellate, and basket neurons. In the present study, total GAD activity was lowest in the cerebellum when compared to other brain tissues, for example, the optic tectum.

In mouse, the cerebellum also appears to contain a lower amount of GAD activity compared to other brain regions (Sheikh et al., 1999). Staining for GAD65 and GAD67 mRNA was more abundant and dense in the forebrain areas compared with the hindbrain. In the telencephalon of the armed grenadier *Coryphaenoides (Nematonurus) armatus,* Trudeau et al. (2000a) report that the expression of both GAD65 and GAD67 mRNA were approximately 2-fold higher when compared to the cerebellum.

GABA producing cells in the optic tectum are extremely abundant and are located predominately in the periventricular grey zone. This has been reported previously in the rainbow trout (Anglade et al., 1999). This study showed that the optic tectum had a high total GAD activity, suggestive of a correlation between GAD mRNA expression and protein activity. Indeed, previous studies observed high correlations between specific GAD mRNAs and *in vivo* GABA synthesis rates in goldfish brain (Hibbert et al., 2004; 2005).

2.4.4. GABA-T expression in the goldfish brain

GABA-T mRNA was detected in all areas of the goldfish brain investigated, including the telencephalon, preoptic area, midbrain regions, optic tectum, and cerebellum. These regions were also high in GAD65 and GAD67 staining. Jeon et al. (2000) detected high levels of GABA-T mRNA with Northern dot-blot analysis in homologous regions in the human brain such as the cerebral cortex, hypothalamus, and cerebellum. GABA-T is localized to the inner membrane of the mitochondria and is a major enzyme degrading GABA to the metabolic precursor succinic semialdehyde, which is further metabolized to succinate by SSDH. Immunohistochemical localization of

GABA-T protein in the rat neostriatum showed that GABA-T protein is localized to subpopulations of neurons as well as astrocytes within the neocortex (Yung et al., 1998). This study detected GABA-T positive cells in regions that contain GABA producing neurons, for example, in the preoptic area and hypothalamus. The regulation of GABA-T expression during development and the reproductive cycle in vertebrates is currently not well understood. GABA-T mRNA is reported to be lower in fetal humans compared to adults, suggesting that GABA-T expression is modulated throughout development (Jeon et al., 2000). Low expression of GABA-T appears to be important in ensuring high levels of GABA during brain development. Sherif et al. (1991) reported that male rats had a higher GABA-T activity than female rats. I did not compare male and female goldfish in GABA-T enzyme activity but showed that GABA-T activity appeared lowest in the cerebellum when compared to other brain regions.

2.4.5. Conclusion

GAD65, GAD67, and GABA-T mRNA are prominent in ventral areas of the telencephalon, preoptic area, and nuclei of the hypothalamus. The overall pattern of GAD65 and GAD67 mRNA staining generally overlap. Each GAD isoform synthesizes GABA but their differential distributions and regulation points to specific function of these two highly related genes. GAD knockout studies in mice reveal that mice without GAD65 are susceptible to seizures and mice lacking GAD67 have a cleft palate and do not survive past the first morning of birth (Asada et al., 1997). Thus, the relative contribution of GAD65 and GAD67 to the hypophysiotropic control of LH release and sexual behaviour in teleosts remains to be studied.

Chapter 3

Phylogenetic classification of $GABA_A$ receptor subunits in the goldfish and early evolution of GABA receptor subunits

Adapted from: Christopher J. Martyniuk, Guy Drouin, Stéphane Aris-Brosou, Joel Cahn, and Vance L. Trudeau. 2006. Early evolution of ionotropic GABA receptors and positive selection of the mammalian-specific theta and epsilon subunits (*in prep*)

3.1. Introduction

Gene duplication followed by gene divergence is one of the major genetic mechanisms underlying the evolution of vertebrates. One particular class of proteins which has undergone such a divergence are cationic (e.g., acetylcholine, serotonin) and anionic (e.g., glycine, γ-aminobutyric acid) ligand-gated channels, estimated to have occurred before the origin of eukaryotes. Gene duplication is a mechanism that can generate the new gene sequences necessary for the evolution of the more complex and specialized central nervous system (CNS) of vertebrates. Therefore, understanding the evolutionary history of ligand-gated receptors may provide insight into their structural and functional complexity in the central nervous system.

GABA is the major inhibitory neurotransmitter found in the vertebrate brain and is involved in CNS development and organization (Kellogg, 1998), neuroendocrine function (Arvat et al., 2002), and neural processes such as learning and memory (Myhrer, 2003). GABA is also present in the nervous system of non-vertebrate taxa, for example, flatworms (Eriksson and Panula, 1994), arthropods (Orona et al., 1990; Darlison, 1992) and early chordates (Anadon et al., 1998). GABA synaptic transmission is achieved through membrane bound postsynaptic receptors. Currently, there are three major classes of GABA receptors identified in the mammalian CNS (designated A, B, and C) and each

receptor has unique characteristics based on the composition of the receptor, pharmacology, and localization. The ionotropic $GABA_A$ receptors are ligand-gated Cl^- channels consisting of several high abundance receptor subunits (α1-6, β1-4, γ1-3, δ) and low abundance subunits (ε, θ, and π) (Whiting, 1999). Changes in GABA receptor subunit abundance and in receptor composition have been linked to differences in receptor sensitivity and responsiveness (Rabow et al., 1995; Costa et al., 2002). The current structural model of the $GABA_A$ receptor is a pentameric receptor containing two binding sites for the ligand GABA and a separate binding site for receptor modulation by benzodiazepines, neurosteroids, ethanol, and barbiturates (Bormann, 2000). The metabotropic $GABA_B$ receptors are members of the seven transmembrane domain family and are coupled to downstream Ca^{2+} and K^+ channels via G-proteins. GABAc receptors are ionotropic Cl^- channels that show similar membrane topology as the $GABA_A$ receptors. However, GABAc receptors have unique functional and electrophysiological characteristics, including a slower Cl^- conductance and insensitivity to bicuculline and other $GABA_A$ modulators (Bormann and Feigenspan, 1995; Bormann, 2000). The ionotropic GABAc receptors are composed of ρ subunits that are highly expressed in the vertebrate retina and preferentially localized to bipolar cells (Koulen et al., 1997) but are also found in the spinal cord and pituitary (Johnston, 2002). Both spatial and temporal regulation of GABA receptor subunit expression provides functional diversity to the GABA receptor family.

Previous phylogenetic analyses show that ionotropic GABA receptor subunits form two monophyletic groups, the (α, γ, and ε) and (ρ, β, δ, θ, and π) clades (Ortells and Lunt, 1995; Xue, 1998). The ancestral $GABA_A$ receptor gene cluster is hypothesized to

have been a β-α-γ subunit motif that expanded in the genome by a tandem duplication event, followed by translocation of the GABAA receptor gene cluster within the genome (Russek, 1999; Darlison et al., 2005). Evidence for this is observed in the human (*Homo sapiens*) genome, where GABA receptor subunits are organized into discrete GABAA receptor gene clusters located on different chromosomes (4, 5, 15, and the X chromosome) which show conserved gene order and orientation (Russek, 1999). However, Escriva et al. (2002) suggest that two major genome duplications, before and after the divergence of the agnathan and the gnathostome lineages, have contributed largely to the presence of duplicated genes and gene families, although there have been extra gene duplications and gene loss throughout evolution. The GABAc ρ subunits are separated from the GABAA gene clusters suggesting a different ancestral origin than the GABAA receptor family (Cutting et al., 1992). The distinct physiology, kinetics, and relatively modest amounts of genetic variation in GABA receptor subunit families provide an interesting multigene family to study the evolutionary history of ion channels.

The aforementioned studies on the evolution of ionotropic GABA receptor subunits largely focused on mammalian sequences. Due to gene duplication events in teleost fish (Taylor et al., 2001) and high nucleotide sequence similarity between teleost GABA receptor subunits, I needed to identify cloned goldfish GABAA receptor subunit transcripts for Chapter 4 by phylogenetic analysis. This study was able to further examine the phylogenetic relationship of ionotropic GABA receptor subunits by accessing newly available genome databases, i.e., those of the sea squirt *Ciona intestinalis* and the pufferfish *Takifugu rubripes*. In this chapter, I cloned goldfish sequences, studied the phylogenetic relationship between various ionotropic GABA

receptor subunit families, and tested whether these different families are evolving at similar rates.

3.2. Materials and Methods

3.2.1. Genome Database Searches

Homologous gene searches were performed on the National Center for Biotechnology Information (NCBI; *http://www.ncbi.nlm.nih.gov*) server. BLASTN and BLASTP searches (Altschul et al., 1990) were used to find homologous genes of full length GABA receptor subunits from rat (GABA α1-6, β1-3, γ1-3, δ, ε, θ, and π) present in the completed genome project deposited in GenBank (August, 2005). The *Ciona intestinalis* sequences were obtained from the Joint Genome Institute at http://genome.jgipsf.org/ ciona4/ciona4.home.html.

3.2.2. Teleost GABA subunit receptor phylogeny

The cloning of goldfish $GABA_A$ receptor subunits is described in detail in Chapter 4 (section 4.2.3.) along with cloning of other genes involved in GABA synaptic transmission. $GABA_A$ receptor subunit amino acid sequences were aligned in DAMBE (Data Analysis in Molecular Biology and Evolution) (Xia and Xie, 2001) using ClustalW and phylogenetic trees generated using the neighbor-joining method. Bootstrap sampling was used with 100 iterations. This phylogeny included only goldfish $GABA_A$ receptor subunits sequences and $GABA_A$ receptor subunits from pufferfish, mouse *Mus musculus*, and chicken *Gallus gallus*. Goldfish $GABA_A$ receptor amino acid sequences (ranging from 100-130 amino acids) were too short to be informative in the following amino acid

sequence analyses of invertebrate and vertebrate GABAA receptor subunits and were excluded from subsequent phylogenetic analyses and the analysis of differential selection pressures among receptor subunits.

3.2.3. GABA receptor phylogeny

The GABAA receptor subunit phylogenetic analysis and evaluation of evolutionary rates among subunits were performed in collaboration with Drs. Guy Drouin and Stéphane Aris-Brosou. In order to study the phylogenetic relationships of the different GABA receptors, we obtained and aligned a data set of 81 homologous protein sequences of this receptor. This data set is composed almost exclusively of complete protein sequences and the alignment contains 953 aligned amino acid positions. To test the stability of these analyses with respect to benzodiazepine (BZ) specificity of the GABA receptor, and to determine the phylogenetic relationships of sea squirt and teleost sequences, we increased the species sampling of receptors with or without benzodiazepine binding sites. Here we follow the convention of Xue (1998) for simplicity and describe the two major clades previously described by Xue (1998) as BZ+, describing the majority of subunits that form a benzodiazepine binding site (α, γ, ε) and BZ-, describing the majority of subunits that are not involved in benzodiazepine binding (ρ, β, δ, θ, and π) of the $GABA_A$ receptor. We point out that Whiting et al. (1997) showed that the $GABA_A$ receptors containing ε subunits are benzodiazepine insensitive. We sampled 55 chordate species including sea squirt and vertebrates for the GABAA protein sequences containing benzodiazepine binding sites (1,007 aligned amino acid

positions) and 55 chordates species including sea squirt and vertebrate protein sequences which did not contain benzodiazepine binding sites (795 aligned amino acid positions).

Phylogenetic trees were inferred using either the maximum likelihood method as implemented in PHYML (Guindon and Gascuel, 2003) or the Bayesian approach as implemented in MrBayes (Ronquist and Huelsenbeck, 2003). In both approaches the inferred phylogeny of the 81-sequences data set was rooted with acetylcholine receptor protein sequences (Ortells and Lunt, 1995; Xue, 1998). Based on the results from the analysis of the 81-sequences data set (Figure 3.2.), the benzodiazepine binding site data sets (Figures 3.3. and 3.4, respectively) were rooted with the lobster β1 (accession number AY098945). Maximum likelihood analyses assumed the JTT model of amino acid substitution (Jones et al., 1992), with among-site rate variation modeled by a discrete Γ distribution with eight rate categories (Yang, 1994). This maximum likelihood model was fitted independently to each data set.

Because the JTT model of substitution might not be the best fitting model to study GABA receptors, the maximum likelihood analyses were complemented by a Bayesian analysis. However, rather than selecting for an *a priori* empirical model of substitution, a reversible-jump Markov chain Monte Carlo (MCMC) was constructed to integrate over model uncertainty (Huelsenbeck et al., 2004). Models with equal prior probability were: Poisson, JTT, Dayhoff, MtREV, MtMAMM, WAG, rtREV, CpREV, VT and BLOSUM62 as described and implemented in MrBayes 3.11 (Ronquist and Huelsenbeck, 2003). Our estimate of the GABA receptor phylogeny is then in proportion of the posterior probability of each of the *a priori* models included in the analysis, and is therefore not dependent on one single model. Among-site rate variation was modeled

using a discrete Γ distribution with five rate categories (Yang, 1994) plus a class of invariable sites. Under this mixed model of protein evolution, four independent MCMC samplers were run for ten million steps using different starting values. To decrease autocorrelation of the samples taken from the target distribution, steps along the chain were sampled every 1,000 accepted steps, a method known as thinning (e.g., Yang, 2005). To improve mixing, each sampler consisted of four chains, three of which were heated to different temperatures (e.g., Yang, 2005). By raising the likelihood function to a power > 1, deep valleys of the likelihood surface become shallow, which facilitates their crossing by the sampler and hence improves the ability the program to explore the parameter space. Sampling was realized from the non-heated chain. Burn-in length and convergence of the samplers were checked by plotting time series plots and checking that the average standard deviation of split frequencies was lower than 0.01 (Ronquist and Huelsenbeck, 2003). The chains appeared to have converged by 10,000 steps; to be conservative, 100,000 steps were discarded as a burn-in.

3.2.4. Analysis of selective pressures

The analyses based on the protein data suggested that the θ and ε paralogs have undergone a period of accelerated evolution following the duplication event. Such periods of accelerated evolution could be due to an episode of diversifying selection affecting these branches. To test this hypothesis, we used a codon substitution model, which measures selective pressures by estimating the nonsynonymous to synonymous rate ratio. This ratio is denoted ω, with $\omega = 1$, < 1 and > 1 indicating neutral evolution, purifying or diversifying selection, respectively (Goldman and Yang, 1994). Codon data

were obtained and split into two smaller datasets, one for each of the paralogous clade of interest, θ and ε. Each dataset contained human (*Homo sapiens*), rat (*Rattus norvegicus*) and mouse (*Mus musculus*) copies of θ and ε paralogs, as well as the corresponding members of the group in which this clade was located: β for θ and γ for ε. Each tree was rooted by the closest human paralog (δ and α, respectively). A statistical approach was then used to detect functional divergence at individual codon sites within the pre-specified branches, based on a procedure similar to that by Bielawski and Yang (2004): For each paralog the null hypothesis (H_0) was that of no variable selective pressure among branches and among sites (Figure 3.5.). This hypothesis was contrasted by means of likelihood ratio tests against three potential alternative modes of evolution. In H_1, only the branch leading to the θ or to the ε clade was allowed to evolve at a different ω rate ratio. This branch is said to have a foreground rate, while all the other branches have the same background rate. However, the θ or the ε clade may evolve at a rate that differs from both the foreground and the background rates (H_2). Finally in H_3, we allowed each paralog to evolve at its own rate after duplication.

In some cases, only a few sites are affected by an episode of diversifying selection within a given branch. A more powerful approach to detecting sites undergoing diversifying selection in such a case is to allow ω to vary among sites within the branch of interest (Yang and Nielsen, 2002). In this case, the ω ratio of the sites in this branch followed a discrete distribution with three categories estimated as free parameters. To test for the specific existence of sites under variable selective pressures with this branch of interest, this model was tested against H_1 by means of a likelihood ratio test. All analyses used PAML version 3.14b (Yang, 1997).

3.3. Results

3.3.1. Goldfish GABA$_A$ receptor subunits

Phylogenetic analysis of goldfish GABA$_A$ receptor subunits identified the cloned gene sequences as the following goldfish GABA$_A$ receptor subunits; α2 (α1), β2, β4, γ1, and γ2 (Figure 3.1.). The regulation of these subunits by GABA was subsequently studied and is detailed in Chapter 4. It should be pointed out that the position and bootstrap values are not strongly supported for some goldfish sequences because of the short amino acid sequence used in the analysis. For example, goldfish GABAA α1 groups with GABAA α2 (bootstrap 39), however, other phylogenetic analysis using maximum likelihood estimation classifies this subunit as a GABAA α1 (data not shown).

3.3.2. GABA$_A$ Receptor Phylogeny

As previously reported by Ortells and Lunt (1995) and Xue (1998), our estimates of the GABA$_A$ receptor phylogeny also shows two major monophyletic clades (Figure 3.2.). This topology is robust as it was obtained by both a maximum likelihood and a Bayesian approach, the latter integrating over different substitution models. The phylogenetic tree of vertebrate sequences containing the GABA$_A$ α, γ, and ε subunits, shows that these receptor subunits do not result from non-orthologous gene displacement but appear to be derived from a common ancestor (Figures 3.2 and 3.3). The GABA$_A$ α subunits are divided into two strongly supported groups: A clade composed of GABA$_A$ α4 and α6 subunits and the other composed of GABA$_A$ α1-3 and α5 subunits. The GABA$_A$ γ1 and γ2 subunits form a strongly supported group, whereas the GABA$_A$ γ3 sequences group with the GABA$_A$ ε subunits. This suggests that the GABA$_A$ ε subunits

are derived from GABA$_A$ γ subunits. These results are consistent with those obtained from the analysis of the 81-sequences data set with lower taxon-sampling (Figure 3.2.). These results are in agreement with Darlinson et al. (2005) in that the GABA$_A$ γ4 subunit of chicken and the GABA$_A$ ε subunits found in mammals are orthologs.

The relationship of GABA receptor subunits without the presence of a benzodiazepine binding site, i.e., GABA$_A$ ρ, β, δ, θ, and π subunits, are quite different depending on whether invertebrate GABA/glycine-like receptor sequences are included or not in the analysis. With the invertebrate GABA/glycine-like receptor sequences included (81-sequences data set), the GABA$_A$ δ and π subunits are shown as being the sister group to the other GABA sequences in this clade (Figure 3.2. and 3.4.). In contrast, the tree estimated without the invertebrate GABA/glycine-like sequences (55-sequences data set) shows that the GABAc ρ subunits are the sister group to other receptor subunit families within this clade (Figure 3.4.). In both trees, the GABAc ρ subunits, the GABA$_A$ θ and GABA$_A$ β subunits, and the GABA$_A$ π and δ subunits, form three strongly supported groups (Figures 3.2 and 3.4.). However, these two phylogenies show different relationships with regards to the GABA$_A$ θ and GABA$_A$ β subunits. Whereas the phylogeny with invertebrate GABA/glycine-like sequences (Figure 3.2.) shows the GABA$_A$ β subunits to be paraphyletic, the phylogeny without invertebrate GABA/glycine-like sequences (Figure 3.4.) shows the GABA$_A$ β subunits to be monophyletic. Thus, evolutionary relationships of these groups cannot be confidently resolved with the sequences currently available and the phylogenetic reconstruction methods used.

3.3.3. Analysis of rates of evolution and of selective pressures

Results of the analyses of selective pressures are presented in Table 3.1. As expected from the phylogenetic reconstruction, the comparison of a null codon model where selective pressures are constant both along lineages and among sites (H_0) against a branch-specific ω or "free-ratio" model (denoted B in Table 3.1.) showed an extensive variation of the ω rate ratio among branches in both datasets.

Results from the analysis of the θ dataset suggested that the $GABA_A$ θ subunit were subjected to diversifying selection. Branches and clades are numbered following conventions set in Figure 3.5A. Allowing selective pressures to differ specifically in the branch 5 leading to this paralog (model H_1) did not significantly explain the data better (at the 1% level) than the constant ω rate ratio model (H_0). However, allowing a different rate in branch 5 and allowing ω to vary among sites within this branch (model H_1^{sites}) explained the data significantly better (Table 3.1.) and led to the identification of four sites potentially under positive selection with a posterior probability > 99%. The very large rate estimate for this class (ω_5 in Table 3.1.) suggests that this estimate is quite imprecise, as confirmed by an extensive exploration of the likelihood surface (not shown). Further analyses allowing ω to differ among branches leading to the β paralogs (models H_2 and H_3) are consistent with an episode of diversifying evolution leading to the θ paralogs, followed by a regime of negative selection after diversification of the vertebrates. To summarize, evidence for diversifying selection acting in this branch is strong, but the actual identification of the sites should be taken with caution due to the small sample size studied here.

Likewise, results from the ε dataset show some evidence that the evolution of the $GABA_A$ ε subunit was driven by diversifying selection after a gene duplication event. However, the results are more complicated in this case. Again, branches and clades are numbered following conventions in Figure 3.5B. As with the $GABA_A$ θ subunit, model H_1 is not significant, but allowing ω to vary among sites within branch 5 (model H_1^{sites}) significantly improved the fit of the model to the data. Although the rate estimate for this class (ω_5 in Table 3.1.) is > 1, a thorough exploration of the likelihood surface (not shown) proved the surface to be almost flat in this region of the parameter space. Two sites were assigned to this rate category of ω > 1 by the empirical Bayes procedure, but only with a posterior probability just above 50%. Note that these two sites are distinct of those identified in the θ subunit. However, the relatively long branch leading to the ε paralogs in the protein-data tree is unlikely to be due to relaxation of selective pressures in this branch. A three-ratio model allowing ω to be different in the branch leading to the ε subunit and within the ε subtree (model H_2) was significantly better that both H_0 and H_1 (Table 3.1.). Yet, the estimated branch-specific ratios suggest that strong negative selection was acting on the protein during most of branch 5 ($\omega_5 = 0.006$), and was later slightly relaxed in clade 4 ($\omega_4 = 0.207$). These estimates proved computationally stable. Results from models H_2 and H_3 further show that these rate estimates are, respectively, quite smaller (branch 5) or larger (clade 4) than the rates of the other clades (1, 2 and 3). Taken together, these results are consistent with a scenario where a short period of diversifying selection took place just after the duplication event leading to the pre-ε subunit. This pre-ε subunit conferred a substantial advantage and shifted under strong

diversifying selection that became relaxed after diversification of the vertebrates for some unknown reason.

Figure 3.1. Phylogenetic analysis of goldfish $GABA_A$ receptor subunit protein sequence. GABA receptor subunit notation is as follows; A (α), B (β), G (γ), D (δ), E (ε), T (θ), P (π). Bootstrap values are indicated at the nodes and the scale bar represents 1 amino acid substitutions per site.

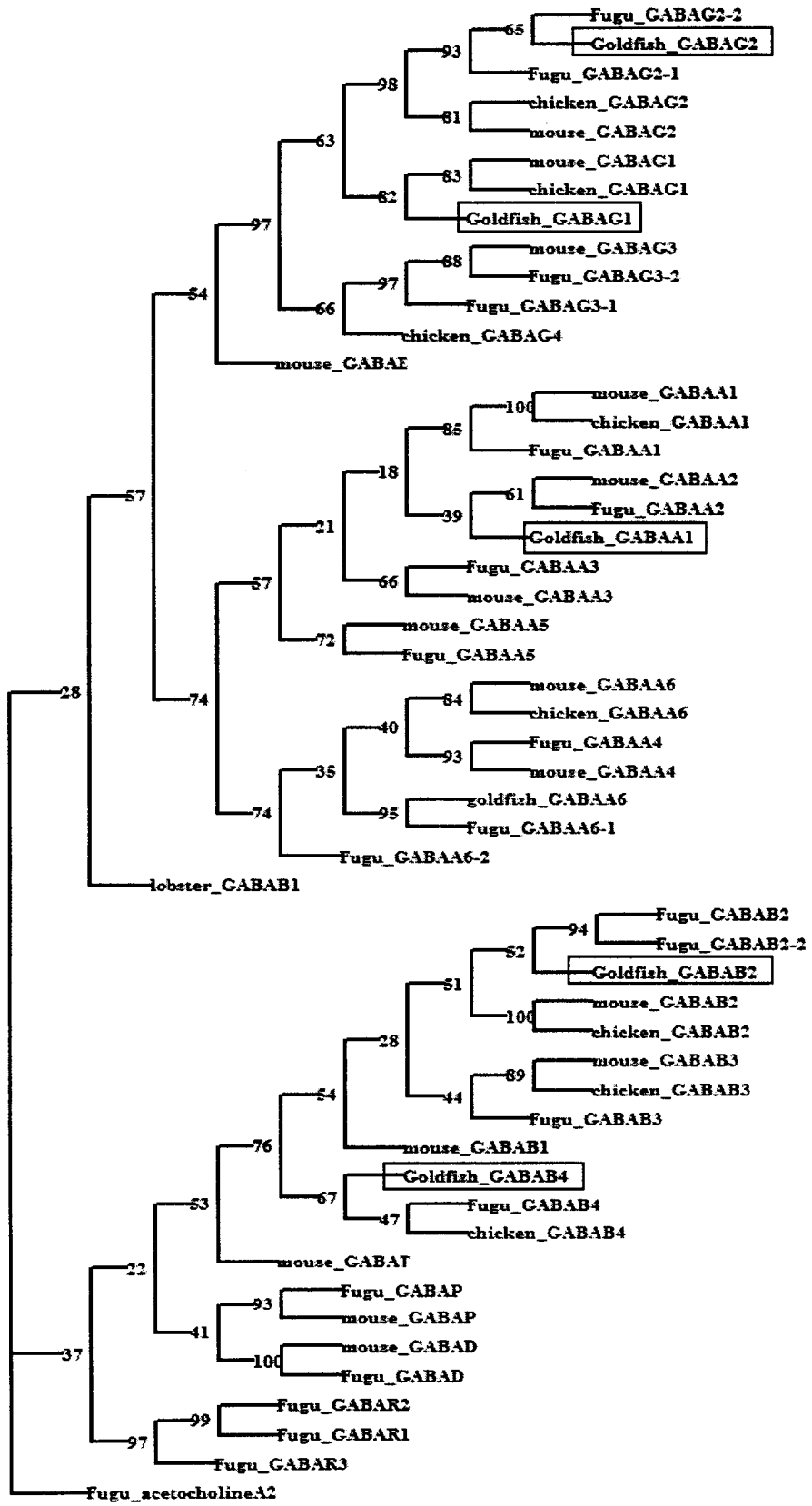

Fugu_GABAG2-2
Goldfish_GABAG2
Fugu_GABAG2-1
chicken_GABAG2
mouse_GABAG2
mouse_GABAG1
chicken_GABAG1
Goldfish_GABAG1
mouse_GABAG3
Fugu_GABAG3-2
Fugu_GABAG3-1
chicken_GABAG4
mouse_GABAE
mouse_GABAA1
chicken_GABAA1
Fugu_GABAA1
mouse_GABAA2
Fugu_GABAA2
Goldfish_GABAA1
Fugu_GABAA3
mouse_GABAA3
mouse_GABAA5
Fugu_GABAA5
mouse_GABAA6
chicken_GABAA6
Fugu_GABAA4
mouse_GABAA4
goldfish_GABAA6
Fugu_GABAA6-1
Fugu_GABAA6-2
lobster_GABAB1
Fugu_GABAB2
Fugu_GABAB2-2
Goldfish_GABAB2
mouse_GABAB2
chicken_GABAB2
mouse_GABAB3
chicken_GABAB3
Fugu_GABAB3
mouse_GABAB1
Goldfish_GABAB4
Fugu_GABAB4
chicken_GABAB4
mouse_GABAT
Fugu_GABAP
mouse_GABAP
mouse_GABAD
Fugu_GABAD
Fugu_GABAR2
Fugu_GABAR1
Fugu_GABAR3
Fugu_acetocholineA2
0.1

Figure 3.1.

Figure 3.2. Phylogeny of GABA receptor protein sequences. The two clades, based on the presence (α, γ, and ε) or absence (ρ, β, δ, θ, and π) of a benzodiazepine binding site, are indicated by BZ+ and BZ–, respectively. Bootstrap values (maximum likelihood analysis) are indicated at all nodes while posterior probabilities (Bayesian analysis) are only indicated when smaller than 100%. The scale bar represents 1 amino acid substitutions per site. Ach: acetylcholine receptor.

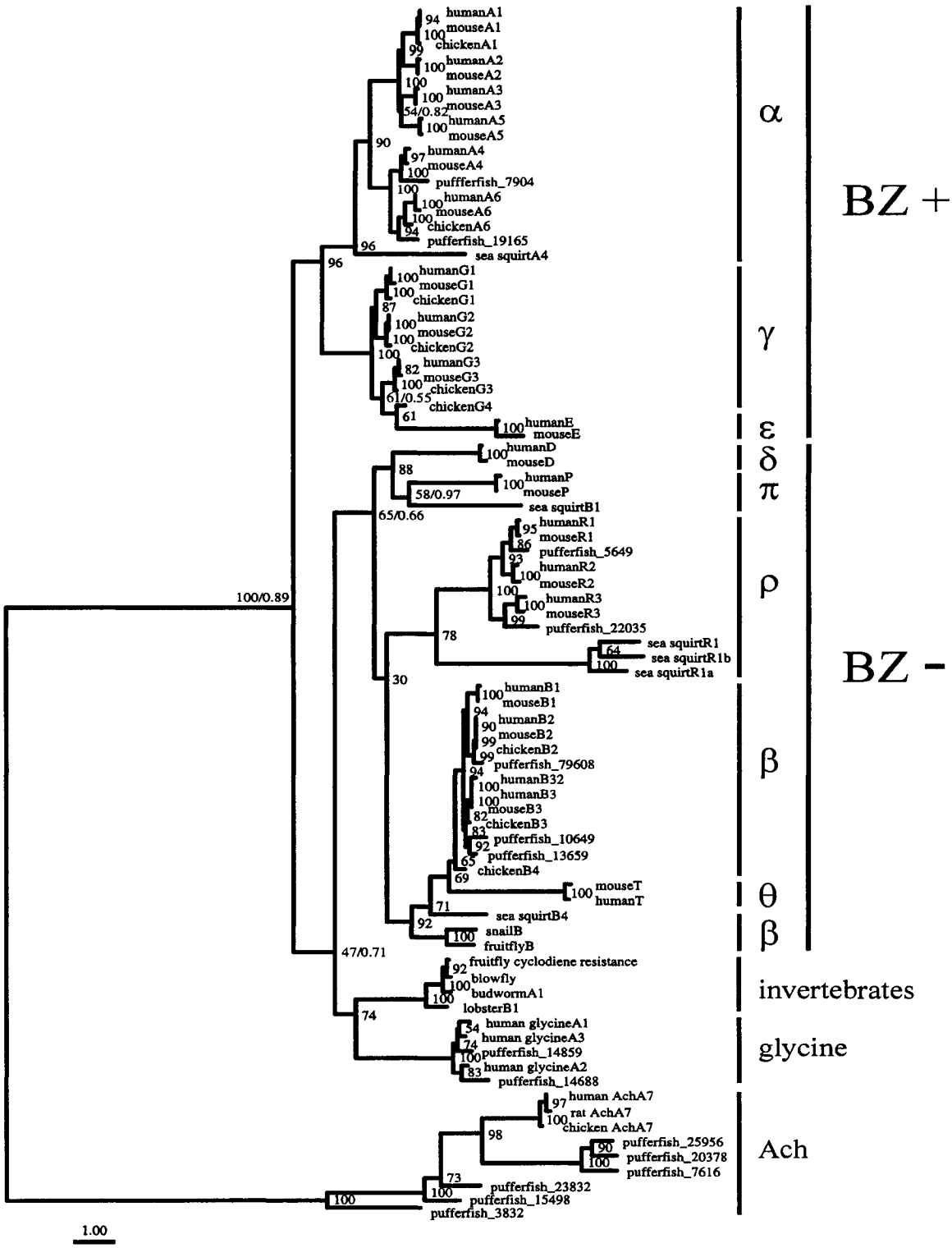

humanA1
mouseA1
chickenA1
humanA2
mouseA2
humanA3
mouseA3
54/0.82
humanA5
mouseA5
humanA4
mouseA4
pufferfish_7904
humanA6
mouseA6
chickenA6
pufferfish_19165
sea squirtA4
humanG1
mouseG1
chickenG1
humanG2
mouseG2
chickenG2
humanG3
mouseG3
61/0.55
chickenG4
humanE
mouseE
humanD
mouseD
humanP
mouseP
58/0.97
sea squirtB1
65/0.66
humanR1
mouseR1
pufferfish_5649
humanR2
mouseR2
humanR3
mouseR3
pufferfish_22035
sea squirtR1
sea squirtR1b
sea squirtR1a
100/0.89
30
humanB1
mouseB1
humanB2
mouseB2
chickenB2
pufferfish_79608
humanB32
humanB3
mouseB3
chickenB3
pufferfish_10649
pufferfish_13659
chickenB4
mouseT
humanT
sea squirtB4
snailB
fruitflyB
47/0.71
fruitfly cyclodiene resistance
blowfly
budwormA1
lobsterB1
74
human glycineA1
human glycineA3
pufferfish_14859
human glycineA2
pufferfish_14688
human AchA7
rat AchA7
chicken AchA7
pufferfish_25956
pufferfish_20378
pufferfish_7616
pufferfish_23832
pufferfish_15498
pufferfish_3832
1.00
α
γ
ε
δ
π
ρ
β
θ
β
invertebrates
glycine
Ach
BZ +
BZ -

Figure 3.2.

Figure 3.3. Phylogeny of vertebrate GABA receptor protein sequences with benzodiazepine binding sites. Bootstrap values are indicated at the nodes and the scale bar represents 1 amino acid substitutions per site.

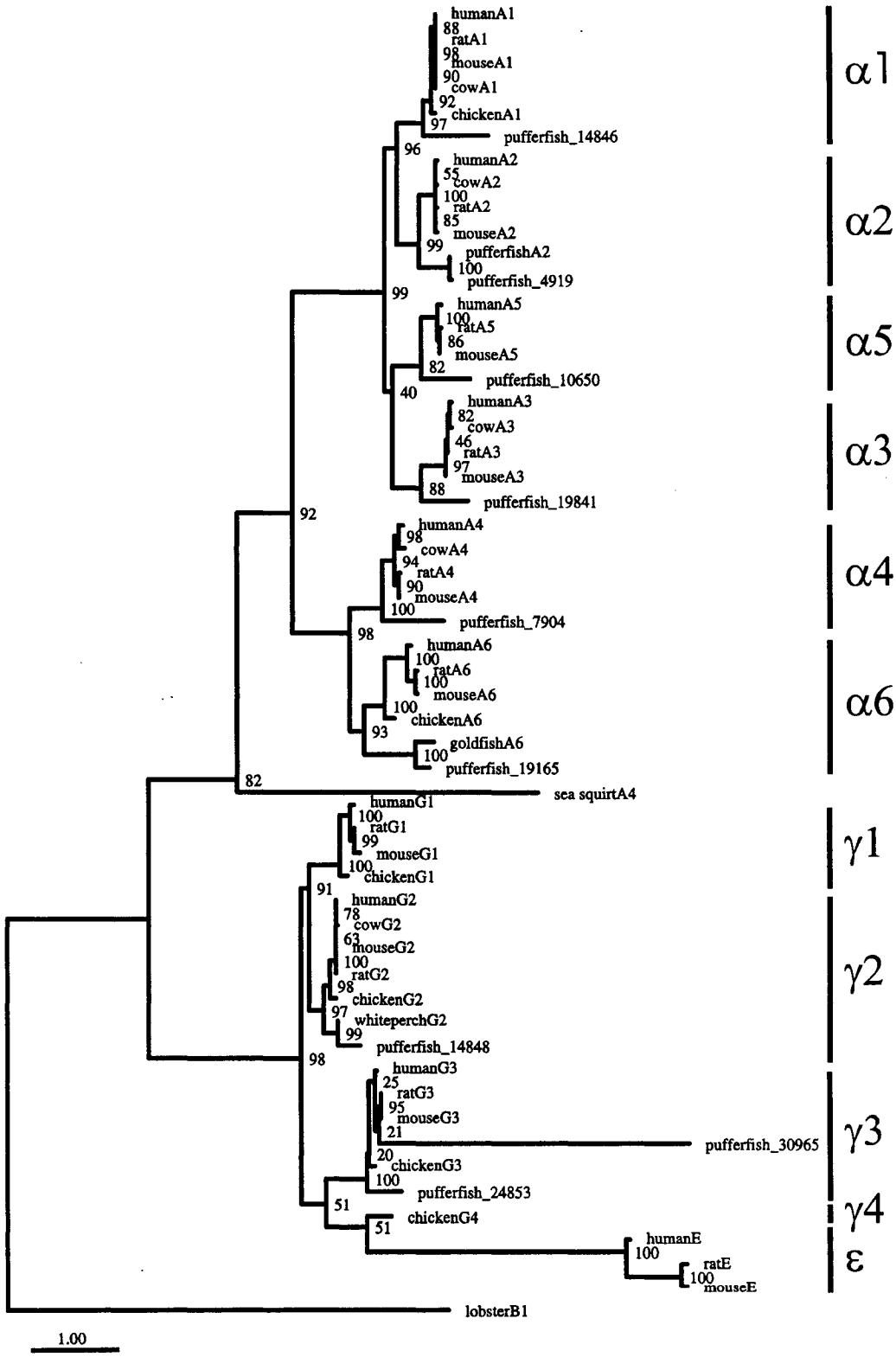

humanA1
88
ratA1
98
mouseA1
90
cowA1
92
chickenA1
97
pufferfish_14846
96
humanA2
55
cowA2
100
ratA2
85
mouseA2
99
pufferfishA2
100
pufferfish_4919
99
humanA5
100
ratA5
86
mouseA5
82
pufferfish_10650
40
humanA3
82
cowA3
46
ratA3
97
mouseA3
88
pufferfish_19841
92
humanA4
98
cowA4
94
ratA4
90
mouseA4
100
pufferfish_7904
98
humanA6
100
ratA6
100
mouseA6
100
chickenA6
93
goldfishA6
100
pufferfish_19165
82
sea squirtA4
humanG1
100
ratG1
99
mouseG1
100
chickenG1
91
humanG2
78
cowG2
63
mouseG2
100
ratG2
98
chickenG2
97
whiteperchG2
99
pufferfish_14848
98
humanG3
25
ratG3
95
mouseG3
21
pufferfish_30965
20
chickenG3
100
pufferfish_24853
51
chickenG4
51
humanE
100
ratE
100
mouseE
lobsterB1
1.00
α1
α2
α5
α3
α4
α6
γ1
γ2
γ3
γ4
ε

Figure 3.3.

Figure 3.4. Phylogeny of vertebrate GABA receptor protein sequences without benzodiazepine binding sites. Bootstrap values are indicated at the nodes and the scale bar represents 1 amino acid substitutions per site.

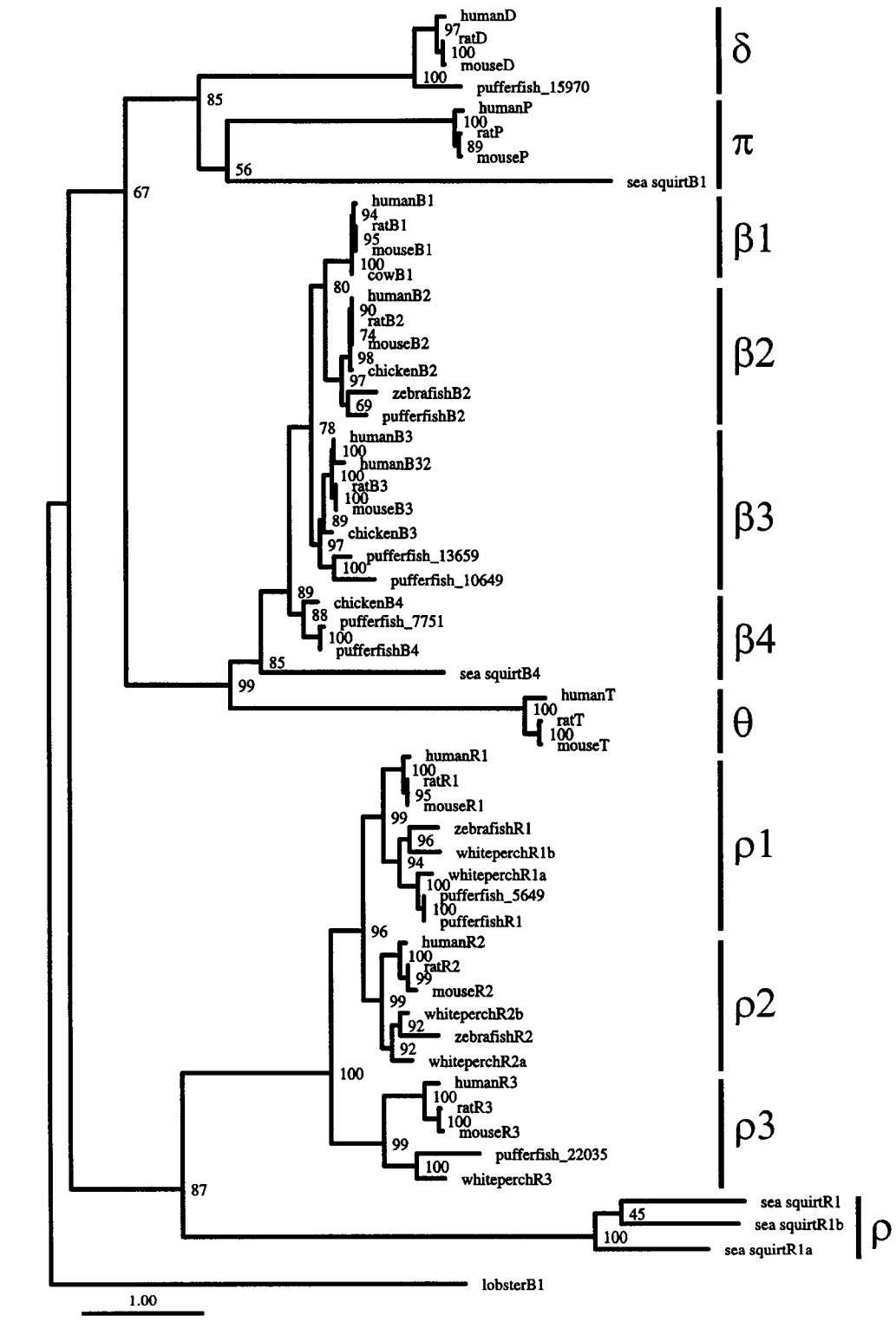

humanD
ratD
mouseD
pufferfish_15970
humanP
ratP
mouseP
sea squirtB1
humanB1
ratB1
mouseB1
cowB1
humanB2
ratB2
mouseB2
chickenB2
zebrafishB2
pufferfishB2
humanB3
humanB32
ratB3
mouseB3
chickenB3
pufferfish_13659
pufferfish_10649
chickenB4
pufferfish_7751
pufferfishB4
sea squirtB4
humanT
ratT
mouseT
humanR1
ratR1
mouseR1
zebrafishR1
whiteperchR1b
whiteperchR1a
pufferfish_5649
pufferfishR1
humanR2
ratR2
mouseR2
whiteperchR2b
zebrafishR2
whiteperchR2a
humanR3
ratR3
mouseR3
pufferfish_22035
whiteperchR3
sea squirtR1
sea squirtR1b
sea squirtR1a
lobsterB1
1.00
δ
π
β1
β2
β3
β4
θ
ρ1
ρ2
ρ3
ρ

Figure 3.4.

Figure 3.5. Phylogenetic trees used to formulate hypotheses about the selective forces that shaped the evolution of: (a) the $GABA_A$ ε and γ subunits; (b) the $GABA_A$ θ and β subunits. Each tree depicts three duplication events and is rooted with the corresponding proto-ortholog that predates the ε / γ and θ / β duplication events. Selective pressures are allowed to vary among sets of branches to test for the *a priori* hypotheses listed at the bottom of the figure: H_0: the null hypothesis that all branch-specific rates are equal; H_1: burst of evolution following the main gene duplication event (ε / γ and θ / β); H_2: also allows for a rate change after the burst of evolution; H_3: extends H_2 to allow for burst of evolution after all duplication events depicted on each tree. Hypotheses test are reported in Table 3.1. The scale bar represents 0.1 substitutions per codon site, with branch lengths estimated under the null model (H_0).

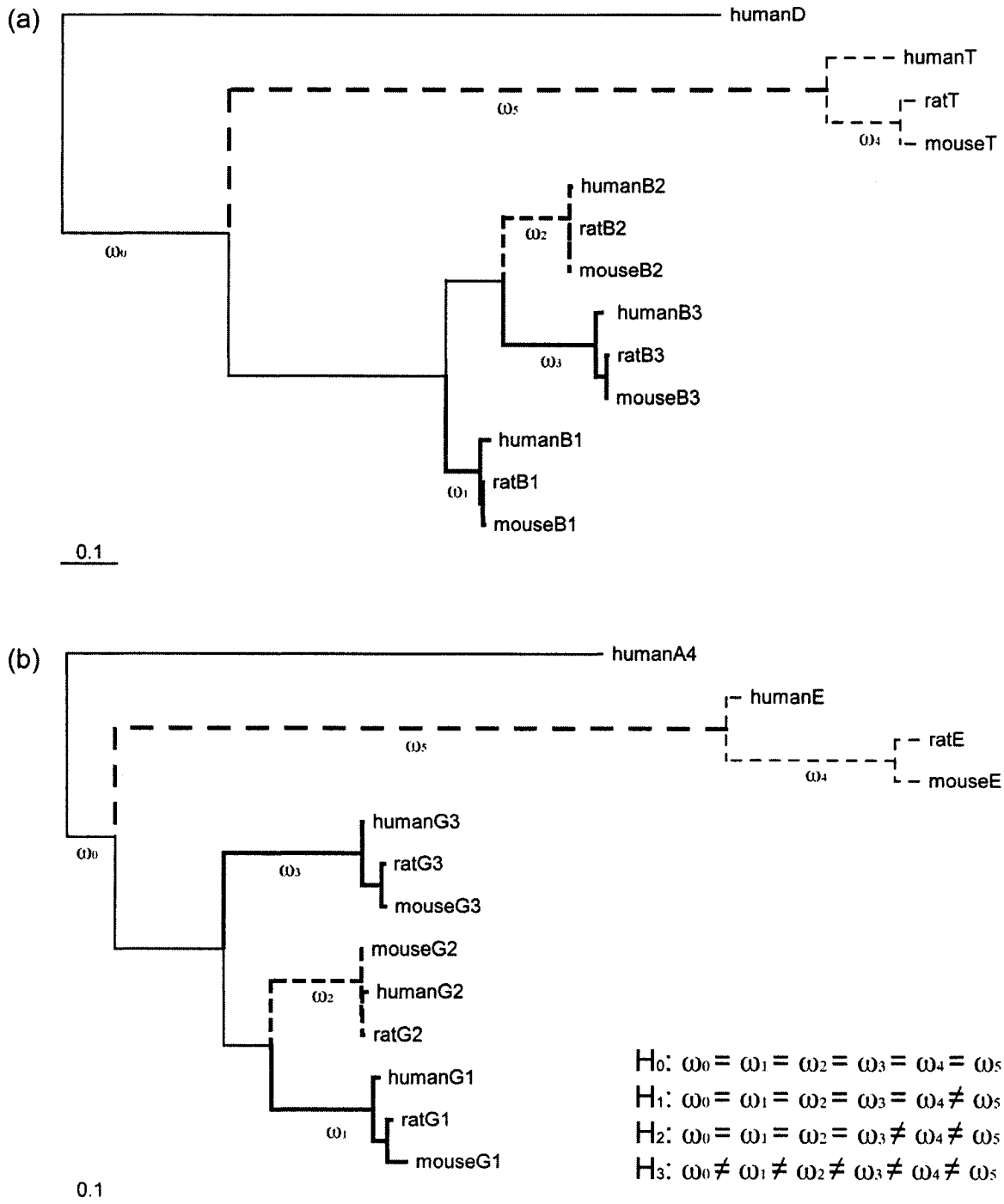

(a)
humanD
humanT
ratT
mouseT
ω5
ω4
humanB2
ratB2
mouseB2
ω2
ω0
humanB3
ratB3
mouseB3
ω3
humanB1
ratB1
mouseB1
ω1
0.1
(b)
humanA4
humanE
ratE
mouseE
ω5
ω4
humanG3
ratG3
mouseG3
ω0
ω3
mouseG2
humanG2
ratG2
ω2
humanG1
ratG1
mouseG1
ω1
0.1
H0: ω0 = ω1 = ω2 = ω3 = ω4 = ω5
H1: ω0 = ω1 = ω2 = ω3 = ω4 ≠ ω5
H2: ω0 = ω1 = ω2 = ω3 ≠ ω4 ≠ ω5
H3: ω0 ≠ ω1 ≠ ω2 ≠ ω3 ≠ ω4 ≠ ω5

Figure 3.5.

Table 3.1. Model comparisons and parameter estimates under models of constant (H_0) or variable ω rate ratios across branches (B), clades (H_1 to H_3) or both clades and sites (H_1^{sites}).

	model	ℓ	np	H_A	*P*-value	ω_0	ω_1	ω_2	ω_3	ω_4	ω_5	sites
θ	H_0	-10977.06	26	–	–	0.075	–	–	–	–	–	none
	B	-10849.41	49	H_0	0.0000	–	–	–	–	–	–	–
	H_1	-10974.15	27	H_0	0.0159	0.075	–	–	–	–	0.629	none
	H_1^{sites}	-10829.11	30	H_1	0.0000	0.015	–	–	–	–	878.76	691L, 784W, 818L, 869R
	H_2	-10869.96	28	H_0	0.0000	0.016	–	–	–	0.251	0.780	none
	H_3	-10864.76	31	H_0	0.0000	0.006	0.021	0.005	0.024	0.252	0.969	none
ε	H_0	-14222.90	26	–	–	0.091	–	–	–	–	–	none
	B	-14114.46	49	H_0	0.0000	–	–	–	–	–	–	–
	H_1	-14220.20	27	H_0	0.0201	0.093	–	–	–	–	0.006	none
	H_1^{sites}	-14000.91	30	H_1	0.0000	0.023	–	–	–	–	2.049	(728S, 849V) (†)
	H_2	-14154.89	28	H_0	0.0000	0.038	–	–	–	0.207	0.006	none
	H_3	-14142.50	31	H_0	0.0000	0.054	0.063	0.013	0.031	0.207	0.006	none

Notes – ℓ : log-likelihood value; np: number of parameters entering the model; H_A: alternative hypothesis to the current model; ω_0 : background rate; ω_5 : foreground rate of branch leading to θ paralogs. Sites were identified using an empirical Bayes procedure at a 99% cut-off level of posterior probability; the reference sequence is mouse (*Mus musculus)* τ for the θ dataset and mouse (*Mus musculus)* γ1 for the ε dataset; position indexes are identical between the two datasets. (†): these sites were putatively identified with a posterior probability > 50% (see text)

3.4. Discussion

Previous phylogenetic analyses and the present study suggest that an ancestral GABA-like receptor subunit gave rise to two monophyletic clades, categorized as receptor subunits containing (α, γ, and ε) or not containing (ρ, β, δ, θ, and π) a benzodiazepine binding site (Ortells and Lunt, 1995; Xue, 1998). Because putative GABA α-, β- and ρ-like receptor subunits are present in the genome of *Ciona intestinalis*, this ancestral duplication most likely occurred before the divergence of urochordates. This result is evidence that benzodiazepine sensitivity evolved early in evolution, as opposed to later as suggested by Hebebrand et al. (1987). Recent electrophysiological data suggests that invertebrates, for example hydra (*Hydra vulgaris*), are responsive to benzodiazepine modulation and this response is similar to the response to GABA (Kass-Simon et al., 2003). However, we acknowledge that presence alone of α and β-like $GABA_A$ receptor subunits in *Ciona* does not necessarily indicate benzodiazepine sensitivity.

Ortells and Lunt (1995) previously suggested that the $GABA_A$ δ subunit is the most primitive receptor subunit within the $GABA_A$ benzodiazepine-absent clade. The present phylogenetic analysis was unable to confidently determine the probable progenitor for receptor subunits in this clade. Within the $GABA_A$ β subunit clade, it is interesting to note that β4 sequences have not yet been reported for any mammalian species and that our genome searches did not identify any β1 sequences in the pufferfish and chicken genomes. It could be that the lineage leading to mammals has lost the β4 sequence and that those leading to pufferfish and chicken lost the β1 paralog. In this respect, it would be interesting to test experimentally whether the β1 and β4 sequences

have similar functions in these different taxa. Similarly, the GABA receptor subunits ε and θ might be specific to the mammalian lineage, as we were unable to find any of these receptor subunits in the genomes of pufferfish, zebrafish, or chicken. Bertrand et al. (2004) describe a similar pattern of evolution by gain and loss in the nuclear receptor gene family in the context of the evolution of the endocrine system, and a number of multigene families are known to be subject to similar processes of evolution by birth-and-death (e.g., Nei, 2005).

Our phylogenetic analyses also indicate that the GABA$_A$ ε subunits are derived from the GABA$_A$ γ subunits. The suggestion that the GABA$_A$ ε genes, found only in mammals, evolved from the GABA$_A$ γ genes is consistent with their chromosomal organization. Human GABA$_A$ ε genes are positioned on a location of the X chromosome that corresponds to the position of GABA$_A$ γ genes on human chromosomes 4, 5 and 15 (Russek, 1999). The higher rate of evolution of the GABA$_A$ ε subunits may explain why GABA$_A$ ε subunits have so far only been found in mammals while the γ4 subunits are present in both birds and reptiles (Darlison et al., 2005).

Although this study could only identify a small number of GABA$_A$ sequences from the sea squirt genome, the current version (as of August 2005) of the pufferfish genome contains orthologs to most of the GABA$_A$ sequences found in mammalian genomes. Furthermore, all the vertebrate genomes searched, including that of the pufferfish, contain a single gene coding for most of the 20 GABA$_A$ receptor subunit families (α1-6, γ1-3, ε, δ, π, ρ1-3, β1-4 and θ). However five of the 20 GABA$_A$ receptor subunit families found in the pufferfish genome contain two paralogous gene copies instead of a single copy. Three of these pairs (i.e., those of pufferfish α2, β4 and ρ1) are

composed of very similar genes that have undergone recent duplication events and, most likely, still have highly similar functions. However, recent gene duplication events of receptors for proglucagon-derived peptides have resulted in differences in ligand binding capabilities between copies (Irwin and Wong, 2005). The presence of two paralogous genes copies in some of the GABA$_A$ subunit families found in the pufferfish genome is consistent with the fact that complete genome duplication events occurred in the pufferfish lineage (Hoegg et al., 2004; Vandepoele et al., 2004). Further studies will elucidate whether duplicated GABA receptor subunits have a functional significance for GABAergic transmission in fish.

Evolutionary rate analyses showed that the GABA$_A$ θ and GABA$_A$ ε subunits experienced diversifying selection in the mammalian lineage (Table 3.1.). The GABA$_A$ θ and ε have a restricted distribution and limited pattern of expression in the CNS. These receptor subunits are also considered to be in low abundance when compared to other GABA receptor subunits (Whiting, 1999). The GABA$_A$ θ subunits are known to co-localize strongly in monoaminergic cell-groups with GABA$_A$ ε subunits in the septum, preoptic areas, hypothalamic nuclei, amygdala, and thalamus of the rat (Moragues et al., 2002). Furthermore the expression of the GABA$_A$ ε subunit in rats is closely associated with the presence of many peptidergic neurons of the rat hypothalamus, such as those producing orexin, oxytocin, and GnRH. This suggests that GABA$_A$ receptors containing ε subunits might have a role in neuroendocrine function, for example, in the control of feeding and reproduction (Moragues et al., 2003). A recent study by Ranna et al. (2006) showed that there was a 100-fold increase in the sensitivity to GABA of human α3β1ε subunits expressed in *Xenopus* oocytes when compared to oocytes expressing the α3β1γ2

subunits. In addition, Ranna and colleagues (2006) provide further evidence that receptors containing the ε subunit show insensitivity to known GABA receptor modulators such as pregnanolone and the barbiturate pentobarbital. Interestingly, these GABA receptor subtypes are predominant in the locus coeruleus. The locus coeruleus contains a large population of noradrenergic positive neurons and when this region is lesioned in rats, there is a disruption in the preovulatory surge of LH, follicle-stimulating hormone (FSH), and prolactin (PRL) (Anselmo-Franci et al., 1997) and a significant reduction in circulating LH (Rocha et al., 2006). It is not known whether or not the rapid divergence of the $GABA_A$ θ and ε subunits played a role in the evolution of the neuroendocrine system in mammals. However, it is plausible that increased control or modulation of GABA receptors in brain regions involved in neuroendocrine function may have been favoured in the evolution of mammals.

We identified four putative amino acid sites 691L, 784W, 869R, and 818L that may be experiencing positive selection in the $GABA_A$ θ family. Examples of positive selection on receptor families in mammals include bitter taste receptor genes (Shi et al., 2003) and olfactory receptor genes (Gilad et al., 2005). Recent studies utilizing point-mutations have shown that single amino acid changes in a GABA receptor subunit will have dramatic effects on the kinetics of the receptor (Derry et al., 2004; Newell et al., 2004) and future studies are needed to determine whether or not changes in these amino acid sites confer significant alterations in GABA receptor kinetics and function.

To conclude, these results show that (1) the two major clades of ionotropic GABA receptors arose before the split from urochordates, (2) the $GABA_A$ receptor family evolved by both gains and losses of subtypes (e.g., teleost β4, chicken γ4, mammalian ε,

θ) and (3) the function of the GABA receptor subunits might have changed twice: First after the duplication event leading to the mammalian-specific $GABA_A$ θ and second, after the duplication event leading to the mammalian ε subunit family. This is supported by our finding that specific amino-acid sites in the mammalian $GABA_A$ θ and ε subunits putatively experienced an episode of diversifying selection after their origin. These episodes of diversifying selection might have played a role in the evolution of neuroendocrine functions controlling feeding and reproduction in mammals. We caution however that further research should be performed to experimentally test the functional divergence hypotheses.

Chapter 4

GABAergic modulation of the expression of genes involved in GABA transmission and stress in the hypothalamus and telencephalon of the female goldfish

Adapted from: Christopher J. Martyniuk, Alyson B. Crawford, Natacha S. Gallant, and Vance L. Trudeau. 2005. J. Neuroendo. 17: 269:275.

4.1 Introduction

GABA is considered to be the most abundant inhibitory amino acid neurotransmitter in the vertebrate central nervous system. GABA has been shown to modulate feeding (van den Pol, 2003) and the stress response (Miklos et al., 2002) in vertebrates. Moreover, although considered an inhibitory neurotransmitter, GABA has a pivotal role in stimulating neural development and differentiation (Ganguly et al., 2001; Borodinsky et al., 2003; Dzhala and Staley, 2003) and reproduction (Trudeau et al., 1993b,d; Mañanos et al., 1999; DeFazio et al., 2002).

Gene expression profiling can be used to identify gene groups that share temporal expression patterns and are involved in a physiological response (i.e. synexpression group) (Niehrs and Pollet, 1999). There is evidence to suggest that GABA enzymes, GABA receptor subunits, and GABA membrane transporters constitute a synexpression group. For example, in the developing rat cervical spinal cord, the majority of $GABA_A$ receptor subunits (e.g. $GABA_A$ α2-4, β1, β3, and γ2) are expressed in a characterized temporal wave of expression along with GAD65, pre-GAD67 (splice variants), and GABA transporter 1 (GAT-1) (Wen et al., 1998). Thus, there is a temporal relationship

between the expression of genes involved in GABA production and genes involved in GABA synaptic transmission.

The objectives of this study were to 1) investigate the effect of GABA on the mRNA expression of a putative GABA synexpression group in the hypothalamus and telencephalon of female goldfish and 2) examine the role of GABA in regulating gene transcripts involved in neuroendocrine processes, for example, reproduction (GnRH, ERα), feeding (NPY, CCK), and stress (CRF) in these same brain tissues. I used the antiepileptic drug vigabatrin, or gamma-vinyl GABA (GVG), to increase GABA levels in the brain during a 24 hour treatment period. GVG is an irreversible inhibitor of the enzyme GABA-T and induces a rapid increase in GABA levels in both rat and human brains (Manor et al., 1996; Petroff et al., 1998). In goldfish, the effects of GVG are also rapid and pronounced, specifically increasing the concentration of GABA in the brain (Trudeau et al., 1993b). For our putative GABA synexpression group, I cloned partial gene sequences for goldfish $GABA_A$ receptor subunits (α1, β2, β4, γ1, γ2), a $GABA_B$ receptor subunit (β1), GABA membrane transporters (GAT-1 and GAT-3), and enzymes involved in GABA synthesis (GAD65, GAD67, and GAD 3) and degradation (GABA-T).

4.2 Materials and Methods

4.2.1. Experimental animals and GVG injection

Common female goldfish were purchased from a commercial supplier (Mount Parnell Farms, Pennsylvania, USA) in July 2003 and allowed to acclimatize over several months to 18 °C under a natural photoperiod. Fish were fed and maintained on standard flaked goldfish food. Goldfish were anesthetised using MS222 (3-aminobenzoic acid

ethyl ester) for all handling and dissection procedures. Care was taken to standardize all handling, injections, and sample protocols. In October 2003, female goldfish were injected in the intraperitoneal (i.p.) cavity with GVG (300 μg/g of body weight; injection volume of 1μl/g) dissolved in 0.6% saline solution. This dose has been used successfully to increase GABA levels in the goldfish brain (Sloley et al., 1994). This study also determined that hypothalamic GABA levels are not affected by anaesthesia. To verify the effects of GVG on GABA levels in the brain, a subset of goldfish were injected with GVG (300 μg/g of body weight) in October (data not shown). We found that GVG treatment significantly increased GABA levels ~ 2.2 fold in both the hypothalamus (n=8; $p< 0.01$) and telencephalon (n=8; $p< 0.01$). The protocol for the measurement of GABA levels in the goldfish brain has been published previously (Sloley et al., 1992). Control groups received an equivalent injection volume of 0.6% saline solution. Goldfish were dissected and brain tissue collected the following day (24 hour treatment period).

4.2.2. RNA isolation

Goldfish tissue was rapidly dissected and frozen on dry ice. Hypothalami and telencephali were dissected and tissue pieces were pooled (4-6/tube) to increase RNA yield prior to isolation of total RNA. Total RNA was extracted from pooled samples using TRIzol Reagent® (Invitrogen Life Technologies, Carlsbad, CA, USA) as per the manufacturer's protocol. Total RNA was resuspended in 30 μl RNAse free water. Concentration of total RNA in each sample was measured in triplicate using GeneQuant® (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Total RNA per sample was ~ 2 μg/μl.

4.2.3. cDNA synthesis, PCR, and cloning of gene sequences

First-strand cDNA synthesis was done using 1-5 μg total RNA from goldfish whole brain in a reaction tube containing 1 μl oligonucleotide dT. The reaction was heated to 70 °C for 10 minutes, quickly chilled on ice, and centrifuged briefly. 4 μl 5X reaction buffer (Invitrogen), 2 μl 0.1M DTT, 1 μl 10mM dNTPs, and 1 μl RNAse inhibitor was added, gently mixed, and heated at 42 °C for 2 minutes. 1 μl Superscript™ II RNase H⁻ Reverse Transcriptase (Invitrogen) was added and the reaction was allowed to continue at 42 °C for 50 minutes. The reaction was inactivated at 70 °C for 15 minutes and stored at –20 °C until used.

Degenerate primers were constructed using ClustalW (EMBL-EBI; http://www.ebi.ac.uk/clustalw/) and Primer3 programs (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) using the most highly conserved regions of the genes. PCR amplification was done using the Mastercycler® gradient Thermal Cycler (Eppendorf, Westbury, NY USA). General PCR conditions used were as follows; 1-2 μl cDNA template in 36.3 μl PCR water, 5 μl 10X PCR reaction buffer, 1.5 μl $MgCl_2$ (50mM), 1 μl dNTPs (10mM), 2 μl forward (F) and reverse (R) primers (10mM), and 0.2 μl Taq® DNA Polymerase (Invitrogen). The initial denaturation step was done at 95 °C for 4 minutes to activate the Taq enzyme. This was followed by 35 cycles with a denaturation step at 95 °C for 30 seconds, an annealing step ranging between 50-60 °C (depending on the primer set used) for 45 seconds, and an extension step at 72 °C for 1 minute. The final extension step was done at 72 °C for 10 minutes.

Amplification products were either ligated directly into the 2.1 TOPO® vector (Invitrogen) or excised from a 1% agarose gel and purified using Qiaquick® Gel

Extraction Kit (Qiagen, Mississauga, ON, Canada) and ligated into the vector. One Shot TOP 10® chemically competent *E. coli* (Invitrogen) were transformed and plated onto LB-agar plates containing ampicillin, X-Gal, and IPTG. Positive colonies were selected and an additional round of PCR was done using M13 primers to ensure the correct insert size was present before sequencing. Colonies were grown overnight in LB broth containing ampicillin, and plasmids were purified using the Wizard® Plus SV Minipreps DNA Purification System (Promega, Madison, WI, USA). Approximately 10 µl of purified plasmid was sent to the Canadian Molecular Research Services (CMRS; Ottawa, Canada) for sequencing. Partial gene sequences for GAD65 and GAD67 were subcloned into 2.1 TOPO® vector using the following sets of gene specific primers; GAD65 F (5' to 3') TGCCAGCCAATGATCTCC; GAD65 R (5' to 3') GCTGGGTTTCGATTCAGC; GAD67 F (5' to 3') CAGACAGCTCCAGGTTGAA; GAD67 R (5' to 3') GGAGATTATCCTGTCGCCTTT. A partial sequence for ERα was obtained using the following primers; ERα F (5' to 3') AGCATTCAAGGTCACAATGA(TC)TA; ERα R (5' to 3') ATCATG(AT)G(CG)AC(CG)AGTTCCTTG.

4.2.4. Reverse northern blots (macroarrays)

The following protocol was adapted from Crump et al., 2002. Briefly, 4 µl of cDNA template was amplified in a 100 µl reaction volume containing 10 µl 5X PCR buffer, 3 µl MgCl (50mM), 2 µl dNTPs (25mM), 3 µl forward M13 primer (5' to 3') CACGCAGTTGTAAAACGAC (10mM) and reverse M13 primer (5'to 3') GGATAACAATTTCACACAGG (10mM), and 0.5 µl Taq® DNA polymerase (5U/µl). PCR cycles consisted of a denaturation step at 95 °C for 30 seconds, followed by an

annealing step at 56 °C for 1 minute, and an extension step at 72 °C for 2 minutes for 34 cycles. Amplified PCR products were purified using MicroSpin S-300 HR Columns™ (Amersham). Final concentrations of purified PCR product ranged between 50-80 ng/µl. PCR products were diluted in a final concentration of 2X SSC buffer and 0.2M NaOH in ddH_2O. Samples were heated at 37 °C for 15 minutes to facilitate denaturation. 200 µl of diluted cDNA (~ 150-200 ng) were spotted onto Hybond™ N+ membranes (Amersham) using a Millipore spotting apparatus. Each sample was spotted in triplicate. cDNA targets were fixed to the membrane by UV crosslinking (115 V; 60Hz, 0.75 amps) for 5 minutes. Membranes were stored at room temperature until used.

4.2.5. cDNA synthesis, radioactive labeling, and hybridization

Membranes were prehybridized in 20 ml of hybridization buffer (0.5 M sodium phosphate buffer, 7% SDS, and 1mM EDTA; pH 7.2) for 2 hours at 65 °C. First-strand cDNA synthesis was done with ~ 10-15 µg of total RNA from the hypothalamus or telencephalon in a reaction tube containing 1.0 µl dNTPs (10mM dATP, dTTP, dGTP, and 0.08mM dCTP) and 1.5 µl random primers (Amersham). After a brief centrifugation, 4 µl 5X reaction buffer, 2 µl 0.1M DTT, 1 µl RNAse inhibitor, 5 µl ^{32}P labelled dCTP (50uCi) (Amersham), and 1 µl MMLV Reverse Transcriptase (Gibco™, Invitrogen) was added. The reaction was incubated at 42 °C for 2 hours. After incubation, samples were briefly centrifuged. To remove RNA from cDNA probes, 1 µl 10% SDS, 1 µl 0.5M EDTA (pH 8), and 3 µl 3N NaOH were added in sequential order and incubated at 68 °C for 15 minutes. Samples were then cooled to room temperature for approximately 2 minutes. Samples were neutralized with 10 µl 0.1M Tris (pH 7.5)

and 30 µl 0.2N HCl. Probes were denatured at 95 °C for 5 minutes then cooled on ice for 1 minute. Denatured probes were added to hybridization tubes with an additional 20 ml hybridization buffer. Hybridization occurred at 65 °C for 48 hours. After hybridization, membranes were washed at 65 °C in 2X SSC, 0.1%SDS for 20 minutes, in 1X SSC, 0.1%SDS for 25 minutes, in 0.5X SSC, 0.1%SDS for 30 minutes, and in 0.2X SSC, 0.1%SDS for 30 minutes. Membranes were then exposed to a phosphoimaging screen (BioRad®,USA) for 5-6 hours.

4.2.6. Data analysis

Densitometry values were obtained for each cDNA spot (triplicate spots for each gene) using Quantity One® (BioRad®,USA) software. All genes used in this study had a signal intensity 2 times above background except for cGnRH2 and all spots were visible after exposure to the phosphoimaging screens. A background hybridization intensity value was obtained for each membrane and this value was subtracted from each cDNA spot on the macroarray. The average signal intensity for each triplicate was calculated. Within each macroarray, a global normalization procedure was implemented by dividing all spots (minus background) by the average of all hybridization intensity values. This was done to reduce the variation due to hybridization and cDNA concentration/spot (Crump et al., 2002). β-actin was used as an internal control and each cDNA spot was further normalized by dividing mean cDNA spot intensity by mean β-actin spot intensity. In the goldfish, previous studies confirm that the expression of β-actin does not change significantly with sex steroid or between the sexes (Bosma et al., 2001). In the cultured neurons of the chicken, Lyons et al., 2000 report that β-actin mRNA expression is also

not affected by persistent exposure (48 hrs) to GABA. Macroarray data did not pass the Kolmogorov-Smirnov test for normality and were therefore analysed using the Mann-Whitney U test. Median signal intensity between control and treatment groups was considered significantly different at $p < 0.05$.

4.3. Results

To study the expression of genes involved in GABAergic signalling in goldfish, we cloned 8 new partial gene sequences, including GABA receptor subunits and GABA transporters. The degenerate primer sequences used to clone partial gene sequences for the putative GABA synexpression group are shown in Table 4.1. We have also included the length of the amplicon generated for the specified gene and the nucleotide sequence similarity to the homologs found in the human genome. Goldfish nucleotide sequence similarity to human ranged between 81-88% for genes in the putative GABA synexpression group. The $GABA_A$ receptor β4 subunit is not present in mammals, therefore the nucleotide sequence is compared to the chicken $GABA_A$ receptor β4 subunit. Each partial gene sequence represents approximately one sixth of the full length coding sequence for the GABA transporters to approximately one third for the GABA receptor subunits. Nomenclature for each gene was assigned according to a nucleotide BLAST search (OMIM; NCBI database). A ClustalW (EMBL-EBI) alignment was used to classify the GABA receptor subunits (data not shown). The sequences reported in this paper have been submitted to GenBank and accession numbers are reported in Table 4.2.

In the hypothalamus, GVG injection significantly reduced the $GABA_A$ β4 subunit 2.2 fold (Figure 4.1A). Each dot on the graph represents an independent sample and the

bar represents the median value in that data set. The $GABA_A$ γ2 receptor subunit is shown as a comparison for the spread around the median (no significant change). This study detected no statistically significant changes in the expression of genes involved in reproduction, feeding, or stress in the hypothalamus. In the telencephalon, increasing GABA levels significantly up-regulated CRF mRNA transcripts 1.5 fold and reduced the $GABA_A$ β2 subunit 1.8 fold (Figure 4.1B). Again, data for the $GABA_A$ γ2 receptor subunit is shown as a comparison. Median fold change values for all genes investigated in the hypothalamus and telencephalon, as well as the direction of the change, are listed in Table 4.2. A value of one is indicative of no change in mRNA abundance between control and treatment groups.

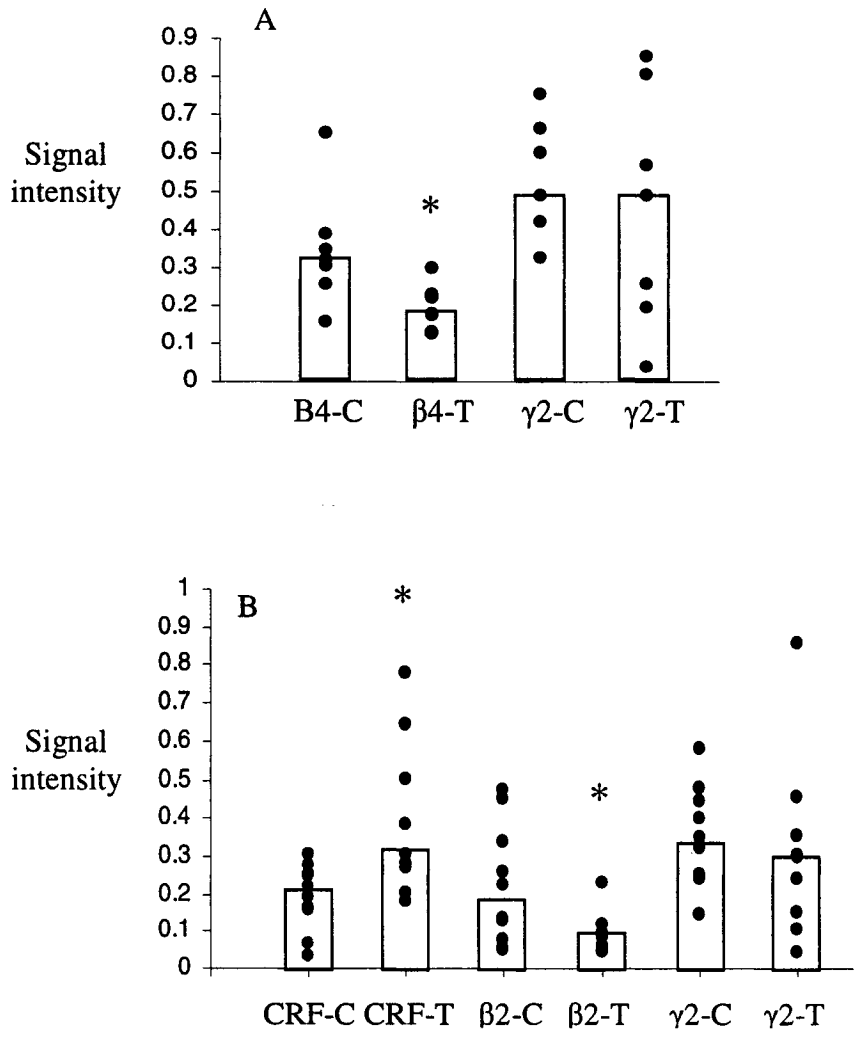


Figure 4.1. Effects of GVG on the expression of A) $GABA_A$ β4 mRNA and $GABA_A$ γ2 mRNA in the hypothalamus (n=7) and B) CRF mRNA, $GABA_A$ β2 mRNA, and $GABA_A$ γ2 mRNA in the telencephalon (n=9). Data on $GABA_A$ γ2 signal intensity is shown to illustrate the variation in signal intensity of a gene that did not significantly change with GVG treatment. Each independent data point is the average signal intensity of the gene adjusted for the average signal intensity of β-actin (per membrane). Control (C; 0.6% saline) and treatment (T; 300μg/g) groups are shown. Bars indicate the median signal intensity for each group. A significant change in mRNA expression ($p<0.05$) is indicated with an asterisk.

Table 4.1. Degenerate primer sequences used to amplify partial sequences for putative GABA synexpression group. Degenerate nucleotide notation is as follows; K = T + G; M = A + C; Y = C + T; R = A + G; B = T + C + G. Asterisk indicates nucleotide sequence compared to chicken.

Gene	Forward Primer (5' to 3')	Reverse Primer (5' to 3')	Amplicon size (bp)	% nt similarity to human
GABA -T	CTG YTT YAG RGT TTG AAT ATG ATG	AAG TTC TCW GGT GGC AGG ATD CC	313	85
GAT 1	ATT GAC AGC CAG TTY TGY AC	GCC AAC CAT CTC CTS RAT RTT	288	81
GAT 3	TAT KTC ACW GCV ACA TTC CCY TA	CCM AGD ACA GAG AAD ATD GCA AA	308	83
$GABA_A$ α1	GGG AAG TCC TCA AGC TGC ATT	TGG GAC GGG CTC TTG GAT	387	83
$GABA_A$ β2+β4*	TCK GAA GTM AAC ATG GAY TAC ACC	GTA KCC ATA GCT YTC GAT CTC MA	327 + 304	82 + 81*
$GABA_A$ γ1	ATY TGG ATY CCA GAC ACT TTT CT	TAK GTC TGR ATG GTY AAG TAK CC	407	88
$GABA_A$ γ2	GAC CTG AYA TMG GAG TBA AAC	GTK CAT CAA TTG GRA ART TGT	360	81
$GABA_β$ β1	TGG TTC CTG ATC GGR TGG TA	GTG CTG TCR TAG TAG CCR ATC TTC	503	82

Table 4.2. Fold changes in gene expression in the hypothalamus (n=7) and telencephalon (n=9) after 24 hour GVG treatment. Median fold change values are shown for each gene after normalization with β-actin. Positive value represents an increase in mRNA abundance while a negative value represents a decrease in mRNA abundance. Genes showing a significant change in mRNA expression ($p<0.05$) are given an asterisk.

Gene	Hypothalamus	Telencephalon	Accession #
$GABA_A$ α1	-1.5	-1.1	AY640225
$GABA_A$ β2	1.6	-2.2*	AY640229
$GABA_A$ β4	-1.8*	-1.0	AY635467
$GABA_A$ γ1	-1.1	1.2	AY640226
$GABA_A$ γ2	1.0	-1.1	AY640227
$GABA_B$ β1	-1.1	1.2	AY640228
GABA Transaminase	-1.1	1.4	AY640231
GAD 3	-1.3	-1.0	AF432155
GAD65	-1.1	1.0	AF043265
GAD67	-1.3	-1.6	AF043266
Ornithine Decarboxylase	-1.0	1.8	AY640230
Glutamine Synthase	1.3	-1.1	AY641442
GABA Transporter 1	1.4	1.4	AY640223
GABA Transporter 3	1.2	1.0	AY640224
Estrogen Receptor α	-1.4	1.6	AY055725
sGnRH	-1.2	-1.7	AB017272
cGnRH2 isoform 1	-1.0	7.0	U40567
cGnRH2 isoform 2	-1.7	1.7	U40567
CCK	-1.2	-1.2	U70865
NPY	-2.1	1.2	M87297
CRF	1.1	1.5*	AY142110
Isocitrate dehydrogenase	1.1	-1.3	DY231529
GAPDH	-1.2	-1.1	AY641443
SNAP 25	-1.1	1.5	AY644725

4.4. Discussion

4.4.1. GABA regulation of genes involved in GABA synaptic transmission

The ionotropic $GABA_A$ receptors are pentameric ligand-gated chloride channels and in mammals seven classes of subunit families have been identified (α_{1-6}, β_{1-4},γ_{1-4}, δ, ε, θ, and π). Pharmacological experiments using both GABA receptor agonists and antagonists have revealed that the $GABA_A$ type receptor is the primary receptor involved in GABA stimulated LH release in the goldfish (Trudeau et al., 2000c). However, the metabotropic G-protein coupled $GABA_B$ receptors may also be involved in mediating the stimulatory action of GABA on gonadotropin release. These results showed that increasing GABA levels in the brain of goldfish resulted in a significant down-regulation of the ionotropic $GABA_A$ receptor β4 subunit in the hypothalamus and the $GABA_A$ receptor β2 subunit in the telencephalon while there were no significant changes in relative mRNA abundance of other $GABA_A$ receptor subunits (α1, γ1, and γ2). This is the first study to report on the regulation of the expression of GABA receptor subunits in fish. Fénelon and Herbison (1996) treated ovariectomized female rats with GVG (150 mg/kg) for three days and found that there was a significant decrease in the abundance of $GABA_A$ receptor γ1 mRNA (35%) in the medial preoptic nucleus and $GABA_A$ receptor α2 mRNA (20%) in the cingulate cortex. Conversely, there was increased levels of the $GABA_A$ receptor γ2 mRNA (17%) in the supraoptic nucleus and $GABA_A$ receptor α1 mRNA (29%) in the globus pallidus. The $GABA_A$ receptor β3 mRNA remained unchanged in all rat brain regions examined. Thus, there is tissue and region specific regulation of the different $GABA_A$ receptor subunits in the brain. In the goldfish, GABA appears to predominantly modulate the expression of $GABA_A$ receptor β mRNA.

However, it should be pointed out that the autoregulation of $GABA_A$ receptor subunit expression by GABA is dependent upon the duration of exposure to increasing GABA. For example, down-regulation of $GABA_A$ receptor α1 mRNA in cultured cortical neurons of chickens occurred after 9 hours of GABA treatment (1mM) while a significant reduction in $GABA_A$ receptor β2S and γ1 mRNA transcription occurred after 48 hours of GABA treatment (Lyons et al., 2000). It is likely that in the goldfish brain as well, changes in $GABA_A$ receptor mRNA expression occur on a temporal scale. Prolonged GABA exposure (100 μM; single dose; 7 days) to developing chick cortical neurons *in vitro* resulted in a reduction between 47% and 65% of all $GABA_A$ receptor subunit mRNA examined (α1, β2, β4, γ1, and γ2) when compared to controls (Baumgartner et al., 1994). In concert with the aforementioned studies in rat and chicken, we suggest that the autoregulation of GABA receptor subunit expression is a conserved mechanism to modulate GABAergic synaptic transmission in response to increasing GABA levels in the vertebrate brain. However, we would like to point out that the present study sampled whole hypothalami and telencephali and was unable to address changes occurring at the level of cell nuclei or specific cell layers. As such, heterogeneous cell populations were investigated and closer examination at the level of specific cell nuclei in the goldfish may result in the observation of greater differences in expression patterns among cell nuclei and brain regions, as in the aforementioned studies in the rat and chicken.

There were no significant changes in the mRNA abundance of enzymes involved in GABA synthesis and degradation, in contrast to studies done in rat which showed a significant down-regulation of GAD67 mRNA in the cortex after a single large i.p. dose of GVG (24 hr; 1mg/g), followed by a decrease of 44 % in GAD67 protein levels (Mason

et al., 2001). There is evidence that the expression of GAD67 mRNA and GAD67 activity are more sensitive to increasing GABA concentrations in the mammalian brain than GAD65 expression (Rimvall et al., 1993, 1994; Sheikh et al., 1998). Similarly, the abundance of GABA transporter mRNA in the hypothalamus and telencephalon did not significantly change with GVG treatment. This is in agreement with a previous study by Herbison et al. (1995) showing that GVG treatment in female rats does not change GAT-1 transporter mRNA abundance in the medial preoptic area or parietal cortex. However, others have shown that GABA membrane transporters are rapidly inserted into the neuronal membrane in response to an increase in extracellular GABA (Bernstein and Quick, 1999). Furthermore, GABA transporter activity has been shown to increase in rat hippocampus following GVG treatment (Overstreet and Westbrook, 2001). These studies suggest that post-transcriptional modulation of GATs may be a more important mechanism in reducing tonic inhibition of neuronal function caused by increasing extracellular GABA than increasing mRNA abundance.

4.4.2. GABA regulation of the expression of genes involved in neuroendocrine function

GABA has multiple effects on neuroendocrine function, including control of reproductive processes (Trudeau et al., 2000c) and studies have reported a correlation between the expression of genes involved in GABAergic signalling and GnRH release (Leonhardt et al., 2000). In the goldfish, GABA stimulates pituitary LH release by stimulating GnRH neurons primarily through post-synaptic $GABA_A$ receptors (Trudeau et al., 2000c). However, there were no significant changes in the expression of GnRH mRNA in the hypothalamus or telencephalon. In the goldfish, GVG treatment caused a significant decrease in the hypothalamic levels of the salmon GnRH, but not in levels of

the chicken GnRH-II peptide (Sloley et al., 1994). Thus, in the goldfish, GABA may not significantly affect transcription of GnRH isoforms but may affect GnRH mRNA stability or GnRH peptide synthesis and release.

The neuroendocrine response to stress in vertebrates is modulated by a number of neuropeptides and is influenced by GABAergic input. GABAergic neurons innervate CRF neurons in the rat hypothalamus (Herman and Cullinan, 1997) and, in general, have a predominantly inhibitory role on CRF secretion both *in vivo* (Plotsky et al., 1987) and *in vitro* (Hillhouse and Milton, 1989). Cultured paraventricular CRF neurons treated with the $GABA_A$ receptor antagonist bicuculline show a potentiated CRF response to excitatory glutamate (Bartanusz et al., 2004). However, treatment with the $GABA_A$ receptor antagonist bicuculline did not alter the expression of CRF mRNA in the hypothalamus periventricular nucleus of the rat, suggesting CRF transcript regulation is not $GABA_A$ mediated (Bali and Kovacs, 2003). Following GVG treatment, we found that CRF mRNA abundance significantly increased in the telencephalon, but did not change in the hypothalamus. The preoptic area in teleost fish is located in the telencephalon and this region contains the majority of CRF producing neurons. For example, Doyon et al., 2003 showed that the preoptic area of juvenile rainbow trout (*Oncorhynchus mykiss*) contains approximately 80% of CRF mRNA in the brain. Pivagabine [4-(2.2-dimethyl-l-oxopropylamino) butanoic acid], a GABA derivative, has been shown to increase CRF mRNA in the hypothalamus and cerebral cortex of rats after 4 days in a dose-dependent manner (Follesa et al., 2000). In contrast, GVG treatment in 9 day old rats results in the down-regulation of CRF mRNA in the paraventricular nucleus of the hypothalamus but not in other limbic regions of the brain (Tran et al.,

1999). Thus, GABAergic feedback on the vertebrate hypothalamic-pituitary-adrenocortical axis is well documented in addition to the effects of stress on the expression of genes involved in GABAergic transmission (Cullinan and Wolfe, 2000 and references therein). It is clear that there is cross-talk between GABAergic and CRF signalling and we show that GABA increases CRF mRNA abundance in a tissue specific manner in the goldfish.

In conclusion, this study suggests that GVG treatment regulates genes involved in GABA synaptic transmission and stress in the hypothalamus and telencephalon of the goldfish and provides the initial groundwork necessary to perform more elaborate physiological, pharmacological, dose and time-course analysis on the GABAergic pathway in goldfish. The modulation of GABA receptor subunit mRNA may be an evolutionary conserved mechanism in vertebrates to mediate GABA synaptic transmission in response to excess intracellular GABA. Teleost fish branched from the main vertebrate line more than 200 million years before present (Larivière et al., 2002 and references therein). With a 24 hour GVG treatment, we observed an approximate 40-50% decrease in mRNA abundance for the $GABA_A$ receptor β2 and β4 subunits. This reduction in mRNA abundance is similar to what others have found in both the rat (Fenélon et al., 1996) and chicken (Baumgartner et al., 1994). $GABA_A$ β subunits are the sites for GABA binding while the $GABA_A$ α and γ subunits are the sites for receptor modulation, for example, by benzodiazepine and ethanol (Costa et al., 2002). Amino acid substitution mutations in the rat $GABA_A$ β2 subunit, when expressed in *Xenopus* oocytes, change the activational properties of the $GABA_A$ receptor (Chang et al., 2003). As well, introduction of plasmids containing rat $GABA_A$ receptor β1 and β3 subunits into

fibroblast cells results in altered sensitivity of the receptor to GABA (Fisher and Macdonald, 1997). Both these studies demonstrate that the stoichiometry of the $GABA_A$ receptor is related to the function of the receptor. Furthermore, there is evidence that GABA itself modulates the electrophysiological properties of the $GABA_A$ receptor. Engel et al. (2001) showed that in cultured rat hippocampal slices, GVG treatment increases the amplitude and frequency of miniature inhibitory postsynaptic current, showing that increased intracellular GABA modulates the plasticity of the neuronal response. Therefore, regulation of GABA receptor subunit mRNA expression may be a mechanism by which GABA modulates its own receptor function and physiology.

Chapter 5

The effects of GABA agonists on GAD, GABA-T, activin, sGnRH, and tyrosine hydroxylase mRNA in the neuroendocrine brain: Molecular mechanisms underlying GABA stimulated LH release in fish

5.1 Introduction

GABA is synthesized by the enzyme glutamic acid decarboxylase (GAD65; GAD67) from glutamate and is degraded by GABA-transaminase (GABA-T) into succinic semialdehyde. GABA stimulates the release of LH (also referred to as GTH-II in fish) from the pituitary in several teleost fish (Kah et al., 1992; Khan and Thomas, 1999; Mañanos et al., 1999) by enhanced GnRH release. In goldfish, there are two major isoforms of GnRH, called salmon GnRH (sGnRH) and chicken GnRH (cGnRH-II) that are expressed in the brain (Lin and Peter, 1996). These two isoforms are the products of different genes and differ in their role in pituitary LH release (Khakoo et al., 1994). GABA will inhibit DA (Trudeau et al., 1993b), which is a potent inhibitor of GnRH stimulated LH release in goldfish, and removes the tonic inhibition of DA on gonadotrophs (Peter et al., 1986). Thus, the control of LH release in teleost fish is largely mediated by a stimulatory GnRH input and an inhibitory DA input. The effect of GABA on LH release is season-dependent and GABA stimulates LH release predominantly when fish are in a period of sexual regression (i.e. gonadal tissue is largely reabsorbed) (Trudeau et al., 1993b,d; Khan and Thomas, 1999). Electrophysiological and pharmacological evidence from goldfish (Trudeau et al., 2000c) and pharmacological evidence from Atlantic croaker (Khan and Thomas, 1999) suggest that GABA stimulates

LH release through actions on the $GABA_A$ receptor. The ionotropic $GABA_A$ receptor is a hetero-oligomeric ligand-gated Cl^- channel, the predominant structural model being a pentameric receptor that consists of several subunits (Whiting, 1999). However, the metabotropic $GABA_B$ receptors may still play a role since baclofen can also increase LH in goldfish (Trudeau et al., 1993b). $GABA_B$ receptors are members of the seven transmembrane domain family and activate second messenger systems, such as phospholipase C and adenylate cyclase. $GABA_B$ receptors are coupled to downstream Ca^{2+} and K^+ ion channels via G-proteins (G-Protein Coupled Receptor superfamily). In general, ionotropic GABA receptors are the mediators of rapid neural responses in the vertebrate brain and the metabotropic GABA receptors are involved in a slower, prolonged neuronal response.

Pituitary LH release in teleost fish is complex and involves a number of additional neuropeptides and neurotransmitters. For example, NPY, NE, and glutamate have been shown to stimulate the release of LH and the effects of various neuropeptides and neurotransmitters are seasonal and sex dependent (reviewed in Trudeau et al., 1997). Activins, also a modulator of LH release, are members of the transforming growth factor beta subfamily and are composed of two beta subunits that form either hetero- or homo-dimers; activin A (βa + βa), activin BA (βa + βb) and activin B (βb + βb). In goldfish, activins will induce the release of LH from dispersed pituitary fragments (Ge et al., 1992) and modulate the transcription of the LHβ subunit (Ge, 2000). In hybrid male tilapia (*Oreochromis niloticus and Oreochromis aureus*), the addition of 20 ng/ml human recombinant activin A resulted in an increase of approximately 12-fold in LHβ mRNA in cultured pituitary cells *in vitro* (Yaron et al., 2001). In the thin-lipped grey mullet, Liza

ramada (Risso), activin BA is located in the telencephalon, midbrain tegmentum, and cerebellum and co-localize with pituitary gonadotrophs (Mousa and Mousa, 2003). MacConell et al. (1998) demonstrated in rat hypothalamus that activin BA is co-localized with GnRH neurons and intracerebroventricular infusion of activin A significantly increased LH secretion. In another study, MacConell et al. (1999) showed that activin A stimulated a reporter gene driven by a minimal GnRH enhancer and promoter element, suggesting that activin regulates GnRH gene transcription. Similarly, Gregory and Kaiser (2004) showed that activin up-regulates GnRH receptor gene expression and stimulates GnRH release from GnRH neurons in the hypothalamus. These studies suggest that activin stimulates reproductive processes in the mammalian hypothalamus through GnRH synthesis and release. Despite anatomical and physiological evidence for the role of activins (and GABA) in regulating LH release in fish and mammals, little work has been done on the interaction between GABA and activin.

To date, the underlying molecular events of GABA stimulated LH release in goldfish remains unclear. Leonhardt et al. (1995) demonstrated that both GnRH mRNA expression and LH release are mediated through the $GABA_A$ channel in the hypothalamus of female rats. However, 2 hours after injection of 10 nmol muscimol (ionotropic GABA receptor agonist) into the lateral ventricle of ovariectomized adult female rats, there was decreased GnRH receptor mRNA in the mediobasal hypothalamus and preoptic area (Seong et al., 1995). The same concentration of baclofen, an agonist of the metabotropic $GABA_B$ receptor, had no effect on GnRH receptor mRNA steady state. I hypothesized that GABA may regulate LH release through transcriptional changes in genes involved in stimulating or inhibiting LH release in the goldfish. In the present study, I selectively

activated the $GABA_A$ receptor and $GABA_B$ receptor *in vivo* using GABA receptor agonists muscimol and baclofen respectively to study the effects on steady state mRNA levels of GABA synthesizing and degrading enzymes (GAD65, GAD67, GABA-T), activin βa and activin βb, sGnRH, and tyrosine hydroxylase (TH) in the hypothalamus and telencephalon, the two major neuroendocrine regions of the brain. I hypothesized that GABA agonists may alter sGnRH transcription because there is evidence that GABA depletes hypothalamic stores of sGnRH and not cGnRH-II stores in goldfish (Sloley et al., 1994) and sGnRH has been shown to increase LHα and LHβ subunit mRNA in sexually regressed goldfish whereas cGnRH-II has little effect on LH subunit transcription at this stage of maturation (Khakoo et al., 1994). sGnRH neurons are also reported to be more abundant in the hypothalamus and telencephalon when compared to cGnRH-II (Yu et al., 1998). TH is the rate-limiting enzyme in the biosynthesis of catecholamines such as dopamine and I hypothesized that GABA reduces TH mRNA expression as a mechanism to reduce inhibitory DA tone on LH release. I chose a dose for each agonist that would produce a comparable response in LH release in female goldfish that were sexually regressed (Trudeau et al., 1993b).

5.2. Materials and Methods

5.2.1. Experimental protocol and RNA extraction

Common adult female goldfish were purchased from a commercial supplier (Aleong's International Inc, Mississauga, ON, CAN) in June 2004 and allowed to acclimatize to 18 °C under a natural photoperiod. Fish were fed and maintained on standard flaked goldfish food. Goldfish were anesthetised using 3-aminobenzoic acid

ethyl ester for all handling and dissection procedures. Care was taken to standardize all handling, injections, and sample protocols. In late August 2004 (muscimol) and early September 2004 (baclofen), gonad-intact, sexually regressed female goldfish were injected in the intraperitoneal (i.p.) cavity with muscimol (1 μg/g of body weight; injection volume of 1 μl/g) or baclofen (10 μg/g of body weight; injection volume of 1 μl/g) dissolved in 0.6% saline solution. Figure 5.1. shows the chemical structures of the agonists used in this study. There was no significant difference in the body weight between control and treatment fish. Both muscimol and baclofen were purchased from Sigma, St. Louis, MO, USA. Control groups received an equivalent injection volume of 0.6% saline solution. Goldfish were sacrificed and brain tissue and blood collected six hours after injection. Hypothalami were pooled (4-6/tube) and total RNA extracted using TRIzol Reagent® (Invitrogen Life Technologies, Carlsbad, CA, USA) as per the manufacturer's protocol. RNA was quantified by measuring the A260/A280 ratio in triplicate using GeneQuant® (Amersham Pharmacia Biotech, Piscataway, NJ, USA). Telencephalon samples were pooled and treated identically.

5.2.2. LH Assay

See Peter et al. (1984) and Zhao et al. (2006) for details on blood handling and the double antibody LH assay.

5.2.3. Real-Time RT-PCR

Total RNA was DNAse treated using the RNeasy® Micro Kit (Qiagen) and first strand cDNA synthesis performed using two micrograms of RNA in a final volume of 11

µl DEPC and primed with 1 µl random hexamer primers (Invitrogen). The reaction was incubated at 65 °C for 10 minutes, quickly chilled on ice, and centrifuged briefly. 4 µl 5X reaction buffer (Invitrogen), 2 µl 0.1M DTT, 1 µl 10mM dNTPs, and 1 µl RNAse inhibitor was added, gently mixed, and heated at 42 °C for 2 minutes. 1 µl Superscript™ II RNase H⁻ Reverse Transcriptase (Invitrogen) was added and the reaction was allowed to continue at 42 °C for 50 minutes. The reaction was inactivated at 70 °C for 15 minutes and stored at –20 °C until used.

Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) was used to design primers. Primers of 18-22 base pairs (bp) with optimal annealing temperature between 59-61 °C were designed to amplify sequences of 100 to 250 bp. Primers were initially tested using goldfish whole brain cDNA and the resultant amplicons were cloned and sequenced to confirm specificity. Real-time RT-PCR analysis of gene expression was carried out on first-strand cDNA derived from DNase treated RNA samples from control and treatment groups in both the hypothalamus and telencephalon. Each PCR reaction contained the following final concentrations; approximately 25 ng first-strand cDNA template, 1X QPCR buffer, 3.5mM $MgCl_2$, 100-150nM gene specific primer (depending on the primer set used), 0.25X SYBR green (Invitrogen), 200µM dNTPs, 1.25U HotStarTaq (Invitrogen), and 100nM ROX reference dye, in a 25µl reaction volume. The primer sets used in this study are reported in Table 1.

The thermal cycling parameters was as follows: initial 1 cycle Taq activation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 58-60°C for 5 seconds (depending on the primer set used), 72°C for 30 seconds, and a detection step at 80°C for 8 seconds. Dilutions (1:10 to 1:31250) of cDNA from each sample were used to

construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was produced starting at 55°C (+1°C/30 seconds) to 95°C. Real-time RT-PCR was assayed on an MX4000® Multiplex Quantitative PCR system (Stratagene) and the accumulation of PCR product was measured in real time as the increase in SYBR green fluorescence. Data was analyzed using the MX4000 Software Package. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified PCR product as a template, and were used to calculate mRNA copy number in each sample. Each sample (n =6-8) represented a pooled sample of three brain tissues. We used beta-2 microglobulin as our reference gene and determined it did not change in response to our treatments (geNORM software; (http://medgen.ugent.be/~jvdesomp/genorm/). Mann-Whitney U tests were used to determine whether there were significant differences in real-time PCR data ($p<0.05$). This test has been shown to be a robust test in identifying differentially expressed genes (Troyanskaya et al., 2002).

5.3. Results

5.3.1. Effects of GABA agonists on serum LH

Both muscimol and baclofen treatments significantly elevated serum LH after 6 hours ($P<0.01$) (Figure 5.2.A,B). Saline injected fish in August had a mean (± SE) serum LH level of 2.5 (0.39) ng/ml and ranged from 0.8 to 4.1 ng/ml. Muscimol injected fish had a mean (± SE) serum LH level of 7.3 (0.87) ng/ml and ranged from 2.2 to 9.9 ng/ml. Saline injected fish in September had a mean (± SE) serum LH level of 3.0 ng/ml (0.44)

and ranged from 1.1 ng/ml to 6.3 ng/ml. Baclofen injected fish had a mean (± SE) serum LH level of 10.8 (1.9) ng/ml and ranged from 5.2 to 15.2 ng/ml.

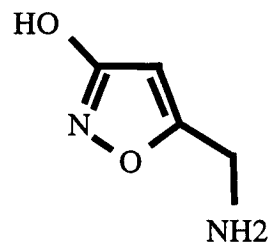
5.3.2. Muscimol: Real-time RT-PCR

Following muscimol injection, GAD65 mRNA in the telencephalon was reduced approximately 10-fold ($p=0.03$) and GABA-T mRNA was significantly decreased in the telencephalon approximately 15-fold ($p=0.03$) (Figure 5.3.A,B) based on comparison of median values. TH mRNA was significantly reduced in the telencephalon after muscimol treatment approximately 2-fold ($p=0.03$) (Figure 5.3.C) based on comparison of median values. There were no changes in activin βa, activin βb, or sGnRH mRNA in the telencephalon. There were no significant changes in the relative abundance of any gene investigated in the hypothalamus after muscimol treatment (data not shown).

5.3.3. Baclofen: Real-time RT-PCR

Following baclofen injection, GAD67 mRNA in the hypothalamus was reduced approximately 2-fold ($p=0.004$) and GABA-T mRNA was significantly decreased in the telencephalon approximately 3-fold ($p=0.004$) (Figure 5.4.A,B) based on comparison of median values. Activin βa mRNA was significantly induced in both the hypothalamus and telencephalon after baclofen treatment approximately 3-4 fold ($p<0.02$) (Figure 5.4.C) based on comparison of median values. There were no changes in GAD65, activin βb, or sGnRH mRNA in the telencephalon. There were no significant changes in the relative abundance of any gene other than activin βa in the hypothalamus following baclofen treatment (data not shown).

A)



B)

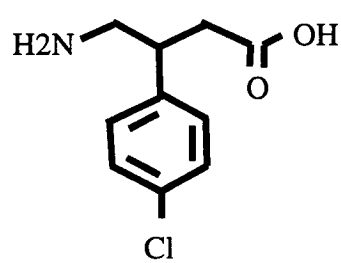
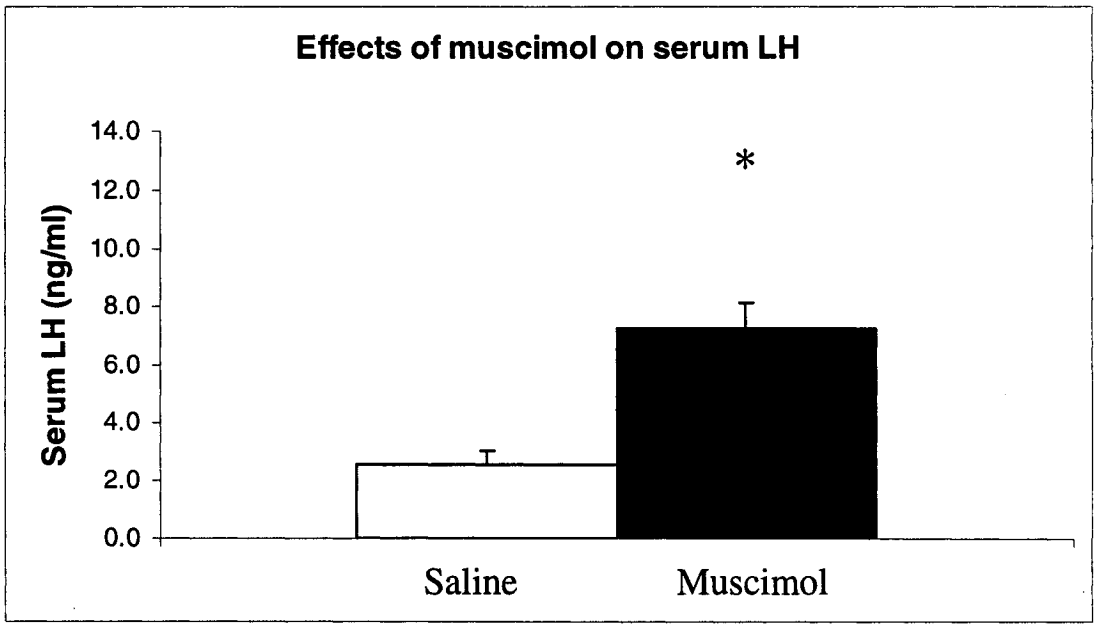


Figure 5.1 Chemical structure of GABA agonists A) muscimol and B) baclofen.

A)



B)

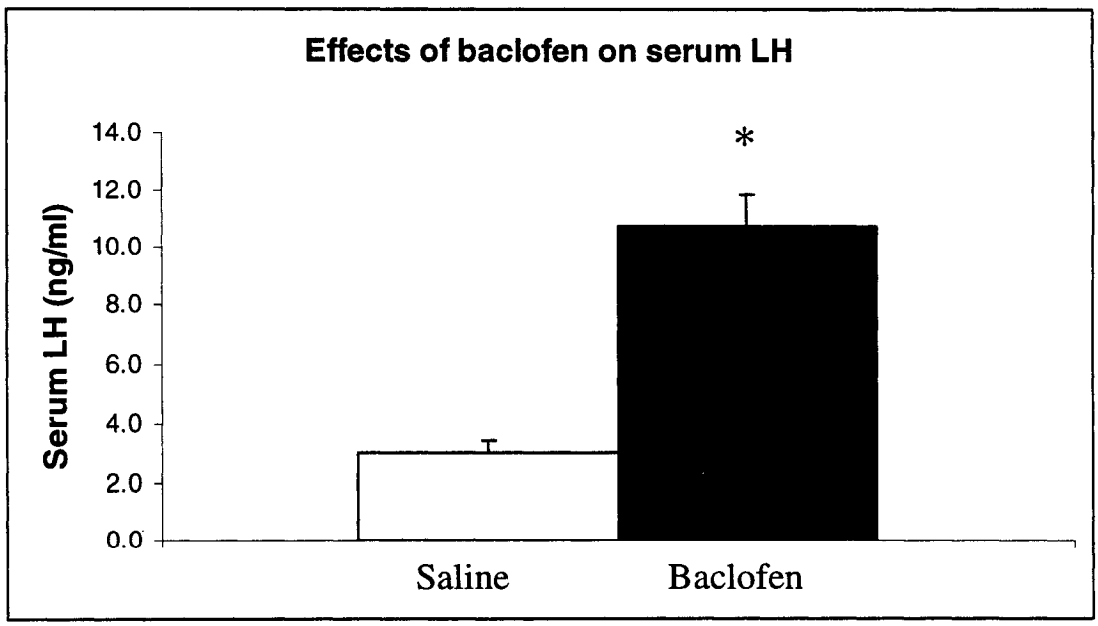
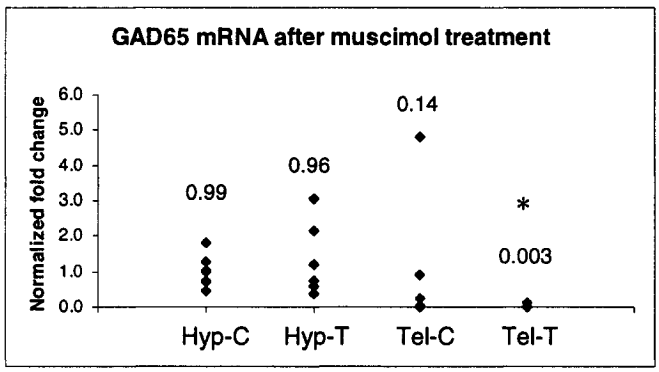
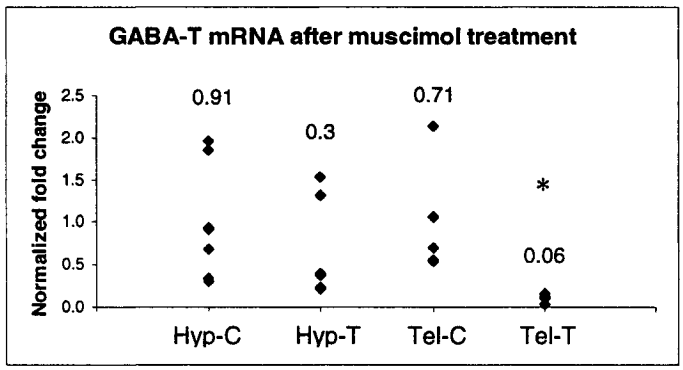


Figure 5.2. A) Effects of muscimol and B) baclofen on serum LH concentration after single i.p. injection after 6 hours. Asterisk denotes significant difference between control and treatment ($p<0.05$).

A)



B)



C)

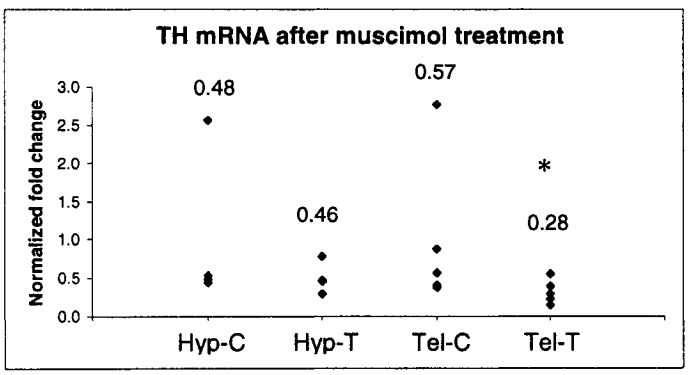
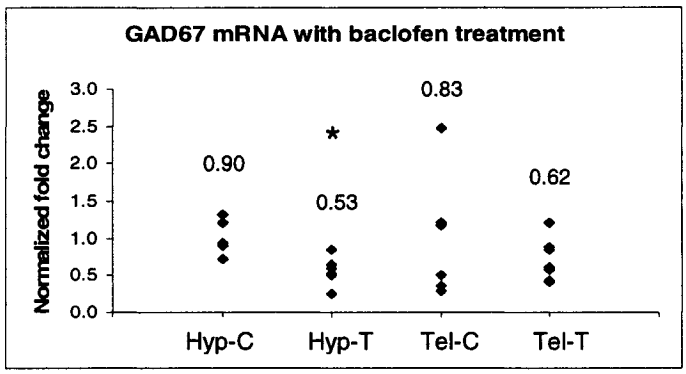
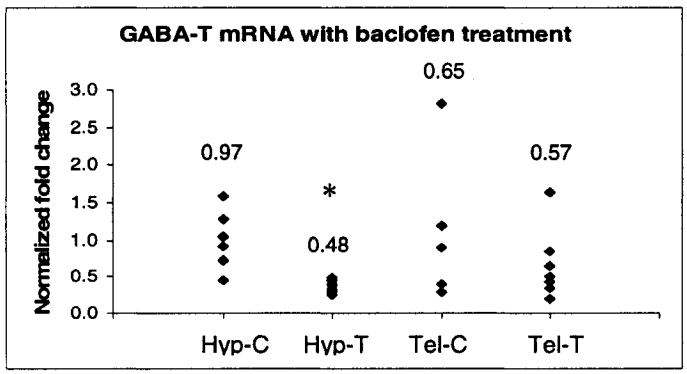


Figure 5.3. Normalized fold changes (using expression of B2M) relative to average fold change of controls in the hypothalamus (Hyp) and telencephalon (Tel) determined by real-time RT-PCR after muscimol treatment (n=5-8); A) GAD65 B) GABA-T and C) TH. Median fold change for each group shown above control (C) and treatment (T) groups. Asterisk denotes significant difference between control and treatment ($p<0.05$).

A)



B)



C)

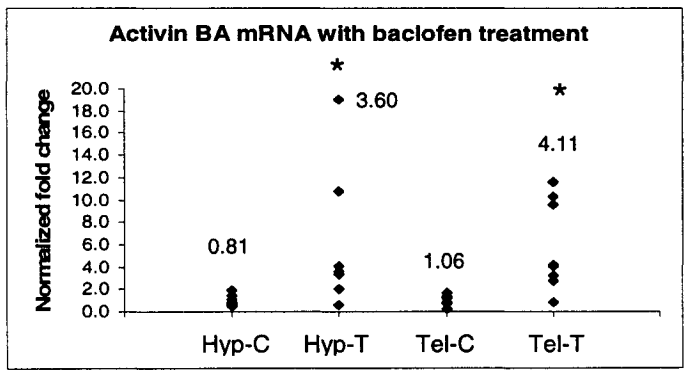


Figure 5.4. Normalized fold changes (using expression of B2M) relative to average fold change of controls in the hypothalamus (Hyp) and telencephalon (Tel) determined by real-time RT-PCR after baclofen treatment (n=6-7); A) GAD67 B) GABA-T and C) activin βa. Median fold change for each group shown above control (C) and treatment (T) groups. Asterisk denotes significant difference between control and treatment ($p<0.05$).

Table 5.1. Primers for real-time RT-PCR

Gene	Forward primer 5' to 3'	Reverse Primer 5' to 3'
B2 microglobin	GCC CTG TTC TGT GTG CTG TA	AAG GTG ACG CTC TTG GTG AG
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
GAD 65	GGA TAC GTG CCG TTC TTT GT	CTC GAC TCC ATT CAG CTT CC
GAD 67	CCA AAG GCA TGT CTG TAG CA	CCC TTC TGT TTG GCA TCA AT
GABA-T	TGC TGTGCC AGG TCC AAA	TGA TTG TAA CCG ATG GGG ATG
sGnRH	CTG GTC ATA CGG TTG GCT TC	CAT CAG CAT CCA CTT CAT TCA C
Tyrosine hydroxylase	AGC ACA CTG GTC AGC TCT CTC	GCC ATG TTT CGA TCT CCTCT

5.4. Discussion

5.4.1. Muscimol and baclofen stimulate LH release in sexually recrudescent goldfish

I observed a significant increase in the concentration of serum LH after GABA agonist i.p. injection. Both muscimol and baclofen increased LH approximately 3-fold after a 6 hour period indicating that GABA stimulated LH release is mediated by both GABA receptor subtypes. Trudeau et al. (1993b) showed that female goldfish injected with muscimol and baclofen in January-February exhibit a dose response in LH. Muscimol, at a dose of 1.0 ug/g body weight, raised LH levels to approximately 45 μg/ml and baclofen, at the same dose, raised LH levels to approximately 20 μg/ml. A higher dose of baclofen (10 μg/g body weight) raised LH to similar levels as 1.0 μg/ml muscimol. This effect of GABA agonists on serum LH was also rapid, occurring after only 30 minutes.

5.4.2. GAD and GABA-T steady-state mRNA in the goldfish following GABA agonist injection

After muscimol treatment, GAD65 and GABA-T steady state mRNA significantly decreased in the telencephalon after 6 hours. A reduction in GABA synthesis via modulation of the expression of GAD65 may be a response to increases in neuronal activity of GABA. I observed a similar result in the hypothalamus with baclofen treatment, however, it was GAD67 and GABA-T mRNA that were significantly reduced after 6 hours. GABA agonists may have different transcriptional effects on genes involved in GABA synthesis and degradation in neuroendocrine tissues in the goldfish. Studies in both mammals (Rimvall et al., 1993; Mason et al., 2001) and fish (Martyniuk

et al., 2005) have investigated the effect of increasing GABA levels following GVG injection on GAD expression, but none that I am aware of has investigated the transcriptional response of these enzymes to specific GABA receptor agonists. Studies have reported that the expression of GAD67 mRNA transcription and GAD67 activity are more sensitive to increasing GABA concentrations in the mammalian brain than GAD65 (Rimvall et al., 1993, 1994; Sheikh et al., 1998). This study demonstrates that the transcription of GADs and GABA-T in the neuroendocrine brain can be differentially sensitive to GABA agonists in the goldfish.

What is interesting is how GAD65 and GAD67 mRNA steady state is altered with the two different agonists. GAD65 and GAD67 show differences in regulation, abundance, and function within the CNS (Kaufman et al., 1991; Feldblum et al., 1993; Asada et al., 1997). There is evidence to suggest that GAD65 is more involved in the synaptic release of GABA and is localized to the nerve terminals whereas GAD67 is involved in the regulation of the GABA metabolic pool (Waagepetersen et al., 2001). In the present study, both GABA agonists decreased steady state mRNA of GAD65 (muscimol), GAD67 (baclofen) and GABA-T (both agonists). The overall effect of decreasing the transcription of both the synthesizing and degradative GABA enzymes on the level of synaptic or metabolic GABA present is not known. In the context of reproduction, GABA increases the release of GnRH from goldfish pituitary fragments with intact nerve terminals in a dose-dependent manner (Kah et al., 1992) and in this study, a significant increase in LH was observed. It is plausible that regulating GABA levels through GAD and GABA-T gene expression has downstream effects on pituitary LH release, however, but this must be evaluated further.

5.4.3. Activin βa, but not activin βb, mRNA is significantly up-regulated in the brain after baclofen treatment

Transcription of activin βa, a member of the transforming growth factor-beta superfamily (TGF-beta), was significantly induced by baclofen in both the hypothalamus and telencephalon. There were no changes in activin βb mRNA levels in the neuroendocrine tissues examined with either muscimol or baclofen, suggesting that activin βa and βb mRNA are under different regulatory factors in the CNS, similar to what has been reported in the gonads (Wang and Ge, 2004). Few studies have investigated the possible role of GABA in the transcriptional regulation of activins in fish. Activin βa mRNA is highly expressed in layers of the mammalian neocortex (II/III and V/VI) and appears to be induced by excitatory input in developing brains as shown by glutamate agonists and $GABA_A$ antagonist bicuculline (Andreasson and Worley, 1995).

In neuroendocrine regions of the goldfish, the rapid and substantial increase in activin βa mRNA, in concert with the increase in LH release, suggests that this may be an important mechanism by which GABA affects LH release in fish. In mammals, activins have been shown to stimulate GnRH release from GnRH neurons in the hypothalamus and also directly affect FSH and LH secretion from the pituitary (see Gregory and Kaiser, 2004). In a mouse pituitary gonadotroph cell line, activin A *in vitro* was able to up-regulate GnRH receptor expression (GnRHR-Luc construct) approximately 2.5 fold after 12 hours (Fernandez-Vazquez et al., 1996). In a mouse GnRH secreting neuronal cell line (GT1-7), activin A stimulated GnRH mRNA transcription (MacConell et al., 1999). The authors also used rat hypothalamic explants and demonstrated that recombinant

activin A induced both GnRH mRNA transcription after 6 hours and increased GnRH peptide secretion approximately 80% after 1 hour relative to control. The effects of activins on GnRH transcription appear to involve a paracrine mode of action. Florio et al. (2000) observed a significant release of activin A from cultured GnRH neuronal cells collected from human olfactory neurons. This study demonstrates that GABA *in vivo* up-regulates activin βa transcription through the $GABA_B$ receptor and this may be one mechanism by which GABA stimulates LH release in teleosts. In the goldfish, Ge et al (1992) demonstrated that porcine activin A acutely induced LH release from primary cultures of dispersed goldfish pituitary cells. Increased activin production in GnRH neurons and surrounding cells may in turn stimulate GnRH release leading to enhanced LH release. However, we detected no change in the hypothalamus or telencephalon in sGnRH mRNA expression with baclofen or muscimol. A previous study using an irreversible inhibitor of GABA, gamma vinyl GABA (GVG), did not detect any significant changes in sGnRH or cGnRH-II mRNA to increased levels of GABA (Chapter 4; Martyniuk et al., 2005). These studies suggest that GnRH release may be more influenced by GABA than GnRH transcription in the goldfish.

5.4.4. Decreased effect of inhibitory DA on GnRH stimulated LH release through $GABA_A$ receptors?

DA is the major neurotransmitter inhibiting LH release in fish. Trudeau et al. (1993b) showed that after 24 hours, increased GABA levels following GVG injection did not alter static levels of DA in the hypothalamus, telencephalon, or pituitary. However, GVG treatment reduced DA turnover in sexually regressed fish in the hypothalamus and

pituitary. Domperidone (1 μg/g), a DA type2 receptor antagonist, potentiated muscimol stimulated LH release *in vivo*. In another study investigating the effects of dopamine depletion on GABA synthesis in goldfish, it was shown that there was a 30% increase in hypothalamic GABA levels after αMPT treatment, an inhibitor of tyrosine hydroxylase, that was accompanied by an increase in GAD67 mRNA in this tissue (Hibbert et al., 2005). The authors suggested that the increase in GABA synthesis after dopamine depletion may contribute to increased LH release. This study showed that muscimol decreases TH mRNA transcription in the telencephalon, which may contribute to decreased DA turnover observed after GABAergic manipulation (Trudeau et al., 1993b). Interestingly, DA partially reduces the stimulation of LH release from goldfish pituitary by activin A (Ge et al., 1992). Thus, a decrease in dopaminergic inhibition may lead to an increased stimulatory effect of activin on GnRH release as well.

In summary, this study shows 1) GABA may regulate its own synthesis and degradation differentially through the $GABA_A$ and $GABA_B$ receptors *in vivo,* 2) that goldfish activin βa, and not βb, mRNA in both neuroendocrine tissues investigated is rapidly induced by baclofen treatment *in vivo,* 3) the effects of GABA agonists on LH release may not be through sGnRH transcription but rather GnRH synthesis and release, and 4) decreased DA synthesis in the telencephalon via $GABA_A$ receptors may reduce inhibition of GnRH neurons by dopamine in this region. A putative model for GABA stimulated LH release in the goldfish is presented in Chapter 7.

Chapter 6

Gene expression profiling in the neuroendocrine brain of male goldfish (*Carassius auratus*) exposed to 17α-ethinylestradiol

Adapted from: Christopher J Martyniuk, Huiling Xiong, Kate Crump, Suzanne Chui, Ravinder Sardana, Ashlie Nadler, Emily Gerrie, Xuhua Xia, and Vance L Trudeau. 2006. (accepted to Physiological Genomics, August 2006).

6.1. Introduction

There has been considerable attention in recent years to the detrimental effects of endocrine disrupting chemicals (EDCs) and pharmaceuticals on wildlife populations (Trudeau et al., 2005). The contraceptive estrogen, 17α-ethinylestradiol (EE2), is a pharmaceutical of concern because it is constantly being introduced into the environment (i.e. pseudopersistant). EE2 is detectable in the final effluent of some municipal wastewater treatment plants at concentrations approaching 40 ng/L (~ 0.2 nM) (Ternes et al., 1999; Yin et al. 2002) and a recent study of 139 U.S. rivers reported maximum concentrations of 830 ng/L (~ 2.8 nM) for EE2 at the point source of sewage effluent (Kolpin et al. 2002). High levels of EE2 (>1000 ng/L) have been shown to induce severe morphological deformities in fish such as skeletal abnormalities (e.g. lordosis and craniofacial) and soft tissue swelling (Boudreau et al., 2004). At lower concentrations, EE2 disrupts sex determination at ~ 15 ng/L (Andersen et al. 2003) and reduces successful reproduction in fish at ~ 100 ng/L, thereby reducing the lifetime reproductive fitness of an individual (Kristensen et al., 2005). It has also been reported that the potency of EE2 *in vivo* in fish may be up to 30-fold higher than the natural estrogens,

17β-estradiol (E2) and estrone (E1) (Thorpe et al., 2003). Thus, EE2 has the potential to disrupt reproductive processes in fish at relatively low concentrations.

In vitro and *in vivo* assays are now widely used to detect the effects of EDC exposure in aquatic animals. The yolk precursor protein vitellogenin (Vtg) in the liver is commonly used as a sensitive and robust *in vivo* biomarker for estrogenic exposure in male fish (Sumpter and Jobling, 1995; Rose et al., 2002). However, data collected from multiple endpoints should be evaluated in order for environmental monitoring programs to be reliable, sensitive, and robust in detecting EDC exposure. Gene expression profiling offers an advantage over more traditional endpoints by screening large numbers of genes simultaneously and gene arrays for teleost fish have been used to study the genomic response in cold acclimation (Ju et al., 2002; Gracey et al., 2004), hypoxia (Ton et al., 2003), and pharmaceuticals of concern, such as chlorpromazine (van der Ven et al., 2005). Most data available for the effects of estrogenic compounds on the transcriptome in fish have largely considered a limited number of genes in peripheral tissues such as liver (Larkin et al., 2003; Brown et al., 2004). To our knowledge, no studies report on the effects of waterborne EE2 in the brain of a teleost fish.

This chapter describes the strategy used to construct a goldfish brain derived cDNA array to study neuroendocrine function in the goldfish. The goldfish brain cDNA array was produced using suppressive subtractive hybridization and contains approximately 1200 partial gene fragments. In addition, these gene fragments were printed along with genes isolated from a mixed tissue carp (*Cyprinus carpio* L.) microarray (see Gracey et al., 2004 for details on the carp array). The common carp is a cyprinid fish that is closely related to the goldfish, diverging less than 10 million years

ago (Zardoya and Doadrio, 1999). Rise et al. (2004) constructed an EST library derived from a number of salmonid species and tissues and investigated the applicability of using cross-species hybridizations. The authors found that approximately 76% of ESTs that were aligned between Atlantic salmon (*Salmo salar*) and rainbow trout (*Onchorhynchus mykiss*), two salmonids that diverged from a common ancestor approximately 8-20 million years ago, had greater than 90% similarity at the nucleotide level. Therefore, a cross-hybridization approach is useful for studies involving closely related fish species.

To date, there is limited data on the impact of environmental pharmaceuticals on normal neuroendocrine function in non-target aquatic organisms. However, this is important considering that coordination of the HPG axis is critical to sexual differentiation and normal reproductive function. The effects of waterborne EE2 in the neuroendocrine brain of male goldfish was investigated using a brain enriched cDNA goldfish-carp microarray. The effects of EE2 were studied because of its high potency, pseudopersistance, and presence in many water systems. I chose two nominal concentrations of 0.1 nM (29.6 ng/L) and 1.0 nM (296 ng/L) EE2. The low dose was similar to environmental concentrations of EE2 and the higher dose was used to represent the highest amount reported in some river systems in the US (Koplin et al., 2002). I studied the response of the transcriptome to EE2 in the hypothalamus using our goldfish-carp microarray because this tissue is high in brain aromatase (cyp19b) activity in teleosts (Pasmanik and Callard, 1985), suggesting that the teleost brain is sensitive to E2, and potentially to xenoestrogens. The expression of a subset of genes was also investigated in the telencephalon because this region of the brain contains the preoptic area and in fish plays a significant role in the CNS control of reproduction.

6.2. Materials and Methods

6.2.1. Experimental design and EE2 exposure

Common adult male goldfish (Aleong's International Inc, Mississauga, Ontario, Canada) were purchased in October 2004. Fish were acclimatized over several months to 18 °C under a natural photoperiod and maintained on standard flaked goldfish food. Goldfish were anesthetised using MS222 (3-aminobenzoic acid ethyl ester) for all handling and dissection procedures. Care was taken to standardize all handling, injections, and sample protocols. In March 2004, gonad-intact male goldfish were separated into 15 fish per 70 L tank and exposed to nominal concentrations of 0.1 nM EE2 and 1.0 nM EE2 for 15 days. EE2 was dissolved in 95% ethanol and added to treatment tanks every three days (static incubation; final ethanol concentration was <0.001% volume of tank). All control tanks received an equal amount of ethanol throughout the experiment. At the end of every third day, fish were removed from tanks and placed into new tanks with a fresh dose of EE2. After treatment, a subset of goldfish was weighed and a blood sample taken by puncture of the caudal vasculature using a 25-gauge needle attached to a 1 mL syringe. Serum was collected by spinning the blood at 7500 rpm at 4 °C for 15 minutes. Serum was stored at -20 °C until used for the radioimmunoassay. Gonad weight was also recorded for the aforementioned subset of fish. The hypothalamus and telencephalon for all fish were rapidly dissected, pooled (2-3 tissues/tube) and stored at -80 °C until total RNA extraction.

6.2.2. Testosterone (T) and E2 RIA

Steroids were extracted and measured following McMaster et al. (1992). Percent cross reaction of antigen with the steroid of interest was >99% (Medicorp Inc., QC, CAN). Unpaired Students T-test was used to test for significant differences between the control and each of the treatment groups.

6.2.3. Subtracted brain goldfish cDNA library

I would like to acknowledge Ravinder Sardana, Suzanne Chui, Kate Crump, and Emily Gerrie, all who had a significant role in the production of the microarray. To produce our brain enriched cDNA array, we extracted total RNA from the hypothalamus, telencephalon, and muscle of ten (5 males and 5 females) goldfish using the RNeasy® Mini Kit (Qiagen, Mississauga, Ontario, Canada) following the manufacturer's protocol. mRNA was isolated from total RNA using Oligotex mRNA Mini Kit (Qiagen). To generate a subtracted library for genes expressed in the hypothalamus and telencephalon, a PCR-select cDNA Subtraction kit (BD Clonetech, Mississauga, Ontario, Canada) was used. Hypothalamus and telencephalon mRNA were used as the "tester" and muscle mRNA as the "driver". 2 μg mRNA from tester and driver was heated with 10 mM cDNA synthesis primer (final volume of 5 μL) to 70 °C for 2 minutes and then cooled on ice for 2 minutes. Each reaction mixture was made up to 10 μL by adding 2 μL 5X first strand buffer, 1 μL dNTP (10 mM each), 1 μL sterile water and 1 μL AMV reverse transcriptase. The reaction mixtures were incubated at 42 °C for 1.5 hours in an air incubator. Following first strand synthesis, second strand synthesis was performed by adding 48.4 μL sterile water, 16 μL 5X second strand buffer, 1.6 μL dNTP (10 mM) and

4 μL 20X second strand enzyme cocktail. Reactions were incubated at 16 °C for 2 hours. The double stranded cDNA was blunted by the addition of 2 μL T4 DNA polymerase, followed by incubation at 16 °C for 30 minutes before the reaction was terminated by the addition of 4 μL 20X EDTA/glycogen mix. Double stranded cDNA was purified by phenol/chloroform extraction. Double stranded cDNA from the tester and driver were separately digested with Rsa I at 37 °C for 1.5 hours. The digested fragments were purified by phenol/chloroform extraction. cDNA fragments from the tester were linked to adaptor 1 and adaptor 2 by T4 ligase. For the first hybridization, cDNA from tester with adaptor 1 and adaptor 2 was mixed with driver cDNA separately, and hybridized at 68 °C for approximately 8 hours. Additional fresh driver was added directly to the pooled mix of the two previous hybridizations and incubated at 68 °C for 20 hours for the second hybridization.

Differentially expressed cDNA were selectively amplified during the following two PCR reactions. In the first amplification, only double stranded cDNA with different adaptor sequences on each end are amplified with PCR primer (5'-CTAATACGACTCACTATAGGGC-3'). In the second amplification, nested PCR was used to further reduce background and enrich for differentially expressed sequences with nested primers (5'-TCGAGCGGCCGCCCGGGCAGGT-3' and 5'-AGCGTGGTCGCGGCCGAGGT-3'). The nested PCR cDNA mix was further incubated at 72 °C for 1 hour with additional dATP Taq DNA polymerase to ensure that most of the cDNA fragments contained A overhangs. Approximately 100 ng PCR cDNA was ligated into 50 ng pCR®II-TOPO® vector and transformed in *Escherichia coli*

competent cells using the TA-cloning kit (Invitrogen Life Technologies, Carlsbad, CA, USA).

6.2.4. Goldfish brain cDNA array

Approximately 1100 positive white colonies were selected at random and cultured overnight at 37 °C in 3 mL LB medium for storage. In addition to the random selection of clones from the library, we targeted approximately 30 genes for the array, including several genes involved in neurotransmitter function. We also obtained an additional 20 gene fragments from collaborators, such as genes coding for receptors and neuropeptides. To amplify cDNA fragments, 4 μL of template was added to a 100 μL reaction containing 1X PCR buffer, 1.5 mM MgCl, 150 μM dNTPs, 300 μM forward primer (5'-CACGCAGTTGTAAAACGAC-3') and reverse primer (5'-GGATAACAATTTCACACAGG-3'), and 2.5 U Taq polymerase (Invitrogen). PCR cycles consisted of a denaturation step at 95 °C for 15 seconds, followed by an annealing step at 52 °C for 30 seconds, and an extension step at 72 °C for 45 seconds for 38 cycles. Amplified PCR products were purified using Millipore PCR clean-up kit or MicroSpin columns™ (S-300HR, Amersham Pharmacia Biotech, Piscataway, NJ, USA) and resuspended in a final concentration of 3X SSC in 50 μL DNase/RNase free H_2O in 96 well plates. Final concentrations of purified PCR product ranged between 50-80 ng/μL. Partial sequences were sent to Canadian Molecular Research Services (CMRS, Ottawa, Ontario) for sequencing. Nucleotide sequences were compared to known sequences in GenBank in the National Center of Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov) database. Goldfish clones were sent to the University of

Liverpool Microarray Facility, United Kindgom (http://www.liv.ac.uk/lmf/root/Liverpool%20Microarray%20Facility) for printing with approximately 8000 carp genes. All genes were printed in duplicate onto poly-L-lysine coated glass sides. Our cDNA microarray also included the Stratagene SpotReport™ Alien™ Array Validation System (Stratagene, La Jolla, CA, USA). Goldfish clones have been annotated and sequence information has been deposited in GenBank (dbEST). More information about the goldfish clone database can be found on www.auratus.ca.

6.2.5. Microarray hybridization and scanning

For microarray hybridizations, total RNA was extracted using TRIzol® Reagent (Invitrogen) as per the manufacturer's protocol. Total RNA was resuspended in 30 μL of RNase-free water and quantified using a GeneQuant® spectrophotometer. We pooled total RNA from approximately 30 fish into a reference control RNA pool. Three separate pools of RNA from treated fish were hybridized to the printed microarrays, and a fourth hybridization was a replicate dye-reversal of one of the three RNA pooled samples. This was done with hypothalamic RNA from both treatments and resulted in a total of 8 microarrays (four arrays/EE2 dose). We used the Genisphere Array 900MPX™ cDNA microarray labeling kit (Genisphere, Hatfield, PA, USA) for all microarray hybridizations. This indirect labeling kit uses Cyanine 3 (Cy3) and 5 (Cy5) as the fluorescent dyes. The complete hybridization protocol is found at (http://www.genisphere.com/pdf/array900mpx_protocol_v06-22-04.pdf). We used 2 μg total RNA for the first strand synthesis. The 2X formamide based hybridization buffer

was used for the microarray pre-hybridization step and the 2X SDS based hybridization buffer was used for the light capture reaction (3DNA hybridization step).

Microarrays were scanned at full speed 10 μm resolution using the ScanArray 5000 XL system (Packard Biosciences/PerkinElmer) using both red and blue lasers. Images were obtained with ScanArray Express software using automatic calibration sensitivity varying PMT gain (PMT starting at 65% for Cy5 and 70% for Cy3) with fixed laser power at 80% and the target intensity set for 90%. Microarray images were opened using QuantArray (Packard Biosciences/Perkin Elmer) and raw signal intensity values obtained for duplicate spots of genes. Raw intensity values for all microarray data and microarray platform information has been deposited to the NCBI Gene Expression Omnibus database (platform accession # GPL3735; series accession #GSE4868).

6.2.6. Data normalization and identification of differentially expressed genes

I would like to acknowledge Huiling Xiong and Dr. Xuhua Xia, both of which had a significant role in the microarray database construction and data analysis. The array quality filter test (AQF) (Sauer et al., 2005) was first applied to check the raw data. The AQF values of all slides were less than the threshold of 0.5. Spots that had been manually flagged due to poor hybridization and spots in which the estimated fluorescence intensity was below or equal to the estimated background signal intensity in either channel were removed prior to further analysis. Several normalization strategies, including intensity-dependent Lowess normalization, within-tip-group Lowess normalization and global normalization, were compared (Yang et. al., 2002) through examining normalization effects on M/A plots where M is log ratio and A is overall

signal intensity from both two channels. The intensity-dependent Lowess normalization with span 0.4 was chosen for normalization because there was no significant variance between the print tips. Box plots of the Lowess $\log_2$ ratios for each of the 4 slides were centered at zero and had similar spreads (Figure 6.1.), therefore no further normalization procedure between slides was carried out. Significance analysis of microarray (SAM) method was performed to assess the significance of differential expression of the genes (Tusher et al, 2001). This technique computes a statistic for each gene and measures the strength of the relationship between gene expression and the response variable. Repeated permutations of the data determine whether the expression of a specific gene was significantly different between test groups. The criterion for significance was $q<5$ which is based on the false detection rate (FDR). FDR is the percentage of significant genes identified by chance while the q value is the minimum false discovery rate at which the gene is significant ($q<5$). The q value is an adjusted p-value, and it is designed for the FDR analysis using SAM.

6.2.7. Real-Time RT-PCR

All primers were designed using Primer3 (http://frodo.wi.mit.edu/cgi-bin/primer3/primer3_www.cgi) and synthesized by Invitrogen. Primers of 18-22 base pairs (bp) with optimal annealing temperature between 58-62 °C were designed to amplify sequences of 100 to 250 bp within the gene region printed on the array. Primers were initially tested using goldfish whole brain cDNA and the resultant amplicons were cloned and sequenced to confirm specificity.

Real-time RT-PCR analysis of gene expression was carried out on first-strand cDNA derived from DNase treated RNA samples from control and treatment groups. We also tested whether genes identified as being regulated by microarray analysis in the hypothalamus were also regulated in the telencephalon. Each PCR reaction contained the following final concentrations; approximately 25 ng first-strand cDNA template, 1X QPCR buffer, 3.5 mM $MgCl_2$, 100-150 nM gene specific primer (depending on the primer set used), 0.25X SYBR green (Invitrogen), 200 μM dNTPs, 1.25U HotStarTaq (Invitrogen), and 100 nM ROX reference dye, in a 25 μl reaction volume. The primer sets used in this study are reported in Table 1.

The thermal cycling parameters were an initial 1 cycle Taq activation at 95°C for 15 minutes, followed by 40 cycles of 95°C for 15 seconds, 58-60°C for 5 seconds (depending on the primer set used), 72°C for 30 seconds, and a detection step at 80°C for 8 seconds. Dilutions (1:10 to 1:31,250) of cDNA from each sample were used to construct a relative standard curve for each primer set. After the reaction was complete, a dissociation curve was produced starting at 55°C (+1°C/30 seconds) to 95°C. Real-time RT-PCR was assayed on an MX4000® Multiplex Quantitative PCR system (Stratagene) and the accumulation of PCR product was measured in real time as the increase in SYBR green fluorescence. Data was analyzed using the MX4000 Software Package. Standard curves relating initial template copy number to fluorescence and amplification cycle were generated using the amplified PCR product as a template, and were used to calculate mRNA copy number in each sample.

We also chose four candidate control genes with differing biological functions (β-actin, β-2 microglobulin, ribosomal 18 subunit, and elongation factor 1 (EF-1) as possible

controls to normalize our expression data. We used geNORM software (http://medgen.ugent.be/~jvdesomp/genorm/) to determine the most stable housekeeping gene. To further evaluate the sensitivity of our microarray, we chose three additional genes as negative controls to investigate with real-time RT-PCR that 1) were not identified by our microarray analysis as being regulated by EE2 and 2) show seasonal variation and sexual dimorphism in mRNA expression. These included glutamic acid decarboxylase (GAD) 65 and 67 (Bosma et al., 2001; Larivière et al., 2005) and the neuropeptide isotocin (Ota et al., 1999). We did this to further demonstrate and increase our confidence in the ability of our microarrays to identify regulated transcripts. This is rarely done in microarray analysis. GAD is the enzyme that converts glutamate into GABA, a neurotransmitter that stimulates LH release in fish (reviewed in Trudeau et al., 2000c). Isotocin is highly expressed in the teleost brain and is involved in reproductive behavior in fish (Goodson and Bass, 2000). An unpaired Students T-test on transformed data was used to determine whether there were significant differences in expression between the control and treatment groups. When data could not be normalized, a Mann-Whitney U test was used.

6.3. Results

6.3.1. Gonadosomatic index (GSI) of EE2 treated male goldfish

Body weight in control and treatment groups did not significantly differ from each other and ranged from 11 g to 40 g for both control and treatment fish. GSI is defined as (gonad weight / body weight) x 100 %. Male goldfish treated with 1.0 nM EE2 had significantly smaller gonads than goldfish treated with 0.1 nM EE2 ($p<0.01$) and ethanol

treated controls ($p<0.02$) (Figure 6.2.). There was no significant difference in GSI between control goldfish and the 0.1 nM EE2 treatment group.

6.3.2. Serum T and E2 levels

Male goldfish exposed to both levels of EE2 had significantly reduced levels of circulating T when compared to control animals (Figure 6.3A) ($p<0.02$). Mean T (± SE) levels in control goldfish were 1600 ± 580 pg/ml and ranged between 800 to 3300 pg/ml (n=5). In males exposed to 0.1 nM EE2, mean T was 550 ± 140 pg/ml and ranged from 210 to 770 pg/mL. In males exposed to 1.0 nM EE2, mean T was 390 ± 60 pg/ml and ranged from 210 to 480 ng/L.

Male goldfish exposed to both levels of EE2 had significantly reduced levels of circulating E2 when compared to control animals (Figure 6.3B) ($p<0.01$). E2 was detected at very low levels in the blood of all males sampled. Mean (± SE) E2 levels in control goldfish were 210 ± 54 pg/ml E2 and ranged between 33 to 460 pg/ml (n=7). In males exposed to 0.1 nM EE2, mean E2 was 5 ± 2 pg/ml and ranged from not detectable to 14 pg/ml. All samples in the high EE2 treatment were below the detection limit of the assay and were assigned the lowest detectable concentration value of the assay. Thus, we were not able to determine the absolute pg/ml concentration of circulating E2 in treatment males. However, there was a dramatic and significant reduction in treatment groups when compared to controls.

6.3.3. Microarray and real-time RT-PCR expression data

The goldfish brain derived portion of the current microarray contains approximately 5% receptor related genes (e.g. ERs, GABA receptor subunits, dopamine receptors), 15% structural proteins (e.g. ependymin, tubulin), 5% metabolic-related, 10% neuropeptide or neurotransmitter-related (e.g. GnRH, transporters, isotocin), 2-3% enzymes (e.g. TH, GADs), and 31% cell cycling, transcription factors. Approximately 1/3 of the goldfish genes are not characterized. We are continuing to update sequences on the microarray and investigating the function of ESTs using bioinformatic techniques (see http://dambe.bio.uottawa.ca/goldminer.asp) for more information on goldfish sequences). Additional brain derived genes are being added to the goldfish-carp microarray for future experiments from both goldfish and carp.

In this first experiment, our strategy was to be strict with our microarray data analysis and focus on genes important for neuroendocrine and reproductive function. Regulated known transcripts identified by microarray analysis are listed in Table 6.2. At a nominal dose of 0.1 nM EE2, we did not detect significant gene targets (q>5) (A). However, we do list the genes that showed the lowest q value with our analysis for a comparison to the 1.0 nM treatment. Candidate genes we identified in the low EE2 treatment group were reduced. Some of these genes, for example, ceruloplasmin, sp1 transcription factor, and interleukin 6 signal transducer are known to be responsive to estrogens. Transcripts that are identified and showed a significant change (q >5) are listed for the 1.0 nM group, in addition to the estimated fold change (B). We are continuing to update sequences on the microarray and investigating the function of ESTs using bioinformatic techniques (http://dambe.bio.uottawa.ca/goldminer.asp).

Real-time RT-PCR was used to verify a subset of genes that were identified as being regulated by EE2 exposure. We selected genes with different functions that showed 1) high fold-changes and/or 2) were of interest to the neuroendocrine control of reproduction. These included aromatase, secretogranin-III, ependymin, and interferon-related developmental regulator 1 (IFDR-1). Using gene expression data from both the hypothalamus and telencephalon, we determined that β-actin and β-2-microglobulin transcription are modulated by EE2 exposure and thus, were not appropriate to normalize our data. Larkin et al. (2003) previously reported that β-actin is estrogen responsive in the liver of sheepshead minnows (*Cyprinodon variegatus*). We determined that elongation factor 1 (EF-1) (accession #AB056104) did not significantly change with the treatment and used this gene to normalize our real-time RT-PCR data. Brain aromatase transcription was significantly induced (approximately 6-fold in the 1.0 nM EE2 exposure in the hypothalamus and approximately 3-fold in the telencephalon (Figure 6.4 A,B). In the present study, secretogranin-III (Sg-III) is significantly up-regulated in the hypothalamus at 1.0 nM EE2 but significantly reduced at both doses of EE2 in the telencephalon (Figure 6.4 C,D). IFDR-1 mRNA was significantly induced in the telencephalon in the high dose EE2 only (Figure 6.4 E,F). There were no significant changes in the mRNA abundance of ependymin, GAD65, GAD67, and isotocin in the hypothalamus or telencephalon.

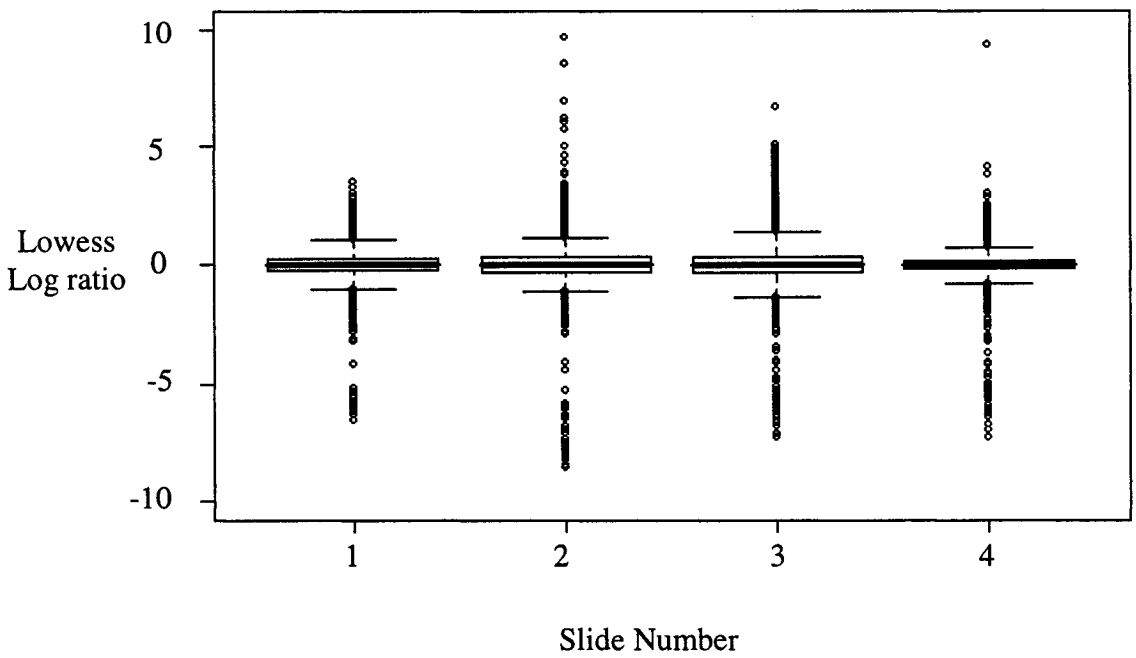


Figure 6.1. Box-plot displaying the log ratio for different microarray replicates after Lowess normalization.

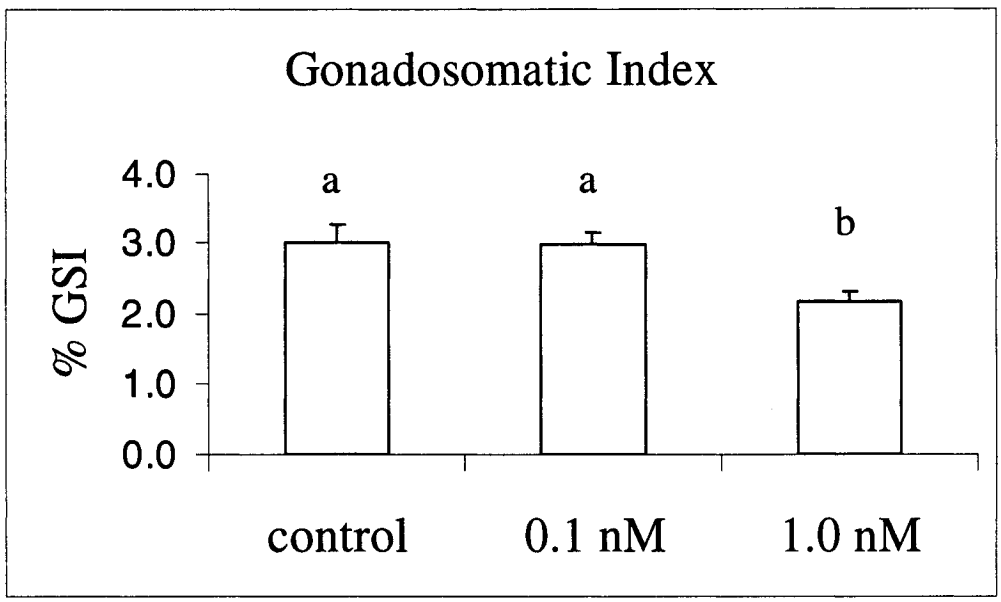
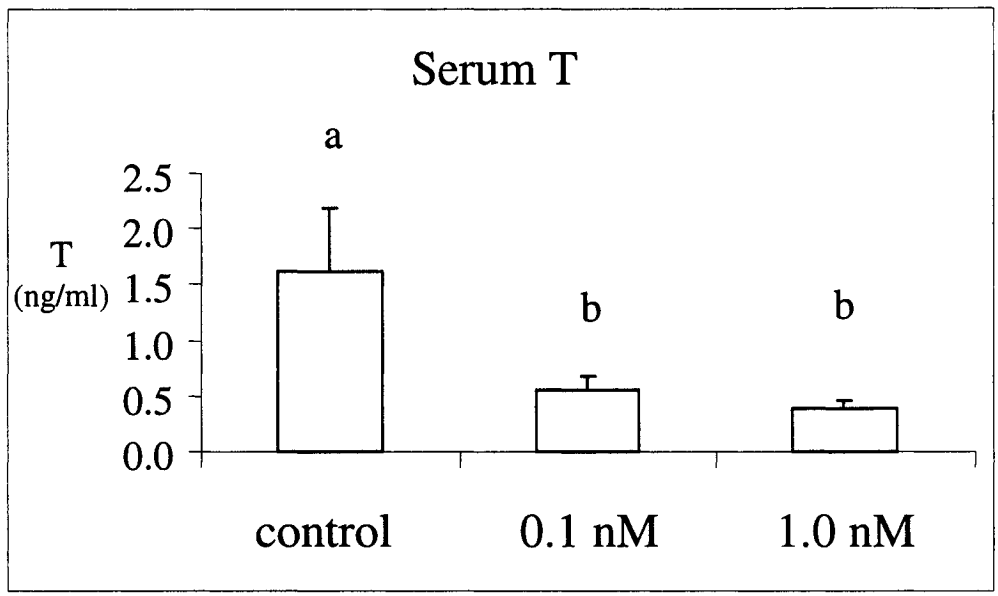


Figure 6.2. Mean (± S.E.) gonadosomatic index goldfish in control and treated (0.1 and 1.0 nM) goldfish. Asterisk denotes significant difference ($p<0.05$; Unpaired Students T-test) compared to controls (n=9-10).

A



B

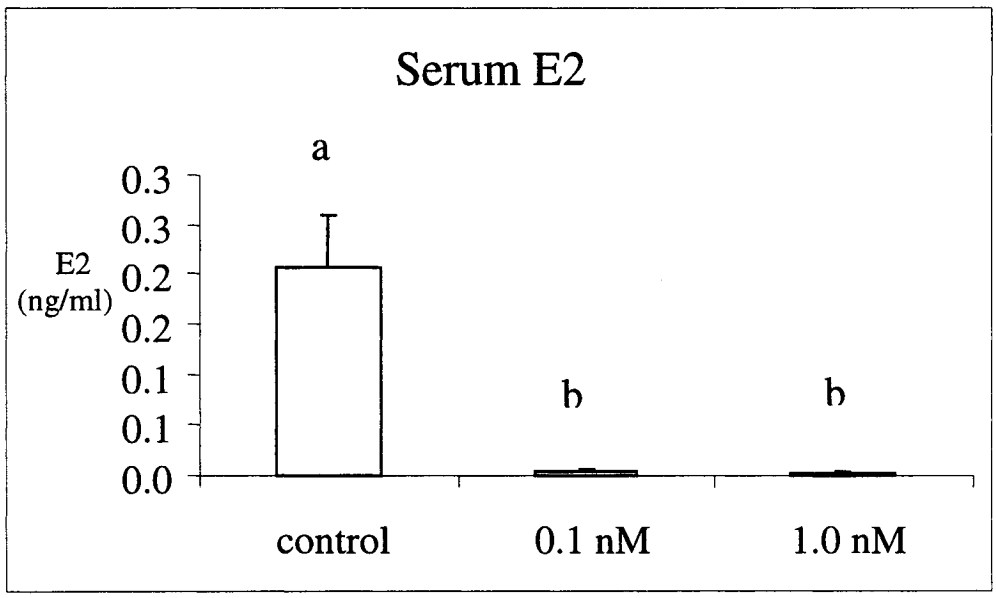


Figure 6.3. Mean (± S.E.) serum concentration of A) T (n=5) and B) E2 (n=7) in control and treated (0.1 and 1.0 nM) goldfish. Asterisk denotes significant difference ($p<0.05$) compared to controls.

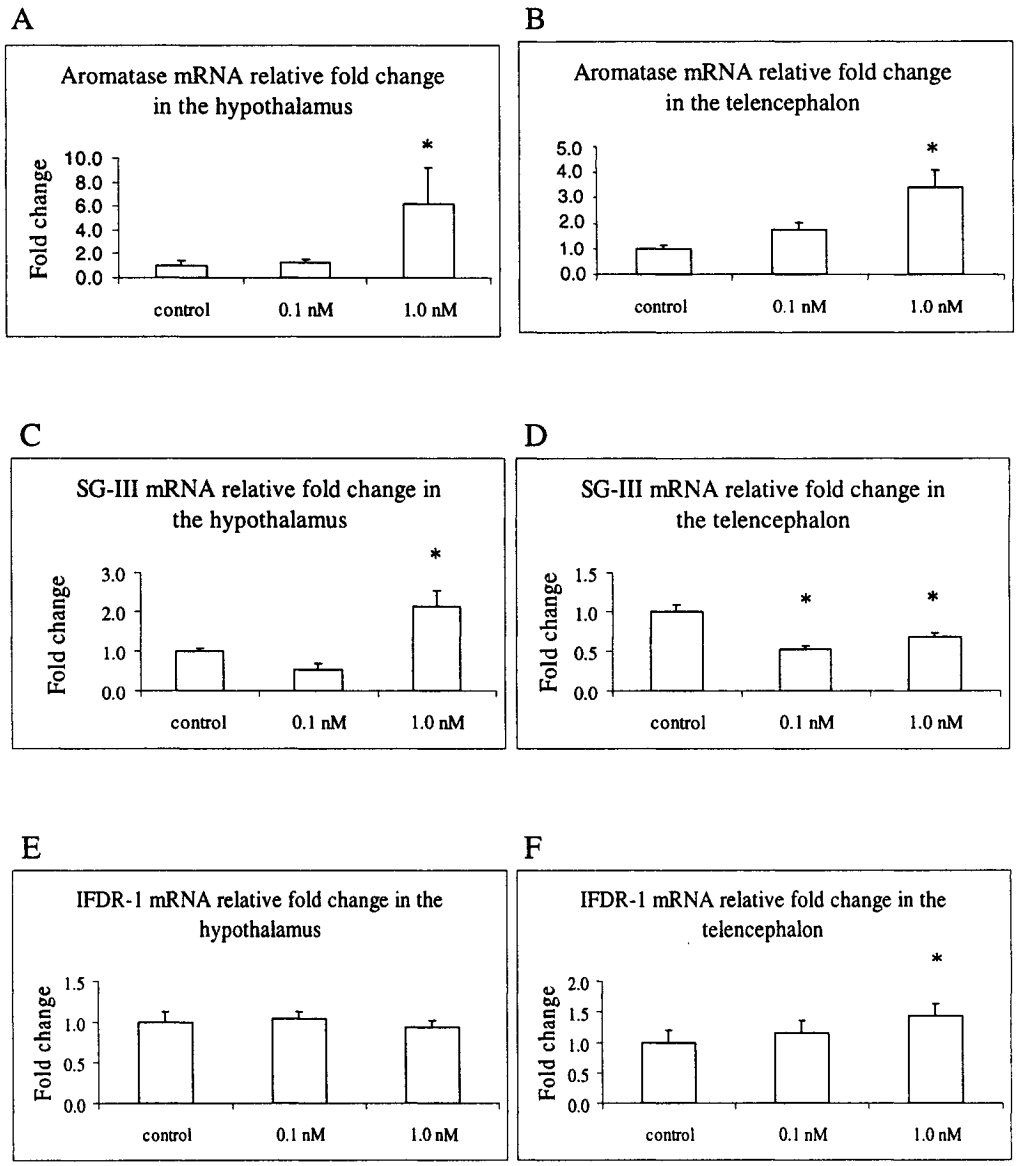


Figure 6.4. Relative fold changes (± S.E.) determined by real-time RT PCR SYBR green assay of a) aromatase mRNA b) Sg-III mRNA c) IFDR-1 mRNA in the hypothalamus and d) aromatase mRNA e) Sg-III mRNA f) IFDR-1 mRNA in the telencephalon. Asterisk denotes significant difference ($p<0.05$) compared to controls (n=5-6).

Table 6.1. Primers for real-time RT-PCR

Gene	Forward primer (5' to 3')	Reverse Primer 5' to 3'
elongation factor-1	GAT TGT TGC TGG TGG TGT TG	GCA GGG TTG TAG CCG ATT T
beta actin	CTG GGA TGA TAT GGA GAA GA	CCA GTA GTA CGA CCT GAA GC
B2 microglobin	GCC CTG TTC TGT GTG CTG TA	AAG GTG ACG CTC TTG GTG AG
ribosomal 18s	AAA CGG CTA CCA CAT CCA AG	CAC CAG ATT TGC CCT CCA
brain aromatase	TGC TGA CAT AAG GGC AAT GA	GGA AGT AAA ATG GGT TGT GGA T
secretogranin III	TTT GCG CTT ATA GAG GAG AGG	TTA CAG CAA GGA CAA CCA CAA
IFDR-1	GCT CCA CAC AAA CGC TCT C	GTC TCC CCA GCA GCA ATC
GAD65	GGA TAC GTG CCG TTC TTT GT	CTC GAC TCC ATT CAG CTT CC
GAD67	CCA AAG GCA TGT CTG TAG CA	CCC TTC TGT TTG GCA TCA AT
isotocin	ATCTTGGCTACTGGCAGGTT	GTATCTGCTGTGGTGAAGGT
ependymin	TGA GCG GAA CAA TGA AAG TG	TCA GAC TCG TGA GTG GCA TC

Table 6.2. List of candidate genes identified by microarray analysis in the 0.1 nM EE2 group (A) and the in the 1.0 nM EE2 group (B). Accession numbers for BLAST hits are given for goldfish or carp (if available) and/or the closest similar sequence after a BLAST search. Not listed are sequences that showed no nucleotide or amino acid similarity to proteins in the NCBI database.

A)

Gene	Fold change	Accession # (nucleotide blast)
Reduced Genes		
sp1 transcription factor	-2	BC067713.1
*similar to Stathmin (Phosphoprotein p19) (pp19) (Oncoprotein 18) (Op18)	-1.93	NM_001017850.1
mitochondrion-hypothetical 18K protein	-1.79	AY714387.1
histone 2, H2, like	-1.6	BC059463.1
amyloid beta precursor protein binding protein 1	-1.57	BC055513.1
aldolase A fructose-bisphosphate	-1.57	AY394966.1
*similar to NEFA-interacting nuclear protein NIP30	-1.54	BC056511.1
*prophenoloxidase activating factor	-1.53	BX842643.1
*hypothetical protein PC000284.00.0	-1.48	CR318588.10
*hypothetical protein LOC496821	-1.48	AC108830.13
nucleobindin 2a	-1.47	BC046077.1
secretogranin III	-1.45	BC059577.1
*hypothetical protein	-1.44	BX548249.13

similar to zinc finger protein 262 (LOC559580)	-1.44	XM_682936.1
poly(rC) binding protein 2	-1.4	AY398394.1
ceruloplasmin	-1.39	BC064000.1
similar to interleukin 6 signal transducer (LOC558568)	-1.36	XM_681798.1

B)

Gene	Fold Change	Accession # (nucleotide blast)
Up-regulated Genes		
GAPDH	2.25	XM_679205.1
†Aromatase	1.96	AB009335.1
fibrinogen, gamma polypeptide	1.82	BC045868.1
fibrinogen, B beta polypeptide	1.77	BC066629.1
similar to apolipoprotein (A-1)	1.66	XM_696149.1
similar to Apolipoprotein C-I precursor	1.65	XM_694143.1
protein tyrosine phosphatase, receptor-type, F interacting protein, binding protein 2	1.62	BC055935.1
†dopamine D1/beta receptor	1.59	AJ005433.1
splicing factor, arginine/ serine-rich 5	1.5	BC063235.1
*Early growth response	1.45	BX470252.10

protein 1 (Krox-24 protein) nerve growth factor		
troponin T3b, skeletal, fast	1.39	BC065452.1
*similar to 1-aminocyclo propane-1-carboxylate synthase	1.37	BX321900.10
MD18 GAPDH mRNA	1.37	AY818346.1
similar to ribosome binding protein 1	1.34	XM_684962.1
ependymin II	1.34	J04986.1
nucleobindin 2a	1.34	BC046077.1
Reduced Genes		
similar to interferon-related developmental regulator 1	-2.26	XM_701614.1
*apolipoprotein A-I binding protein	-2.26	BC075969.1
*Ferredoxin 2	-1.49	BX005286.6
bone morphogenetic protein-15	-1.41	AY860977.1

* indicates genes that were not similar to known nucleotide sequence after BLAST search but showed similarity to known protein after translation of nucleotide sequence.
† indicates a goldfish sequence on the goldfish-carp microarray.

6.4. Discussion

The presence of xenoestrogens in both aquatic and terrestrial environments has led to a number of studies evaluating the risks associated with endocrine disruption. To date, however, few studies have investigated the effects of EDCs on brain tissue. Here I exposed fish to the pharmaceutical EE2 for a 2 week period and, along with my collaborators, evaluated physiological parameters in addition to gene expression changes in the brain.

6.4.1. Testis size is reduced in high EE2 exposure group

This study found that male goldfish exposed to 1.0 nM had smaller testis (approximately 33% reduction), relative to absolute body weight, than both control fish and fish exposed to 0.1 nM EE2. This is comparable to what has been reported in other teleost fishes exposed to waterborne EE2. In sexually maturing rainbow trout exposed to varying concentrations of EE2 in water for 62 days, only trout exposed to higher concentrations of EE2 (100 ng/L or ~ 0.34 nM) showed a reduction in testis mass (Schultz et al. 2003). Sexually maturing trout exposed to 10 ng/L (~ 0.034 nM) did not show a reduction in testis size. Goldfish exposed to a nominal concentration of 1-10 μg/L E2 in water had approximately a 50% reduction in testis size after 24-28 days of treatment (Bjerselius et al., 2001). In the fathead minnow (*Pimephales promelas*), low doses of EE2 (0.034 nM) did significantly reduce testis size after a three week treatment period (Pawlowski et al., 2004). Goldfish are seasonal reproducers and our study used sexually maturing goldfish in May. It appears as though a higher exposure to EE2 is

needed to significantly reduce testis size when T and E2 are relatively high in male goldfish (i.e. sexually mature).

6.4.2. Sex steroids are depressed after EE2 treatment

Serum T was significantly reduced after 2 weeks in both EE2 exposure groups compared to control fish. Reduction of circulating serum T in males after estrogenic exposure has been previously documented in fish. Male goldfish implanted with E2 silastic implants (100 μg/g body weight) showed approximately a 3-fold reduction in both T and 11-ketotestosterone (11-KT) (Trudeau et al., 1993e). In studies investigating effects of waterborne exposures to EE2, male mummichogs showed depression of T and 11-KT at 7 and 15 days static exposure to >250 ng/L EE2 (MacLatchy et al., 2003). Tilton et al. (2005) showed that T production was significantly depressed at an exposure to 500 ng/L EE2 in Japanese medaka (*Oryzias latipes*). These studies demonstrate that E2 negatively regulates androgen production and is consistent with the observed effects in the present study that males exposed to waterborne EE2 have T levels 60-70% reduced when compared to controls.

Endogenous E2 in male teleost fish plays a critical role in spermatogenesis and gametogenesis (Miura et al., 1999). Our study detected an average of approximately 210 pg E2/ml in control fish, similar to what has been reported for other male teleost fish (Rinchard et al., 2001; Sisneros et al. 2004). We found that levels of E2 were significantly lowered in both EE2 exposure groups when compared to the control animals after EE2 exposure. In contrast, male channel catfish (*Ictalurus punctatus*) injected with 1 mg/kg EE2 had a significant increase in serum E2 concentrations after 7 days post-

injection (Tilton et al., 2001). When exposing to estrogenic compounds, low doses appear to induce or promote physiological responses whereas higher doses of estrogens inhibit or impair normal reproductive function. For example, MacLatchy et al. (2003) showed in female mummichogs (*Fundulus heteroclitus*) that high EE2 concentrations depressed ovarian production and circulating E2 levels but EE2 concentrations <100 ng/L caused increased E2. Current evidence suggests that there is a threshold effect of EE2 in which EE2 no longer stimulates, but inhibits, gonadal steroid production. This threshold concentration of exposure to estrogens will depend upon multiple factors such as age, sex, time of season, and species sensitivity to estrogens.

6.4.3. EE2 and gene expression in the brain

There were smaller expression changes in the brain after EE2 exposure when this study is compared to other tissues investigated in the literature. It has been previously shown that changes in mRNA transcripts in the brain are low compared to other tissues and typically change less that 2-fold (Marvanova et al., 2004; Bosetti et al., 2005). This study observed similar fold changes in the hypothalamus using our goldfish-carp microarray, which typically ranged between 1.2 to 2-fold. However, these small changes in mRNA abundance may have pronounced downstream effects on brain function. Trudeau et al. (2005) recently demonstrated using polyethylenimine-mediated *in vivo* somatic gene transfer of an estrogen response element-thymidine kinase-luciferase (ERE-TK-LUC) construct into the brain that male goldfish exposed to EE2 (10 nM) had significantly elevated brain luciferase activity (2-fold). This indicates that waterborne estrogenic chemicals found in the environment can significantly modulate gene

transcription in the brain. Transcripts we identified as being significantly regulated by EE2 can be categorized into cell signalling and transduction, cellular metabolism (protein and nucleic acid metabolism, fatty acid), cell structure and growth, transcription/ translation, and others.

In general, the microarray data tended to underestimate the magnitude of the fold change when compared to real-time RT-PCR data, an observation that has been documented previously after comparing the two techniques (Yuen et al., 2002). Relative changes in brain aromatase, Sg-III, and IFDR-1 mRNA were confirmed by real-time RT-PCR. GAD65, GAD67, and isotocin mRNA, genes that have an important role in reproduction in fish, were not identified by microarray analysis as being differentially regulated and this was confirmed with real-time RT-PCR. This increased the confidence in the ability of our miroarrays to identify estrogen-responsive targets. This study identified genes in the brain previously reported to be estrogen responsive in other tissues in both the low and high EE2 treatment groups. For example, Pinto et al. (2006) report that in the testis of the sea bream (*Sparus auratus*), apolipoproteins and fibrinogen beta and gamma are up-regulated after E2 injections. These genes were identified as being up-regulated in the brain after exposure to 1.0 nM EE2.

Neural aromatase, the enzyme that converts T into E2, is pronounced in the teleost brain and may be up to 100-1000 times greater than in the mammalian brain (Pasmanik and Callard, 1985). The 5' flanking region in the promoter of the brain aromatase gene in goldfish contains 2 estrogen response elements (EREs) and a half ERE site (Callard et al., 2001). This is in contrast to the gonadal form of aromatase (cyp19a) which does not have EREs in its promoter region. Local production of neuroestrogen in

the brain can therefore be regulated by effects of locally produced estradiol and well as estradiol from gonadal sources on aromatase transcription (Martyniuk et al., 2006). Menuet et al. (2005) have shown in adult male and female zebrafish that E2 increases aromatase expression via ERs in radial glial cells in the preoptic area and mediobasal hypothalamus. Japanese medaka fry exposed to 5.5 μg/L o,p-DDT, a compound with estrogenic activity, also had increased brain aromatase mRNA and activity (Kuhl et al., 2005). The doses of o,p-DDT used in the aforementioned study also induced male to female sex reversal. There was significant variation in aromatase B mRNA levels in the brain of both control and treated fish. This is similar to what has been reported in the brain and gonad of male fathead minnows (*Pimephales promelas*) (Halm et al., 2002) and in the brain of male zebrafish (*Danio rerio*) (Trant et al., 2001). Goldfish exposed to 1.0 nM EE2 had a significant increase in brain aromatase steady state mRNA in both the hypothalamus and telencephalon, however, there were no changes in brain aromatase mRNA in the lower EE2 dose. Lyssimachou et al. (2006) report that aromatase mRNA in the brain of juvenile Atlantic salmon is induced by lower EE2 (<50 ng/L) exposure after a 3 day treatment. This contrasting data may reflect the age of the animals used or the length of the exposure. There is other evidence to suggest that endogenous E2 and exposure to environmental estrogens induce aromatase mRNA transcription in the teleost brain. *In vivo* steroid treatment in the goldfish with E2 and aromatizable androgens increase levels of brain aromatase mRNA 8- and 4-fold in the forebrain and hindbrain respectively (Gelinas et al., 1998). Similar findings of an induction in aromatase transcription in zebrafish 4-10 days post hatch (Trant et al., 2001) and activity in Japanese medaka 14 days (Contractor et al., 2004) after EE2 exposure have been

reported. In the present study, aromatase was induced in the brain despite the significant reduction in circulating serum T and E2. Forlano and Bass (2005) report recently that in female plainfin midshipman (*Porichthys notatus*), higher doses of EE2 were correlated with lowered E2 levels and an increase in brain aromatase expression. The authors also observed a dose effect of aromatase induction with low concentrations of EE2, however, in our study this was not observed. The induction in aromatase mRNA when circulating E2 levels are low suggests that brain aromatase mRNA regulation may also be independent of circulating E2. Interestingly, there were no changes in estrogen receptor alpha, beta, or gamma mRNA abundance (data not shown) in either tissue, suggesting that the regulation of aromatase transcription is direct and not dependent upon increased ER transcription. The induction in aromatase transcription may be a response of the male brain to maintain local production of neuroestrogen when circulating serum sex steroids are rendered low following EE2 exposures. Social behaviour in fish has been correlated with brain aromatase activity. In the bluebanded gobies (*Lythrypnus dalli*), a socially induced decrease in brain aromatase activity in males was associated with increased aggressive behaviour (Black et al., 2005). Alterations in brain aromatase expression during exposure to estrogenic compounds in the environment could also result in impaired sexual behaviour in male fish.

Real-time RT-PCR data showed that Sg-III was significantly up-regulated in the hypothalamus at 1.0 nM EE2 but were reduced significantly at both doses of EE2 in the telencephalon. This suggests that there is tissue specific regulation of Sg-III that is dependent upon the dose of EE2. Microarray analysis did not detect differential gene expression in Sg-III mRNA in the 1.0 nM EE2 dose in the hypothalamus. This gene was

a possible target identified in the lower dose in the hypothalamus and demonstrates that microarrays are not as sensitive as real-time RT-PCR when detecting small fold changes in mRNA. Sg-III is a member of the granin family which also includes chromogranin A, B and secretogranin II (Sg-II). Chromogranin and/or secretogranin family members are often concentrated in secretory granules in CNS neurons. These proproteins are actively processed into small neuroactive peptides that can influence neurotransmitter release. Hosaka et al. (2005) demonstrated that Sg-III has a role in prohormone-processing and is able to bind proopiomelanocortin (POMC) derived peptides, in addition to cholesterol rich secretory granules in endocrine cells. The authors suggest that the targeting role of Sg-III and other proteins facilitates the efficient release of peptides at exocytosis. To date, little is known about the regulation of Sg-III and this is the first report of Sg-III being sensitive to estrogens. Previous work with other members of the granin family in mammals suggests that, in general, transcription of this family is negatively responsive to estrogen feedback (Anouar et al., 1991; Lloyd et al., 1992). In the telencephalon, this study shows that exposure to EE2 decreases Sg-III mRNA, consistent to what has been shown in the mammalian pituitary for Sg-II mRNA. However, there was a 2-fold increase in Sg-III in the hypothalamus of the 1.0 nM EE2 treatment group. In the goldfish, Samia et al. (2004) found that goldfish pituitary Sg-II expression levels vary with the seasonal reproductive cycle, independent of sex steroids. The authors suggest that Sg-II modulation is most likely through other neuropeptides important for reproduction, for example, gonadotropin-releasing hormone (GnRH). In the mammalian pituitary, Sg-II is co-localized with LH and appears to be involved in secretory vesicle packaging in gonadotrophs (Cozzi and Zanini, 1986). This raises the question of whether

exposure to environmental estrogens disrupts synaptic transmission in the brain, specifically neurotransmitters with a prominent role in reproduction.

Microarray analysis identified IFDR-1 mRNA as being reduced in the hypothalamus after 1.0 nM ng/L EE2 exposure, but we were unable to verify this with real-time RT-PCR in this tissue. However, there was a significant up-regulation in the telencephalon of IFDR-1 mRNA after real-time RT-PCR. In the developing rat, this gene shows homology with interferon-γ (IF-γ) and is expressed in differentiated tissues such as nervous tissue, kidney, and lung (Buanne et al., 1998). IFs belong to the cytokine family and will act as mitogens and growth factors. There is evidence that a large number of IFs are regulated by estrogens and that they mediate reproductive physiology and development and remodeling of reproductive tissues (Kimmins et al., 2003; Hayashi and Spencer, 2005). Moreover, human interferon regulatory factor-1 (IRF-1), in addition to a number of cytokines, plays a role in signalling networks in breast cancer (Zhu et al., 2006). Disruption in the expression of these proteins by EE2 during critical phases of seasonal reproduction could have downstream consequences for reproductive neuroendocrine function.

6.5. Conclusions

This study outlines the production of a brain enriched cDNA goldfish microarray and demonstrates its use in evaluating risks associated with endocrine disruption in aquatic vertebrates. The goldfish-carp microarray is now being used to study neuroendocrine function in response to neurotransmitter receptor agonists and the effects of pharmaceuticals in the vertebrate brain. In the present study, a number of candidate

genes in the brain were identified as being estrogen responsive. Gene profiling, coupled to more traditional estrogenic endpoints such as vitellogenin induction, will provide more sensitive and robust data for evaluating risks associated with endocrine disruption.

Chapter 7
GENERAL CONCLUSIONS AND PERSPECTIVES

"Science never solves a problem without creating ten more"
- *George Bernard Shaw*

7.1. Significance of thesis

I investigated neuroendocrine regulation of gene expression by GABA and E2 feedback using different molecular and bioinformatic techniques. The major objective of this thesis was to develop a goldfish brain derived cDNA microarray and a number of steps were done to achieve this goal. The distribution of GAD and GABA-T mRNA, the enzymes responsible for synthesizing and degrading GABA, were described using non-radioactive ISH. This technique will be used by others to study the expression distribution of known and novel genes identified by microarray analysis. I chose to optimize this technique using the GADs and GABA-T because the regulation of the GAD isoforms in goldfish by sex steroids was studied previously and the mRNA distribution in the CNS of these three genes were not yet described previously for a teleost fish. GABA producing cells were located in brain regions that contained reproductive neuropeptides (i.e. GnRH) and ERs, neuroanatomical support that GABA is involved in reproduction. Before moving to a larger scale microarray, a reverse northern technique was first used to study the effects of GABA on genes involved in GABA synaptic transmission and genes related to reproductive processes. A number of genes involved in GABA synaptic transmission were cloned, including GABA receptor subunits that were later categorized through phylogenetic analysis. It was determined that GABA autoregulates GABA receptor subunit mRNA expression but not GnRH or ERα mRNA after treatment with

GVG, a irreversible inhibitor of GABA-T. After increased confidence in our ability to study gene transcript changes with reverse northern blots, a larger scale approach (microarray) was used to identify transcripts mediated by the estrogenic pharmaceutical EE2 and exogenous E2 (collaboration with Ph.D. students Vicki Marlatt and Dapeng Zhang). After EE2 exposure, a number of genes differentially regulated by estrogens, for example, brain aromatase, secretogranin-III, apolipoproteins, fibrinogen isoforms, and interferon-related developmental regulator 1 were identified by microarray analysis. Microarray analysis after EE2 exposure did not detect changes in relative mRNA abundance of genes involved in GABA synaptic transmission. This is not to suggest that estrogens do not modulate the transcription of genes such as GABA receptor subunits and GATs, rather we not observe transcriptional changes under the particular experimental conditions. Studies in mammals have shown that E2 potentially regulates brain-derived neurotrophic factor expression and activity possibly via GABAergic interneurons (Blurton-Jones et al., 2004) and there is evidence that in rats, E2 regulates the relative mRNA abundance of GABA receptor subunits (Herbison and Fénelon, 1995) and GATs (Herbison et al., 1995). GAD expression has been shown to also be differentially regulated by estrogens and is seasonal and sex dependent (Larivière et al., 2005). Subsequent studies are needed to determine to what extent E2 regulates the GABAergic system and vice versa in fish. Section 7.5. discusses the regulation of GADs by E2 in experiments done as part of this thesis.

The production of the goldfish brain derived cDNA is a significant contribution to fish neuroendocrinology. Microarrays continue to be a powerful tool in evaluating physiological, pharmacological, and environmental issues of concern. The goldfish

cDNA microarray is now used by other students in the laboratory to study the dopaminergic system in relation to Parkinson's disease, E2 feedback in the CNS, and the neuroendocrine effects of waterborne pheromones and Prozac^{TM}, a pharmaceutical that is detected in water systems and has the potential to depress the reproductive axis in fish.

7.2. GABAergic modulation of a putative GABA synexpression group

I originally hypothesized that GABA and E2 will influence GABAergic synaptic transmission by modulating the transcription of genes such as GATs, GABA receptor subunits, and enzymes involved in GABA synthesis and degradation. Although there is strong evidence for the temporal regulation of genes involved in GABA synaptic transmission during rat spinal cord development (Wen et al., 1998), my study suggests that GABA does not influence transcription of these genes in a temporal manner in the adult goldfish brain at the time and dose examined. It is likely that the temporal expression of a synexpression group as defined by Niehrs and Pollet (1999) is dependent upon the physiological process and it should be cautioned that synexpression should be clearly defined on a specific temporal scale. In teleost fish, there is evidence that gene duplication has lead to differences in tissue regulation between two duplicated genes and the partitioning of protein function (Loh et al., 2004; Smith et al., 2006). It is presently not known if in teleost fish, multiplicity of GABA receptor subunits have led to *de novo* functions of various subunits. The phylogenetic analyses presented in this thesis have revealed that teleost fish contain unique subunits of the $GABA_A$ receptor (e.g. β4). The mammalian GABA receptor subunits ε and θ have unique distributions in the vertebrate

brain, also suggesting functional differences. Studies may address how GABA receptors containing teleost specific subunits respond to GABA and GABA modulators.

Although a synexpression group may be expected to be under strong selection pressure to co-evolve, the uncoupling of some gene members from a synexpression "wave" may occur if duplicated genes are no longer needed to perform specific functions within a synexpression group. Thus, increased regulatory control of gene expression may occur if genes can be expressed both as a group, to coordinate the genomic response to a signal, and then individually expressed to perform modulator or subsidiary roles. I would hypothesize that in the adult vertebrate brain, there is both temporal coordination and temporal separation in the expression pattern of genes involved in GABA synaptic transmission. The neonatal brain may require these genes to be more cohesive during sensitive periods of development (i.e. more tightly regulated during organizational periods). Studies investigating gene expression profiles or waves at different periods in the adult and juvenile brain may reveal differences in the clustering of gene expression.

7.3. A model for GABA stimulated LH release in fish?

Based on studies presented in this thesis, I propose a model for GABA stimulated LH release in the goldfish (Figure 7.1.). Increases in GABA levels in the neuroendocrine brain via modulation of genes involved in GABA synthesis and degradation during spawning may lead to increased release of pituitary LH. GABA stimulated LH release in teleosts is dose dependent, with higher concentrations of GABA agonists eliciting a larger release of LH from the goldfish pituitary (Trudeau et al., 1993b). Joy et al. (1999) showed in catfish (*Heteropneustes fossilis*) that GABA levels in the hypothalamus and

telencephalon are significantly higher in the prespawning and spawning period than in the preparatory postspawning period. Injection of GABA during the prespawning and spawning period were shown to elevate LH levels, but only in fish treated with GABA + GnRH analogue or GABA + DA_2 receptor antagonist (pimozide). GABA autoregulation of its production and metabolism appears to occur in both the hypothalamus and telencephalon. GAD65-/GAD65- and GAD67-/GAD67- knockout studies in mice suggest that each GAD isoform has distinct functional roles (Asada et al., 1997). I have shown that there are tissue differences in the expression of the GADs in the goldfish neuroendocrine brain (Chapter 2). Future studies should address the relative contributions of the GADs to reproduction and LH release.

GABA may also regulate its own synaptic transmission through regulation of its receptors. I demonstrated that increasing GABA levels in the goldfish results in a reduction in $GABA_A$ receptor subunit relative mRNA abundance in neuroendocrine tissues (Martyniuk et al., 2005). This has also been observed in other vertebrates, for example, rat (Fénelon and Herbison, 1996) and chicken (Baumgartner et al., 1994).

The significant and rapid induction of activin βa transcription may enhance LH release through the stimulation of GnRH release by activin A or BA. This study suggests that induction of activin βa in the hypothalamus and telencephalon is mediated through the $GABA_B$ receptor. However, without further time-course and dose response analysis, the potential role of $GABA_A$ receptor in the regulation of activin mRNA can not be discounted at the present time. An increase in GnRH release is hypothesized to be a predominant mechanism in the present model because we did not detect any changes in

the expression of sGnRH mRNA with GABA agonists. Similarly, Martyniuk et al. (2005) did not observe transcriptional changes in sGnRH or cGnRH-II with increasing GABA levels, suggesting that GnRH synthesis and release may be more affected by GABA than is GnRH transcription.

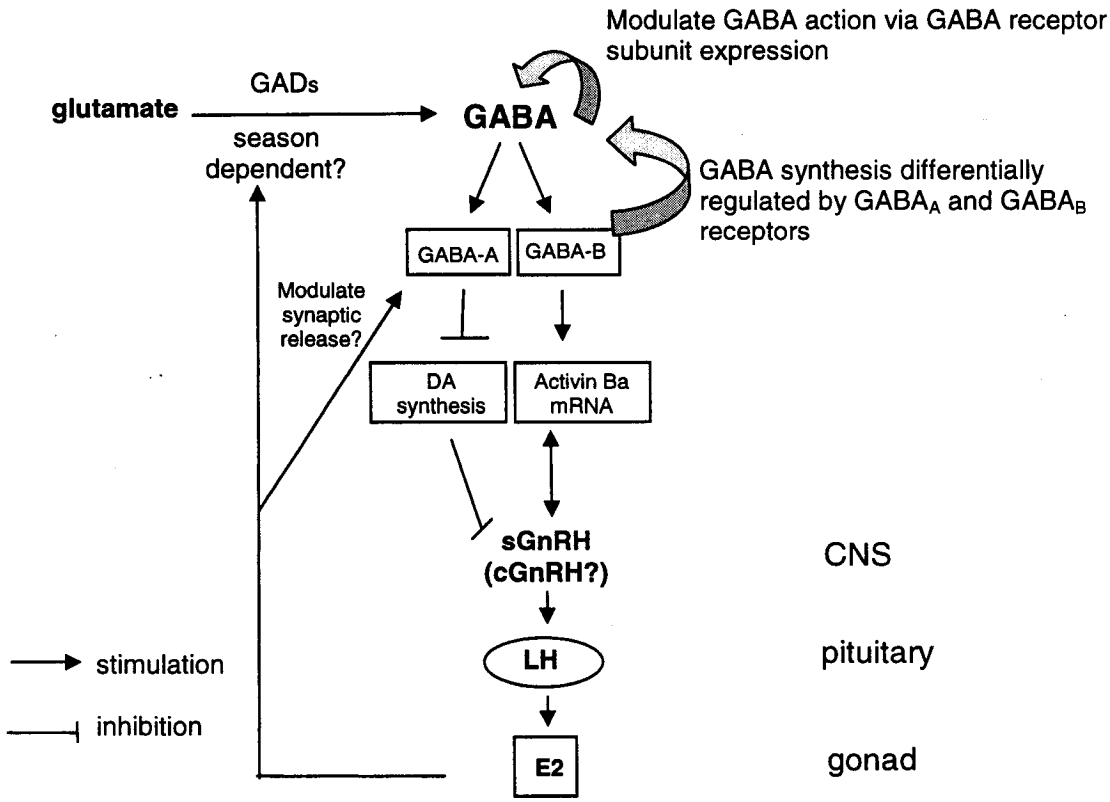


Figure 7.1. A model for GABA stimulated LH release in sexually regressed goldfish based on studies presented in this thesis

I observed a decrease in TH mRNA, the rate limiting enzyme in catecholamine synthesis in the telencephalon. The telencephalon contains the preoptic area in fish and this area is high in GnRH neurons (Yu et al., 1998). There is also direct innervation of the pituitary by GnRH neurons from the preoptic area (Kah et al., 1987). A decrease in the production of DA and inhibitory DA tone on GnRH neurons in the preoptic area, in addition to a larger effect of activin A, is hypothesized to result in enhanced GnRH stimulated LH release in fish.

In future studies, I would investigate more closely the interaction between activins and GABA. What I was not able to do was to examine whether or not there was a dose or time response of activin βa transcription to baclofen. I hypothesize that GABA increases activin βa transcription during sexual recrudescence. There was no effect of the $GABA_A$ agonist muscimol on activin βa transcription, suggesting that $GABA_B$ receptors may be the predominant GABA receptor mediating activin βa transcription. However this should be investigated more closely. To pursue this I would 1) first study the seasonal regulation of activins to determine if activin transcription (βa and βb) is highest in periods of sexual recrudescence, 2) complete a dose response for activin transcription with each GABA agonist, 3) determine if GABA antagonists (bicuculline, saclofen) reduce the response of activin transcription, and 4) and progress to more elaborate studies blocking activin action (siRNA, follistatin antagonism of activin) to determine if GABA is still able to stimulate LH release in teleost fish. These studies are becoming possible with recent work identifying important amino acids involved in activin binding and activity (Cook et al., 2005). Future work would investigate the effects of GABA on the protein abundance of activins.

7.4. E2 feedback in the neuroendocrine brain

E2 feedback occurs in the CNS to regulate reproduction. In collaboration with Ph.D. students Vicki Marlatt and Dapeng Zhang, we treated male goldfish with i.p. 100 μg/g E2 implant for a 24 hour and a 7 day exposure to study the genomic response to E2 in the neuroendocrine brain. Similar to waterborne exposure to 1.0 nM EE2, brain aromatase expression was significantly increased ~3 fold in both the hypothalamus and telencephalon after 24 hours E2 exposure period and remained induced at one week (Figure 7.2.). Unlike the EE2 waterborne exposure in which E2 in males was reduced

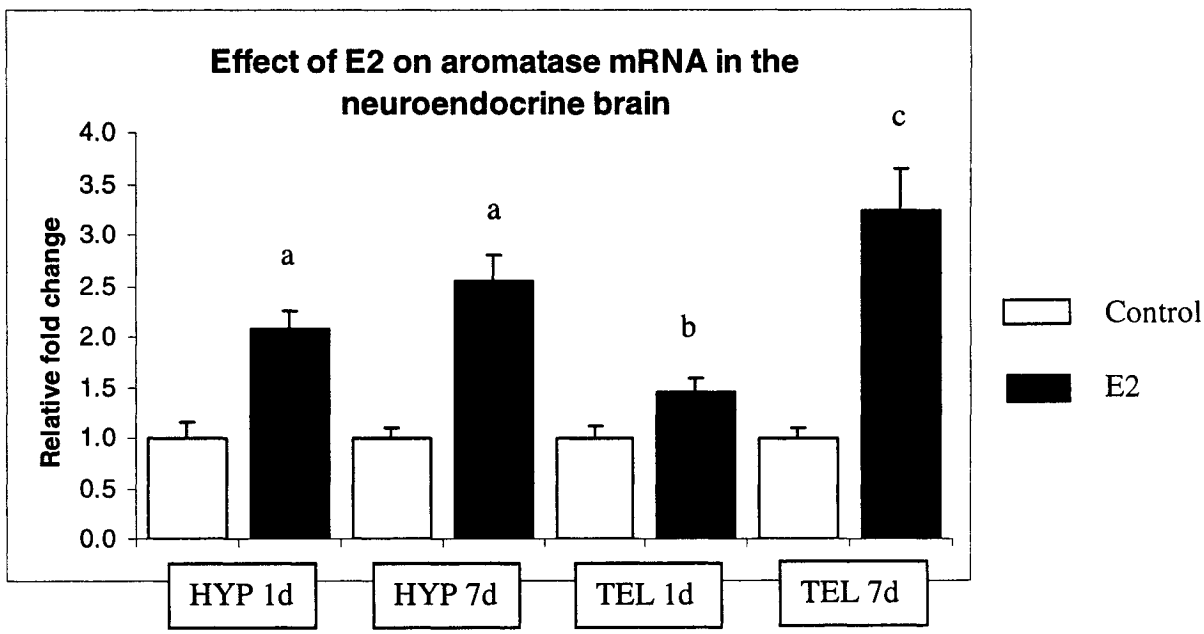


Figure 7.2. Brain aromatase mRNA expression in the hypothalamus and telencephalon after 1 day and 7 day silastic implants (Marlatt, Martyniuk, and Zhang, unpublished real-time RT PCR data)

~100-fold, E2 levels were significantly elevated after both 1 and 7 days approximately 10-fold and brain aromatase steady state mRNA was elevated with both treatments.

ER steady state mRNA was largely unchanged in the hypothalamus and telencephalon after E2 implantation at both 1 day and 7 days. Similarly, there were no significant changes in the mRNA relative abundance of ERs after exposure to waterborne EE2. This suggests that aromatase may be the predominant factor regulating local E2 production in the brain and local production of E2 in the brain is tightly regulated. The increase in aromatase in both the EE2 and E2 experiments is interesting because in the E2 implantation study, E2 levels increased whereas in the EE2 exposure experiment, serum levels of E2 and T were significantly decreased. The goldfish brain aromatase gene contains two estrogen response elements (EREs) in addition to an ERE half-site (Callard et al., 2001). Therefore, brain aromatase mRNA expression is sensitive to E2 feedback. Increases in both E2 and T with silastic implants in goldfish resulted in an increase in aromatase activity in hypothalamic regions and the telencephalon in both males and females (Pasmanik et al., 1988). In our E2 implant study, aromatase mRNA was induced. These studies suggest that brain aromatase transcription and activity is in part regulated by circulating sex steroids (positive feedback). In the EE2 exposure, we observed a larger relative increase in brain aromatase mRNA with lowered endogenous circulating E2 and T. The importance of E2 to normal brain function is well documented in the literature (reviewed in Fink et al., 1996) and this may be a mechanism in the brain to maintain local production of E2 despite marked declines in sex steroids. However, the regulation of aromatase is most likely complex and, although in part mediated by circulating sex steroids via ERs, is most likely regulated by other factors.

We have used the goldfish-carp microarrays as a tool in two different experiments investigating E2 feedback in the brain. We observed that the gene expression profile is largely different depending on treatment (E2 vs. EE2), dose (EE2: 0.1 nM vs. 1.0 nM), and duration (E2: 1 day vs. 7 day). We identified fewer genes as being differentially expressed in 1) the lower dose of waterborne EE2 and 2) the longer treatment period (7 days) of exogenous E2 implantation (data not presented). The adult brain, in these studies, appears to be relatively resistant to gene expression changes at low doses of waterborne EE2 and longer exposures to exogenous E2. Exposures to environmental estrogens at low doses may not affect the brain as significantly as other tissues. However, it is plausible that small changes in the transcriptome not detected with our 9K microarray may have profound downstream consequences to neural transmission and processing. Similarly, longer periods of exogenous E2 exposure resulted in fewer changes at the transcript level. These observations may suggest that the adult brain 1) is relatively resistant to low estrogenic exposures and 2) may reach a level of homeostasis after prolonged exposures to estrogens (i.e. initial positive feedback of E2 followed by negative feedback).

Despite the different gene profiles between the E2 and EE2 experiments, there were some interesting gene families that showed similar regulatory patterns. For example, apolipoproteins, which are involved in lipid mobilization and transport have been reported previously to be E2 responsive (Pinto et al., 2006). When serum levels of E2 were high (E2 implantation), a large number of these genes were reduced as identified by microarray analysis (Table 7.1.). In contrast, after EE2 exposure and a significant reduction in circulating sex steroids, members of this gene family were up-regulated.

This trend was similar to fibrinogens, estrogenic-responsive genes involved in the formation of blood clotting via platelet formation. Fibrinogen contains two sets of three different chains (α, β, and γ) that are linked to each other by disulfide bonds.

	Gene	Relative fold change	Serum E2
E2	Apolipoprotein A-1	↓ 1.9	Increased
	Apolipoprotein A-2	↓ 1.5	Increased
	Apolipoprotein Eb	↓ 1.3	Increased
	fibrinogen β	↓ 1.3	Increased
EE2	Apolipoprotein A-1	↑ 1.7	Decreased
	Apolipoprotein C-1	↑ 1.7	Decreased
	fibrinogen β	↑ 1.8	Decreased
	fibrinogen γ	↑ 1.8	Decreased

Table 7.1. Expression patterns in the hypothalamus of apolipoproteins and fibrinogens that were identified as differentially expressed with microarray analysis in both the E2 silastic (Zhang, Martyniuk, and Marlatt, unpublished data) and the EE2 exposure experiments (Chapter 6).

7.5. E2 feedback and GABA synaptic transmission

Previous studies have shown that GAD transcription in the brain is modulated by E2 in both fish (Bosma et al., 2001) and mammals (McCarthy et al., 1995; Szabo et al., 2000). I tested this hypothesis in both EE2 and E2 exposed animals using real-time RT-PCR. These two experiments did not result in significant changes to the expression of GAD isoforms in either the hypothalamus or telencephalon (data not shown). Bosma et al. (2001) reported that in sexually regressed gonad-intact males, both hypothalamic GAD65 and GAD67 expression were modulated by E2 implantation after a 5 day

treatment. Levels of GAD65 mRNA in the hypothalamus decreased ~ 4-fold, whereas GAD67 mRNA decreased 2-fold. The lack of change in GAD expression with EE2 waterborne treatment may reflect seasonally variable responses to sex steroids. Male goldfish in my EE2 exposure study (Chapter 6) were sexually mature (March) and Larivière et al. (2005) recently showed that GAD transcription in sexually mature animals with silastic implants (November and February) is not significantly affected by sex steroid feedback. Secondly, circulating levels of T and E2 were decreased, and not increased, in males exposed to waterborne EE2.

E2 silastic implants in sexually regressed male goldfish (August) for a 1 day and 7 day treatment did not support the findings of Bosma et al. (2001) and a reduction of GAD65 and GAD67 in the hypothalamus. We detected no change in either GAD isoform in the hypothalamus. However, similar to Bosma et al. (2001) we did not detect any change in GAD expression in the telencephalon. It is possible that E2 modulation of GAD expression occurs after a 24 hour period, followed by an increase to basal levels after 1 week. Larivière et al. (2005) showed that E2 implantation in males did not significantly alter GAD steady state mRNA at all periods of the year examined. E2 feedback in the neuroendocrine brain was only studied in male goldfish for this thesis and tissue-specific sexual dimorphism in GAD mRNA levels may have been observed in females (Bosma et al., 2001). E2 may not significantly modulate the transcription of GAD65 and GAD67 in the telencephalon of male goldfish but it is still unclear to what extend E2 modulates GAD transcription in the hypothalamus.

Although the experiments (EE2 exposure and E2 silastic implant) presented in this thesis did not detect expression changes in genes involved in GABAergic signalling,

further studies may focus on the seasonal modulation of GABA receptor subunits and GAT expression by E2 given 1) the importance of GABA in regulating LH release in teleosts, 2) evidence in mammals that these genes are regulated by E2, and 3) the significant E2 feedback in the CNS to regulate reproduction in vertebrates.

7.6. Concluding Remarks

I investigated the molecular mechanisms underlying GABA stimulated LH release in the goldfish and coordinated the development of a brain enriched cDNA microarray. I have demonstrated that there are conserved effects of GABA on the transcription of genes involved in GABAergic synaptic transmission in fish (Chapter 4), similar to what has been shown in chicken and mammals. In addition, there appear to be conserved effects of estrogenic feedback in the teleost brain. For example, brain aromatase, fibrinogen β and γ, apolipoproteins, ceruloplasmin, and other genes identified in experiments here are also estrogen responsive genes in various tissues in mammals as well as other teleost fish.

The power of a microarray is that it allows one to generate new hypotheses about underlying genomic mechanisms of physiology. Based on the development and application of the goldfish cDNA array, the following hypotheses are postulated as examples;

1) Exposure to environmentally relevant concentrations of EE2 (or other estrogenic compounds) disrupts normal neurotransmitter release. Microarray analysis identified E2 responsive genes that have not yet been well characterized, for example

secretogranin-III, a recently discovered protein involved in synaptic vesicle docking and in the facilitation of neurotransmitter release. This thesis is the first to report that Sg-III is regulated differentially by exposure to estrogens and that it shows tissue specific regulation. Therefore, additional studies may investigate the relationship between pharmaceuticals found in the environment and neurotransmitter systems.

2) The expression of the reproductive neuropeptide isotocin is disrupted by environmentally relevant concentrations of fluoxetine (Prozac^{TM}). This was a collaboration with honours student Jan Mennigan using the goldfish-carp microarray to study the effects of injected fluoxetine, a selective serotonin reuptake inhibitor (SSRI), in the teleost brain (unpublished data; appendix1). Based on our microarray and real-time RT PCR results, I would hypothesize that concentrations of fluoxetine found in the environment disrupt sexual behaviour in fish, predicting that exposure to this pharmaceutical at low levels significantly decreases isotocin mRNA expression in the neuroendocrine brain.

To conclude, this thesis 1) gives evidence that GABA producing cells are located in goldfish brain regions involved in the control of reproduction, 2) presents a putative model for GABA stimulated LH release in fish and, 3) outlines the production of a brain derived cDNA microarray. The goldfish microarray will continue to identify candidate genes underlying processes of reproduction and used as a tool to study how pharmaceuticals and environmental contaminants alter these processes.

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Appendix I (additional manuscripts not included in this thesis)

Mennigen J, **Martyniuk CJ**, Crump K, Xiong H, Nadler A, Trudeau VL. The effects of fluoxetine on neuroendocrine function of the goldfish (*Carassius auratus):* Fluoxetine – a potential neuroendocrine disruptor? *In prep.*

Martyniuk CJ, Gallant NS, Marlatt, VL, Wiens S, Woodhouse A, Trudeau VL. 2006. Recent perspectives on estrogen and estrogen receptors in teleost fishes. (book chapter) *Fish Endocrinol.*

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