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Multiplicity of Glutamic Acid Decarboxylase Genes: molecular evolution and steroidal regulation

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ABSTRACT

Gamma-aminobutyric acid (GABA) is a key neurotransmitter for reproductive control. A major site for the control of GABA synthesis is the regulation of synthesis and activity of glutamic acid decarboxylase (GAD), which exists in three isoforms in goldfish. Previous sequence data suggested that GAD_{65} and GAD_{67} originated prior to teleost divergence. We have shown that this event occurred prior to 450 million years ago, before the emergence of Chondrichthyes. Previous data showed that estradiol and testosterone modulate GAD65 and GAD67 mRNA levels in sexually regressed goldfish brain. No previous data are available on the regulation of GAD3 in any animal. We found that levels of all three GADs vary seasonally. Steroid administration of testosterone, progesterone and estradiol had seasonal, brain-region and sex specific effects. This is the first evidence for seasonal variations in GAD3 and we show a likely role for progesterone in down-regulating GAD_{65} and GAD_{67} in goldfish brain.

RÉSUMÉ

L'acide gamma-aminobutyrique (GABA) est l'un des principaux neurotransmetteurs impliqués au contrôle de la reproduction. La synthèse du GABA est contrôlée, en grande partie, par la régulation de la synthèse et de l'activité de la décarboxylase du glutamate (GAD), qui existe en trois isoformes chez le poisson rouge. Des analyses ultérieures suggéraient que la divergence du GAD_{65} et du GAD_{67} se serait produite avant la divergence des téléostéens. Nous avons démontré que ces deux gènes ont divergé il y a plus de 450 million d'années, avant l'apparition des Chondrichthyens. Il a déjà été démontré que les ARNm de GAD_{65} et de GAD_{67} sont susceptibles à la régulation par l'oestrogène et la testostérone. Il n'existe par contre aucune information concernant la régulation du GAD3. Nous avons trouvé que les niveaux des ARNm des GADs varie de façon saisonnière. Les traitements à la testostérone, a l'estrogène et à la progestérone ont eu des effets spécifiques selon la saison, le sexe et la région du cerveau à l'étude. Ceci démontre pour la première fois des variations saisonnières chez l'expression de GAD3 et révèle un rôle pour la progestérone dans la régulation du GAD_{65} et du GAD_{67} au sein du cerveau des poissons rouges.

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The road that has led me to the completion of my M.Sc has given me the opportunity to meet a great number of people. Several were of great help to my progress and are thanked here. First I would like to mention all those who had their influence on this project in the background; friends and family who stood by me through all the ups and downs of research. In the lab, Géraldine Majchrzak, Valerie Greco and Lisa MacEachern are all students that helped with GAD phylogeny work as part of their Honours studies. Although life has now taken them to different paths, I am sure they will all remember the joys of cloning GADs. Suzanne Chiu, the perennial presence in our lab, helped in this research not only with cloning, but with the work she does to keep the lab and her webpage running year after year. Dr Guy Drouin was invaluable to the analysis of much of the data produced by these phylogenetic studies and I would like to thank him for his help. Bill Fletcher was essential to the care regimen of the experimental goldfish. Marwan Samia and I designed the animal protocols together and our dissections were performed cooperatively. He has been invaluable in my studies as both a resource person and a research partner. I would also like to thank Ben Hibbert for his help with fish dissections as well as his assistance with RNA isolations. All members of the Trudeau Lab during my stay were also an important part of this research. The technical staff of the Department of Biology also deserve a mention here, as they helped with the daily running of the lab. Dr Drouin as well as Dr Jack Kelly played an important role in this project as members of my M.Sc committee. Finally, I must thank Dr Vance Trudeau, my supervisor. He was invaluable to my research progress and provided me with support, fabulous opportunities and good advice.

TABLE OF CONTENTS

LIST OF ABBREVIATIONS

ARC-ME	arcuate nucleus-median eminence
CNS	central nervous system
CTP	cytidine triphosphate
cDNA	complementary DNA
DA	dopamine
DBB	diagonal band of Broca
DMH	dorsomedial nucleus of the hypothalamus
DNA	deoxyribonucleic acid
E2	estradiol
ER	estrogen receptor
FSH	follicular-stimulating hormone
GABA	gamma-aminobutyric acid
GABA-T	GABA transaminase
GAD	glutamic acid decarboxylase or glutamate decarboxylase
GAT	GABA transporter
GnRH	gonadotropin-releasing hormone (also known as LHRH, luteinizing hormone releasing hormone)
GSI	gonado-somatic index
GTH	gonadotropin
GVG	gamma-vinyl-GABA
HPG axis	hypothalamal-pituitary-gonadal axis
HYP	hypothalamus
LH	luteinizing hormone
MBH	medio-basal hypothalamus
ME	median eminence
MPN	medial preoptic nucleus
mRNA	messenger RNA
NE	norepinephrine
ovx	ovariectomized

P4	progesterone
PLP	pyridoxal-5'-phosphate
POA	pre-optic area
PRL	prolactin
RNA	ribonucleic acid
RPA	RNase protection assay
RT-PCR	reverse transcription-polymerase chain reaction
SEM	standard error on the mean
SSADH	succinic semi-aldehyde dehydrogenase
T	testosterone
TEL	telencephalon
UTP	uridine triphosphate
VMH	ventro-medial hypothalamus
Vs	pars commissuralis of the telencephalon
Vv	pars ventralis of the telencephalon

LIST OF TABLES AND FIGURES

Chapter 1: Introduction

1.1 General overview of the project

Reproduction is one of the most fundamental processes of living organisms. In vertebrates, levels of circulating gonadal steroids regulate sexual development by actions on the brain, pituitary and reproductive organs. The secretion of these steroids, which include testosterone (T) and its androgenic metabolites, estradiol (E2; formed when T is aromatized by the enzyme aromatase) and progesterone (P4) and other progestogens, is under the control of the gonadotropins secreted from the pituitary. In mammals, the gonadotropins are follicle-stimulating hormone (FSH), which stimulates ovarian maturation and spermatogenesis and luteinizing hormone (LH), which induces ovulation, estrogen and progesterone production and, in males, production of testosterone. In fish the gonadotropins are designated as GTH-I and GTH-II and are homologues of mammalian FSH and LH, respectively. These gonadotropins, secreted from the gonadotrophs in the anterior lobe of the pituitary (PIT) in both fish and mammals, are released after stimulation by gonadotropin-releasing hormone (GnRH) (see Figure 1.1 for an overview). GnRH is released by neurons from the hypothalamus (HYP) and pre-optic area (POA) which project into the mammalian median eminence (ME). From the ME, GnRH is carried via a portal blood system to the gonadotrophs of the pituitary. Teleosts lack a functional hypothalamo-pituitary portal blood system and the anterior pituitary is directly innervated by neurons producing a multitude of neuropeptides, neurotransmitters that regulate GTH-II release (Peter et al. 1990; Kah et al. 1993). This is one of the unique features of the teleost reproductive axis which make it an interesting model for study.

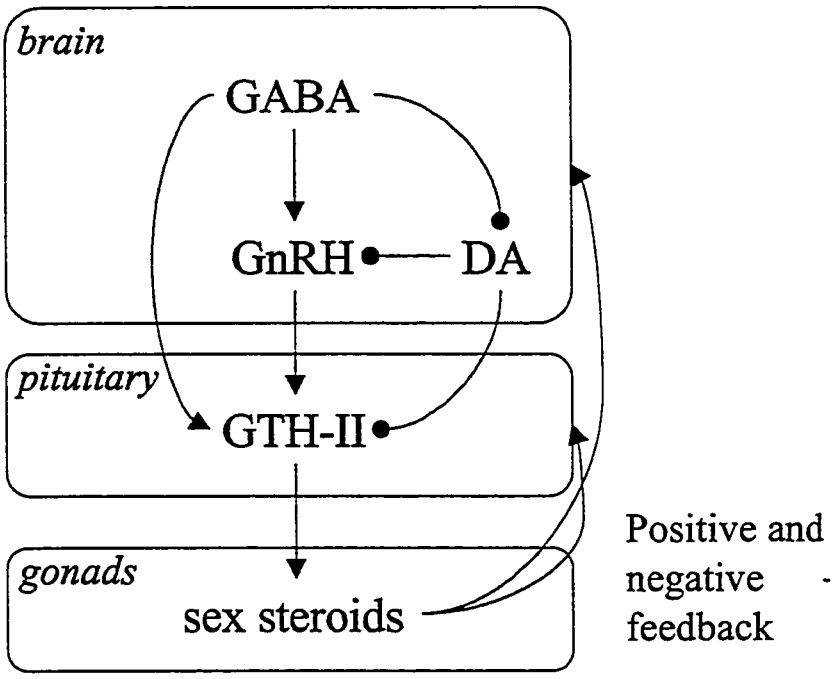

Figure 1.1: Simplified model of the neuroendocrine control of reproduction in fish. This diagram illustrates the stimulation and inhibition of the hypothalamo-pituitary-gonadal axis by GABA and DA and the multiple targets for steroidal feedback. Adapted from Trudeau et al. (2000b). ➤=stimulation; ●=inhibition

In fish, dopamine (DA) has been identified as the sole inhibitor of GnRH and GTH-II release (Peter et al. 1986). Dopamine has been shown to directly inhibit the release of GTH-II from the pituitary in fish (Peter et al. 1986; Kah et al. 1993; Melamed et al. 1996; Peter and Yu 1997) through a mechanism mediated by DA D2-type receptors (van Asselt et al. 1988). Data from some of these studies also shows seasonal variation of responses to DA. Furthermore, DA has also been shown to inhibit GnRH release from both pituitary nerve terminals and in the preoptic-hypothalamic region (Yu and Peter 1990; Peter et al. 1990; Yu and Peter 1992). The role of DA in the simplified model of reproductive control is presented in Figure 1.1. The dual inhibitory effects of DA make it an important player in teleost reproductive control.

In fish, gamma-aminobutyric acid (GABA) also plays a significant role in the regulation of GTH-II release by both stimulating GnRH release from the brain and inhibiting dopamine turnover (Trudeau 1997). In mammals however, GABA plays a predominantly inhibitory role whereas in fish it has mostly been found to be stimulatory (Trudeau et al. 2000b). GABAergic neurons are targets for steroid action and thus are an integral part of the mechanisms of sex steroid feedback on gonadal release.

My studies have focused on the GABA-synthesizing enzyme GAD and the effects of gonadal steroids on its gene expression. We set out to determine the probable timing of the gene duplication which gave rise to GAD_{65} and GAD_{67} and to further elucidate vertebrate evolution of the GAD genes. We also set out to study seasonal variations in GAD mRNA levels and the regulation of GAD mRNA expression by sex steroids to

determine their role, if any, in sex steroid feedback on the reproductive axis. In order to contextualize these studies, research on GABA, GAD and their role in reproductive control is discussed in the following literature review.

1.2 GABA is an inhibitory neurotransmitter

GABA (γ-aminobutyric acid) is an amino acid present in organisms from every kingdom. Although many molecules can be considered to modulate the inhibitory action of GABA, its abundance in the brain and its widespread action lead many to regard it as the major central inhibitory neurotransmitter. GABA is known to be a local regulator of neuronal activity. Most GABAergic neurons in the brain are therefore intrinsic neurons or interneurons. In addition to its role as a neurotransmitter, GABA can act as a paracrine signalling molecule, a metabolic intermediate and a trophic factor (Tobet et al. 1996; Pinal and Tobin 1998). It has also been shown to play an important role in synaptic plasticity and development (Wolff et al. 1978; Waagepetersen et al. 1999; Barbin et al. 1993; Tobet et al. 1996). The many post-synaptic actions of GABA are mediated by three main classes of receptors: the chloride-channel type $GABA_A$ and $GABA_C$ (Enz and Cutting 1998) receptors and the metabotropic $GABA_B$ receptors, which are members of the G-protein linked family (Enz and Cutting 1998; Chebib and Johnston 1999; Bormann 2000). GABA can also be secreted from the pancreas where defects in this process can lead to diseases such as diabetes in certain individuals (Lernmark 1996). Furthermore, auto-antibodies to the GABA-synthesizing enzyme glutamic acid decarboxylase are involved in "Stiff-man Syndrome" (Lernmark 1996), rare cases of encephalitis (Marchiori

et al. 2001) and cerebellar ataxia (Honnorat et al. 2001) in humans. Defects in GABAergic neurotransmission can also lead to epilepsy (During et al. 1995).

1.3 GABA synthesis

A major site for the control of the synthesis of GABA is the regulation of glutamic acid decarboxylase (GAD; EC 4.1.1.15) synthesis and activity. GAD catalyses the formation of GABA from glutamate. Upon neuronal stimulation, GABA is released from synaptic vesicles and acts on its receptors or can be metabolized to succinate by GABA transaminase (GABA-T; EC 2.6.1.19) and succinic semi-aldehyde dehydrogenase (SSADH). Some GABA is taken up from the synaptic cleft by a family of specific GABA transporter proteins (GATs), which serves to atetnuate GABAergic neurotransmission and recycle GABA. Research has also shown that GABA synthesis is not affected by substrate (glutamate) concentrations (Martin and Rimvall 1993) but levels of GABAergic neuron activity have been correlated with GAD mRNA levels (Litwak et al. 1990; Erlander and Tobin 1991b). In mammals, GAD exists in at least two forms, designated GAD_{65} and GAD_{67} according to their respective molecular weights of 65 and 67 kDa (Spink et al. 1987; Kaufman et al. 1991; Erlander et al. 1991a). Each form is produced from a separate gene (Erlander et al. 1991a; Erlander and Tobin 1991b; Martin and Rimvall 1993; Lemmark 1996). These two forms also have different intraneuronal distributions (Kaufman et al. 1991; Erlander et al. 1991a) and differing interaction with their cofactor pyridoxal-5'-phosphate (PLP)(Martin and Rimvall 1993). The GAD genes possess a highly divergent N-terminal domain which appears to be responsible for mediating GAD_{65} membrane association as well as sub-cellular targeting and the

formation of heterodimers (Chen et al. 2000) (Christgau et al. 1992; Dirkx et al. 1995). GAD_{67} has only been found to associate with membranes in the presence of GAD_{65} through the formation of heterodimers (Dirkx et al. 1995).

1.4 GAD activity

GABA synthesis occurs at a rate much lower than total GAD activity. This seems to indicate a large reserve capacity for GABA production (Martin and Rimvall 1993). *In vivo*, GAD activity is limited by co-factor binding; GAD is dependent on PLP for its activation from the inactive apo- form to the active holo- form. Most of the brain's GAD is present in the inactive apo-GAD form (Miller et al. 1977). PLP binds to the NPHK (Asn-Pro-His-Lys) site present in the centre of all known forms of GAD (Bosma et al. 1999). The interconversions between these two forms are catalysed by GAD and are highly regulated (Martin and Rimvall 1993). The rates at which each of the two forms interconverts from apo- to holo-GAD vary; GAD_{65} seems to cycle faster than GAD_{67} (Spink et al. 1987). For this reason, there is a larger proportion of GAD_{65} in the apo-GAD form than of GAD_{67} in neurons (Martin et al. 1991; Martin and Rimvall 1993) and the activity of GAD_{65} is affected by the availability of PLP to a greater degree than the activity of GAD_{67}. Co-factor binding data along with results showing a higher percentage of GAD_{67} in cell bodies lead some to believe this form may have a role in the use of GABA as a metabolic substrate through the GABA shunt. This may have been the original function for GAD in primitive organisms (Lernmark 1996). However, GAD_{67} maintains a role in the GABA synthetic pathway and may be most important in producing GABA for baseline synaptic function and in tonic GABAergic neurons, those that are

involved in constant secretion (Erlander and Tobin 1991b). Conversely, the presence of higher levels of GAD_{65} in the axonal terminals of GABAergic neurons as well as results showing its more rapid apo- to holo- interconversion rate suggests GAD_{65} may synthesize GABA that may play a larger role in rapid response models and in phasically active (burst secretion) GABA neurons (Henry and Tappaz 1991; Erlander et al. 1991a; Erlander and Tobin 1991b; Martin and Rimvall 1993). However, the presence of some GAD_{67} in nerve terminals (Erlander et al. 1991a; Erlander and Tobin 1991b) does cast some doubt over this putative separation of roles for the two studied GAD isoforms. Furthermore, the discovery of GAD heterodimers (Christgau et al. 1992; Dirkx et al. 1995; Sheikh and Martin 1996) and their great abundance within GABAergic neurons would indicate that the putative roles outlined above for each GAD may not be as distinct as hypothesised.

1.5 GAD and development

In experiments involving gene knock-out mice, it was found that the two studied forms of GAD were important for different reasons. The results show GAD_{65}-/- mice have normal development, locomotion and reproduction. When GAD activity was determined in GAD_{65}-/- mice holo-enzyme levels remained the same in the absence of GAD_{65}, but apo-enzyme levels were much lower. Despite the similar holo-GAD levels in these mice they are more prone to chemical and stress-induced epilepsy than mice expressing both forms of GAD (Asada et al. 1996; Kash et al. 1997). This susceptibility may be due to the lower levels of apo-GAD available to be activated in times of sudden demand for GABA. These results also show that GAD_{67} can provide a certain portion of

the apo-GAD pool and that GAD_{67} can, to a certain degree, assume the functions of GAD_{65}. In other studies, it was found that GAD_{67}-/- mice did not live more than a day after birth. GAD_{67} is therefore necessary for life beyond the fetal stage. GAD_{67}-/- mice are born with a cleft palate and other severe birth defects (Asada et al. 1997; Condie et al. 1997). Seven percent of wild-type GABA content and less than 20% GAD activity is identifiable in GAD_{67}-/- mice. The GAD knock-out studied seem to indicate that the major GAD contributing to GABA synthesis in developing animals is GAD_{67} (Asada et al. 1997). This would support GAD_{67} as the more essential form of the two genes, a hypothesis that remains to be rigorously tested.

1.6 Molecular evolution of GAD

Until recently, only several mammalian GADs and one *Drosophila* GAD had been sequenced, providing little information on the evolution of the enzyme in vertebrates. In 1999, Bosma *et al.* published sequences from a protochordate, a turtle, fish, a bird, and compared them to mammalian GADs. Using this new sequence information, they were able to produce the first phylogenetic tree including most of the major vertebrate classes for this gene. They also isolated a short 547 nt cDNA fragment of a novel third form of GAD from a deep-sea fish, the armed grenadier (*Coryphaenoides (Nematonurus) armatus)*, and designated it GAD3. This sequence was quite different from the two other forms, only 65% identical at the nucleotide level to both GAD_{65} and GAD_{67}, but was clearly related to GAD. This third form may have appeared as a result of a second gene duplication. According to the Hox gene theories on chordate evolution

(Holland and Garcia-Fernandez 1996; Meyer and Malaga-Trillo 1999) there have been up to 3 gene duplications in the course of the evolution of the teleosts. These duplications followed by certain GAD gene losses by convergent or divergent evolution could perhaps explain the presence of three forms of this gene, but more work remains to be done before any one theory can be solidly supported. The existence of a third GAD further complicates the question of how GABA is synthesized *in vivo*.

1.7 GABA and the neuroendocrine control of reproduction

The role of GABA in the neuroendocrine regulation of reproduction has been studied in a number of ways. In rat, certain GnRH neurons have been found to possess GABA receptors (Jung et al. 1998) and GABAergic neurons form direct synaptic connections with GnRH neurons (Leranth et al. 1985). Differences in brain concentrations of GABA in rat have been associated with different reproductive behaviours. High levels of GABA in the medial HYP were shown to be permissive to lordosis whereas increases in GABA levels in POA were associated with a low level of lordosis response (McCarthy et al. 1990; McCarthy et al. 1991). It was also found that blocking GABA synthesis in HYP using GAD antisense oligonucleotides reduced lordosis (McCarthy et al. 1994). In male rats, there were no brain sites at which GABA was facilitatory to sexual behaviour (McCarthy 1995a). Increasing brain GABA concentrations by artificially blocking its degradation by GABA-T have shown to cause a decrease pulsatile LH release whereas bicuculline, a GABA antagonist prevented this decrease and enhanced mean plasma LH levels (Donoso and Banzan 1984). LH release from the pituitary was also shown to be decreased after intracerebroventricular injections

of GABA (Vijayan and McCann 1978; Lamberts et al. 1983) and after direct injection into the POA (Herbison et al. 1991a; Herbison and Dyer 1991b) but not after GABA injections to the pituitary of the rat (Ondo 1974). Studies on gene expression have also showed an inhibitory effect of GABA on GnRH mRNA expression when injected into the POA (Bergen et al. 1991). Developmental studies have also shown that the activation of the GABA system in immature female rats can delay sexual development by decreasing GnRH, LH and E2 release (Feleder et al. 1999). These experiments indicate a specific effect of GABA on GnRH expression and clearly show that in mammals, GABA is an inhibitor of reproduction and reproductive behaviour. Under certain conditions, GABA can also induce membrane depolarization, a mechanism that is likely important for neuronal development (Barbin et al. 1993; Owens et al. 1996). Teleost fish, where GABA is mostly stimulatory to GnRH and LH release, provide an ideal model to study the novel stimulatory action of GABA on reproduction.

In fish, research has been more limited, yet there appears to be major difference in the role of GABA in the control of reproduction when compared to the mammalian model. In goldfish (*Carassius auratus*), intraperitoneal injection of GABA has been shown to increase serum GTH-II levels in sexually regressed or fish in early recrudescence, but not in fish in late recrudescence (Kah et al. 1992). Increasing GABA levels by use of a GABA-T inhibitor was also found to increase serum GTH-II levels, in a dose-dependant manner (Kah et al. 1992; Trudeau et al. 1993a). GABA-stimulated release of GTH-II has been found to be mediated via a $GABA_A$ type receptor mechanism (Trudeau et al. 1993a). In pituitary slices which contain GnRH nerve terminals, GABA

was also found to stimulate GnRH release in a dose-dependant manner (Kah et al. 1992). In rainbow trout (*Oncorhynchus mykiss*), GABA injection was found not to affect basal GTH-II release in spermiating males and immature fish, but increases in plasma GTH-II were observed in mature and recrudescent females after similar treatment (Mananos et al. 1999). Furthermore, GABA was also shown to stimulate GTH-I release from trout pituitary cells in vitro (Mananos et al. 1999). In Atlantic croaker (*Micropogonias undulatus*), stimulation of GTH-II release upon intraperitoneal injection of GABA was observed in regressed and pre-recrudescent fish, but a dose-dependent inhibition of GTH-II release was observed in fish with fully recrudesced gonads (Khan and Thomas 1999). Mechanisms of GABAergic stimulation of GnRH release were found to occur via $GABA_A$ receptors, as the $GABA_B$ agonist bicuculline did not induce GnRH release in catfish (Joy et al. 1999). It has been shown that the anterior lobe of the goldfish pituitary is prominently innervated by GABAergic nerve terminals (Kah et al. 1992). Moreover, GABAergic fibres were also found to be adjacent to GTH cells in rainbow trout (Mananos et al. 1999), indicating a possible role for direct effects of GABA on GTH-II secretion. However, administering GABA to dispersed goldfish pituitary cells was found to be without effect (Kah et al. 1992). In teleosts, inhibition of reproductive processes occurs through the action of dopamine (DA) on the release of GnRH and GTH-II (Fig 1.1) Dopamine and dopamine-receptor agonists were shown to reduce induced GTH-II secretion (Van Goor et al. 1998) and the dopamine agonist apomorphine was shown to inhibit basal and GnRH-induced GTH-II release (Lin et al. 1993). In sexually regressed goldfish, increasing neuronal GABA content by inhibiting GABA-T action with GVG was found both to stimulate LH release and to suppress DA turnover rates in both the

HYP and the PIT (Trudeau et al. 1993a). By acting both to inhibit DA and to stimulate GnRH, GABA is a potent stimulator of GTH-II release in fish. Elevated GTH-II levels following injection of the GABA-transaminase inhibitor gamma-vinyl-GABA (GVG) in male goldfish also provokes sex steroid production (Sloley et al. 1994), providing additional evidence of the importance of GABA in reproduction.

1.8 GABA and sex-steroids

Steroids are known to have feedback effects on the reproductive axis. However, there is no conclusive evidence of any direct effect of these steroids on GnRH neurons; although researchers have attempted to identify estrogen receptors (ER) on GnRH neurons they have for the most part been unsuccessful. More recent evidence shows limited immunocytochemical evidence for the presence of beta-type ERs on certain GnRH neurons, but the discovery of these receptors remains controversial (Herbison, 1998). This supports a more significant role for neurotransmitters in the feedback regulation of GnRH release, of which GABA is one of the most significant.

There exist several lines of evidence for an important role for GABA in mediating the feedback actions of gonadal steroids on GnRH and gonadotropin release. First, at the morphological level, a direct synaptic connection has been shown to exist between GABAergic fibres and GnRH neurons in rat brain (Leranth et al. 1985; Jung et al. 1998). In rat medial pre-optic area and anterior hypothalamus, two brain regions critical in reproductive control, a large proportion of GABAergic neurons possess E2 receptors (Flugge et al. 1986). GABA transporters (GATs) can also be sites for control of

GABAergic neurotransmission. Moreover, there is evidence that their activity and synthesis may be regulated by hormones such as insulin and adrenal and gonadal steroids (Figlewicz 1999). The expression of GAT-1, in particular, has been shown to be modulated by sex steroids (Herbison et al. 1995) showing an intimate connection between steroidal and GABAergic control mechanisms. Furthermore, it is known that the action of sex steroids can modulate the action of GABA by membrane-mediated or by classic genomic action; by affecting gene expression or by affecting membrane transport or ion permeability. Among other effects, sex steroids such as E2 can enhance or reduce GABA receptor binding capability (McCarthy 1995a). Effects of sex steroids on GABAergic neurotransmission can thusly be expected but mechanisms of action may be numerous.

Sexual dimorphisms of GABA and GAD levels in brain also point towards a potential role for gonadal steroids in the control of GABAergic transmission. Sex differences have been found in GABA concentrations in the medial POA and the ventro-medial HYP of the rat (Frankfurt et al. 1984). In rat brain, hormonally-regulated developmental GAD mRNA sex-differences were also found (Davis et al. 1996). In grenadier, a deep sea fish, hypothalamic expression of GAD_{65} mRNA was found to be 1.8 times higher in males than in females (Trudeau et al. 2000a).

In several different ways, researchers have begun to elucidate the effects of sex steroids on the GABAergic system and the role of GABA in the mediation of steroidal negative and positive feedback on gonadotropin release in mammals. These methods

include both studies of natural variations in gene expression, enzyme activities and neurotransmitter concentrations and manipulation of endogenous gonadal steroids vertebrates by gonadectomies or injection/implantation of exogenous sex steroids. During the ovulatory LH surge in rats, contradictory results have been obtained. Herbison et al. (1992) noted a decrease in GAD_{67} mRNA in the POA during the preovulatory peak. Leonhardt et al. (2000) however, observed an increase in GAD_{67} mRNA in the POA during the LH surge, with no increase in GAD levels in the medio-basal HYP (MBH). It was also found that E2 decreased GAD mRNA levels in the POA 42 hours but not 24 hours after administration, showing a time-dependent effect of E2 on GABAergic neurons (Wagner et al. 2001). It was hypothesized (Wagner et al. 2001) that E2 was acting to negatively regulate GnRH release during the initial suppresive phase (negative feedback) and subsequently reduced GABA function during the LH surge (positive feedback). Studies on GABA levels however, revealed an increase in GABA content of the ventro-medial nucleus of the HYP (VMH) when E2 levels were increased with no effect observed in the POA under the same conditions (Luine et al. 1997). In ovariectomized (OVX) rats, E2 was shown to increase GAD_{65} mRNA levels and decrease GAD_{67} mRNA levels in the magnocellular POA. In the dorsomedial nucleus of the HYP (DMN), E2 had an opposite effect increasing GAD_{67} mRNA levels and decreasing GAD_{65} mRNA levels (McCarthy et al. 1995b). This correlates well with the behavioral data which showed opposite effects of GABA increases in the POA and HYP (McCarthy et al. 1990; McCarthy et al. 1991). In OVX estrogen-primed rats, P4 was shown to have an inhibitory effect on GAD_{67} mRNA levels in the POA at the start of the LH surge (Unda et al. 1995). E2 was also found to increase GABA concentrations and GAD

activity in the arcuate nucleus/median eminence (ARC-ME), an effect that was reversed by the administration of a prolactin (PRL) antiserum (Lasaga 1994). In male rats, castration was shown to decrease single-cell GAD mRNA levels in the diagonal band of Broca (DBB) and the sexually-dimorphic nucleus of the POA, with effects being more rapid and dramatic in the POA than in the DBB (Sagrillo and Selmanoff 1997). This suggests different involvement of the two brain regions in steroidal feedback mechanisms (Sagrillo and Selmanoff 1997). In males, blocking T action by antiandrogen microimplants decreased GAD single-cell levels in medial pre-optic nucleus (MPN) and (DBB) (Grattan et al. 1996). Removing T via castration decreased GABAergic neuronal activity and increased LH secretion (Sagrillo and Selmanoff 1997). Both these studies indicate a role for T in maintaining high GABA levels to inhibit LH release in the rat.

Common to most of the mammalian data is the involvement of the POA and the MBH in the majority of steroid effects on GABA. Moreover, steroids are found to have dramatically different effects depending on the sex of the animal and the endogenous steroid levels in the reproductive states examined. Control of GABAergic neurotransmission is subtle, and involves a highly complex system of interactions. What can be concluded from this is that GABA occupies a critical role in reproductive control, and is a major target of gonadal steroid feedback.

In fish, effects of steroids on the GABAergic system have also been found, but evidence is more limited. Analysis of results in fish also requires taking into account dual role of GABA in reproduction, both a direct stimulator of GnRH release and an inhibitor

of DA which itself inhibits GnRH release. In fish, variations in the role of GABA during seasonal reproductive cycles have been documented. In rainbow trout, GABA was shown to increase GTH-II plasma levels in mature females, but not in immature fish (Mananos et al. 1999). After E2 or T steroid implantation, regressed fish did respond to GABA injections with increases in plasma GTH-II (Mananos et al. 1999). In sexually regressed and sexually recrudescent female goldfish, T enhanced GABA stimulated LH release (Kah et al. 1992). Experiments on sexually regressed females showed that E2 enhanced HYP GABA synthesis but had no effect in the TEL (Trudeau et al. 1991a). In these experiments, T was found to decrease GABA synthesis in the TEL, but not in the HYP. More recent studies have found that in sexually regressed female goldfish, E2 increased GAD_{65} but not GAD_{67} mRNA levels in the HYP, whereas T increased GAD_{65} but not GAD_{67} in the TEL (Bosma et al. 2001). In sexually regressed male goldfish, E2 and T decreased GAD_{65} and GAD_{67} mRNA content in the HYP, but did not affect GAD_{65} or GAD_{67} levels in the TEL (Bosma et al. 2001). Therefore, in fish there are important sexually dimorphic responses of GAD to sex steroids. It is now important to determine the actions of sex steroids at other times of the year when endogenous steroid levels vary with seasonal gonadal development.

1.9 Goals of this research

This study set out with two goals: to elucidate both the evolution of GAD in vertebrates and regulation of the three known GADs in goldfish brain. At the outset of this project, GAD sequence data showed a single GAD isoform in *Ciona*, a urochordate, and both GAD_{65} and GAD_{67} isoforms in vertebrate classes from teleosts to mammals. We

set out to determine the time of divergence of GAD_{65} and GAD_{67} by sequencing GADs from chondrichthyes, lamprey, a hagfish and amphioxus, a cephalochordate. A third form of GAD has been discovered in grenadier in recent years (Bosma et al. 1999). We attempted to obtain further sequence information for this novel GAD3 from other species. We expect all GAD3 seqeunces to group together. We also attempted to add to available sequence information by sequencing GADs from alligator, axolotl and South American lungfish. Furthermore, much remains to be discovered in regards to the effects of steroids on the GABAergic system in fish, and more particularly on GAD expression in fish of varying reproductive states, in particular in recrudescing and mature fish. Goldfish were chosen as an experimental model as most work on fish reproduction has been carried out with this species. We set out to study the effects of T and E2 on GAD expression in recrudescent and mature goldfish. Variations in both GAD_{65} and GAD_{67} mRNA levels have been observed after administration of these steroids to regressed fish and we expect to also see differences in GAD mRNA levels at other points of the reproductive cycle of the goldfish with similar treatment. Although most studies on fish reproduction do not consider the importance of P4, significant levels of this steroid have been found in some fish (Degani et al. 1998; Garnier et al. 1999; Tricas et al. 2000). Progesterone is known to suppress GAD_{67} mRNA levels in the POA of rats (Unda et al. 1995) and decreases pituitary GABA synthesis rates in sexually regressed goldfish (Trudeau et al. 1993a). We expect to find P4 plays a role in the regulation of the GADs at some point in the yearly reproductive cycle of the goldfish. We therefore included P4 administration in our study. The newly discovered GAD3 was also of interest to this study of fish GAD expression, notably due to its discovery in a fish species. This gene was cloned from goldfish as part

of our evolutionary studies. This study of the GABAergic system in vertebrates has provided new information on both evolutionary and regulatory aspects of GAD.

Chapter 2 - Multiplicity of glutamic acid decarboxylase in chordates: early gene duplication and novel forms.

2.1 Introduction

The amino acids glutamate and γ-aminobutyric acid (GABA) mediate fast synaptic excitatory and inhibitory neurotransmission in the CNS, respectively. GABA is synthesized from glutamate in a single enzymatic step by glutamic acid decarboxylase (GAD) and has been shown to control various physiological processes as well as holding an important role in synaptic plasticity and development (Wolff et al. 1978; Barbin et al. 1993; Waagepetersen et al. 1999). GABA induces postsynaptic membrane hyperpolarization through activation of $GABA_A$, $GABA_B$ or the recently discovered $GABA_C$ receptors. Both the A and C subtypes are chloride channel type receptors whereas the B subtype receptors are metabotropic (G-protein-linked) receptors (Chebib and Johnston 1999; Bormann 2000). GABA can also induce membrane depolarization, a mechanism that is likely important for neuronal development (Barbin et al. 1993; Owens et al. 1996). Upsets in GABA synthesis have been known to underlie several neurodegenerative conditions including epilepsy (During et al. 1995; Treiman 2001; Chapouthier and Venault 2001). Autoantibodies to GAD are also involved in stiff-man syndrome and insulin-dependent diabetes mellitus (IDDM; (Lernmark 1996; Yoon et al. 1999; Baekkeskov et al. 2000; Balasa et al. 2001). In addition to its role as a neurotransmitter in higher brain centres, GABA is involved in the regulation of neuroendocrine function by acting on the hypothalamo-pituitary axis (Kah et al. 1992; Trudeau et al. 2000b; Bosma et al. 2001).

Glutamic acid decarboxylase (GAD; EC 4.1.1.15) is the rate limiting enzyme catalyzing the synthesis of GABA from glutamate. At least two isoforms of GAD exist in vertebrates, which have molecular weights of 65 kDa (GAD_{65}) and 67 kDa (GAD_{67}). In mammals, both GAD_{65} and GAD_{67} are co-expressed in GABAergic neurons, but they differ in cellular distribution, regulation, and in their interaction with the cofactor pyridoxal 5' phosphate, PLP (Erlander et al. 1991a; Erlander and Tobin 1991b; Bu et al. 1992; Martin and Rimvall 1993; Sheikh et al. 1999). Co-factor interaction is also known to be modulated in both GADs by several conditions including increased neuronal activity and changing cellular GABA levels. (Chen et al. 2000).

The genes coding for GAD_{65} and GAD_{67} show highly similar deduced amino acid sequences. The similarity of their intron/exon organization led Bu and Tobin (1994) to suggest that these genes derive from a common ancestral GAD gene. GAD_{65} and GAD_{67} are encoded by two distinct genes located on human chromosomes 10 and 2, respectively (Erlander et al. 1991a; Bu et al. 1992; Edelhoff et al. 1993). They are thought to have arisen due to a gene duplication event early in vertebrate evolution (Bu and Tobin 1994; Bosma et al. 1999). These two isoforms were first cloned from mammalian brain, while partial GAD sequences were recently cloned from the brains of goldfish, armed grenadier, zebrafish, red-eared slider turtle, and zebra finch (Bosma et al. 1999). Further work has also lead to partial sequences for leopard frog, snapping turtle, and a diverse group of teleosts (Trudeau et al. 2000b). GAD_{65} and GAD_{67} were found in each of these species, demonstrating for the first time that representatives of teleosts, amphibians, reptiles, and birds express both isoforms of this gene. Only a single highly divergent

GAD, considered a derivative of the archetypal chordate gene, was cloned from the urochordate *Ciona intestinalis*. From these results, it was concluded that the possible gene duplication events leading to the presence of dual forms of GAD occurred after the branching off of the ascidians (i.e., *Ciona intestinalis*), but before the divergence of the teleosts from the main vertebrate lineage.

A third form of GAD, designated GAD3, was also cloned from the armed grenadier (*Coryphaenoides (Nematonurus) armatus*), a deep-sea fish (Bosma et al. 1999). It shows only 64% identity to either GAD_{65} or GAD_{67} at the amino acid level whereas grenadier GAD_{65} and GAD_{67} are 73% identical to each other at the amino acid level. This novel GAD was shown to group separately from the other GAD isoforms in phylogenetic analyses, however its evolutionary relationship with GAD_{65} and GAD_{67} could not be resolved (Bosma et al. 1999). It was suggested that the ancestral GAD may have undergone two phases of gene duplication events to produce the three expressed forms of GAD (Bosma et al. 1999).

This study set out to further examine some of the important events occurring in GAD evolution by cloning and sequencing several new vertebrate cDNA fragments. First, we examined the evolution of GAD around the putative time of divergence of GAD_{65} and GAD_{67}. In order to do this we studied amphioxus, three representative species from the agnathans and four species from the Chondrichthyes. No GAD sequence information was previously available from any of these three groups. Single forms of the GAD gene were identified from amphioxus and each agnathan species whereas two

forms were cloned from two Chondrichthyan species. The addition of lungfish GAD sequence information along with GAD sequences from the axolotl, a neotenic salamander, have allowed us to further examine GAD evolution during the vertebrate water to land transition. We also show that a close evolutionary relationship exists between reptile and bird GAD isoforms. Within the teleosts, multiple forms in eel and the discovery of a new GAD3 sequence from goldfish increases the multiplicity of known GADs in chordates.

2.2 Materials and Methods

2.2.1 Tissue Collection

Amphioxus (*Branchiostoma lanceolatum*) were collected by Dr. John Bishop (Plymouth Marine Laboratory, England) and front ends were pooled on dry ice. Hagfish (*Myxine glutinosa*) and hedgehog skate (*Raja erinacea*) were captive animals located at the University of Guelph (Guelph, Ontario) and were provided by Dr. Jim Ballantyne. The lampreys were collected by Dr. Claude Renaud (Canadian Museum of Nature; Ottawa, Ontario). *Petromyzon marinus* is a parasitic lamprey, while *Ichthyomyzon unicuspis* is a non-parasitic lamprey. Greenland sleeper shark (*Somniosus microcephalus*) brain was collected in Cumberland Sound, Baffin Island, by Dr. Aaron Fisk (Canadian Wildlife Services). Whole shark brain tissue was collected and transported in RNAlater (Ambion) on dry ice. Ratfish (*Hydrolagus colliei*) were obtained from the Bamfield Marine Station, British Columbia, and brains were collected by Dr. S. Perry (University of Ottawa). Dogfish (*Squalus acanthias*) were collected by V. L. Trudeau near Aberdeen, Scotland. Eels (*Conger conger*) were collected by V. L. Trudeau near Scalloway, Shetland and I. Napier (NAFC) provided trawling and aquarium services. Electricfish (*Apternotus leptorhynchus*) were provided by W. Ellis and L. Maler (University of Ottawa). Lungfish (*Lepidosiren paradoxa*) were caught in northern Argentina courtesy of Dr. G. Somoza (Universidad de San Martin, Argentina). Alligator (*Alligator mississippiensis*) brain stored in RNAlater (Ambion) was provided by Dr. L. Guillette (University of Florida). Axolotls (*Ambystoma mexicanum*) were provided by the Toronto Zoo (Toronto, Ontario). Brains were dissected and pooled on dry ice. All tissues were stored at -80°C until RNA extraction.

2.2.2 RNA extraction and RT-PCR

Total RNA was isolated from the frozen tissue samples using the acid guanidinium thiocyanate method (Chomczynski and Sacchi 1987; Chomczynski and Mackey 1995). Messenger RNA was isolated from *S. microcephalus* and *R. erinacea* total RNA using the Oligotex mRNA mini kit (Qiagen, USA). Messenger RNA was isolated directly from *L. paradoxa* tissue and from *A. mexicanum* total RNA using the Straight As kit (Novagen). First-strand cDNA was synthesized from 1-5μg poly(A)+ RNA (*S. microcephalus, R. erinacea, L. paradoxa* and *A. mexicanum*) or total RNA (*I. unicuspis* and hagfish) using Oligo (dT) primer and Superscript II reverse transcriptase (Gibco BRL) and stored at -20°C until used. Partial GAD sequences of GAD_{65} and GAD_{67} were amplified using the protocol described by Bosma et al. (1999) which is based on the use of degenerate primers designed from highly conserved regions of the GAD genes including the putative PLP co-factor binding site. The nucleotide sequences of the two forward primers were as follows: RAC DGC MAA YAC BAA YAT GTT YAC HTA TGA (GADALL.FW1) and RAC DGC MAA YAC BAA YAT GTT HTA YGA (GADALL.FW3). The reverse primer nucleotide sequence was as follows: CAT CAT YTT RTG NGG RTT CCA NGT (GADALL.REV2). Annealing temperatures ranging from 43°C to 51°C and a standard $MgCl_2$ concentration of 1.5mM were used in attempts to obtain GAD isoforms from every species. Goldfish GAD3 was amplified using the following primers: gGAD3 f/r2 (f: TGT GGG ATG GTG CGA GGA AGA G; r2: GTC TGC TAC AAG AGT GCA ACC AGC TG) with an annealing temperature of 63°C. Desired bands of approximately 600bp were extracted from 1% agarose gel using the QIAquick gel extraction kit (Qiagen). Following PCR, DNA was extracted as above

and ligated into the pCRII-TOPO cloning vector (Invitrogen) and used to transform One Shot competent cells (Invitrogen). Plasmids were isolated using the Wizard Miniprep kit (Promega). Plasmids were sequenced by the Canadian Molecular Research Services (Ottawa, Canada); both strands of three to six inserts were sequenced for each GAD consensus sequence obtained.

2.2.3 Sequence Analysis

All nucleotide consensus sequences obtained were translated to predicted amino acid sequences using the ExPASy proteomic server of the Swiss Institute of Bioinformatics. Sequences were compared to previously known sequences using BLAST (Altschul et al. 1997). Sequence alignments were performed using ClustalW (Thompson et al. 1994). Accession numbers of previously published sequences are as follows: goldfish (*Carassius auratus*) GAD_{65} and GAD_{67}-AF043266 and AF043265; grenadier (*Coryphaenoides armatus*) GAD_{65}, GAD_{67}, and GAD3-AF043268, AF043267, and AF043269; anglerfish (*Lophius piscatorius*) GAD_{65} and GAD_{67}-AF202123 and AF202124; slickhead (*Alepocephalus baiirdi*) GAD_{65} and GAD_{67}-AF202121 and AF202122; zebrafish (*Danio rerio*) GAD_{65} and GAD_{67}- AF017265 and AF042374; leopard frog (*Rana pipiens*) GAD_{65} and GAD_{67}- AF202125 and AF202126; *Xenopus laevis* GAD-U38225; rat (*Rattus norvegicus*) GAD_{65} and GAD_{67}-M72422 and X57572; human (*Homo sapiens*) GAD_{65} and GAD_{67}-M81882 and M81883; pig (*Sus scrofa*) GAD_{65} and GAD_{67}-D31848 and D31849; red-eared slider turtle (*Trachemys scripta*) GAD_{65} and GAD_{67}-AF043272 and AF043274; snapping turtle (*Chelydra serpentina*)GAD_{65} and GAD_{67}-AF202127 and AF202128; zebra finch (*Taeniopygia*

guttata) GAD_{65} and GAD_{67}-AF043270 and AF043271; and *Ciona intestinalis* GAD-AF043275.

Maximum-likelihood trees were calculated using the TREE-PUZZLE version 5.0 program (available at http://www.tree-puzzle.de/; Strimmer and von Haeseler 1996). The JTT model of amino acid substitutions (Jones et al. 1992), and one fixed and eight-gamma distributed rates of heterogeneity were used. The tree was rooted with *Ciona intestinalis* GAD, a urochordate known to be an outgroup to all other species analyzed. Alternative amino acid tree topologies were compared using user-defined trees, the JTT model of substitutions (Jones et al. 1992), and eight gamma distributed rates of heterogeneity. The RRTree version 1.1.10 program was used to perform relative rate tests on the GAD amino acid sequences (available at http://pbil.univ-lyon1.fr/software/rrtree.html; Robinson et al. 1998; Robinson-Rechavi and Huchon 2000).

2.2.4 Data submission

The new sequences reported here were submitted to GenBank. Accession numbers are as follows: amphioxus (*Branchiostoma lanceolatum*) GAD- AF432147; skate (*Raja erinacea*) GAD_{65} and GAD_{67}- AF432148 and AF432149; sleeper shark (*Somniosus microcephalus*) GAD_{67}- AF432150; axolotl (*Ambystoma mexicanum*) GAD_{65} and GAD_{67}- AF432151 and AF432152; lungfish (*Lepidosiren paradoxa*) GAD_{65} and GAD_{67}- AF432153 and AF432154; goldfish (*Carassius auratus*) GAD3- AF432155; hagfish (*Myxine glutinosa*) GAD- AF432156; *Petromyzon marinus* GAD- AF432157;

Ichthyomyzon unicuspis GAD- AF432158; ratfish (*Hydrolagus colliei*) GAD_{65} and GAD_{67}- AF432159 and AF432160; alligator (*Alligator mississippiensis*) GAD_{65} and GAD_{67}- AF432161 and AF432162; eel (*Conger conger*) GAD_{65}Aand GAD_{65}B- AF432164 and AF432163; dogfish shark (*Squalus acanthias*) GAD_{67}- AF432165; electricfish (*Apternotus leptorhynchus*) GAD_{65} and GAD_{67}- AF432166 and AF432167.

2.3 Results

2.3.1 Multiple GAD sequences from vertebrates

Twenty-one new partial GAD sequences 541 to 547bp in length from 14 different species were obtained. These sequences represent one-third of the coding sequence of the GAD gene, and are located in the centre of these sequences. Two forms were obtained from alligator, axolotl, lungfish, electricfish, eel, ratfish, and skate. Single forms were cloned from dogfish shark, greenland shark, both lamprey species, hagfish and amphioxus. In addition, a GAD3 sequence 588bp in length was obtained from goldfish brain. This sequence is 86% identical both at the nucleotide and at the amino acid level to grenadier GAD3. These two GAD3 sequences are equally similar at the amino acid level (~62%) to both GAD_{65} and GAD_{67} from goldfish.

2.3.2 Relative rates of evolution

Relative rate tests show that the different GAD genes evolve at similar rates (Table 2.1). Although two comparisons (GAD3 versus GAD_{67} and GAD3 versus agnathan GADs) are significantly different at the 5% level, neither of these comparisons are significant at the 0.5% level when the alpha level is corrected for the ten comparisons conducted (Rice 1989).

2.3.3 Phylogenetic analysis

Phylogenetic trees were created using the predicted partial amino acid sequences obtained from each gene. *Ciona* GAD, a protochordate sequence, was used as an outgroup. Amphioxus GAD was found to group outside the other GADs alongside the *Ciona* sequence (Figure 2.1). The tree shows five major GAD groups. The first group

contains the two outgroups, *Ciona* and amphioxus. The four other groups, GAD3, GAD_{65}, agnathan GADs and GAD_{67} appear to group separately from each other, but support values for these groupings are low. Furthermore, alternative tree topologies were tested to determine whether the inferred phylogenetic position of the GAD3 and agnathan GAD genes was significant. None of the alternative topologies we tested were significantly different from the topology shown in Figure 2.1 (Table 2.2). The lamprey and hagfish sequences group together, as do the GAD3 sequences. The GAD_{65} and GAD_{67} sequences, which include most known vertebrate GADs, form two well-defined

Table 2.1. Relative rate tests of GAD evolution.

group 1	group 2	dK	s.d.	P
amphioxus GAD	GAD3	-0.111	0.081	0.168
amphioxus GAD	GAD_{65}	-0.009	0.077	0.907
amphioxus GAD	GAD_{67}	0.027	0.075	0.722
amphioxus GAD	agnathan GADs	0.024	0.076	0.750
GAD3	GAD_{65}	0.102	0.063	0.107
GAD3	GAD_{67}	0.138	0.063	0.030
GAD3	agnathan GADs	0.135	0.069	0.050
GAD_{65}	GAD_{67}	0.036	0.048	0.458
GAD_{65}	agnathan GADs	0.033	0.049	0.500
GAD_{67}	agnathan GADs	-0.003	0.047	0.956

Notes. The relative rate tests were performed on the GAD amino sequences, the *Ciona* GAD sequence was used as the outgroup sequence in all analyses and the sequences were equally weighted. GAD3 = grenadier and goldfish GAD3; GAD_{65} = all GAD_{65}; GAD_{67} = all GAD_{67}; agnathan GADs = *I. unicuspis*, *P. marinus* and hagfish GADs. dK = difference in the number of substitution per site; s.d. = standard deviation, P = P-value.

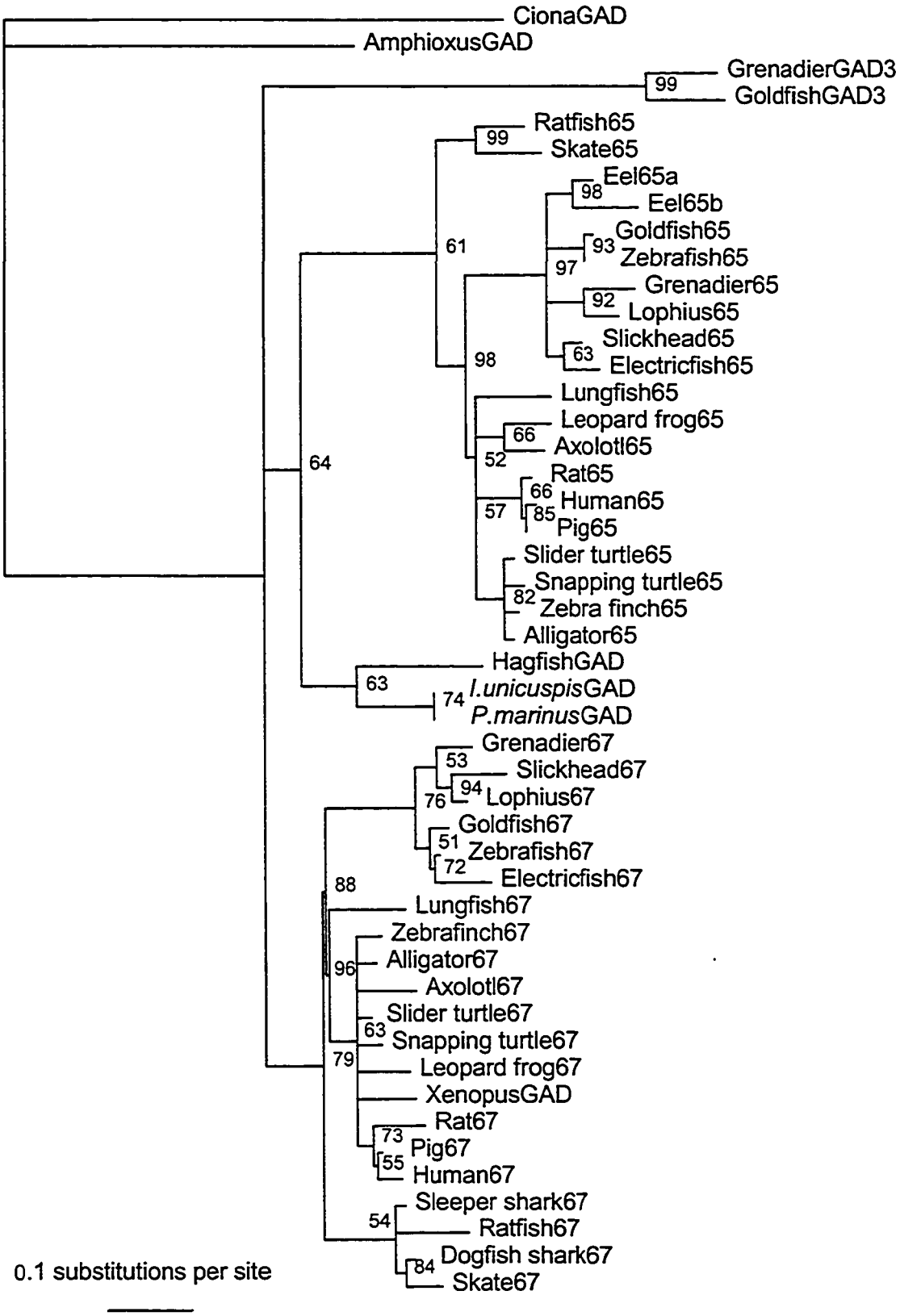

Figure 2.1: Maximum-likelihood tree for chordate GAD. Support values are shown next to their respective nodes.

Table 2.2. Maximum-likelihood results of alternative topologies of GAD trees

tree	log L	difference	s.e.	significantly worse
Tree shown in Figure 2.1	-3865.34	0.00	--	--
GAD3 outgroup to GAD_{65}, GAD_{67} and lamprey	-3865.63	0.29	4.39	no
GAD3, GAD_{65}, GAD_{67} and lamprey unresolved	-3868.93	3.59	3.38	no
GAD3 ancestral to GAD_{65}	-3866.88	1.54	4.41	no
GAD3 ancestral to GAD_{67}	-3866.50	1.15	2.03	no
GAD3 ancestral to lamprey	-3868.93	3.59	3.38	no
Lamprey sister group to GAD_{67}	-3865.60	0.26	4.44	no
Lamprey outgroup to GAD_{65}, GAD_{67} and GAD3	-3868.93	3.59	3.38	no

Notes. GAD3 = grenadier and goldfish GAD3; GAD_{65} = all GAD_{65}; GAD_{67} = all GAD_{67}; lamprey = *I. unicuspis*, *P. marinus* and hagfish GADs; logL = log likelihood; difference = difference in log likelihood; s.e. = standard error. These tests (5% significance) follow Kishino and Hasegawa (1989).

groups within the tree, each of these groups following the general model of vertebrate evolution. Within the GAD_{65} grouping, Chondrichthyes branch off first, followed by the teleosts, which group together in a highly supported branch. The lungfish sequences subsequently branch off, followed by the tetrapod sequences. The tetrapod sequences are divided into tree groups: an amphibian group, a reptilian and bird group, and a mammalian group. The GAD_{67} sequences follow a topology that is similar to that of GAD_{65} sequences. However, these GAD_{67} sequences are not resolved into well defined groups, likely because of their higher similarity when compared to other GAD sequences.

2.4 Discussion

A few mammalian GAD gene sequences have long been available. However, GADs from species representing the other major groups of vertebrates have only recently been sequenced (Bosma et al. 1999; Trudeau et al. 2000b). By sequencing a number of new GADs from species related to key events in vertebrate evolution, we have further resolved the molecular evolution of this important GABA synthesizing enzyme. This has allowed us to push back the known time of divergence of this family of genes by approximately 200 million years by showing that both GAD_{65} and GAD_{67} are present in cartilaginous fish species. Furthermore, we have studied the existence of multiple forms of GAD in vertebrates including GAD3, first identified in a deep-sea fish (Bosma et al. 1999), and which we were able to clone from an unrelated teleost, the goldfish.

We sequenced GAD from the amphioxus, which is considered to be the direct ancestor of all modern vertebrates (Holland and Chen 2001). We found that the amphioxus GAD groups separately from the sequences for both GAD_{65}/GAD_{67} and agnathan GADs, but directly adjacent to *Ciona* GAD, a protochordate sequence. Bosma et al. (1999) had previously suggested that the GAD expressed by *Ciona* is most likely a derivative of the archetypal chordate form. The single form we cloned from amphioxus is probably derived from the same ancestral gene. In order to further elucidate the time of divergence, we cloned a single GAD form from a parasitic lamprey, a non-parasitic lamprey, and a hagfish. Our data support a hagfish/lamprey clade, all three species grouping together. However, the sequence data available are not sufficient to resolve the phylogenetic position of the agnathans with either the GAD_{65}, GAD_{67} or GAD3 groups

(Table 2.2). Although studies on mitochondrial DNA have yielded phylogenies grouping lampreys with tetrapods, with hagfishes grouping separately (Rasmussen et al. 1998), more recent studies on ribosomal rDNA sequences have shown moderate to strong support for the monophyly of these three cyclostome sequences (Mallatt and Sullivan 1998). Among the lamprey GADs, only minor DNA sequence differences were observed, which did not result in any amino acid differences (results not shown). This demonstrates a remarkable conservation of the GAD gene between these two species despite their very different life histories. Our research indicates that the agnathan form of GAD evolved separately from GAD_{65} and GAD_{67} and may represent a descendant of an ancestral form of the gene. Furthermore, the two isoforms of GAD found in mammals and other vertebrates were identified in the two extant subclasses of the chondrichthyes; the holocephalans (ratfish) and the elasmobranchs (skate). These two GADs clearly group with GAD_{65} and GAD_{67} in this study. This supports the presence of a genome duplication early in vertebrate evolution, prior to the emergence of the cartilaginous fishes.

Many genes appear to have undergone duplications early in vertebrate evolution. The patterns of evolution of certain homeobox genes show some similarities to the hypothesized GAD evolutionary history we have presented. For example, the *Hox* gene family encodes a helix-turn-helix transcription factor involved in developmental patterning, which has been used to examine animal evolution in several studies. Evidence from *Hox* gene studies seems to indicate that the emergence of the vertebrates occurred after at least one large scale genome duplication event (Amores et al. 1998; Stellwag 1999). Data from studies with the *Hox* gene show a single cluster of genes in amphioxus

as well as in *Ciona* (Garcia-Fernandez and Holland 1994; Holland and Garcia-Fernandez 1996) which became four clusters in mammals. Studies done on other genes such as the hedgehog gene family (Shimeld 1999a) and the *Emx* homeobox gene class (Williams and Holland 2000), also support the presence of single copies of certain genes in amphioxus, where multiple forms are found in mammals. These conclusions would support our recent findings of a single, ancestral form of GAD in amphioxus. The study of *Emx* and hedgehog genes also support the presence of at least one genome duplication event which would have occurred prior to the emergence of ray-finned fishes (Shimeld 1999b; Williams and Holland 2000) whereas the study of *Hox* genes suggests two duplications (Bailey et al. 1997). Three *Hox* clusters have been found in lampreys and hagfish (Pendleton et al. 1993), as opposed to the four clusters in tetrapods and the single cluster found in chordates. The manner in which these three clusters formed is not presently known. Had the GAD genes diverged to form the 65 and 67kD forms during the second putative genome duplication, the presence of single forms of GAD in agnathans could be explained by the fact that an ancestral form was preserved in the jawless fishes (Shimeld 1999a). The two forms of GAD present in other vertebrates appear in Chondrichthyes. This shows that the duplication event giving rise to GAD_{65} and GAD_{67} occurred prior to the emergence of the Chondrichthyes and places the time of divergence of these two major forms of GAD to approximately 400 million years ago.

We have found that lungfish sequences consistently group as intermediates between fishes and amphibians for both GAD_{65} and GAD_{67} in phylogenetic trees. Lungfish GADs group with the amphibians and they are placed between the fishes and

tetrapods in the tree, supporting the proposal that terrestrial vertebrates have evolved from lobe-finned fishes (subclass Sarcopterygii) (Zardoya and Meyer 1997; Longhurst and Joss 1999). Only six species of lungfish and the coelacanth, a single lobe-finned fish, now represent this subclass. The South American lungfish is the first of these species from which GAD has been cloned. Our expansion of amphibian sequence information by the addition of axolotl GAD_{65} and GAD_{67} has provided more data to support this tree topology. Some recent controversial studies have shown lungfish as the most ancestral of the fishes, with Chondrichthyes occupying a terminal position in the piscine tree (Rasmussen and Arnason 1999a; Rasmussen and Arnason 1999b). Our study does not support this conclusion, but shows trees placing Sarcopterygians as the sister group to tetrapods as being more likely.

We also sequenced the alligator GAD_{65} and GAD_{67} genes. Both were shown to group with known turtle and bird sequences. Using the available sequence information, it was not possible to group together anapsids (e.g., turtles) and diapsids (e.g., crocodilians and birds), however the common ancestry of the GAD genes from these two groups is clear. We obtained two different forms of GAD_{65} from the brain of a conger eel that differ by 16 amino acids in the sequence portion obtained. Whether these are allelic forms or separate genes is unknown.

Previous phylogenetic studies identified a third form of GAD from a deep-sea fish, the armed grenadier (Bosma et al. 1999). In our study, we were able to clone this form of GAD from goldfish. The additional sequences added to our phylogenetic analysis

have yet to resolve the origin of GAD3, as trees grouping GAD3 alone, or with either of the 3 vertebrate GAD groups are not statistically different (Table 2.2). Furthermore, relative rate tests have shown that GAD3 from goldfish does not evolve at a rate significantly different from GAD_{65} or GAD_{67}. These results are consistent with previous relative rate test analyses on a smaller GAD sequence dataset (Bosma et al. 1999). The position of this novel GAD on the phylogenetic tree is suggestive of an early duplication event. Had GAD participated in both genome duplication events as earlier described, four GADs would be expected. With the presence of three GADs, we hypothesize that the other form is silent in all classes, and that the GAD3 form has gone silent in many of the vertebrate classes. A novel function may be reasonably assumed for this GAD, as this gene likely would have degenerated into a pseudogene had it not a separate function (Shimeld 1999a). Furthermore, differential distribution for GAD3 has been established in the brain of the deep-sea grenadier. It has been found to be more highly expressed in the cerebellum than other brain regions, a pattern not consistent with the distribution of the GAD_{65} or GAD_{67} which are more abundant in telencephalon (Trudeau et al. 2000a). This differential distribution also implies a novel function for this third form of GAD.

This study presented evidence for the existence of multiple forms of GAD in species that diverged after the agnathans. The lamprey and hagfish sequences were found to group together separately from the other forms of GAD. It was also found that Chondrichthyes possess both GAD_{65} and GAD_{67} and the time of divergence of the two main forms of GAD can therefore be placed prior to the divergence of the cartilaginous fishes. Lungfish GADs were sequenced, and were shown to be a sister group to the

tetrapods on the GAD tree. We also cloned alligator GADs, which allowed us to confirm the grouping of birds and crocodilians in the same lineage in the GAD evolutionary tree. A new representative of the GAD3 family was sequenced from goldfish, providing additional data for the analysis of this novel GAD group. Further studies of this third GAD will allow more data regarding its phylogeny and function to be elucidated. The topology of the tree we obtained is consistent with the commonly accepted evolutionary relationships of vertebrate taxa. Our data also support the proposal that the neural GAD gene is a useful marker to study vertebrate evolution (Bosma et al. 1999).

Chapter 3 - Seasonal variation and sex steroidal regulation of glutamic acid decarboxylase expression in goldfish hypothalamus and telencephalon

3.1 Introduction

γ-Aminobutyric acid (GABA) is an abundant neurotransmitter in the vertebrate brain. It has been shown to play important roles in development, neuronal plasticity as well as the neuroendocrine control of reproduction. In addition to its role as a neurotransmitter, GABA can act as a paracrine signalling molecule, a metabolic intermediate and a trophic factor (Pinal and Tobin 1998). The many post-synaptic actions of GABA are mediated by three main classes of receptors: the ionotropic $GABA_A$ and $GABA_C$ receptors and the metabotropic $GABA_B$ receptors (Chebib and Johnston 1999; Bormann 2000). Defects in the GABAergic system can lead to diseases such as diabetes, stiff-man syndrome and epilepsy in certain individuals (Lemmark 1996).

In vertebrates, reproduction is regulated by a series of hormonal signals. The decapeptide GnRH is the most important and acts on gonadotrophs in the pituitary. In mammals, GnRH and other neurohormones are carried to the gonadotrophs in the anterior pituitary via the median eminence (ME) portal system. In teleosts, the anterior lobe of the pituitary is directly innervated by a multitude of neuroendocrine neurons including GnRH, DA and GABA (Kah et al. 1986; Kah et al. 1987a; Kah et al. 1987b), presenting a unique model system for the study of vertebrate reproductive neuroendocrine regulation. In the fish pituitary, directly innervated clusters of gonadotrophs can release gonadotropins, gonadotropin-I (GTH-I) or gonadotropin-II (GTH-II), which are respectively the functional analogues of follicular stimulating hormone (FSH) and luteinizing hormone (LH) in mammals. These gonadotropins

released into the general circulation stimulate gonadal sex steroids, sperm and egg production. GABA has been shown to play an important stimulatory role on GTH-II release in goldfish, by stimulating GnRH release and inhibiting the turnover of dopamine in neuroendocrine nerve terminals residing in the pituitary (Trudeau 1997). Inhibitory effects of GABA on the hypothalamo-ptituitary-gonadal (HPG) axis in teleosts have only been clearly demonstrated in the Atlantic croaker where GABA can play both an inhibitory or stimulatory role on GTH-II release (Khan and Thomas 1999). In mammals, GABA has also been shown to have either a stimulatory or inhibitory effect on gonadotropin release (Virmani et al. 1990; Martinez et al. 1994; Feleder et al. 1996). In these animals, the effects of GABA are highly dependent on the developmental stage and endogenous steroid levels of the animals studied. Furthermore, evidence exists in goldfish that GABA is involved in the mediation of sex-steroid feedback at the level of the hypothalamus (Trudeau et al. 1993a; Trudeau 1997).

A major site for the control of the synthesis of GABA is the regulation of glutamic acid decarboxylase (GAD) synthesis and activity. GABA is synthesised from glutamate by the rate-limiting enzyme GAD in a single step. GAD exists in two forms in mammals, designated GAD_{65} and GAD_{67} according to their respective molecular weights of approximately 65 and 67 kDa (Spink et al. 1987; Kaufman et al. 1991; Erlander and Tobin 1991b). These two enzymes are products of two separate genes, have different intracellular distributions and different interaction with the cofactor PLP (Martin and Rimvall 1993). More specifically, GAD_{65} has been shown to be more strongly regulated by co-factor binding whereas GAD_{67} activity is controlled to a greater degree by its faster

turnover rate as determined in the pancreas (Christgau et al. 1991; Michelsen et al. 1993). A third form of GAD was discovered in the armed grenadier, a deep-sea fish, and designated GAD3 (Bosma et al. 1999). More recently, we partially sequenced GAD3 from goldfish and seek to learn more about its regulation in this study.

As seasonal breeders, goldfish undergo yearly cycles of gonadal growth and regression, with the period of maturity and spawning occurring in spring, when conditions for fry survival are highest. Spawning seasons are in large part influenced by photoperiod and temperature (Randall et al. 1998; Joy and Senthilkumaran 1998). It is therefore crucial to study neurohormonal changes at several of these reproductive states, as responsiveness of gonadotrophs, GABAergic neurons and other neurohormonal cells is altered cyclically throughout the year.

Although there has been a great deal of research on the effects of GABA on the neuroendocrine control of reproduction in the laboratory, studies have not been conducted to study the GABAergic system throughout the reproductive cycle in fish, or most other animal models. Furthermore, the third GAD isoform GAD3 has hitherto not been studied at the regulatory level in any animal. We set out to elucidate the effects of sex steroids on the expression of the GAD genes. We examined levels of GAD mRNA in hypothalamus (HYP) and telencephalon (TEL). In goldfish the TEL includes the pre-optic area (POA), a known sex steroid target expressing GADs in rodents. The effects of the sex steroids estrogen (E2) and testosterone (T) on GAD mRNA expression were investigated. These gonadal steroids are known to feedback on the HPG axis to regulate

LH release (Trudeau 1997). Furthermore, detection of progesterone in some fish (Degani et al. 1998; Garnier et al. 1999; Tricas et al. 2000), its ability to potentiate E2 action on GnRH-stimulated GTH-II release in goldfish (Trudeau et al. 1991b) and its role in mammalian reproduction have led us to also include P4 in our study of the effects of sex steroids on GAD mRNA levels in goldfish brain. The effects of these steroids on fish with a range of endogenous steroid levels and thus different physiological status was achieved by selecting early and late recrudescent fish and sexually mature fish. Our results show that in early recrudescent fish, GAD mRNA levels are increased in the HYP of both males and females. In the TEL, we find increases in steady-state levels of GAD mRNA in mature fish, just prior to the spawning season. After steroid implantation for 5 days, there was no effect of E2 on GAD mRNA levels in recrudescent and mature goldfish. Testosterone decreased GAD_{65} and GAD_{67} levels in the TEL of male goldfish. Furthermore, we observe a significant lowering of GAD_{67} mRNA levels in mature female goldfish and of both GAD_{65} and GAD_{67} in mature male goldfish after P4 implantation. These contrasting effects of sex steroids on males and females also indicates important sex differences in the regulation of the GADs.

3.2 Materials and Methods

3.2.1 Animals and treatment

Mount Parnell goldfish of approximately 16-35g were obtained from ABC Aquarium Services (Montréal, Canada) in April, October and January. Fish were sexed upon arrival and held in dechlorinated, oxygenated City of Ottawa tap water at 18°C with a simulated natural photoperiod for a minimum of 2 weeks prior to experimentation. They were fed with standard flaked or pelleted goldfish food. At three separate timepoints, both male and female fish were implanted intraperitoneally with silastic pellets containing E2, T, or P4 (100μg/g body weight) or with a control pellet containing no hormone. This technique has been described by Trudeau et al. (1991a) where physiological levels of 20ng/ml were achieved in goldfish. Five days after implantation, fish were weighed, then killed by spinal transection after MS222 (ethyl 3-aminobenzoate methanesulfonate salt or Tricaine; Sigma) anaesthesia. Gonads were removed and weighed. HYP and TEL from 4-10 fish were dissected out, pooled on dry ice and stored at -80°C until RNA extraction. Four to seven pools for each treatment group were obtained.

3.2.2 RNA isolation, quantification of gene expression by RNase protection assay

Total RNA was isolated from collected tissues by the acid guanidinium thiocyanate-phenol-choloroform extraction (Chomczynski and Sacchi 1987; Chomczynski and Mackey 1995). Desired sequences were amplified from known goldfish GAD clones using specific PCR primers containing T7 or SP6 RNA polymerase promotor sequences for use as probe templates. The RPA method was adapted from

Current Protocols (Ausubel et al, 2000). Radiolabelled RNA probes were transcribed from PCR products using T7 RNA polymerase (Ambion) and 32P-labeled CTP (for β-actin and GAD3; Amersham) or UTP (for GAD_{65} and GAD_{67}; Amersham). Probes were DNase digested and gel-purified to eliminate short partial transcripts and template DNA. . Validation protocols were conducted and the RPA signal was shown to be linear for all four protected fragments between 6 and 10μg total RNA after testing values from 1μg to 15μg. Therefore, 8μg of total RNA from each sample pool was hybridized overnight with 2×10^4cpm of each of the GAD_{65}, GAD_{67}, GAD3 and β-actin RNA probes at 56°C and then digested with RNaseA/T1 (Sigma) at 30°C, followed by a proteinase K (Roche) digestion. Protected RNA fragments were phenol:chloroform extracted, ethanol precipitated and separated on a 5% denaturing polyacrylamide gel. Gels were dried and exposed to phosphor screens (Kodak K-screen) overnight for quantification. β-actin levels were identical throughout experimental groups and were therefore valid for standardizing GAD levels.

3.2.3 Data analysis

Exposed phosphor screens were scanned using the Molecular Imager FX (Bio-Rad). Bands were identified and quantified using the Quantity One software package (Bio-Rad). Statistics and graphs were performed using Systat 8 (SPSS Inc.). Initially, two-way ANOVAs (Sex*Month) were conducted to determine the effects of the variables. Sex and month were found to be significant ($P < 0.05$) and were therefore analyzed separately. One-way ANOVAs were performed on data for each GAD for each month and tissue tested, followed by Fisher's test for comparisons where $P<0.05$. Two-

way ANOVAs (Sex*Treatment) followed by Fisher's test were performed to determine significant changes due to steroid treatment.

3.3 Results

3.3.1 Sexual maturity of experimental fish

Fish used in the experiment were found to have average GSI values that varied between 1% and 10% (Figure 3.1). Both sexes showed similar seasonal trends with highest GSI in May (mature fish), lowest GSI in November (early recrudescence), and intermediate GSI values being observed in February (late recrudescence). This corresponds with natural spawning times and previous results from goldfish observed by Sohn et al. (1999).

3.3.2 Seasonal variations in GAD expression in females

The GAD mRNA levels in goldfish hypothalamus and telencephalon (which in goldfish includes the POA) were quantitated using an RNase protection assay. In females, distinct seasonal patterns of GAD expression were observed in both HYP and TEL (Figure 3.2). In the HYP highest levels of GAD expression occurred in November, when GSI is at its lowest. Lowest GAD expression occurred in February; GAD_{65} mRNA levels showed a 40% decrease from November to February. HYP GAD_{67} mRNA levels in February and May were similar and were about 25% lower than the peak levels recorded in November. HYP GAD3 mRNA expression showed a pattern similar to that of GAD_{65} with highest expression in November. Seasonal GAD expression patterns in the TEL showed some striking differences from that observed in the HYP. GAD_{65} and GAD3 levels in the TEL were highest in May, lowest in November and intermediate in

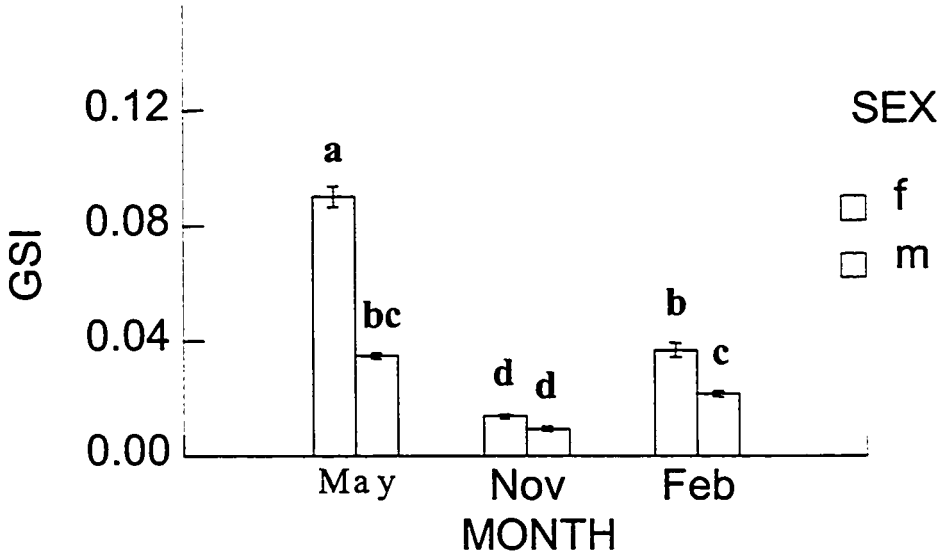

Figure 3.1: Mean GSI values (gonadal wt/body wt) ± SEM for untreated female (f) and male (m) fish at three times of the year (n=27-51). Values marked with different lowercase letters are significantly different ($p<0.05$) as determined by Fisher's test.

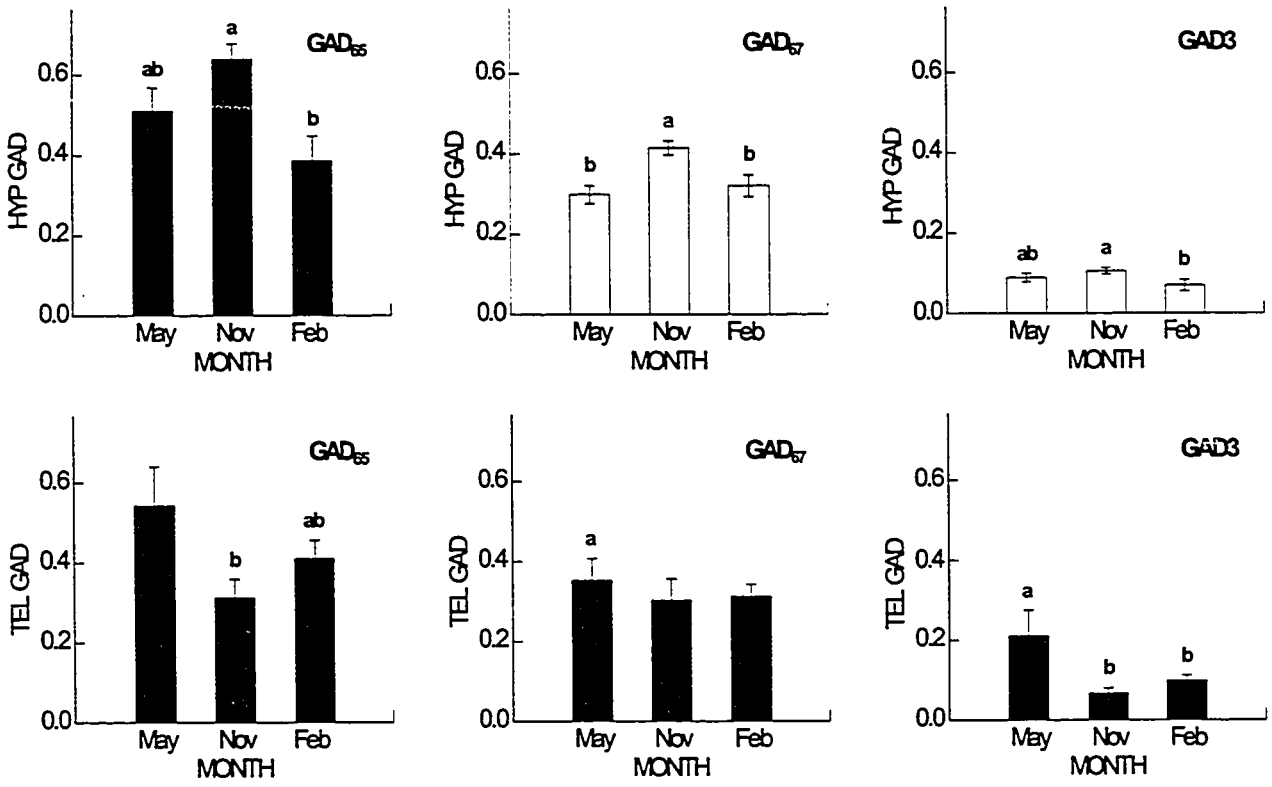

Figure 3.2: Mean GAD mRNA levels for untreated female fish at three times of the year. Data is shown as GAD expression/ β-actin expression ± SEM (n=4-7). Values marked with different lowercase letters are significantly different ($p<0.05$) as determined by Fisher's test.

February. GAD3 expression in May was two-fold higher than expression during the other two months. No seasonal differences were observed in GAD_{67} expression in the TEL of the female goldfish studied. Generally in females, seasonal trends for GAD mRNA expression in the TEL follow the same pattern as those for GSI whereas in the HYP, GAD mRNA levels are highest when GSI is lowest.

3.3.3 Seasonal variations in GAD expression in males

Males showed similar patterns of GAD expression to those observed in females (Figure 3.3). In the HYP, GAD expression was highest in November. GAD_{65} mRNA levels were 30% higher in November when compared to February values in the HYP, similar to data obtained from females. GAD_{67} expression in males showed an increase of 35% between May and November. In this case, intermediate and statistically different levels of male HYP GAD_{67} were observed in February. There were no statistically significant differences in GAD3 mRNA levels between the 3 timepoints studied. Overall patterns of seasonal changes of GAD gene expression in the HYP were similar in males and females. In the TEL, male GAD_{65} and GAD3 expression were highest in May, and were two- to three-fold more than GAD_{65} and GAD3 expression in February and November. GAD_{67} expression in TEL was also high in May, nearly two-fold higher than in February and November. Similarly to that observed in females, GAD expression in male TEL appears to follow the same pattern as GSI levels, with highest levels being detected when GSI is highest and lowest when GSI is low. An important sex difference in TEL GAD_{67} gene expression was observed. No seasonal variations were detected in the females whereas there was a distinct peak of TEL GAD_{67} expression in males in May

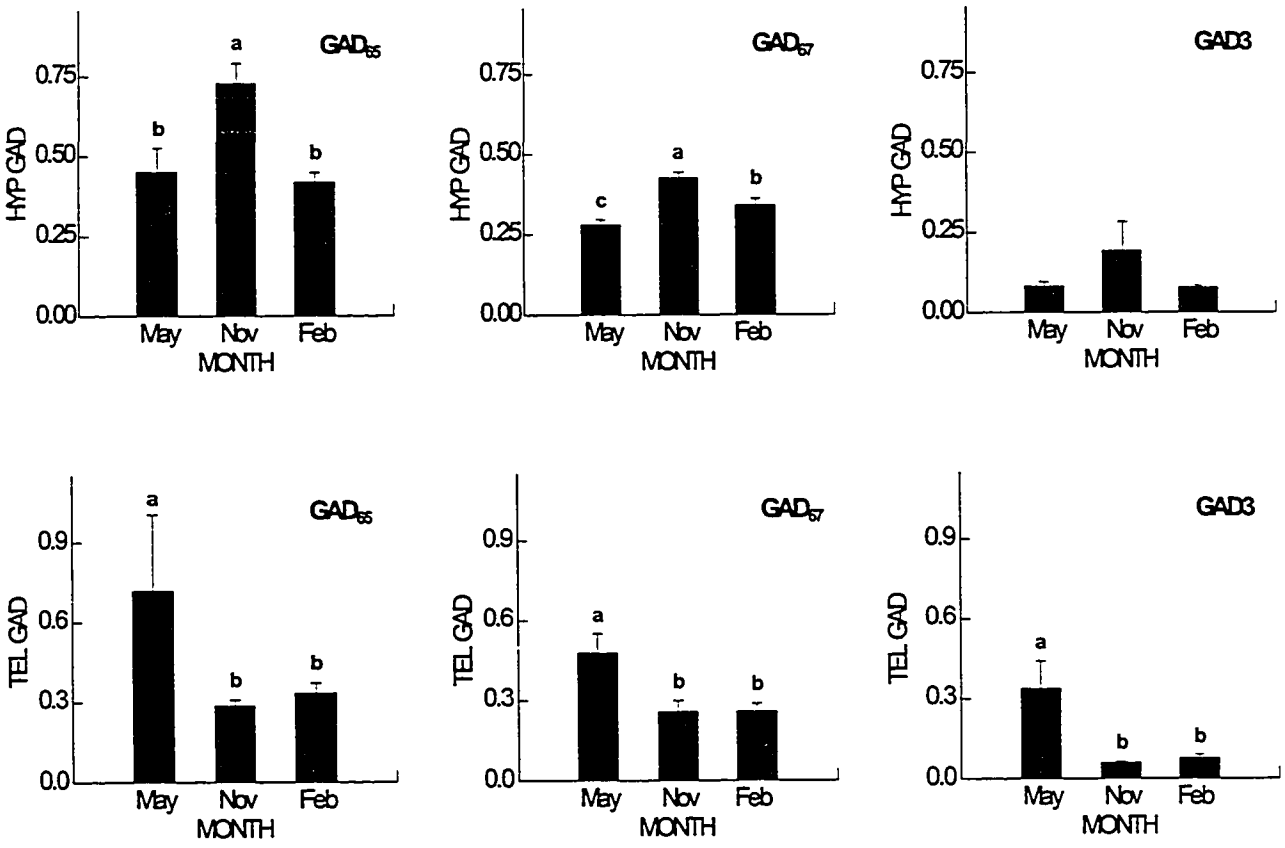

Figure 3.3: Mean GAD mRNA levels for untreated male fish at three times of the year. Data is shown as GAD expression/ β-actin expression ± SEM (n=4-7). Values marked with different lowercase letters are significantly different ($p<0.05$) as determined by Fisher's test.

when compared to February and November. Absolute levels of GAD mRNA between males and females however, were similar throughout.

3.3.4 Effects of steroid administration on HYP and TEL GAD expression

Testosterone, E2 and P4 were administered by intraperitoneal implantation for 5 days to fish in May, November and February and results are presented in Table 3.1. No significant effects of any of the steroids tested were observed in either November or February for either males or females. In May, however, significant decreases were observed in GAD_{65} and GAD_{67} gene expression in the female HYP after P4 administration when compared to control. GAD3 mRNA levels in the HYP were not affected by any of the sex steroid treatments. In contrast to the results in the female HYP, sex steroids did not affect GAD_{65}, GAD_{67} or GAD3 levels in males in May.

Several differences in gene expression were found in the TEL of males after steroid treatment when compared to control. GAD_{67} expression was reduced by 37% when either T or P4 were administered to mature animals in May. Reductions of 50% were observed in GAD_{65} mRNA expression with P4 administration and of 35% with T. Sex steroid implantation for 5 days did not affect steady-state mRNA levels of GAD3. No effect of steroid implantation was found on GAD gene expression in the TEL of females.

Table 3.1: GAD gene expression in the goldfish HYP and TEL at three points in their yearly reproductive cycle after 5 days of sex steroid implantation.

A May 2000

Treat-ment	HYPOTHALAMUS						TELENCEPHALON					
	GAD_{65}		GAD_{67}		GAD3		GAD_{65}		GAD_{67}		GAD3	
	f	m	f	m	f	m	f	m	f	m	f	m
Ctrl	0.509 ±.053	0.450 ±.063	0.298 ±.020	0.280 ±.013	0.089 ±.009	0.080 ±.012	0.720 ±.188	0.931 ±.262	0.353 ±.048	0.478 ±.064	0.210 ±.056	0.338 ±.090
T	0.447 ±.019	0.476 ±.057	0.262 ±.005	0.258 ±.010	0.075 ±.003	0.070 ±.004	0.486 ±.050	**0.596* ±.170**	0.321 ±.010	**0.306* ±.030**	0.220 ±.056	0.157 ±.044
E2	0.510 ±.044	0.569 ±.096	0.289 ±.014	0.301 ±.028	0.092 ±.017	0.110 ±.023	0.555 ±.107	0.712 ±.235	0.308 ±.024	0.374 ±.066	0.181 ±.062	0.218 ±.067
P4	**0.344* ±.008**	0.472 ±.043	**0.239* ±.006**	0.271 ±.016	0.063 ±.001	0.080 ±.007	0.322 ±.035	**0.464* ±.065**	0.259 ±.012	**0.301* ±.025**	0.108 ±.012	0.176 ±.048

B November 2000

Treat-ment	HYPOTHALAMUS						TELENCEPHALON					
	GAD_{65}		GAD_{67}		GAD3		GAD_{65}		GAD_{67}		GAD3	
	f	m	f	m	f	m	f	m	f	m	f	m
Ctrl	0.638 ±.036	0.728 ±.056	0.414 ±.016	0.426 ±.017	0.105 ±.007	0.192 ±.081	0.268 ±.037	0.241 ±.019	0.303 ±.048	0.257 ±.037	0.067 ±.012	0.060 ±.003
T	0.616 ±.026	0.768 ±.110	0.389 ±.010	0.425 ±.023	0.105 ±.005	0.142 ±.328	0.426 ±.158	0.295 ±.022	0.312 ±.066	0.256 ±.037	0.084 ±.033	0.054 ±.006
E2	0.787 ±.089	0.663 ±.050	0.456 ±.022	0.411 ±.022	0.132 ±.019	0.112 ±.009	0.290 ±.022	0.290 ±.019	0.281 ±.038	0.262 ±.035	0.056 ±.008	0.052 ±.004
P4	0.599 ±.038	0.780 ±.066	0.379 ±.010	0.451 ±.025	0.122 ±.011	0.138 ±.012	0.275 ±.057	0.339 ±.052	0.248 ±.031	0.208 ±.048	0.051 ±.005	0.069 ±.011

C February 2001

Treat-ment	HYPOTHALAMUS						TELENCEPHALON					
	GAD_{65}		GAD_{67}		GAD3		GAD_{65}		GAD_{67}		GAD3	
	f	m	f	m	f	m	f	m	f	m	f	m
Ctrl	0.385 ±.055	0.420 ±.026	0.320 ±.024	0.341 ±.018	0.069 ±.012	0.077 ±.006	0.411 ±.040	0.334 ±.034	0.312 ±.027	0.260 ±.025	0.098 ±.012	0.076 ±.010
T	0.377 ±.025	0.476 ±.076	0.317 ±.015	0.352 ±.025	0.066 ±.007	0.088 ±.018	0.415 ±.135	0.354 ±.030	0.282 ±.064	0.268 ±.018	0.091 ±.032	0.081 ±.010
E2	0.372 ±.020	0.401 ±.062	0.306 ±.011	0.329 ±.030	0.057 ±.003	0.073 ±.010	0.319 ±.019	0.348 ±.030	0.248 ±.013	0.277 ±.019	0.068 ±.007	0.078 ±.009
P4	0.346 ±.031	0.367 ±.048	0.306 ±.009	0.316 ±.015	0.063 ±.003	0.068 ±.009	0.300 ±.018	0.345 ±.052	0.238 ±.012	0.268 ±.037	0.067 ±.005	0.083 ±.017

Data shown are mean GAD expression/ β-actin expression ± SEM (n=4-7). Values marked with an asterisk (in bold) are significantly different from control ($p<0.05$) determined after a two-way ANOVA (Sex*Treatment). Ctrl=control, T=testosterone, E2=estradiol and P4=progesterone. Control values are repeated from Figs. 3.2 and 3.3.

The results of the steroid implantation experiments demonstrate that P4 consistently reduces GAD mRNA levels at the times and locations where it has an effect. Also, effects of gonadal steroids are shown to differ between brain regions, sexes and level of sexual maturity of the fish studied.

3.4 Discussion

This study has shown that GAD mRNA expression in the goldfish can be affected by gonadal steroids, and that these effects vary depending on the sex and physiological state of the fish. Seasonal variations were evident for all GADs in both the HYP and the TEL, with males showing higher GAD expression than females in most cases. Patterns of GAD expression in the TEL followed GSI values, with higher levels in mature fish with larger GSI and lower expression in recrudescent fish with lower GSI. Trends in the HYP were opposite of TEL expression, with GAD being expressed at a higher level in November in fish with the lowest GSI values studied. We have also found that GAD expression is not affected by exogenous T, E2 or P4 in early or late recrudescing male or female fish. This is in marked contrast to results obtained in more mature fish. In mature females, P4 was found to have an inhibitory effect on GAD_{67} mRNA expression in the HYP only. In males, both T and P4 were found to decrease GAD_{65} and GAD_{67} mRNA expression in the TEL.

Seasonal trends in both HYP and TEL were found to correlate with GSI values. Results in TEL are concurrent with the known role of GABA as a stimulator of GTH-II release, with high levels of GAD occurring in May at the highest measured levels of sexual maturity. Observed expression of GADs in HYP showed lowest GAD expression in November, in early recrudescent fish, which contrasts with results obtained from the TEL. Reverse effects of the GABAergic system when comparing the HYP and POA in mature versus post-spawn or regressed/recrudescent animals were also observed in rats. McCarthy et al. (1991) observed higher HYP GABA levels and lower POA GABA levels

in postreceptive females when compared to receptive females (McCarthy et al. 1991). In fish, GABA is mostly stimulatory to the reproductive axis whereas in rats and other mammals, GABA is mostly inhibitory to GnRH and LH release. This can perhaps help explain our results, opposite to those found in rats, of reduced GAD mRNA levels in HYP and elevated GAD mRNA levels in TEL in sexually mature goldfish. Furthermore, elevated levels of GAD expression in the HYP in November when GSI is low would seem to indicate an important role for the GAD genes in the onset of gonadal recrudescence. Conversely, elevated levels of GAD mRNA in the TEL in May when GSI is high, may indicate a role for GADs and GABA in the TEL in the regulation of GTH-II during the final stages of gonadal development required for spawning.

Previously, Bosma et al (2001) demonstrated that implantation of both T and E2 decreased HYP GAD_{65} and GAD_{67} mRNA levels in sexually regressed male goldfish. This study was performed at a period of the sexual reproductive cycle when endogenous circulating sex steroid levels are at their lowest. In the present study, T, E2 and P4 did not affect HYP GAD_{65}, GAD_{67} or GAD3 mRNA levels in sexually recrudescent (Nov, Feb) or sexually mature (May) males. In the female HYP, a different pattern of steroidal regulation of GAD expression is evident. Our previous study (Bosma et al. 2001) on sexually regressed females in the summer demonstrated that HYP GAD_{65} was upregulated by both T and E2 whereas GAD_{67} was not affected. In sexually recrudescent or mature females in the present study, T and E2 were without effect on HYP GADs, but P4 decreased GAD_{65} and GAD_{67} expression in female HYP. It appears that GAD_{65} and

GAD_{67} in male HYP but not in female HYP are responsive to sex steroids only in the sexually regressed phase, when endogenous steroids are low.

In comparing the effects of exogenous T and E2 on GAD_{65} and GAD_{67} expression it is evident that the responsiveness of the TEL and HYP varies according to the physiological state of the goldfish. For example, in females, T and E2 did not affect HYP GAD_{65} in mature or recrudescent animals (this study) but both steroids increased GAD_{65} levels in regressed fish (Bosma et al. 2001). A second example of this distinct seasonal variation in brain responsiveness to sex steroids was also evident in males where T did not affect TEL GAD_{65} or GAD_{67} in regressed males (Bosma et al. 2001), T did reduce TEL GAD_{65} and GAD_{67} in mature males. It is likely that the mechanism by which E2 and T affected GABA synthesis and GAD mRNA levels in regressed fish is altered by the elevations in endogenous serum sex steroid levels which occur during recrudescence and high levels present at the mature stage.

Seasonal and sex differences in the regulation of HYP GAD_{67} are also evident in goldfish. Whereas both T and E2 decreased male HYP GAD_{67} in regressed animals (Bosma et al. 2001), only P4 affected HYP GAD_{67} in the present study. An interesting sex difference was observed in the seasonal regulation of GAD3 in which did not vary throughout the year. GAD_{67} mRNA levels were also shown not to vary seasonally, but only in female TEL.

Sexually dimorphic regulation of TEL GAD mRNA levels were also observed in sexually regressed and sexually mature animals. Both T and E2 increase TEL GAD_{65} in sexually regressed females but not in regressed males (Bosma et al. 2001). In contrast to this earlier study (Bosma et al. 2001), sex steroid treatments were without effect on TEL GAD_{65} and GAD_{67} in recrudescent and mature females in the 3 months studied here. In the current analysis, both T and P4 did not affect TEL GAD_{65} in recrudescent males in either Nov or Feb. However, both T and P4 suppressed GAD_{65} and GAD_{67} in mature males.

Similarly to results from the HYP, seasonal and sex differences in GAD_{65} and GAD_{67} mRNA levels were also observed in the goldfish TEL. In sexually mature males, T suppressed both GAD_{65} and GAD_{67}, whereas T had no effect on GAD in the TEL of sexually mature females. In males, the ventral area of the telencephalon, more specifically in the vicinity of the pars commissuralis (Vs) and pars ventralis (Vv), is critical to the control of male sexual behavior in the goldfish. Lesions to the Vv/Vs area have been shown to impair male spawning behavior in goldfish (Kyle and Peter 1982). Moreover, high levels of GAD_{65} have been observed in this area of the TEL in trout (Anglade et al. 1999). Patch clamp electrophysiological studies have demonstrated that certain GABAergic neurons from the Vv/Vs project directly to the POA of male goldfish (Trudeau et al. 2000b). In rats, both GABA and sex steroids modulate sexual behaviour in addition to controlling LH release (McCarthy 1995a). In male fish, T also controls sexual behaviour and GTH-II release (Blazquez et al. 1998). Perhaps our observations on

T-regulated GAD_{65} and GAD_{67} levels have special relevance to the control of behaviour in mature males in the spawning season.

In females as well as in males, GAD_{67} levels are lower in mature untreated fish (May), when compared to recrudescent fish in November. Progesterone treatment was shown to further decrease GAD_{67} in female HYP in these mature fish. This seems to indicate P4 may play a role in decreasing HYP GAD levels in mature fish. In males, the inhibitory effects of P4 are not observed in the HYP, but rather in the TEL. Male fish, however, exhibit a seasonal decrease in GAD levels in May in the HYP but not in the TEL, in a manner similar to females. The varying effects of P4 on males and females despite similar seasonal variations of the GADs indicates a sex and brain region specific effect of P4. Although P4 has not been previously considered to be a major modulator of reproduction in fish, it is a major controller of reproductive hormone release and sexual behaviour in mammals. However, fish produce P4 in levels comparable to T and E2, and P4 has been shown to potentiate the positive effect of E2 on GnRH-stimulated GTH-II release (Trudeau et al. 1991b). Given this important effect of P4 on GAD mRNA levels in areas of the goldfish brain central to reproductive control, perhaps it too plays a role in feedback mechanisms or in control of sexual behaviour.

Seasonal variations and sex steroidal regulation of GAD_{65} and GAD_{67} were observed in both male and female sexually mature goldfish. However, the mechanism by which this modulation occurs is not yet clear. The promoter structure of goldfish GAD genes is currently unknown and therefore steroid response elements have not been

identified, however steroids could affect GAD levels indirectly as well. Several other systems are known to be affected by steroids and to modulate GABAergic activity, including NE and DA (Trudeau et al. 1993a; Trudeau et al. 1993b). Any of these neurotransmitters could be mediating the feedback effects of sex steroids on GAD mRNA levels. Furthermore, although there were clear seasonal changes in the levels of TEL and HYP GAD3 in females and TEL GAD3 in males, none of the sex steroid treatments used altered GAD3 levels. On the other hand, GAD_{65} and GAD_{67} seem to be co-regulated, our results showing decreases in mRNA levels for both with P4 and T in mature male TEL and with P4 in mature female HYP. The factors responsible for the regulation of GAD3 remain to be determined, and will most likely show a different role for this novel gene in reproductive function and in other hormonally regulated processes.

The gonadal steroids studied were shown to have an effect only when administered to sexually mature fish, in May. In other parts of the reproductive cycle, endogenous steroid levels are increasing as the gonad grows in preparation for spawning. It may be that responsiveness to sex steroids is at its maximum during this phase. When the fish have attained reproductive maturity, it is at the peak of their their endogenous serum sex steroid and GTH-II levels and perhaps a shift occurs towards negative steroid feedback to trigger regression in the post-spawning phase. A similar biphasic mechanism by which negative feedback shifts to positive feedback to allow the LH surge in rats has been previously described (Wagner et al. 2001). Increasing the already high steroid levels in these fish may trigger the suppressive affects of P4 in females and T and P4 in males.

From this work it is clear that in the brain of the goldfish, GAD mRNA varies seasonally and that P4 and T can regulate GAD mRNA in sexually mature fish. GAD mRNA levels were shown to vary seasonally, in patterns that follow gonadal growth and regression. The administration of exogenous sex steroids affected GAD mRNA levels differently at different reproductive states, with GAD expression only in sexually mature fish being responsive to negative feedback by T and P4. These effects vary by brain region, and are likely to be tied to the function of each of these areas in reproduction (i.e., control of gonadotropin release and sexual behaviour). The sex of the fish was also shown to be important as endogenous steroid levels are different in males and females. This work complements similar work done in sexually regressed fish (Bosma et al. 2001), which found both stimulatory and inhibitory effects of steroids on GAD mRNA expression in the brain of sexually regressed goldfish. Further work focusing on the action of steroids on GAD mRNA levels would help further characterize the mechanism by which GAD is affected.

Chapter 4: Major conclusions and research areas of future interest

At the outset of this work, our goals were to further study both the evolution of the vertebrate GADs and the regulation of GADs in goldfish. It is now clear that the GAD_{65} and GAD_{67} isoforms diverged prior to the Chondrichthyes branching off from the main vertebrate tree, more than 450 million years ago. Furthermore, we obtained GAD3 sequence information from goldfish, only the second partial sequence available for this GAD isoform. With our studies on GAD regulation, we demonstrated differential steroidal influence on GAD mRNA expression in both HYP and TEL. These effects are dependant on the stage of sexual maturity and sex of the goldfish studied. We also found seasonal variations in GAD baseline values. We have also shown the first evidence of seasonal regulation of GAD3 in both males and females. This research provides us with new insights into the the effects of reproductive hormones in gene expression in the vertebrate brain and presents new opportunities for further research in this field.

We were able to push back the known time of divergence of GAD_{65} and GAD_{67} to over 450 million years ago. However, it is not possible, using our current methods, to conclusively determine whether lampreys or hagfish have only one form, or rather have multiple forms which we have not yet detected. Further sequence data from this group of organisms would help determine the absence of multiple isoforms in these species with more certainty. There appears to be four major groups of the vertebrate GADs; GAD_{65}, GAD_{67}, GAD3 and lamprey/hagfish GAD. However, it is still difficult to define specifically where each of these GADs first appeared. In order to clarify this tree, more sequence information would be required, either by sequencing a larger portion of the

GAD gene from key species, or by increasing the number of species from less-represented groups in the study.

The study of the regulation of the GADs showed not only seasonal variations but also varying steroidal effects on goldfish at three different stages of sexual maturity. This research complements similar work done in sexually regressed fish by Bosma et al (2001) and other experiments done on GABA synthesis by Trudeau et al (1993). At the cellular level, there exist several levels of control in the process of transcribing DNA and translating new proteins which can affect final neurotransmitter release and these two studies leave a large gap in knowledge of the process which controls GABA release in neurohormonal control of reproduction. Work done on other regulatory steps in the protein synthesis pathway, for example measures of GAD protein levels or GAD activity would help to better understand the mechanisms of GABAergic release in the context of reproductive control.

Progesterone decreased GAD mRNA expression only in sexually mature fish in May. However, previous research on goldfish has shown a role for P4 in potentiating the action of E2 (Trudeau et al., 1991b). Perhaps administering a combination of these two gonadal steroids to fish of varying reproductive states may reveal other aspects of the control of GAD gene expression. Furthermore, administration of a non-aromatizable analogue of T such as hydroxytestosterone or 11-ketotestosterone would help determine whether the observed effects of T on GAD are through T or one of its metabolites.

The RPA designed for the regulatory study was set up to study relative levels of three goldfish GAD mRNAs. By using only three genes, our view on the reproductive control and steroidal regulation on GABA synthesis is limited. A larger number of mRNA targets could be added to the assay, perhaps by employing a reverse-Northern or microarray technique, to screen differences in a larger amount of genes thought to be involved in the process. For example if the GABA degrading enzyme GABA-T may also vary seasonally, which would contribute to the regulation of GABA levels, and thus the action of GABA. By studying the regulation of sets of GABA-related genes, perhaps GAD, GABA-T and GABA transporter molecules (GATs) to start, we could gain an understanding of the integrative process of GABAergic neurotransmission as it relates to fish reproduction.

This research is the first to address GAD3 in a species other than the armed grenadier from which it was first isolated. The partial GAD3 sequence obtained from goldfish provides an excellent starting point for several other avenues of research. We were not able to obtain a functioning genomic Southern assay to determine the presence of GAD3 in other species, or to begin to characterize the gene itself. This step is critical in the study of GAD3 as it will help determine the scope of its presence in vertebrates. Furthermore, only a partial sequence is now available. Full-length sequencing, followed by insertion into an expression system and characterization of enzyme activities are essential to verify the functionality of this putative new enzyme. We have shown here that expression of GAD3 mRNA varies with the stage of the seasonal cycle, suggesting a role for GAD3 in reproduction. Preliminary in situ hybridization studies in collaboration

with Dr. Tom Finger (Denver, Colorado) indicate a differential brain distribution of GAD3 compared to the other GADs, further emphasizing the novelty of GAD3. Many questions on the evolution, structure, function and regulation of GAD3 remain to be answered.

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